OLIGOKITOSANO ULTRAPURUETAN OINARRITUTAKO POLIPLEXOAK: DISEINUA, KARAKTERIZAZIOA ETA TERAPIA GENIKORAKO APLIKAZIOAK

POLIPLEXOS BASADOS EN OLIGOQUITOSANOS ULTRAPUROS: DISEÑO, CARACTERIZACIÓN Y APLICACIONES PARA TERAPIA GÉNICA

POLYPLEXES BASED ON ULTRAPURE OLIGOCHITOSANS: DESIGN, CHARACTERIZATION AND APPLICATIONS FOR GENE THERAPY

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Lo que sabemos es una gota de agua
lo que ignoramos es el océano

Isaac Newton

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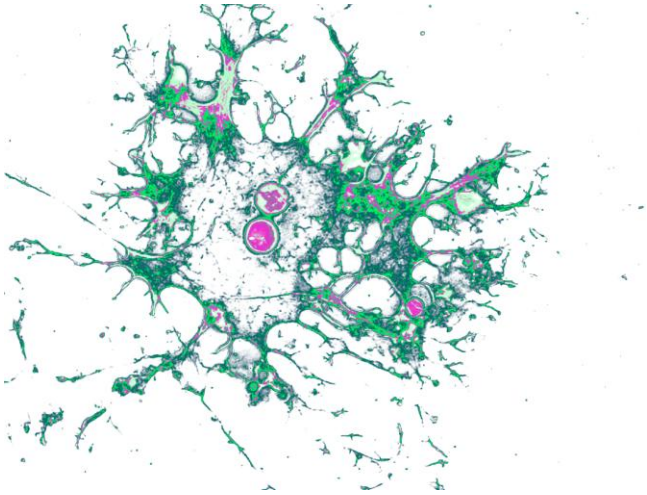


Sarrera

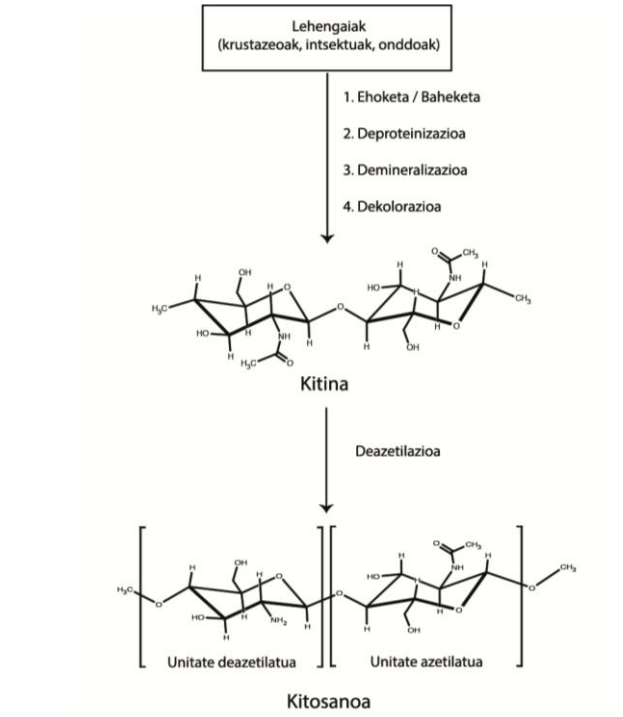
Introducción

Introduction

Pisu molekular baxuko kitosano (LMWC)-etan oinarritutako poliplexoen erabilera pDNA-aren garraiorako

Terapia genikoa arrakastatsua izateko gene terapeutikoa biodegradatua izan aurretik zelulan sartu eta transkribatu egin behar da. Helburua lortzeko garraiolari lanak egingo dituen bektore egokia beharrezkoa da, genea itu-zelularaino garraiatuko duena eta nukleasen degradaziotik babestuz nukleorainoko bidean zelularen mintz ezberdinak gainditzen lagunduko diona. Egun, oraindik ere terapia genikoan bektore biralekin emaitza eraginkorragok lortzen dira. Hala ere, immunogenizitatea eta mutagenizitatea bezalako segurtasun arazoek kolokan jartzen dute bektore biralen erabilera klinikoa [1, 2]. Hori dela eta, bektore ez-biralak gero eta gehiago ikertuak izaten ari dira alternatiba seguruagoa direlako azido nukleikoen garraiorako [3, 4]. Bektore ez-biralak bi talde nagusitan sailkatzen dira: (a) lipoplexoak, lipido kationikoetan oinarrituak; (b) poliplexoak; polimero kationikoetan oinarrituak. Esan bezala, nahiz eta lipido kationikoak izan gehien ikertzen diren bektore ez-biralak, toxikotasun txikiagoa dutelako ordezko aukera gisa emaitza oso interesgarriak lortzen ari dira polimero kationiko naturalekin [5]. Polimero naturalen artean, kitosanoaren (Ch) erabilera oso zabaldua dago ikerlarien artean [6-10], izan ere biobateragarria, biodegradagarria eta ia toxizitaterik gabekoa da [11]. Kitina, krustazeoen, intsektuen eta onddoen exoeskeletotik eratorritako polisakarido nitrogenatua da. Kitina da, zelulosaren atzetik, naturan gehien dagoen polimeroa eta elikagaien industriaren hondakinetatik urtero tonak ekoizten dira [12]. Kitina, beraz, errentagarritasun ekonomiko handiko materiala izaki, birziklagarria ere bada. Hala ere, kitina ez da solugarria ez uretan ezta disolbatzaile organikoetan ere, eta gainera kimikoki ere inertea denez, aplikazio biomedikoetarako ez da erabilgarria. Kitinaren deazetilaziotik sortzen da Ch. Ch lotura (1–4) glukosidikoarren bidez elakrtutako β-D-glukosamina and β-*N*-azetil-D-glukosamina azpiunitateek osatzen duten polisakarido kationiko natural eta lineala da. Ch-ren amina taldeen pKa-ren balioa

6,5 da [13-15] eta honek, pH fisiologikoetatik behera protonatuta egoteko aukera ematen dio. Karga positiboa duten amina taldeei esker, interakzio elektrostatiko bidez, Ch erraz lotzen da karga negatiboa duten DNA-ren fosfato taldeekin eta poliplexoak eratzen dira. Era berean, Ch-aren ekoizpena eta interesatzen zaigun kate luzera lortzea ez da batere zaila, nahikoa da kitina osatzen duten sakarido unitateak lotzen dituzten lotura glukosidikoa suntsitzearekin. 1. irudian ikusi daiteke depolimerizazio prozesuaren adibidea.



1. Irudia: Pisu molekular baxuko kitosanoen manufakturazio prozesuaren eskema.

Ch-an oinarritutako bektoreen transfekzio eraginkortasunean faktore asko daude murgilduta, eta horien artean garrantzitsuenetakoak dira polimeroaren pisu molekularra eta deazetilazio maila (DDA) [17]. Polimeroaren pisu

molekularraren araberakoak izan daitezke poliplexoen partikula tamaina, polimeroaren eta azido nukleikoaren arteko lotzeko erraztasuna, poliplexoak zelulan sartzeko duen gaitasuna, DNA-ren askapena eta, azken batean, transfekzioaren eraginkortasuna. Frogatuta dago terapia geniko ez-birala arrakastatsua izateko ezinbestekoa dela DNA babesteko eta zelula barnean askatzeko gaitasunen arteko oreka mantentzen duten poliplexoak eratzea [18].

Pisu molekular handiko Ch-ekin (HMWC) (›150 KDa) egiten diren poliplexoek DNA lotzeko gaitasun handiagoa dute. Hala ere, zelulan barneratu ostean lotuta daramaten DNA askatzea gehiago kostatzen zaie. Aldiz, pisu molekular txikiko Ch-ekin eratutako poliplexoak (LMWC) ezegonkorragoak direnez alderantzizkoa gertatzen da, babesteko gaitasun txikiagoa dutela baina errazago askatzen dutela DNA [19]. Hala ere, zenbait ikerketak erakutsi duten bezala LMWC-ekin HMWC-ekin egindako bezain poliplexo eraginkorrak ekoiztu daitezke [20, 21]. Horrez gain, Kopping-Hoggard eta kideen argitalpenaren arabera 15 eta 24 bitarteko polimerizazio gradua duten Ch oligomeroek transfekziorako propietate ezin hobeak dituzte eta gai dira modu eraginkorrean p lotzeko, degradaziotik babesteko eta transgenea inolako kalterik gabe askatzeko [22].

DDA da Ch-aren transfekzio gaitasunean eragin garrantzitsua duen bigarren parametro fisikokimikoa. DDA-ren balioak adierazten du Ch molekularen egituran deazetilatuta dauden amina primarioen portzentajea, edo beste era batera esanda, azido nukleikoarekin interakzioa egiteko prest dauden karga positibodun aminen portzentajea. DDA altuko (›%90) Ch-ek karga positiboagoa izatea eragiten dute eta ondorioz, poliplexoa errazago eratzen da eta egonkorragoa da. Baina DDA baxuko Ch-ekin lotura elektrostatiko ahulagoa duten poliplexoak eratzen dira eta arrisku handiagoa izaten da askatu eta degradatzeko [19]. Hain zuzen ere, Kiang eta kideek frogatu zuten DDA baxuko Ch-etan oinarritutako poliplexoekin *in vitro* eginiko saioan geneen espresio maila baxuagoa lortzen dela, batez ere poliplexoen

ezegonkortasunagatik [23]. Ildo berean, pisu molekular eta DDA egokia duen Ch erabilita posible da babesteko eta askatzeko gaitasunaren arteko oreka bilatzea eratutako poliplexoan. Badirudi pisu molekular zehatz bakoitzari DDA balio egoki bat dagokiola eta Ch-aren eta DNA-ren arteko loturan bi parametro fisikokimiko hauek garrantzi handia dutela [24]. Beraz, esan daiteke LMWC-ek ezaugarri interesgarriagoak dituztela erabilera klinikorako, adibidez disolbagarritasun handiagoa pH fisiologikoan [20]. Horrez gain, LMWC-ek mikrobioen aurkako eragina, gaitasun immunoestimulantea, antioxidantea eta minbizien hazkuntza inhibitzeko gaitasuna ere badute [25]. Laburbilduz, errebisio honetan LMWC-etan oinarritutako poliplexoak terapia genikoan bektore ez-biral gisa erabiliak izateko garapen prozesua deskribatuko da; zehazki, eraketa, karakterizazioa eta *in vitro* eta *in vivo* transfekzioaren deskribapena egingo dira.

1. LMWC-pDNA poliplexoen eraketa eta karakterizazioa

1.1 LMWC-pDNA poliplexoen eraketa prozesua

LMWC-pDNA poliplexoak eratzeko metodorik arruntena nahasketa zuzena da karga positiboa duen kitosanoaren eta karga negatiboa duenaren artean. Egindako ikerketen arabera nahasketan erabilitako metodoak eta inkubazio baldintzek azkenengo gene espresioan eragina izan dezakete; hain zuzen ere, asko dira prozesu honen optimizazioan lan egiten diharduten taldeak. Lavertu eta kideen arabera, egokiena Ch pri gehitu ondoren gora eta behera mantso pipeteatzea litzateke [26]. Beste batzuek aldiz, bortizki irabiatzea egokiagoa dela uste dute [27-30]. Inkubazio denbora da kontuan izan beharreko beste faktore bat, izan ere, LMWC-pDNA poliplexoak eratu eta ordu batzuetara ezegonkortu eta agregatzeko joera dutelako [31, 32]. Zehazki, 15 eta 30 minutu bitarteko inkubazio denbora da ikerlari gehienek aukeratzen dutena poliplexoen eraketarako egokiena bezala [27, 30, 33, 34]. Nahiz eta Lavertu eta kideek eginiko zenbait ikerketen

arabera 30 minututik 2 orduraino inkubatzen edukitako poliplexoekin ere gene espresio egokiak lortu [26].

Ch-an oinarritutako poliplexoen elaborazio prozesuan nahasketa estekiometria ere garrantzitsua da. N/P ratioa erabiltzen da Ch molekularen katean protonatu daitezkeen aminen (N) eta azido nukleikoko karga negatiboa duten fosfatoen (P) arteko proportzioa adierazteko. Transfekzio mailarik altuena ziurtatzeko ezinbestekoa da ikertzaileek N/P ratio ezberdinekin frogak egitea. Normalean, poliplexoak eratzerakoan Ch proportzio handiagoa erabiltzen da, N/P ratioa batetik gorakoa. Aurrerago 2.5 atalean azaltzen da N/P ratioak transfekzio prozesuan duen eragina.

Argitalpen ezberdinen arabera, disoluzioen pHa nahasketa prozesuan parametro garrantzitsua da, zehazki Ch-aren ionizazioan eragiten du eta ondorioz eratzen diren poliplexoen ezaugarri fisikokimikoetan (tamaina, masa, dentsitatea, morfologia) [35]. pHaz gain nahasketa ordenak eta ionizazio mailak ere badute zeresana poliplexoen eraketa prozesuan. Baita nahasketa disoluzioko azido nukleikoaren kontzentrazioak ere; hain zuzen ere, zenbat eta handiagoa izan kontzentrazioa poliplexoen tamaina ere handiagoa izaten da. Baina sarrerako atalean azaldu den bezala, parametro guztien artean bi garrantzitsuenak Ch-aren Mw eta DDA dira, eta transfekziorako egokiena Mw baxukoak eta DDA altukoak dira [22, 36]. Dena den, poliplexoen eraketa prozesuko zehaztasunen azterketa sakonagoa egitea komeni da, zeren oraindik ere formulazioetako parametroek poliplexoen ezaugarri fisikokimikoetan duten eraginaren gaineko emaitza kontrajarriak irakurri genitzake literaturan [10].

Laburbilduz, Ch poliplexoak eta orokorrean geneen garraiorako sistema ez-biralak prestatzeko Ch eta pDNA soluzioen bolumen txikiak erabiltzen dira. Gainera, nahasketarako erabiltzen diren soluzioak oso diluzio handikoak dira,

horrela lortzen diren esekidura koloidalen egonkortasuna eta homogeneotasuna bermatzen delako. Baina, aldi berean, nahasketarako soluzio hain diluituak erabiltzea aplikazio klinikorako oztopo bihurtzen da, izan ere dosi egokia lortzeko bolumen handiak behar izaten dira. 5. atalean arazo honentzako soluzio posiblea eztabaidatzen da.

1.2. LMWC-pDNA poliplexoen karakterizazio fisikokimikoa

LMWC-pDNA garraiatzaileen karakterizazio fisikokimikoan tamaina, zeta potentziala, morfologia, lotura-gaitasuna, buffer-gaitasuna eta egonkortasun koloidala neurtzen dira. Karakterizazioak sortutako poliplexoaren transfekzio-gaitasuna aurreikusteko balio du, nahiz eta transfekzio froga egin arte ezin den ezer konfirmatu. Karakterizaziorako erabiltzen diren ohiko tekniken laburpena 1. taulan ikusi daiteke.

1. Taula: LMWC/pDNA poliplexoen karakterizazio metodoak

Karakterizazio teknika	**Parametroa**
Agarosa gelean elektroforesia	Lotura-gaitasuna Askapen ahalmena Degradaziotik babesteko ahalmena
Azido-base orekatze entsegua	*Buffer* gaitasuna
Dynamic light scattering	Tamaina Egonkortasun koloidala
EtBr-ren desplazamenduaren metodoa	Egonkortasuna
Indar atomiko bidezko mikroskopia	Tamaina Morfologia
Laser Doppler velocimetry	Zeta potentziala
Mikroskopia elektronikoa	Tamaina Morfologia
Nanoparticle tracking analysis	Tamaina Pisu molekularra Kontzentrazioa
Titrazio isotermikoaren kalorimetria	Lotura-gaitasuna Egonkortasuna

1.2.1- Tamaina

Normalean tamaina dynamic *light scattering* (DLS) bidez neurtzen da.

Argitalpen ezberdinen arabera Ch-pDNA poliplexoak tamaina askotarikoak izan daitezke, hasi hamar nm ingurukoekin [18] eta ehun nm baino handiagokoetaraino [30]. Tamaina faktore ezberdinen menpe dago, besteak beste, N/P ratioa, inguruneko pHa, eta aukeratutako Ch-aren eta pDNA-ren ezaugarriak. Mikroskopia elektronikoa ere erabiltzen da tamaina neurtzeko. DLS-arekin mikroskopia elektronikoarekin baino tamaina txikiagoak neurtzen dira, zeren eta DLS neurketarako partikulak ingurune urtsuan esekiduran egoten dira [37] eta neurketa zehatza izateko partikulak esferikoak izan behar dira, eta sarritan partikulak ez dira erabat esferikoak izaten [38].

Elkarrekin nahastu behar diren Ch-aren eta pDNA-ren soluzioen kontzentrazioak ere eragiten du poliplexoaren partikula tamainan. Zenbait lanetan ikusi da pDNA soluzioaren kontzentrazioa igota poliplexoen tamaina ere handitu egiten dela [17, 39]. Pisu molekularrari dagokionez ondorio ezberdinak daude argitaratuta. Koping-Hoggard eta kideek 60ko N/P ratioarekin prestatutako poliplexoen tamaina 68tik 174nm-ra handitzen zela ikusi zuten 2 edo 7 KDa-eko kitosanoa erabilita [22]. Gure laborategian duela gutxi egindako ikerketan pisu molekular handiko kitosanoekin (214 kDa), N/P 20 ratioa erabilita, pisu molekular baxuko kitosanoak (5,3 kDa; 7kDa) erabilita baino 4 aldiz tamaina handiagoko poliplexoak lortu ziren [30]. Hala ere, beste argitalpen batzuek kontrakoa ere adierazi dute [19]. Desadostasun hauen azalpena, poliplexoak sortzeko erabiltzen den metodoan egon daiteke. N/P ratioaren kasuan ere, ez dago argi poliplexoaren tamainan duen eragina. Gehien onartzen den teoria da N/P ratioa igotzerakoan Ch eta pDNA elkarrekin lotzea errazagoa dela eta konpaktatuago dagoen poliplexoa lortzen dela, ondorioz tamaina txikiagoko poliplexoa lortzen da. Baina gure ikerketa taldeak ondorioztatu duenez, oligokitosanoekin egindako poliplexoetan N/P ratioak ez du poliplexoaren tamainan modu esanguratsuan eragiten, seguru aski N/P ratioaz gain, aurrerago aipatu diren faktoreek ere zeresan handia

dutelako partikularen azken tamainan (2A. Irudia) [29,30,33]. Poliplexoaren tamainaren arabera gorputzeko organo batera edo beste batera joan daiteke. Adibidez, 100 nm baino txikiagoko partikulak gai dira gibeleko hodi fenestratuak zeharkatzeko eta hepatozitoetara heltzeko; aldiz, 200 nm inguruko partikulak birikietako kapilareetan gelditzen dira harrapatuta [40]. Argi dago beraz, interesa dugun organo edo gune anatomikora poliplexoak heldu daitezen, tamaina aztertu behar dela.

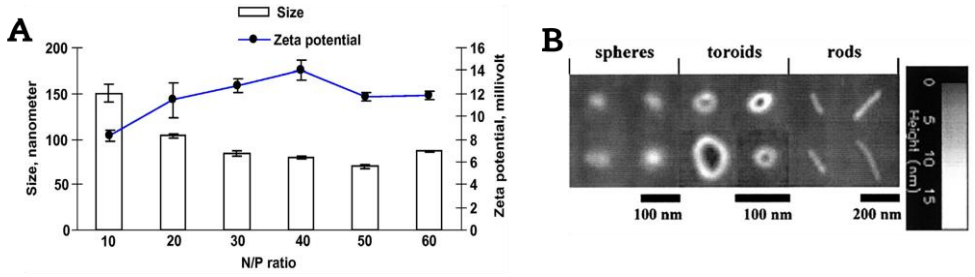
1.2.2. Zeta Potentziala

Poliplexoen gainazaleko karga neurtzen duen zeta potentziala *Laser Doppler Velocimetry* (LDV) teknikaren bidez neurtzen da. Parametro hau poliplexoen esekidura urtsuaren egonkortasun koloidalaren adierazle izateaz gain, LMWC-pDNA poliplexoen bideragarritasunean, zelula-barneratzean eta transfekzio-gaitasunean erabakigarria izan daiteke [30,41]. Asko dira LMWC-etan oinarritutako poliplexoen zeta potentzialean eragin dezaketen faktoreak, ondorioz aukera ugari daude nahi den gainazal karga lortzeko estrategietan. Ch-aren pKa 6,5 ingurukoa denez, poliplexoak eratzeko ingurunearen pHa 7,4tik 6,5era jaisten denean gainazal karga positiboagoa bihurtzen da. Zehazki, gure laborategian 6 mV positiboago ziren poliplexoak lortu genituen LMWC ultrapuruekin poliplexoen eraketan pHaren jaitsierarekin [30, 41]. Lehen aipatu bezala, parametro honen menpe egon daiteke poliplexoak zelula barnera sartzea edo ez, beraz, prestaketa prozesuan jarraipena egin behar zaion ezaugarria dela argi dago.

1.2.3. Morfologia

LMWC-etan oinarritutako poliplexoen morfologia zehazteko ekorketa bidezko mikroskopia elektronikoa (SEM), transmisio bidezko mikroskopia elektronikoa (TEM) eta indar atomiko bidezko mikroskopia dira gehien erabiliak. Ch-pDNA poliplexoek morfologia anitz izan ditzakete, batez ere, azetilatutako

unitateen eta polimerizazio graduaren arabera [42]. Ikertzaile batzuek konplexu esferikoak deskribatu zituzten [33, 43], baina beste batzuek garatutako poliplexoak toroideak, zizare itxurakoak edo globularrak ere izan dira [38, 39]. Danielsen eta kideek frogatu zuten poliplexoen itxura zehazteko interakzioak oso garrantzitsuak direla. Horrez gain, polimerizazio gradu ezberdineko Ch-ekin egitura beretsuko poliplexoak lortzea posible da, baina polimerizazio maila altuko Ch-en kasuan kantitate txikiagoa behar da helburu berdina lortzeko [42]. 2B. irudian ikusi daitezke LMWC-ekin eratutako poliplexoek izan ditzaketen morfologia ezberdinak.

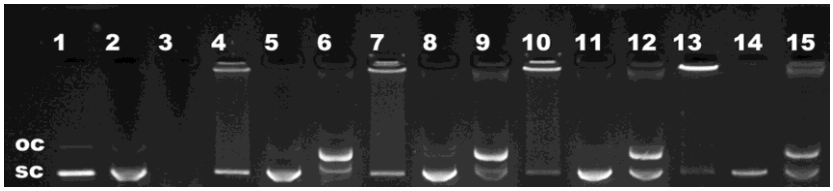


2. Irudia: (A) Oligokitosano/pDNA poliplexoen tamainan N/P ratioak duen efektua erakusten duen grafika. [33] erreferentziatik jasoa *Elsevier*ren baimenarekin. (B) Esfera-, toroide- eta zizare-itxurako poliplexoak. Poliplexo hauek, 13.3 μg/ml-ko kontzentrazioan eta 60:1 ratioan eratu eta indar atomikoko mikroskopioan aztertu ziren. Barra horizontalak 100 nm (esfera- eta toroide-itxurakoak) eta 200 nm (zizare-itxurakoak) adierazten ditu. Barra bertikalak poliplexoen altuera adierazten du. [22]. erreferentziatik jasoa *Natur*en baimenarekin.

1.2.4. Lotura-gaitasuna

LMWC eta pDNA-ren arteko lotura-gaitasuna mugarria da polimeroak azido nukleikoa biltzeko, askatzeko eta babesterako orduan. Ondorioz, transfekzio-gaitasunean ere eragina dauka. Teknika ezberdinak daude LMWC eta pDNA-ren arteko lotura-gaitasuna neurtzeko. Lotura-gaitasuna neurtzeko gehien erabiltzen den metodoa agarosa gelaren entsegua da. Elektroforesi mota honen bitartez N/P

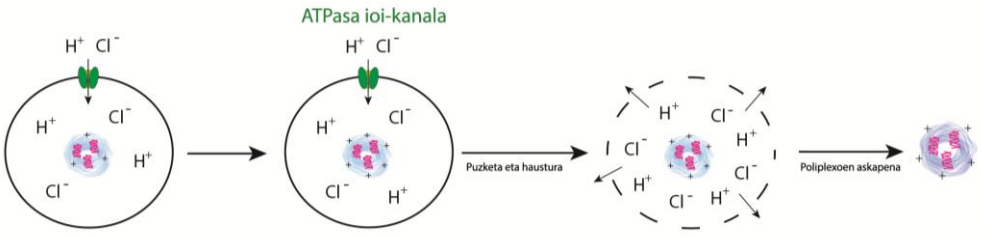
ezberdinetan eratutako poliplexoetatik pDNA-ren askapenaren azterketa egiten da (3. irudia). Ikerketa ezberdinen arabera, DDA baxua duten Ch-ekin eta N/P ratio txikiarekin sortutako LMWC-pDNA poliplexoak oso ezegonkorrak dira eta pDNA errazago askatu dezakete [18,22]. Hala ere, frogatuta dago LMWC-ak gai direla azido nukleikoa bildu, askatu eta babesteko, nahiz eta printzipioz formulazioa ezegonkorragoa izan [29, 34]. Lotura-gaitasuna zeharka neurtzen duen beste teknika ezagun bat Etidio Bromuroaren (EtBr) desplazamenduaren metodoa da. EtBr DNA-ren helize bikoitzeko base pareetan txertatzen da molekula fluoreszentea bihurtuz. Beraz, EtBr-arekin markatutako azido nukleikoa poliplexoa eratzeko polikatioiarekin kontaktuan jartzean lotzen bada, EtBr desplazatu eta konplexuak fluoreszentzia galtzen du. EtBr-aren desplazamendua edo fluoreszentziaren jaitsiera neurtu daiteke eta jaitsiera horrek erakusten digu polimero kationikoaren eta pDNAren arteko lotura-gaitasuna [27]. Gutxiago erabiltzen den beste teknika bat titrazio isotermikoaren kalorimetria, ingelesez *isothermal titration calorimetry* (ITC) eta *single set of identical sites model* (SSIS) metodoen konbinazioa da. Azken teknika honen bidez Ch-aren eta DNA-ren arteko lotura-konstanteak neurtzen dira. Neurketa horiek pHaren, erakarpen ionikoaren eta Ch-aren estruktura ezaugarrien araberakoak dira [24].



3. Irudia: Agarosa gelean egindako elektroforesia non oligokitosano eta DNA-ren arteko loturaren efizientzia eta DNase I entzimen degradaziotik bababesteko ahalmena erakusten den. OC: DNA-ren "open circular" forma, SC: DNA-ren forma superkoloidala. 1-3 lerroak: DNA askea; 4-6, N/P 5; 7-9, N/P 10; 10-12, N/P 20; 13-15, N/P 30. Poliplexoak dodezil sulfato sodikoarekin (SDS) (2, 5, 8, 11 eta 14. lerroak) eta DNAasa I entzimarekin tratatu ziren (3, 6, 9 eta 15. lerroak). [29]. erreferentziatik jasoa *Elsevier*ren baimenarekin.

1.2.5. *Buffer*-gaitasuna

Zelulan sartu ondoren endosoman barneratzen dira poliplexoak eta endosomatik ihes egiteko protoien puzte-efektua literaturan deskribatuta dago. Protoein puzte-efektu hori Ch-aren buffer-gaitasunaren menpekoa da. Mekanismo honetan endosoman edo lisosoman barneratu diren poliplexoek ingurunearen azidotzea eragiten dute garraiatzen dituzten protoien eraginez; era berean, kloro ioien barneratzea gertatzen da protoien karga orekatzeko eta honek presio osmotikoaren igoera eragiten du eta besikula puztu egiten da apurtu arte. Apurtzean poliplexoen kanporatzea baimentzen da endosoma/lisosoma barneko entzimen eraginez degradatuak izan aurretik (4. irudia).



4. Irudia: Protoien puzte-efektuaren irudikapen eskematikoa.

Ch-aren *buffer*-gaitasuna azido-base orekatze entseguaren bidez neurtzen da [30, 44]. Ikertzaile batzuen arabera Ch-arekin egindako poliplexoak, polietilenimina (PEI) bezalako beste polimero kationikoekin egindakoen aldean, ezinezkoa da endosomatik protoien puzte-efektuaren bidez ateratzea, izan ere *buffer*-gaitasun txikiagoa dute. Hala ere, Bushcmann eta kideek frogatu zuten Ch eta PEI molaritatearen arabera konparatuz gero eta ez masa-kontzentrazioaren arabera, polimero naturalak PEIak baino buffer-gaitasun handiagoa duela endosomen/lisosomen pH balioetan [45]. Hain zuzen ere, gure laborategian eginiko esperimentuetan pisu molekularraren menpeko *buffer*-gaitasuna ikusi

genuen kitosanoen kasuan, zehazki oligokitosanoek pisu molekular altuko kitosanoek baino gaitasun handiagoa erakutsi zuten. Ondorioz, hau izan daiteke LMWC-ek HMWC-ek baino transfekzio-gaitasun handiagoa izateko arrazoia [30].

1.2.6. Egonkortasun koloidala baldintza fisiologikoetan

LMWC-pDNA poliplexoen mugarik handienetakoa, baldintza fisiologikoetan duten egonkortasun koloidal baxua da [46]. Hain zuzen ere, odoleko proteinak, heparinak edo glukosaminoglukanoak (GAG) bezalako polianioiek, poliplexoaren zatiketa eta terapiarako organora heldu baino lehenagoko pDNAren askapena eragin dezakete [47, 48]. Arrazoi honengatik ikertzaileak ahalik eta egonkortasun handieneko bektoreak diseinatzeko ahaleginetan dabiltza; hori da eraldatutako kitosanoekin (mCh) eginiko poliplexoen (mCh-pDNA) kasua. Egonkortasun koloidala zehazteko tamaina hidrodinamikoa neurtzen da denbora tarte jakin batean eta egoera fisiologikoak simulatzen dituzten inguruneetan. Frogatuta dago LMWC-pDNA poliplexoen tamaina 100 nm-koa izatetik mikra bat baino handiagokoa izatera pasatzen dela fosfatozko gatz-tanpoian (PBS) ordu bat inkubatzen izan ostean [46]. Horrez gain, 2 mg/mL dituen behiaren albumina serumaren (BSA) soluzioarekin Ch-DNA poliplexoak inkubatuz gero, soluzioa arretu egiten da poliplexoen agregazioagatik. Aitzitik, Ch laktosilatuarekin eginiko konplexuen kasuan egonkortasuna mantentzen da 24 ordutan zehar BSArekin inkubatu ostean [49].

LMWC-ez eginiko poliplexoen egonkortasun koloidal murritzak *in vivo* izan dezaketen portaera mugatzen du, batez ere era sistemikoan administratu ostean. Egonkortasuna handitzeko eta odolean zirkulazio denbora areagotzeko intentzioarekin polimeroaren modifikazio kimiko ezberdinak proposatzen dira: PEGilazioa [50], kuaternizazioa [51] eta glikolizazioa [52], esate baterako.

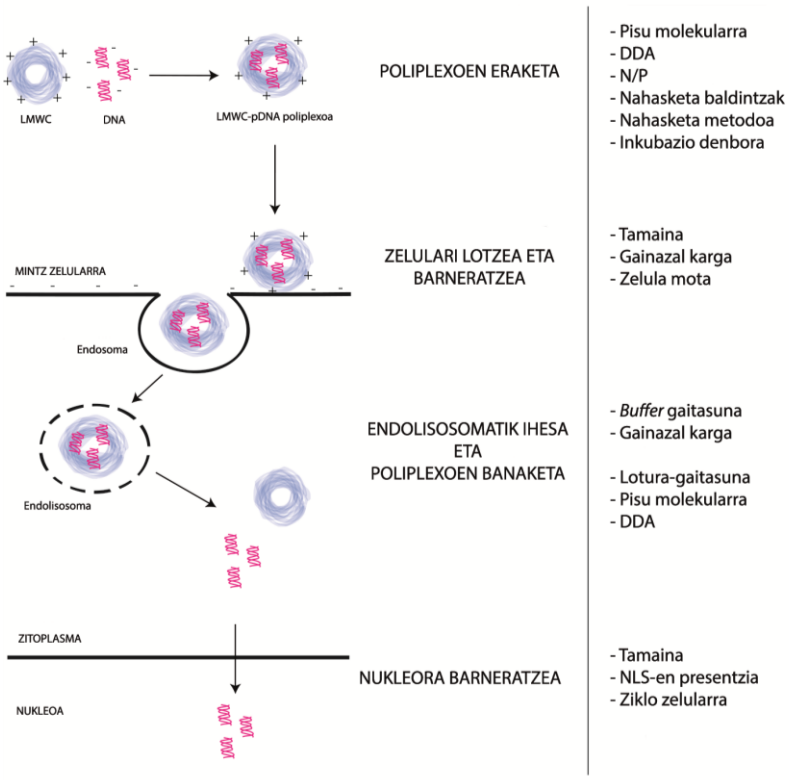
2. LMWC-pDNA poliplexoen transfekzio prozesuaren *in vitro* ebaluazioa

Transfekzio-gaitasunean pDNA-ren garraiatzaileak, LMWC poliplexoak kasu honetan, transgenearen espresioa eragiteko duen gaitasuna neurtzen da. Garraiatutako geneen azken espresioa neurtzean datza eta horretarako gene erreportariak erabiltzen dira; besteak beste, proteina berde fluoreszentea (GFP), luziferasa edo galaktosidasa kodetzen duten geneak. Transfekzio-gaitasuna neurtzeko gehien erabiltzen den teknika fluxu-zitometria da. Fluxu-zitometriaren bidez, GFP bezalako transgene fluoreszentea espresatzen duten zelulen neurketa kuantitatiboa egin daiteke, edo luziferasa eta galaktosidasaren kasuan, ekoiztutako entzimaren aktibitatearen neurketa kuantitatiboa egin daiteke. Bestalde, era kualitatiboan fluoreszentziazko mikroskopia erabiltzen da gehien.

Transfekzio prozesuak pausu anitz ditu, parametro ezberdinek eragin dezakete eta oso konplexua da (5. irudia). Transfektatutako zelulak azken proteina ekoiztu aurretik bektore ez-biralak mugarri asko gainditu behar ditu: zelularen mintzera atxikita eta zelulan barneratu, endolisosomatik ihes egin eta poliplexoa zatitu eta azkenik, pDNA nukeloan barneratu behar da. Beraz, arreta berezia jarri behar zaie nukleoraino heldu aurretik gainditu beharreko oztopo hauei, ondo ikertu behar dira ahalik eta eraginkorrenak diren poliplexoak diseinatzeko.

2.1. Zelulari lotu (binding) eta zelulan barneratu (uptake)

Itu-zelulara inguratzen denean poliplexoa zelularen gainazalean itsasten da. Baldin eta ligando espezifikoekin moldatzen ez bada, poliplexoaren eta zelularen arteko lotura interakzio elektrostatiko inespezifikoen bidez gertatzen da; hau da, poliplexoaren karga positiboaren eta zelula-mintzaren karga negatiboaren arteko elkarrekintzak eragiten du poliplexoa zelula-mintzean atxikitzea [53]. Zelularen



5. Irudia: Transfekzio prozesuaren eta pausu bakoitzean eragina duten parametro/faktoreen irudikapen eskematikoa.

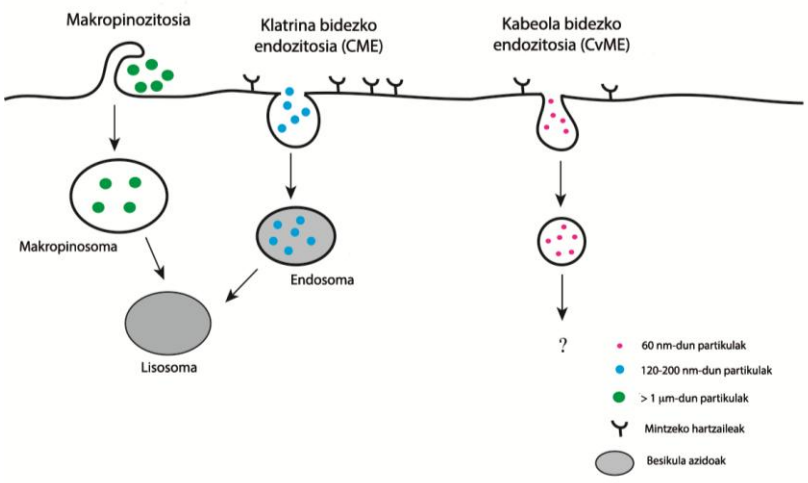
inguruko baldintzek garrantzi handia dute zelularekin loturan eta ondorengo barneratzean. Adibidez, LMWC-pDNA kasuan, ingurunea azidoa baldin bada eta pHa poliplexoaren pKa baino baxuagoa bada, poliplexoaren aminak protonatuagoak egongo dira eta poliplexoaren zeta potentziala positiboagoa izango da; ondorioz, zelularekin elkarrekintza indartsuagoa eta errazago lotu eta barneratuko da [30, 41, 54].

Poliplexoak zelulan batez ere endozitosi bidez sartzen dira. Hain zuzen ere, 5 endozitosi mota ezberdin daude deskribatuta: fagozitosia, makropinozitosia,

klatrina bidezko endozitosia (CME), kabeola bidezko endozitosia (CvME) eta klatrinarekiko zein kabeolinarekiko independientea den endozitosia (6. irudia) [55]. Fagozitosia eta makropinozitosia gehienbat espezilizatutako zeluletan gertatzen da; besteak beste, monozitoetan, zelula dendritikoetan eta zelula antigeno-erakusleetan. Bestalde, CME eta CvME dira gene terapia ez-biralaren arloan gehien ikertutako endozitosi mekanismoak, nahiz eta CvMEaren kasuan, oraindik ere argitu gabeko nahiko galdera dauden planteatuta [56]. Argitalpen gehienen arabera LMWC-etan oinarritutako poliplexoek bi endozitosi mekanismo hauek erabili ditzakete zelulan barneratzeko; hala ere, ezin daiteke esan kasu guztietan erabateko adostasuna dagoenik [53, 57]. Ch-pDNA poliplexoen barneratze mekanismoa faktore askoren menpe dagoen prozesua da eta emaitza ezberdineko ikerketak aurkitu genitzake. Partikulen tamainaren arabera, barneratzea endozitosi bide batetik edo bestetik gerta daiteke. Kasu honetan, argitaratuta dago partikula oso txikiak (60 nm inguru) CvME bidez barneratzen direla, erdibideko tamaina dutenak (120 nm inguru) eta handiagoak direnak (200nm inguru) CME bidez barneratzen direla eta mikra batetik gorako (›1 μm) partikulak, aldiz, CME edo makropinozitosia erabili dezaketela [58].

Poliplexoaren gainazaleko kargak zelulan barneratze prozesuan eragina dauka. Karga positiboa duten poliplexoek modu eraginkorragoan burutzen dute zelula-mintzarekin elkarrekintza eta barneratzea errazagoa izaten da [54]. Horrez gain, zelularen mintzaren fenotipoan izan daitezkeen aldaketen eta espresatu daitezkeen hartzaileen arabera, konplexuek endozitosi bide ezberdina erabili dezakete; hau da, endozitosi-bidea zelularen araberakoa izan daiteke. Douglas eta kideek argitaratu zuten Ch poliplexoak (150 nm inguru) CME eta CvME bidez barneratzen zirela 293T zeluletan, baina txinako hamsterraren obulutegietako (CHO) zeluletan CvME bidea bakarrik erabili zutela [59]. Poliplexoen barneratzea zelulan aurkitzen den lehenengo hesia bada ere, ez da transfekzio prozesuan

gainditu beharreko oztopo bakarra. Zenbait ikerketak frogatu dute ez dela beti poliplexoen barneratze mailaren eta transfekzioaren eraginkortasunaren arteko korrelazio zuzena ematen [18, 30, 53]; izan ere, genea espresatu aurretik, nahiz eta zelula barnean egon, nukleora heldu arte beste hainbat muga gainditu behar direlako.



6. Irudia: Geneen garraioan parte hartu dezaketen bide endozitiko desberdinen irudikapen eskematikoa

2.2. *Endolisosomatik ihesa eta poliplexoaren zatiketa*

Poliplexoa zelulan sartu den endozitosi bideak zelula barnean jarraituko duen bidea eta ondorioz, transfekzioaren arrakasta baldintzatuko du. Jarraitutako endozitosi bidea endozein dela ere, poliplexoek endosomatik lisosomara pasatu aurretik ihes egin behar dute lisosoman degradatuak ez izateko. Esate baterako, CME bidea jarraitu duten poliplexoak endosometan barneratzen dira eta endosomen heltze prozesua pairatzen dute; ingurunea azidotu egiten da, endosoma berantiar bihurtzen dira eta azkenik, lisosoma izatera pasatzen dira [56].

Endosoma azidotu aurretik eta lisosoma bihurtu aurretik poliplexoak ihes egitea funtsezkoa da transfekzio prozesua gertatu dadin [57]; izan ere, endosomaren ingurune azidoak eta lisosomaren entzimek pDNA suntsitu dezakete. 2.2.5 atalean azaldu den bezala, LMWC-ez eginiko poliplexoek protoien puzte efektuaren bidez egin dezakete ihes endosomatik eta hori gertatu dadin ingurune azidoa izateak mesede egiten die protonatuago egon daitezkeelako. Beraz, CME bidez zelulan barneratzen diren poliplexoek endosometatik errazago ihes egingo dutela uste da. Badago endosomaren azidotasunarekin erlazionatutako ihes egiteko beste mekanismo bat, eta kasu honetan ere, CME bidearen lehentasuna defendatzen duena. Hau da, endolisosomako ingurune azidoak poliplexoen gainazal karga handitzen du positiboago bihurtuz, ondorioz endosoma-mintzeko fosfolipidoak desegonkortu egiten dira eta zitoplasmako alderantz dagoen lisosomaren muturra barnerantz dagoen muturrarekin trukatu egiten da. Honek mintzaren antolaketa apurtzen du eta poliplexoek ihes egin dezakete [60]. Zenbait ikertzaileren arabera, endosomak erabiltzen ez dituzten endozitosi bideak, CvME adibidez, ez dira egokiak pHari erantzuteko gaitasuna duten Ch bezalako polimeroentzako. CvME bidez barneratzen diren poliplexo horiek ezin dituzte aprobetxatu endosomak ihes egiteko ematen dizkien aukerak [61] eta besikuletan harrapatuta gelditzen dira eta pDNA degradatu egiten da [62].

Ch-arekin eginiko poliplexoetan DNA-ren banaketa oso pausu garrantzitsua da transfekzio prozesuan. Thibault eta kideen aburuz, poliplexoaren banaketa zinetika formulazioaren menpe dagoen zelulabarneko prozesu kritikoena da. Poliplexoko bi elementuen banaketaren garrantziak agerian jartzen du Ch-aren ezaugarri fisikokimikoen (Pisu molekular eta DDA) eta transfekzio prozesuaren arteko erlazioa [53]. Ch-aren eta pDNAren arteko erakargarritasuna oso handia bada, zaila izan daiteke bi molekulen banaketa. Horrela, LMWCek pisu molekular txikiagoa dutenez, HMWCekin baino poliplexo ezegonkorragoak eratzen dira eta

DNA-ren banaketa errazagoa izan beharko luke.

2.3. Barneratze bideak aztertzeko tresnak

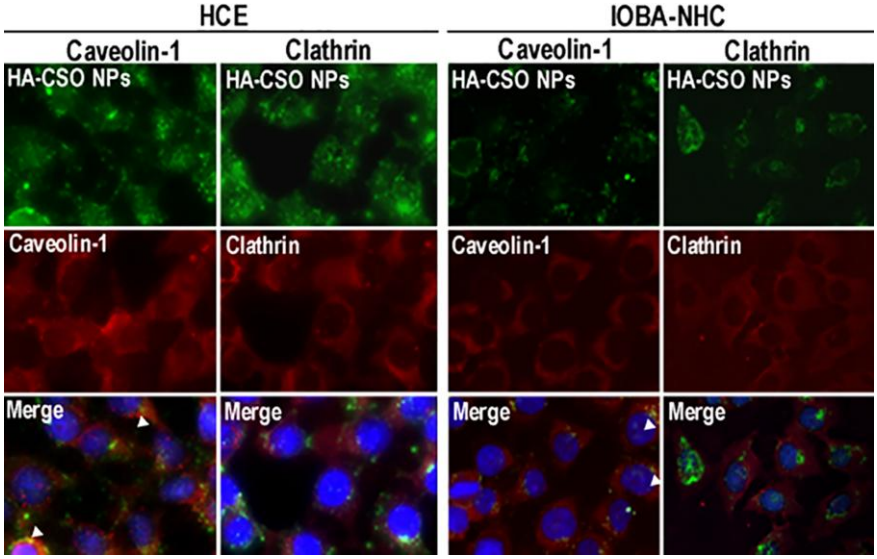
Transfektatzeko bektore ez-biral egokia ekoizteko zelulara barneratze mekanismoak aztertzea ezinbestekoa da. Gaur egun ez dago tresna edo estrategia bakarra pDNA garraiatzaileen barneratze bidea zehazteko.

Endozitosiaren inhibitzaileak erabiltzen dira endozitosi bide jakin batek poliplexoaren barneratze prozesuan duen pisua aztertzeko. Lehendabizi, endozitosia erabiltzen duten eta endozitosia erabiltzen ez duten bideak ezberdindu behar izaten dira. Horretarako, endozitosia erabiltzen duten bideak energiaren menpekoak direnez, modu azkarrena energia baitzea da; hau da, zelulak 4ºC-tan inkubatzen dira eta energiarik gabeko egoera berri honetan poliplexoak zelulan barneratu diren ikusten da [57,63]. Bestalde, energía behar duten mekanismoen artean; fagozitosia eta makropinozitosia, CME eta CvME barneratze bideengandik ezberdintzeko, amilorida (sodio-protoi ponparen inhibitzailea) eta wortmannina (fosfoinositidaren inhibitzailea) erabiltzen dira. CME bidea ingurune hipertonikoan inkubatuta, zitosola azidotuta edo klorpromazina erabilita inhibitu daiteke. CvME inhibitzeko aldiz, genisteina, nistatina edo filipina dira gehien erabiltzen diren inhibitzaileak [57,63,64].

Zelularen barnean sartu osteko prozesua aztertzeko inhibitzaileak ere badaude. Esate baterako, monesinak edo bafilomizina A-k endosomen azidotzea oztopatzen dute eta ondorioz, endosomak ez dira garatatzen eta ez dira fusionatzen lisosomekin. Era berean, klorokina ere erabili izan da endosomen garapena oztopatzeko. Klorokinak egoera osmotikoan eragiten du eta endozitosi besikulak puztu egiten dira [65].

Zelulan sartzeko bideen inhibitzaileekin lortu genezakeen antzeko informazioa eskuratu genezake froga molekularrekin, markatzaileekin eta

sondekin. Adibidez, transferrina, CME bidearen markatzaile gisa erabiltzen da eta kolera toxinaren beta azpiunitatea CvME bidearena. Markatzaile hauek fluoroforoekin konbinatu daitezke mikroskopia bidez jarraituak izateko. Markatzaile fluoreszente hauen eta fluoroforoekin zigilatutako poliplexoen kolokalizazioak bektoreak zelula barnean jarraitzen duen bideari buruzko informazio garrantzitsua eman dezake (7. irudia). Era berean, organelentzako sonda espezifikoak ere oso erabilgarriak dira bektore ez-biralak zelula barnean jarraitutako bidea ezagutzeko. Sonda horien artean gehien erabiltzen direnak Lysotracker eta Lyso Sensor (lisosomentzako sondak) eta Cell light (endosoma goiztiarrentzako sonda) dira.



7. Irudia: Kabeolin-1 eta klatrinen inmunofluoreszentzia HCE eta IBA-NHC zeluleta, azido hialuroniko-kitosano oligomerikoetan oinarritutako nanopartikulekin (HA-CSO NP) inkubatu ondoren. Irudi elkartuek HA-CSO NP eta kabeolina arteko kolokalizozioa erakusten dute (gezien bidez adierazia). [64]. erreferentziatik jasoa editorialaren baimenarekin.

2.4. *Nukleoan barneratzea*

Genea espresatu aurretik pDNAk gainditu beharreko azken hesia nukleoaren mintza da. Nukleoaren mintzeko lipidoen geruza bikoitzean erregulazio fineko erreten urtsuak daude, nukleoaren poro konplexua (NPC) osatzen dutenak. NPC-ak egoera erlaxatuan 10 nm-ko diametroa du eta molekula txikien difusio pasiboa baimentzen du. Hala ere, molekula handiagoen garraio aktiboa ere posible da, baldin eta molekula horiek nukleoaren lokalizazio zeinuak (NLS) deritzan elementuekin bat egiten badute. LMWC-ez eginiko poliplexoen partikula tamaina normalean 100 nm baino handiagoa da, ondorioz, pDNA ez bada Ch-tik banatzen eta poliplexoak osorik zeharkatu behar badu nukleo-mintza, ezinbestekoa da poliplexoak NLS-ekin elkarrekitea nukleoan sartzeko. Dena den, pDNA zelularen banaketa prozesuan ere heldu daiteke nukleoraino, mintza desagertzen delako. Hain zuzen ere, ondo deskribatuta dago transfekzioaren eraginkortasunaren eta zelula-banaketaren eta zelula-hazkuntzaren arteko korrelazioa [66]. Beraz, asko proliferatzen duten zeluletan transfektatzea errazagoa izan beharko luke normalean mitosirik izaten ez duten zelulekin konparatuz gero. Argitaratutakoaren arabera, esan genezake nukleoan barneratzea transfekzio prozesuan gutxien aztertzen den pausua dela, eta LMWC-pDNA poliplexoekin eginiko transfekzioa arrakastatsua izateko ezinbestekoa dela nukleoan barneratzeko erabiltzen duten mekanismoaren karakterizazio zehatza egitea.

2.5. *LMWC-ez eginiko poliplexoen transfekzioan eragina duten faktoreak*

Deazetilazio maila handia duten LMWC-ekin eginiko poliplexoen transfekzioan formulazioaren parametro askok eragiten dute. Besteak beste, serumak, transfekzio ingurunearen pH-ak, N/P ratioak, pDNA kontzentrazioak eta baita transfektatutako zelula motak ere. Parametro hauek definituko dituzte poliplexoen ezaugarri fisikokimikoak eta azken finean transfekzioaren arrakasta.

Serumari dagokionez, ez dago batere argi LMWC-etan oinarritutako poliplexoen transfekzioan duen eragina. Ikertzaile batzuen arabera, serumaren presentziak handitu egiten du Ch-ekin eginiko poliplexoen transfektatzeko gaitasuna [41,67,68]. Nahiz eta transfekzioaren hobekuntza horren azalpena argi ez egon, ikerketa batzuen arabera serumak zelulen funtzioa hobetu dezake eta honekin batera zelularen banaketa eta endozitosi prozesuak. Adibidez, Nimesh eta kideek serumarekin inkubatutako poliplexoekin zelulara barneratze maila handiagoa lortzen zela ikusi zuten. Beharbada Ch-ak karga negatibodun serumeko proteinekin elkartu eta zelulan erraz barneratzen diren diametro hidrodinamiko txikiko konplexuak eraikitzen dituelako [41]. Baina puntu honetan ikerketa komunitateak dituen zalantzen adierazgarri da, oraindik ere Ch-ekin eginiko poliplexoekin transfektatzeko serumik gabeko Opti-MEM soluzioa izatea erabiliena [28, 29].

Kontuan hartu beharreko beste parametro bat, transfekzio soluzioaren pHa da. Kitosanoa pHaren araberako portaera duen polimeroa da. Honek esan nahi du, bere karga soluzioaren pHaren balioaren araberakoa dela. Literatura zientifikoan argi deskribatuta dago medioaren azidotzeak LMWC-etan oinarritutako poliplexoen zelula-barneratzea eta transfekzio efizientzia areagotzen duela [30, 41]. Gure ikerketa taldeak deskribatu duen bezala, soluzioaren pH-a 7,4-tik 6,5-era jaisteak oligokitosano ultrapuruetan oinarrituriko poliplexoen zeta potentzialaren handipena eta tamainaren murrizketa eragiten du. Karga positiboa duten konplexuak negatiboki kargaturiko mintz zelularrera lotu daitezke, zelulara barneraketa lagunduz eta horrela, transfekzio efizientzia hobetuz. Emaitza hauen arabera, bektore motak hauek, ingurune azidoan aurkitzen diren zelulak transfektatzeko erabiliak izan daitezke, tumore zelulen transfekziorako adibidez.

Poliplexoen prestaketarako erabiltzen den N/P ratioak ere transfekzio prozesuan bere eragina dauka. Poliplexoak pDNA lotzeko egonkortasun nahikoa

izan behar dute, baina aldi berea, behin zelula barnean daudela karga askatzeko gai izan behar dira. Lotura eta askapenaren arteko balantzea lortzeko behar den N/P ratioa, kitosanoaren pisu molekularraren eta DDA-ren araberakoa izango da. Lavertu eta kideek deskribatu zuten, LMWC-ek, pisu molekular altukoek baino N/P ratio altuagoa behar dutela DNA guztiz lotzeko.

3. Kitosanoaren eraldaketa kimikoak transfekzioaren oztopoak gainditzeko

Transfekzio prozesua eraginkorra izan dadin, beharrezkoa da garaturiko bektore ez-biralek ondoren aipatzen diren hezi biologikoak gainditzea: zelulara lotze eta barneratzea, endolisosomatik ihesa eta nukleora barneratzea. Zailtasun biologikoez gain, konplexuen egonkortasun koloidalaren hobekuntza ere transfekzio eraginkor bat lortzeko funtsezkoa da.

Oztopo hauek gainditzeko helburuarekin, LMWC-ak modu desberdinetan eraldatuak izan daitezke. Eraldaketen artean, C2 amina eta C6 hidroxilo taldeetan molekula edo polimeroen txertaketak dira erreakzio ohikoenak. Nahiz eta C6 hidroxiloa erreaktiboa eta erraz funtzionalizatu daitekeen taldea izan, orokorrean, ikerketa lanetan C2 aminaren eraldaketak erabili izan dira.

Kitosanoen talde amino funtzionala erabiltzeak duen eragozpen nagusia erreakzio honek oztopo esterikoa sortzea eta pDNA-ra lotuko diren amina ionizagarrien kopuruaren murrizketa da. Beraz, Ch-aren amina primarioan dauden lotu-gabeko elektroiak hautagai paregabeak dira eraldaketa erreakzioak burutu ahal izateko. Kuaternizazioa, amidazioa eta N-alkilazioa dira transfekzio prozesuaren heziak gainditzeko erabiltzen diren erreakzio kimiko nagusiak.

Kitosanoen kuaternizazioa pH fisiologikoan disolbagarritasuna hobetzeko erabili izan da. 2 dira kuaternizazio metodo arruntenak: N-trialkilazioa CH_3I-rekin [51, 70] eta glizidiltrimetilamonioa kloruroan oinarrituriko erreakzioa da bestea.

Amidazioa Ch-aren amino taldean molekulak eta polimeroak txertatzeko erabiltzen den beste teknika da. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) edo DCC (dicyclohexyl carbodiimide) bezalako konposatu karboxilikoak NHS-rekin batera erabiltzen dira erreakzioa bizkortzeko [27]. Amidazioak, transfekzio prozesuarekin erlazionaturiko zenbait eragozpen gainditzeko baliagarria izan daiteke, aukeratzen den molekularen arabera. Horrela, azido uroniko, imidazol eta histidina bezalako molekulen erabilerak bektoreen ihesa endosomatik hobetu dezake [73-75]. Gainera, Ch-ari arginina lotzeak barneratze zelularra handitzen duela frogatuta dago; bestalde, PEG-succinimidyl ester edo folato-PEG-COOH molekulek farmakozinetikan eragina izan dezakete [76].

Azkenik, N-alkilazio metodoa Ch-aren amino taldean aldehido deribatu bat txertatxeko balio du. Txertatzen diren molekulen artean, konposatu fluoreszenteak [77], fosforilkolina [78, 79], oligosakaridoak [18, 46, 52, 80] eta dextranoa bezalako polimeroak daude [81]. Normalean, N-alkilazioa zitotoxizitatea murriztera eta transfekzio efizientzia handitzera zuzenduta dago. Dextranoen kasuan, konplexuen egonkortasuna handitzeko erabili ohi da. Succinoyl taldeak ere txertatu izan dira Ch-ra terapia genikoko aplikazioetarako [82].

4. Poliplexoen izozte-lehorketa prozesua

Aplikazio klinikorako poliplexoek duten muga nagusiena suspentsio urtsuen denbora luzerako egonkortasun gabezia da [31]. Denborarekin gertatzen den poliplexoen agregazioak transfekzio efizientzia murrizten du, horregatik beharrezkoa da poliplexoak administrazioaren momentu berean prestatzea. Bektoreen eguneroko prestaketak produktuaren kalitatean, segurtasunean eta transfekzioan bariazioak eragiten ditu [83]. Honengatik, transfekzio efizientzia altuko eta egonkorrak diren poliplexoen garapena ezinbesteko pausoa da

formulazio hauen aplikazio klinikoa posible izateko.

Izozketa-lehorketa edo liofilizazioa formulazio lehorrak prestatzeko ohiko metodoa da, aldez aurretik prestaturiko konplexuen denbora luzeko kontserbazioa baimentzen duena [32, 84, 85]. Liofilizazio prozesua hiru ataletan banatu daiteke: izozketa, sublimazioz egindako lehen mailako lehorketa eta bigarren mailako lehorketa. Prozesu hauek, polimeroan zein DNA-an kalteak eragin ditzake. Liofilizaturiko produktuak, hasierako formulazioaren ezaugarri fisiko eta kimikoak mantendu behar ditu, eta denboran zehar egonkorra izan. Izozketa eta lehorketa prozesuek eragin ditzaketen kalteak ekiditeko agente krio/lio babesleak gehitzen dira konplexuen formulaziora. Kriobabesle ezagunenak, egonkortzaile ere deituak, azukreak dira, trealosa, sukrosa, glukosa eta manitola, besteak beste [86].

In vitro entseguekin alderatuz, in vivo esperimentu eta entsegu klinikoetan DNA dosi altuagoak beharrezkoak dira, administraziorako egokia den bolumenean formulatua. Ezaguna da, poliplexoen nahasketa baldintzek bukaerako bektore ez –biralaren ezaugarrietan zer esana dutela, eta oso zaila da poliplexoen prestaketa metodo ohikoekin kontzentrazio altuko formulazioak garatzea. Efektu terapeutikoa lortzeko beharrezkoa den dosia oso altua izanez gero, poliplexoen in vivo aplikazioak eta entsegu klinikoak mugatuta egon daitezke, formulaziorako erabiltzen diren polimero eta plasmido soluzio diluituak direla eta. Beraz, poliplexo suspentsio kontzentratuagoak beharrezkoak dira. Horrela, bektoreen suspentsioak kontzentratzeko eta DNA kontzentrazioa alguagoak lortzeko liofilizazioa metodo abantailatsua izan daiteke. Izan ere, lortzen den formulazio lehorra diluitzailearen bolumen txiki batean formulatzeko aukera ematen du [87, 88]. Gaur egun arte, ez dago literaturan LMWC-pDNA poliplexoen liofilizarioari buruzko lan asko; hala ere, beste konplexu mota batzuekin lorturiko emaitza positiboak izanda, LMWC-etan oinarrituriko

bektoreen liofilizazioa kontuan hartzeko aukera bat da.

5. pDNA-aren garraiorako LMWC-etan oinarrituriko poliplexoen *in vivo* aplikazioak

Behien poliplexoak eratu, karakterizatu eta *in vitro* transfekzio efizientzia frogatu direnean, hurrengo pausoa, hauen efizientzia eta toxizitatea *in vivo* testatzea da. Ezagunak dira LMWC-en biobateragarritasun eta biodegradazioa eta ondorioz, in vivo terapia genikorako asko erabilia izan da polimero hau. Gainera, Ch-ak duen mukoitsagarritasun altuari esker, begi eta birikara zuzendutako azido nukleikoen administrazioetarako hautagai ezin hobea da polimero natural hau.

5.1. Begira zuzendutako garraioa

Begia organo pribilegiatua da era lokalizatu batean bektore ez-biralak begiaren ehun espezifikoetara garraiatu ahal izateko. Gainera, ongi ezagutzen diren hainbat gaixotasun genetiko begian ematen dira. Hainbat ikerketa taldek, LMWC-etan oinarritutako bektoreak aztertzen ari dira pDNA-a begiko ehun desberdinetara garraiatzeko sistema gisa.

Novafect O15 eta O25 oligokitosanoak kornea eta erretinako zelulak in vivo transfektatzeko erabili izan dira. Klausner eta kideek ezaugarrri desberdineko plasmidoekin eraturako Ch-DNA nanopartikulak administratu zituzten korneako estroman. LMWC-etan oinarrituriko poliplexoak, pCpG-GFP plasmidoa korneako keratozitoetara garraiatu eta zelulak GFP-a maila altu batean ekoizteko gai zirela aurkitu zuten [28, 34]. Gure ikerketa taldean ere, Novafect O15 eta O25 kitosanoak erabili dira pDNA-a erretinara garraiatzeko. Lehen aldiz aurkeztu zen LMWC-eta oinarritutako poliplexoak, 10 N/P proportzioan, arratoien erretinako zelula desberdinak transfektatzeko gai direla, administrazio bidearen arabera. Poliplexoak erretinako azpian administratu ondoren, gene espresioa erretinaren pigmentodun epitelioan ikusi zen batez ere. Humore-beirakaran ere administratu

ziren poliplexoak; kasu honetan, GFP espresioa zelulan ganglionarretan eta erretinaren barne geruzetan aurkitu zen (8. Irudia) [29, 33]. Azido hialuroniko eta kitosanoz eraturiko nanopartikulu bioitsaskorrak ere topikoki administratu ziren untxien begietan. Emaitza positiboak lortu ziren kornearen epitelioan administrazio ondorengo 7. egunean [91].

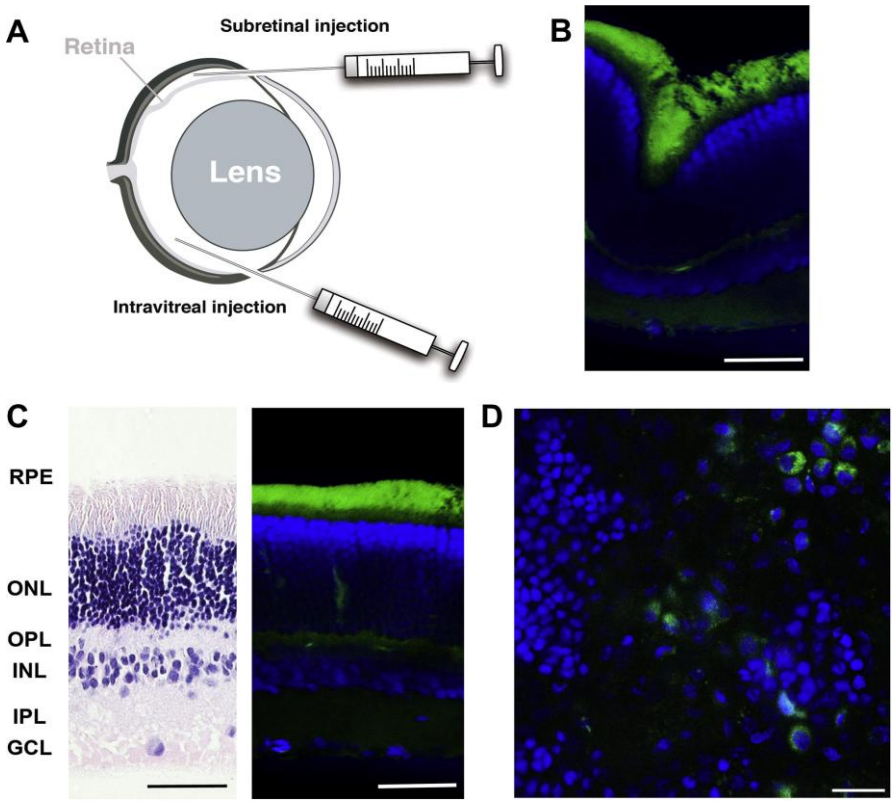
5.2. Birikara zuzendutako garraioa

Birikara zuzenduriko terapia geniko ez-birala, fibrosi kistiko edo birikako kantzerrak tratatzeko hautagai posible bat da. Ch-aren mukoitsagarritasuna abantaiala ezin hobea da pDNAaren garraio organo honetara bideratzeko. Medikamentuak birikara garraiatzeko era ez-inbasibo ohikoena inhalazioa da. Mohammadi eta kideek Ch-DNA nanopartikulak nebulizatu zituzten luziferasa kodifikatzen duen pGL3 plasmidoa saguen biriketara zuzentzeko. 48 igaro ondoren, luziferasa maila altuak neurtu zituzten saguen biriketan. [92].

Inbasiboagoa den trakea barneko administrazioa, partikulak birikara zuzentzeko ere erabili izan da. Kopping-Hoggard eta lagunek, LMWC-etan oinarrituriko poliplexoekin HMWC-ekin baino *in vivo* transfekzio efientzia altuagoak lortzen zirela erakutsi zuten, partikulak trakea barnean administratu ondoren. Hau, pDNA eta Ch-aren artean ematen den elkarrekintza ahulagoari dagokio eta baita pisu molekular baxuko kitosanoek mantentzen duten babes gaitasunari. Honek, gene aproposaren askapen eta garraio eraginkorra ahalbidetzen du [22]. Trisakaridoekin konjokaturiko Ch oligomeroak ere trakea bidez administratuak izan dira. Administrazioa burutu eta 24 ordura saguen biriketa luziferasa genearen espresioa behatu zuten ikerlariek [52].

5.3. Beste emanbide batzuk

Sudur barneko administrazioa interes handiko emanbidea bilakatu da, izan



8. Irudia: GFP-aren *in vivo* gene espresioa oligokitosano/DNA (N/P=10) poliplexoak arratoietan administratu ondoren. (A) Erretina azpian eta beirakara barnean egindako adminitrazioen eskema. (B) Administrazio gunetik gertu dagoen erretinaren zeharkako mozketa. Proteina berde fluoreszentearen (EGFP) espresioa eta zelulen nukleoak Hoechst 33342 tindaketarekin. Eskala marra = 50 μm. (C) Erretinaren geruza desberdinak erakusten dituen zeharkako mozketa baten hematoxilina eosina tindaketa: RPE (Erretinaren pigmentodun epitelioa), ONL (Kanpoko geruza nuklearra), OPL (Kanpoko geruza plexiformea), INL (Barneko geruza nuklearra), GCL (Zelula ganglionarren geruza); eta trataturiko erretina baten 5 μm-tako zeharkako ebaketa baten fluoreszentzia irudia. Eskala marra = 50 μm. (D) Gainjarritako erretinaren RPE geruzaren hainbat sekzioa. Eskala marra = 100 μm. [33] erreferentziatik ateratako irudia, Elservierren baimenarekin.

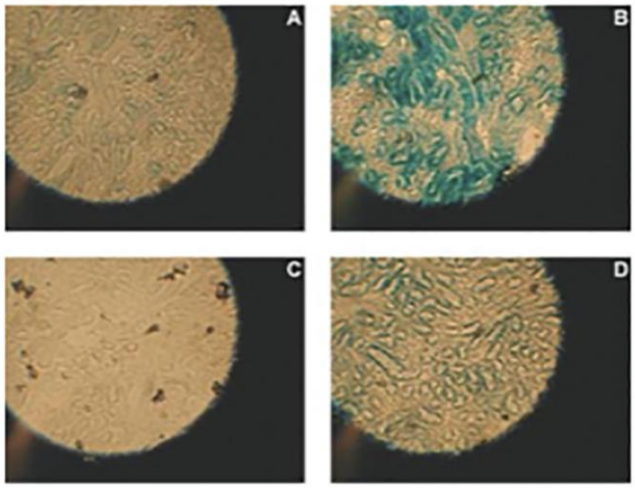
ere, sudur bidetik garraiaturiko DNA txertoak erantzun inmune humoral eta zelular eraginkorra eragiteko gai direla aurkitu dute ikerlariek. Gainera, gaixoek

hobeto onartu eta bete dezaketen min gabeko emanbidea da. Yang eta kideek "cholesteryl ester transfer" proteina (CETP) kodifikatzen duen plasmidoaren *in vivo* garraioaren efizientzian, Ch-aren pisu molekularrak duen garrantzia ikertu zuten. LMWC/pDNA poliplexoen sudur barneko administrazioak, 21 aste baino gehiago iraun zuten anti-CET IgG antigorputz espezifikoen ekoizpena bultzatu zuen [93]. Berriki, manosilatutako Ch-ekin eraturiko nanopartikulak sudur bidetik administratu ziren, anti-GFP IgG antigorputzen ekoizpena sustatu eta GFP peptidoaren menpeko tumore-zelulen hazkuntza inhibitzeko intentzioarekin. Emaitzek Ch hauetan oinarrituriko partikulak DNA txertoen garraiorako baliagarriak direla frogatu zuten eta tumoreen immunoterapeutikorako aukera berri bat eskaintzen dute [94].

Larruazalpeko (SC) eta muskulu barneko (IM) administrazio bideak ere, pDNA-aren garraiorako erabiliak izan dira. Jean eta lagunek Ch/pVax1-4sFGF-2 edota Ch/pVax1-PDGF-bb komplexuen SC administrazioak, FGF-2 eta PDGF-BB proteina errekonbinanteen espresio eta banaketa maila altuak eragiten zituela aurkitu zuten. Era berean, IM administrazioak antigorputz espezifikoen ekoizpen azkarra eragin zuen. Hau, etorkizun handiko estrategia izan daiteke, txerto profilaktiko edo terapeutikoen garapenerako [95]. Ikerketa talde berberak, Ch-etan oinarritutako konplexuek GLP-1 proteina errrekonbinantea kodifikatzen duen pVax1-GLP-1 plasmidoa garraiatzeko duten ahalmena deskribatu zuen, diabetesa duten arratoi eredu batean, hain zuzen ere [96].

Minbiziaren terapia genikoari dagokionez, geneen espresioa ehun tumoralean lortzeko dagoen bide zuzenena, poliplexoen tumore barneko adminstrazio da. Ch/pCMV-Luc poliplexoak larruazalpean sorturiko tumore barnean administratuak izan ziren, transfekzio efizientzia onargarriak lortuz [97]. Azkenik, LMWC-etan oinarrituriko poliplexoen administrazio sistemikoa ere ikertu izan da. Zenbait organo eta ehunetara ezin daitezke konplexuak garraiatu

administrazio lokal batekin; emanbide sistemikoak, organo eta ehun hauetara konplexuen garraioa ahalbidetzen du. Zhang eta kideek, pCMV-Luc generaren garraiorako hidrofobikoki eraldaturiko LMWC-etan oinarriturriko bektoreak eratu zituzten. Buztaneko zainean sistemikoki administratu eta hiru egunetara, genearen espresio indartsua behatu zuten trataturiko saguen giltzurrunetan (9. Irudia) [98]. bPEI-rekin funtzionalizaturiko eta makrofagoetari bideratzeko tuftsinarekin konjokaturiko LMWC-ak ere, pDNA-a sistemikoki administratzeko egokiak direla ere frogatu. HMWC-ekin ez bezala, LMWC-ekin eraturiko poliplexoak administratu eta 7 egunetara, gene espresio maila altuak behatu ziren saguen bare, bihotz eta burmuinean [99].



9. Irudia: LMWC/pDNA konplexuen administrazio sistemikoaren ondoren saguen giltzurrunean aurkitzen den gene espresioa. pUC 19 DNA eta (A) LMWC; (B) % 3-ko eraldaketa hidrofobikoa jaso duen LMWC; (C) % 18-ko eraldaketa jaso duen LMWC eta (D) adardun PEI-rekin eratu ziren poliplexoak N/P 5 ratioan, eta saguen buztaneko zainean injektatu ziren. Adminsitratu eta 3 egunetara giltzurrunak atera ziren.. X200-eko handipena. [98]. erreferentziatik jaso *Wiley*-ren baimenarekin.

2. Taula: LMWC-etan oinarrituriko poliplexoekin egindako *in vivo* entseguen laburpen taula

ADMINISTRAZIO BIDEA	ANIMILIA EREDUA	KITOSANO MOTA	pDNA	AZALPENAK	HELBURUA	ERREFERENTZIA
Kornean injekzioa	Sprague-Dawley arratoiak	Novavect O15 (5.7 kDa, DDA %99) Novafect O25 (7.3 kDa, DDA %99)	gWiz-Luc (luziferasa kodifikatzen duen genea) 1.5 g gWiz-GFP (GFP kodifiatzen duen genea) 1.5 g		Jasotako eta herentziazko korneako gaixotasunen tratamendua	[27]
Kornean injekzioa	Sprague-Dawley arratoiak	Novafect O15 (5.7 kDa, DDA %99)	gWiz,-Luc, pCpG-Luc, pEPI-CMV, pEPI-UbC (luziferasa kodifikatzen dutenak) 1.5 g gWiz-GFP, pCpG-GFP (GFP kodifikatzen duena) 1.5 g		Jasotako eta herentziazko korneako gaixotasunen tratamendua	[33]
Erretina azpian eta beirakara barnean injekzioa	Sprague-Dawley arratoiak	Novafect O15 (5.7 kDa, DDA 9%9)	pCMS-EGFP genea (100ng)		Erretinako gaixotasunen tratamendua	[28]
Erretina azpian eta beirakara barnean injekzioa	Sprague-Dawley arratoiak	Novafect O25 (7.3 kDa, DDA %99)	pCMS-EGFP genea (100ng)		Erretinako gaixotasunen tratamendua	[32]
Administrazio topikoa	Untxiak	Kitosano ultrapuruaren hidrokloruro gatza (113 kDa).	pEGFP genea (25 g, 50 g, 100 g)	Kitosanoa HA gatzarekin nahastu zen, NPak gelifikazio ionotropiko bidez prestatu ziren	Begiko gaixotasunen tratamendua	[89]

Aerosola	Balb/c saguak	Ch Chitoclear (126 kDa, DDA %98)	Luziferasa kodifikatzen duen pGL3- plasmidoa	Indar elektrostatiko bidez eraturiko poliplexoak FAP-B-rekin konjokatu ziren	Birikara zuzendutako garraioa	[90]
Trakea barneko administrazioa	Balb/c saguak	UPC, Protasan UPG 210	gWiz-Luc, pCMV-Luc (luziferasa kodifikatzen duena) 5 g, 10 g, 25 g	Guztiz deazetilaturiko Ch-a depolimerizatu zuten, DPn 25 eta 18-dun oligomeroak lortzeko	Birikara zuzendutako garraioa	[21]
Trakea barneko administrazioa	Balb/c saguak	Guztiz azetilaturiko Ch-a (3.6-7 kDa)	gWiz-Luc, pCMV-Luc (luziferasa kodifikatzen duena)	Ch oligomeroak trisakaridoengatik ordezkatu ziren, %7, 23, 40 amina ordezkatuak dituzten oligomeroak lortuz	Birikara zuzendutako garraioa	[50]
Administrazio intranasala	Sprague-Dawley arratoiak	Ch Mw: 5, 173 kDa	GFP kodifikatzen duen pEGFP-C3; CETP-C kodifikatzen duen pDNA		Arteroesklerosiaren tratamendurako DNA txerto inmunoterapeutikoak	[93]
Administrazio intranasala	C57BL/6 saguak	Ch Mw: 115 kDa, DDA %95	pGRP (0.5mg)	Ch- manosarekin konjokatu zen	Anti-GRP IgG-ren produkzioa eta tumoreen hazkuntzaren inhibizioa	[94]

Muskulu barneko eta larruazalpeko administrazioa	Balb/c saguak	Depolimerizaturiko Ch 92-10 and 80-80 (Mw-DDA)	pVax1-4sFGF-2 eta pVax1-PDGF-BB (FGF-2 and PDGF proteina errekonbinanteak kodifikatzen dituztenak)		Kartilagoko do hezurretako lesioen konponketa	[95]
Muskulu barneko eta larruazalpeko administrazioa	Zucker Diabetic Fatty arratoiak	Depolimerizaturiko Ch 92-10 and 80-80 (Mw-DDA)	GLP-1errekonbinantea kodifikatzen duen pVax1-GLP1 (165 g)		2. motako diabetesaren tratamendua	[96]
Tumore barneko administrazioa	C.B-17/Icr-scid-bg saguak	Ch Mw 15.5 kDa, DDA % 75-85	GFP eta luziferasa kodifikatzen duten pAcEGFP1-C1 eta Luc plasmidoak (100 g)		Kantzerraren tratamendua	[97]
Bena-barneko administrazioa	Saguak		B -galaktosidasa kodifikatzen duen pUC 19	Hifrofobikoki eraldaturiko LMWC-ak		[98]
Bena-barneko administrazioa	Balb/c saguak	Depolimerizaturiko Ch-ak (7 kDa and 10 kDa)	pGL3 luziferasa genea (25 g)	Ch-a LMWP eta tuftsinarekin konjokatu zen		[99]

6. Etorkizuneko erronkak

LMWC-ak DNA-ren garraiorako hamabost urtez ikertu eta garatu izan diren arren, genearen espresio efizientea ahalbidetuko duen bektorea ez dago oraindik nahi bezain garatuta. Bektore ez biralen ekoizpen prozesuan aurkitu daitezkeen erronkei aurre egitea eta hauen azterketa zehatza izan dira LMWC-etan oinarritutako garraiatzaileen erabilera biomedikorako giltza, eta konkretuki geneen garraiorako [100, 101].

In vitro transfekzio entseguetan arrakasta lortzeko beharrezko zenbait oinarri fisikokimiko eta biologikoa argitu dira; DNA-ren entzimen degradazioa saihesteko, mintz zelularraren permeabilitatea hobetzeko, solubilitate baxua hobetzeko eta pH fisiologikoan egonkortasuna handitzeko aurrera pausoak eman dira [22,29]. Hala ere, LMWC-etan oinarritutako poliplexoekin *in vivo* arrakasta lortu ahal izateko, hainbat dira oraindik gainditzeke dauden oztopoak. Material landugabetik, klinikara translazioa hobetu ahal izateko, Ch-aren eraldaketa kimiko eta biologikoetan ari dira lanean ikerlariak. LMWC-etan oinarritutako nanokonplexuei loturiko ligando espezifikoek, ehun espezifikoetara zuzenduko lukete konplexuen garraioa administrazio sistemikoaren ondoren. Hala ere, poliplexoek duten gainazal karga positibo altua dela eta, polianioi biologikoen presentzian ematen den agregazioari aurre egitea da administrazio sistemikorako bektore ez-biral polimerikoen garapenean dagoen erronka nagusietako bat. Honi aurre egiteko, LMWC-ei lotu daitezkeen polimero neutroen ikerketa asko sustatu da [51].

Bena-barneko administrazioarekin erlazionaturiko eragozpenak direla eta, gure ustez, etorkizuneko entsegu klinikoak, garraiatzaileen ehun barneko administrazio zuzenera bideratuko dira. Hala ere, aipatu beharra dago, hainbat patologien tratamendurako onuragarria den administrazio sistemiko batekin,

plasmidoaren barreiadura zabalagoa lortuko litzatekeela; konpartimentu-anitzeko tumore lokalizatu edo metastasidun tumoreen kasuetan adibidez.

Laburbilduz, gisa gaixotasun genikoeentzako arrakastatsua izango den tratamendua lortzeko, ezinbestekoa izango da erregulazio agentzien arauak betetzen dituzten karakterizazio maila altuko nanokonplexak kantitate egokian ekoiztea. Gainera, nanoplexo horiek indikazio kliniko bakoitzerako gaitasun ezberdinak bildu beharko dituzte; besteak beste, transfektatuko diren azido nukleikoen garraiorako gaitasuna, *targeting* gaitasuna eta segurtasuna.

Erreferentzien zerrenda 74-81. orrialdeetan aurkitzen da.

Low molecular weight chitosan (LMWC)-based polyplexes for pDNA delivery: from bench to bedside

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Geneak garraiatzeko bektore ez-biralak gero eta aztertuagoak dira bektore biralen alternatiba seguruago gisa. Polimero naturalen artean kitosanoa (Ch) da ikertuenetakoa eta pisu molekular baxuko Ch-ek (LMWC), zehazki, abantaila ugari dituzte pDNA-aren garraiorako. Oso garrantzitsua da LMWC-pDNA konplexuen eraketarako metodo egokiena zein den jakitea. Era berean, beharrezkoa da baita ere eratutako konplexuen ezaugarri fisiko-kimikoen karakterizazio zehatza egitea, behin administratuak direnean izango duten jarrera aurreikusteko. Dena den, Ch-en transfekzio gaitasuna nahiko baxua denez, ezinbestekoa da transfekzioan eragiten duten prozesu guztiak ahalik eta ondoen aztertuta izatea. Esate baterako, zelulan barneratzea, endosomatik askapena eta nukleoan barneratzea nola gertatzen den ezagutzeak eta prozesu hauetan eragina duten parametroak identifikatzeak, bektore ez-biral eraginkorrak diseinatzen eta garatzen lagunduko digute. Errebisio lan honen helburu nagusiak dira; batetik, LMWC polimeroetan oinarritutako poliplexoen eraketa metodoak eta karakterizazio teknikak deskribatzea; bestetik, in vitro transfektatzeko prozesua deskribatzea eta azkenik, in vivo terapia genikorako LMWC poliplexoek izan ditzaketen aplikazioak azaltzea.

En el área de terapia génica, los vectores no-virales están emergiendo como una alternativa más segura a los virales. Entre los diferentes tipos de vectores no-virales, los de composición lipídica y/o polimérica son los más utilizados. En cuanto a los polímeros naturales, el quitosano (Ch) es el más estudiado hasta el momento, y concretamente, los Chs de bajo peso molecular (LMWC) presentan una serie de ventajas para la vehiculización de ADN. Es crucial determinar el proceso óptimo para la formación de los complejos basados en LMWC y ADN, así como caracterizar sus propiedades fisicoquímicas para comprender el comportamiento de dichos complejos tras su administración. La eficiencia de transfección de los poliplexos basados en Ch es relativamente baja, por ello, el conocimiento del proceso de transfección en sí, desde la captación celular de los poliplexes hasta su transporte al núcleo, junto con los parámetros que afectan dicho proceso, permitiría mejorar el diseño y desarrollo de estos vectores no-virales. En esta revisión introductoria, se pretende describir la formación y caracterización de los poliplexos basados en LMWC, el proceso de transfección in vitro, y finalmente, detallar las aplicaciones in vivo de los poliplexoes basados en LMWC para su aplicación en terapia génica.

Low molecular weight chitosan (LMWC)-based polyplexes for pDNA delivery: from bench to bedside

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ABSTRACT

Non-viral gene delivery vectors are emerging as a safer alternative to viral vectors. Among natural polymers, chitosan (Ch) is the most studied one, and low molecular weight Ch, specifically, presents a wide range of advantages for non-viral pDNA delivery. It is crucial to determine the best process for the formation of Low Molecular Weight Chitosan (LMWC)-pDNA complexes and to characterize their physicochemical properties to better understand their behavior once the polyplexes are administered. The transfection efficiency of Ch based polyplexes is relatively low. Therefore, it is essential to understand all the transfection process, including the cellular uptake, endosomal escape and nuclear import, together with the parameters involved in the process to improve the design and development of the non-viral vectors. The aim of this review is to describe the formation and characterization of LMWC based polyplexes, the *in vitro* transfection process and finally, the *in vivo* applications of LMWC based polyplexes for gene therapy purposes.

Key words

Low molecular weight chitosan (LMWC), pDNA, polyplexes, non-viral vector, gene delivery, transfection efficiency

1. Introduction

The success of gene therapy relies on ensuring that the therapeutic gene enters into the target cell and transcribes without being biodegraded. Thus, a delivery vehicle, referred to as vector, is required to carry the therapeutic gene into the target cell, protecting it from nucleases degradation and facilitating its entry across the cellular membrane. Nowadays, gene therapy is still dominated by viral vectors, which present higher transfection efficiencies compared to non-viral gene delivery systems. However, safety issues such as immunogenicity and mutagenicity hinder the clinical applications of the viral gene delivery systems [1,2]. At this point, non-viral vectors are emerging as a safer alternative to viral vectors for the delivery of nucleic acids, since they are non-pathogenic and non-immunogenic [3,4]. Non-viral vectors can be classified into two main groups: (a) lipoplexes, based on cationic lipids; (b) polyplexes, which are elaborated with cationic polymers. Although cationic lipids are the most studied vectors at the moment, natural cationic polymers have shown promising results and they are being studied as a less toxic alternative to lipid based non-viral systems [5]. Among natural polymers, chitosan (Ch) is one of the most studied polysaccharides [6–10] as it is biocompatible, biodegradable and non-toxic [11]. Chitin is a nitrogenous polysaccharide derived from the exoskeleton of crustacean, insects and fungi. It is the second most ubiquitous natural polymer after cellulose on earth and tones of chitin are produced as food industry waste each year [12], making it an economical and renewable material. However, the use of chitin for biomedical uses is hampered as it is considered chemically inert and insoluble in water and organic solvents. Deacetylation of chitin leads to its main derivative, Ch, a natural linear cationic polysaccharide that consists of β-D-glucosamine and β-*N*-acetyl-D-glucosamine subunits connected by a (1–4) glycosidic bond. The amine groups of the Ch have a pKa value of 6.5 [13–15], making it a pH responsive polymer, which is protonated at pH values below physiological

pH. Thanks to the positively charged amine groups, it is possible to form polyplexes by electrostatic interactions with the negatively charged phosphate groups of DNA. The size of the Ch chain length and thus, the molecular weight (M_W) can be modified by a depolymerization process, which consists of the cleavage of glycosidic linkage between saccharide units [16]. Figure 1 shows a schematic representation of Ch manufacturing process.

Numerous factors affect the stability and transfection efficiency of Ch based vectors, being the most relevant ones the molecular weight (Mw) and the degree of deacetylation (DDA) of the polymer [17]. The Mw of the polymer is an important factor because it influences the particle size of the polyplexes, the binding affinity between the polymer and the nucleic acid, the cellular uptake, the dissociation of the DNA and thus, the transfection efficiency. For a successful non-viral gene therapy an optimized balance between the DNA protection and intracellular DNA unpacking is required [18]. Polyplexes elaborated with

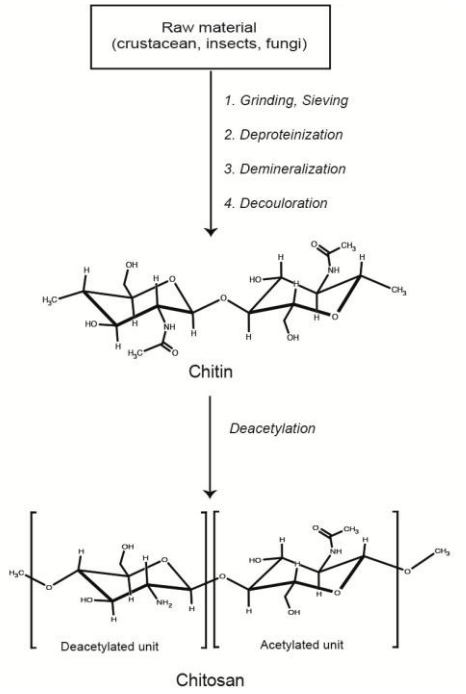

Figure 1. Schematic representation of low molecular weight chitosan (LMWC) manufacturing process.

high molecular weight chitosans (HMWC) (>150 KDa) [10] show better DNA binding, which is beneficial for the protection of DNA. However, the release of the DNA cargo once inside the cell is restricted due to the high stability of the polyplexes. Polyplexes based on low molecular weight chitosans (LMWC) (<150 KDa) are less stable and thus, the protection of the DNA seems to be less effective than the protection

obtained with HMWC based polyplexes [19]. However, it is reported in the literature the ability of LMWC to form plasmid/LMWC complex as effectively as HMWC [20,21]. In addition, Kopping-Hoggard and colleagues reported the capacity of chitosan oligomers with a degree of polymerization (DP) 15 to 24 to retain pDNA, protect it against DNase degradation and efficiently release and deliver the intact transgene [22].

DDA is the second physicochemical parameter of Ch that influences the transfection efficiency of the vectors. The DDA refers to the percentage of deacetylated primary amine groups in the Ch molecule backbone, and thus it determines the average amount of amines available to interact with nucleic acids, and thus, the positive charge of the polymer. High DDA (>90%) results in an increased positive charge, enabling the formation of stable polyplexes with a great binding capacity between the polymer and the DNA. Decreased DDA has been shown to reduce the strength of the electrostatic interactions and cause an easier release and degradation of the DNA [19]. Kiang *et al.* reported decreased overall *in vitro* gene expression levels with low DDA Ch due to the instability of the particles [23]. Consequently, taking both the Mw and the DDA into account, binding affinities between Ch and DNA should be modulated by using Ch with the appropriate Mw and DDA values, in order to obtain a balance between DNA protection and unpacking. In fact, there seems to be an optimal DDA value for each particular Mw chitosan, which indicates the coupling effect between the two parameters being the binding constant between Ch and DNA significantly influenced by Mw and DDA [24]. LMWC, particularly, presents more interesting characteristics for its clinical use, such as higher solubility at physiological pH [20]. In addition, LMWC have antimicrobial, inmunostimulant, antioxidant, and cancer growth inhibitory effects [25]. Therefore, this review will describe the formation, characterization, *in vitro* and *in vivo* transfection process and the future prospects of LMWC based polyplexes as promising non-viral vectors for gene therapy purposes.

2. Formation and Characterization of

LMWC-pDNA Polyplexes

2.1. *LMWC-pDNA Polyplexes Formation Process*

The basic method for the formation of LMWC-pDNA polyplexes is the direct mixing of the positively charged Ch and negatively charged DNA, via a process mainly driven by electrostatic interactions. It has been reported that the mixing technique of Ch and pDNA and the incubation conditions influence the final gene expression; therefore, several groups have worked in the optimization of this process. According to Lavertu and colleagues the best mixing technique was found to be adding Ch over pDNA, pippeting up and down and tapping the tube gently [26]. Other authors, however, have reported that for the correct formation of LMWC-pDNA polyplexes a more vigorous vortex agitation is preferred [27–30]. The incubation time required for the correct formation of the polyplexes is another factor to consider. LMWC-pDNA polyplexes tend to aggregate after several hours of incubation due to the physical instability of aqueous suspensions over time [31,32]. Therefore, an incubation time between 15 and 30 min has been chosen by several authors as the optimal for the correct formation of the polyplexes avoiding the formation of aggregates [27,30,33,34]. Nevertheless, according to Lavertu *et al*. there were no observed differences in the gene expression with incubation times in the range of 30 and 120 min [26].

Much attention is paid to the mixing stoichiometry in the elaboration process of Ch based polyplexes. The N/P ratio is used to define the ratio between the protonable amines in the Ch backbone (N) and the negatively charged phosphate groups in nucleic acid. In order to ensure high transfection efficiencies, polyplexes elaborated at different N/P ratios are tested by the researchers. Generally, Ch-pDNA complexes are prepared using an excess of Ch vs. pDNA (N/P ratios above one). The influence of the N/P ratio on the physicochemical characteristics and transfection efficiency of the LMWC-pDNA polyplexes is described below (Section 3.5).

Based on the literature, the pH of the solution is a very important mixing parameter that affects Ch ionization and

therefore physico-chemical properties (size, mass, density, morphology) [35] of the formed polyplexes. Apart from the pH, other parameters such as the ionic strength and the mixing regime seem to be also important in the formation of the polyplex. The mixing concentration of nucleic acid has also a clear influence on the resulting polyplexes properties: size of polyplexes increase with the increment of the nucleic acid concentration. However, the two main structural parameters that affect the final properties of the formed polyplexes are the MW and the DDA of the Ch, as it has been explained in the introduction section, being highly deacetylation LMWC the most suitable for gene therapy purposes [22,36]. Therefore, a more detailed study of their mechanisms of formation is needed to circumvent this situation, which will resolve several contradictory results reported in the literature regarding the influence of formulation parameters on the resulting polyplexes properties [10]. There are also a significant number of works that present an incomplete description of the polyplex preparation method, which limits the comparison with other studies.

In addition, Ch polyplexes, and non-viral gene delivery systems in general, are usually prepared by the manual mixing of small volumes of Ch and pDNA solution. Moreover, the mixing procedure is performed in diluted conditions in order to ensure a stable and homogeneous colloidal suspension. Thus, the clinical applicability of non-viral vectors is hampered, since the administrable volumes limit the deliverable dose. An approach to overcome this limitation is explained below (Section 5). Taking all this considerations into account, polyplexes elaboration must be carried out considering all the factors that can influence the final characteristics of the resulting polyplexes, in order to development the desired vector for gene delivery.

2.2 Physico-Chemical Characterization of LMWC-pDNA Polyplexes

LMWC-pDNA carriers are characterized by size, zeta potential, morphology, binding-affinity, buffering

capacity and colloidal stability. These physicochemical characteristics are used to, somehow, predict the transfection efficiency of the polyplexes. The methods used for physicochemical characterization and the role of these physicochemical characteristics in the transfection process of LMWC-pDNA polyplexes is described below. In addition, the most commonly employed characterization techniques are summarized in Table 1

2.2.1. Size

Typically, the size of Ch based polyplexes is measured by dynamic light scattering (DLS). It is described in the literature that Ch-pDNA polyplex size can range from a few ten nm [18] to a few hundred nm [30] depending on several factors such as the mixing concentration, the N/P ratio, the pH of the medium and properties of the chosen Ch and pDNA. Electron microscopy has also been employed to measure particle size, but smaller results in particle size are obtained compared to DLS. Some authors have attributed this increment in particle size by DLS characterization due to the fact that, particles are fully hydrated when characterized by DLS [37] or that they are not spherical [38].

The concentration of the mixing solutions of Ch and pDNA has a clear influence on the particle size. It is described in the literature an increase of polyplexes size with increasing the concentration of the nucleic acid solution [17,39]. According to the influence of the M_W on the polyplexes size, contradictory results are described in the literature. Koping-Hoggard *et al.* observed a size increase from 68 to 174 nm when chitosan Mw increased from 2 to 7 kDa for polyplexes prepared at N/P ratio of 60 [22]. Our research group has recently described that particle size of HMWC is around 4-hold higher than the size of polyplexes based on LMWC (5.3 kDa; 7kDa), prepared at N/P of 20 [30]. However, other authors have reported a decrease in size of polyplexes with increased Ch M_W [19]. These differences in the results could be explained in part due to the fact that different methods have been employed for the preparation of the

Table 1: Characterization methods of low molecular weight chitosan (LMWC)/pDNA polyplexes

Characterization technique	Parameter
Acid-base tritation	Buffering capacity
Agarose gel electrophoresis	Binding affinity Release capacity Protection against endonuclease Stability
Asymmetrical flow field-flow fractionation (AF4) coupled with light scattering	Size Stoichiometry
Atomic force microscopy	Size Morphology
Electronic microscopy	Size Morphology
Dynamic light scattering	Size Colloidal stability
Electron microscopy	Size Morphology
EtBr displacement assay	Stability
Isothermal titration calorimetry	Binding affinity Stability
Nanoparticle tracking analysis	Size Mw Concentration
Orange II dye depletion assay (AF4 results confirmation)	Stoichiometry
Polyanion competition assay	Stability
Potentiometric titration	Buffering capacity
Static light scattering	Mw

polyplexes. In relation to the N/P ratio, it is no clear how it affects the particle size of Ch based polyplexes. Some authors have reported that, as the N/P ratio increases, so do the ability of the Ch to complex and compact the pDNA, and thus a decreased size is observed. Nevertheless, our research group has not found a significant influence of the N/P ratio on the size of polyplexes elaborated with oligochitosans, probably because both aforementioned effects equilibrate the final particle size (Figure 2) [29,30,33]. The particle size of the polyplexes will determine the ability of the vectors to reach various organs. For example, particles smaller than 100 nm are required to cross liver fenestrae and target hepatocytes, while particles with 200 nm will be trapped in the lung capillaries [40]. For

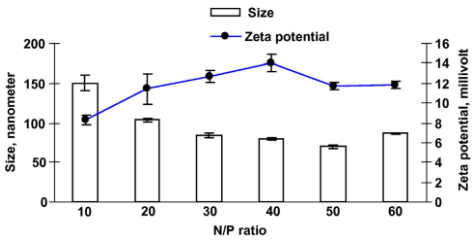

Figure 2. Effect of N/P ratio on the size of oligochitosan/pDNA polyplexes suspended in HEPES medium 10 mM, pH 7.1. Reproduced from [33] with permission from Elsevier.

this reason, polyplexes with the appropriate sizes must be elaborated in order to deliver the genetic material to the desired target organ.

2.2.2 Zeta Potential

The zeta potential of the nanometric polyplexes is measured by laser doppler velocimetry (LDV) and it indicates the value of the polyplexes surface charge. This parameter determines the colloidal stability of the polyplexes aquous suspension and it plays a crucial role in the viability, cellular uptake and transfection efficiency of LMWC-pDNA polyplexes, as it is widely reported in the literature [30,41].

Several factors influence the zeta potential of LMWC based polyplexes and therefore, they should be modulated in order to obtain the desired surface charge. Since the pKa of Ch is about 6.5, the pH strongly influences the zeta potential of Ch based polyplexes. As reported in the bibliography, an increment of the surface charge is observed when the pH of the medium is decreased from 7.4 to 6.5. Recently, our research group has described an increase of around 6 mV in the zeta potential of ultrapure LMWC [30,41]. Considering the zeta potential has an important influence on the cellular uptake of the vectors, as it is explained below, much attention must be paid to the elaboration of the polyplexes in order to get the appropriate surface charge.

2.2.3 Morphology

Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) have been extensively used to determine the morphology of LMWC based polyplexes. According to the bibliography, Ch-pDNA polyplexes can adopt different structures, depending

mainly on the fractional content of acetylated units and the degree of polymerization [42]. Some authors have reported the spherical shape of the complexes [33,43] while toroids, rodlike and globular particles have also been developed [38,39]. Danielsen *et al.* reported that the interactions are important for determining the shape of the polyplexes. Moreover, they revealed that Ch with high and low DP yielded the same structure, but higher amounts of the low DP Ch were needed for this purpose [42]. Figure 3 shows the different morphological shapes that LMWC based polyplexes can adopt.

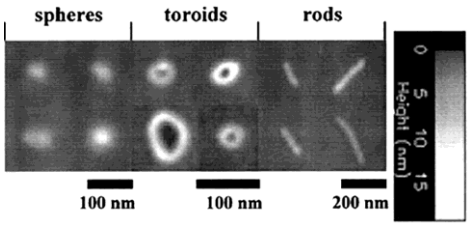

Figure 3. Spherical, toroidal and rod shapes of polyplexes base on fractionated chitosan oligomers. The polyplexes were formed at a concentration 13.3 µg/mL and a charge ratio of 60:1 and examined in the atomic force microscope. The horizontal bars indicate 100 nm (spheres and toroids) and 200 nm (rods). The vertical bar gives the height of the polyplexes. Reproduced from [22] with permission from Nature.

2.2.4 Binding Affinity

The binding affinity between LMWC and pDNA determines the ability of the polymer to complex, release and protect the nucleic acid from degradation, and consequently the transfection efficiency of the corresponding polyplex.

Several techniques have been employed in order to establish the binding affinity of LMWC for DNA. One of the most commonly used methods to qualitatively analyze this parameter is the gel retardation assay. This type of electrophoresis-based assay has been used to monitor the pDNA dissociation from polyplexes formed at different N/P ratios (Figure 4). As reported in the bibliography, LMWC-pDNA polyplexes with low DDA elaborated at low N/P ratio are less stable, and thus they dissociate the pDNA more easily [18,22]. However, the ability of LMWC to complex, release and protect the DNA has been established by several authors [29,34]. Ethidium bromide (EtBr)

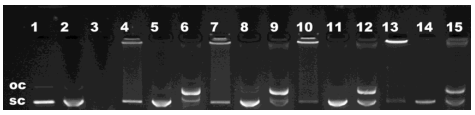

Figure 4. Binding efficiency between oligochitosans and DNA at differnet N/P ratios, and protection capacity from DNase I enzymatic digestion visualized by agarose gel electrophoresis. OC: open circular form, SC: supercoloid form. Lanes 1–3 correspond to free DNA; lanes 4–6, N/P 5; lanes 7–9, N/P 10; lanes 10–12, N/P 20; lanes 13–15, N/P 30. Polyplexes were treated with sodium dodecyl sulfate (SDS) (lanes 2, 5, 8, 11 and 14) and DNase I (lanes 3, 6, 9 and 15). Reproduced from [29] with permission from Elsevier.

displacement is another method that indirectly measures the binding affinity between LMWC and DNA. EtBr intercalates between the base pairs of the DNA double helix yielding a highly fluorescent DNA. Upon the addition of the polycation, EtBr is expelled from the DNA, which results in a decrease of fluorescence. The displacement degree of EtBr provides information about the relative strength of the interaction between the polycation and the pDNA [27]. Another technique employed to quantify the binding affinity of Ch to pDNA is the isothermal titration calorimetry (ITC) combined with the single set of identical sites model (SSIS). This technique allows the quantification of the binding constants of Ch to DNA, which is influenced by the pH, the ionic strength and structural properties of Ch [24].

2.2.5 Buffering Capacity

It has been reported that the mechanism by which polyplexes escape from the endosome, once inside the cell, is the commonly referred *proton sponge effect*, which is determined by the buffering capacity of the Ch. This mechanism is based on the acidification of the endosome or lysosome by the pumping of protons accompanied by the influx of chloride ions that compensates the proton influx into the vesicle. This ion influx causes an increase in the osmotic pressure inside the vesicle and thus a swelling process ruptures the endosome/lysosome, allowing the escape of the polyplexes before being degradated (Figure5). .

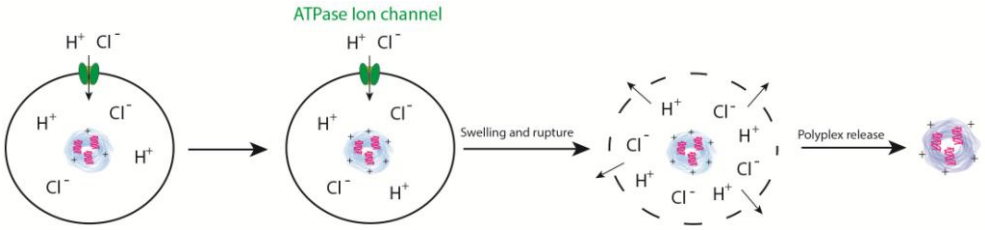

Figure 5. Schematic representation of the proton sponge effect that takes place after the cellular uptake of the polyplexex through an endosomal pathway.

The buffering capacity of Ch has been determined by acid-base titration assay [30,44]. Some authors have concluded that it is unlikely that Ch escape the endosome by proton sponge effect due to its low buffering capacity compared to other cationic polymers with high buffering capacity, such as polyethilenimine (PEI). However, Bushcmann *et al.* have recently reported that comparing Ch and PEI in a molar charge basis, instead of in a mass concentrations basis, the natural polymer has a larger buffering capacity than PEI in the endosomal/lysosomal relevant pH ranges [45]. Interestingly, our research group has established a M_W dependent buffering capacity, being significantly higher for oligochitosans compared to HMWC, which could explain in part the flattering properties of LMWC for the transfection purposes when compared to their HMWC counterparts [30].

2.2.6. Colloidal Stability in Physiological Conditions

One of the main drawbacks related to LMWC-pDNA polyplexes is their poor colloidal stability in physiological conditions [46]. Thus, the presence of polyanions such as blood proteins, heparin or glycosaminoglycans (GAG) in the body may cause the dissociation of the polyplex and the early release of the pDNA before it reaches the desired target tissue [47,48]. For this reason, researchers have focused their attention in the

development of more stable vectors, as it is the case of modified Ch (mCh) based polyplexes (mCh-pDNA).

For the characterization of the colloidal stability, the hydrodynamic size is measured along the time, in different mediums that mimic physiological conditions. It has been reported that the size of LMWC-pDNA polyplexes increased from 100 nm to more than 1 micron after 1 h of incubation in phosphate buffered saline (PBS) [46]. In addition, bovine serum albumin (BSA)-induced aggregation of polyplexes has been assessed by measuring changes in the turbidity of the solution after their incubation with BSA. A significant increase in turbidity was observed after the incubation of Ch-DNA polyplexes with 2 mg/mL BSA. However, lactosylated-Ch based complexes were stable even after the incubation for 24 h [49].

The limited colloidal stability of LMWC based polyplexes may influence their behavior *in vivo*, especially after systemic administration. In order to improve the stability and increase the circulation lifetime of the polyplexes, PEGylation [50], quaternization [51] and glycolyzation [52] of the polymer have been proposed.

3. *In Vitro* Evaluation of LMWC-pDNA Polyplexes Transfection Process

Transfection efficiency refers to the ability of a pDNA carrier, LMWC based polyplexes in this case, to induce transgene expression. Most researchers have focused their attention in the determination of the final gene expression, employing traceable reporter genes such as genes that express Green fluorescente protein (GFP), luciferase and galactosidase. One of the most commonly employed techniques to evaluate the transfection efficiency of the non-viral vectors is flow cytometry. This technique allows the quantitative assessment of the percentage of cells expressing a fluorescent transgene, such as GFP

Moreover, the quantification of luciferase activity by luminometry provides the level of gene expression. Qualitative techniques based on

microscopy are also valuable to determine the expression of the codified reporter protein.

The transfection process is a complex multi-step process influenced by several parameters (Figure 6). Previous to the protein synthesis, there are several barriers that the non-viral vectors must overcome: binding to the cell membrane and uptake, endolysosomal escape and polyplex unpacking and finally, the import of pDNA into the nucleus. Apart from the determination of the final gene expression, considerable attention must be paid to these intracellular barriers to understand the possible limitations of LMWC based polyplexes in order to improve the design of these promising polymers for gene therapy applications.

3.1 Cellular Binding and Uptake

Once in the proximity of the target cells, the polyplexes are bound to the cell surface. Unless the polyplexes are modified with a specific targeting ligand, the binding of polyplexes to the cell membrane occurs by unspecific electrostatic interactions between the positive charge of the polyplexes and the negative charge of the cell surface [53]. The microenvironment around the target cells has to be considered, since it has been described that acidic environments favor the cellular binding and uptake of LMWC-pDNA polyplexes due to an increase in the zeta potential value of the polyplexes at low pH values as a consequence of the protonated amines [30,41,54].

Polyplexes enter the cell mainly via endocytosis. Five types of endocytic pathways have been reported: phagocytosis, macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME) and clathrin- and caveolin- independent endocytosis (Figure 7) [55]. The usage of phagocytosis and macropinocytosis seems very limited, as it only exists in specialized cells such as macrophages monocytes, dendritic cells or antigen presenting cells. Thus, CME and CvME are the best-characterized types of endocytosis in non-viral gene therapy field

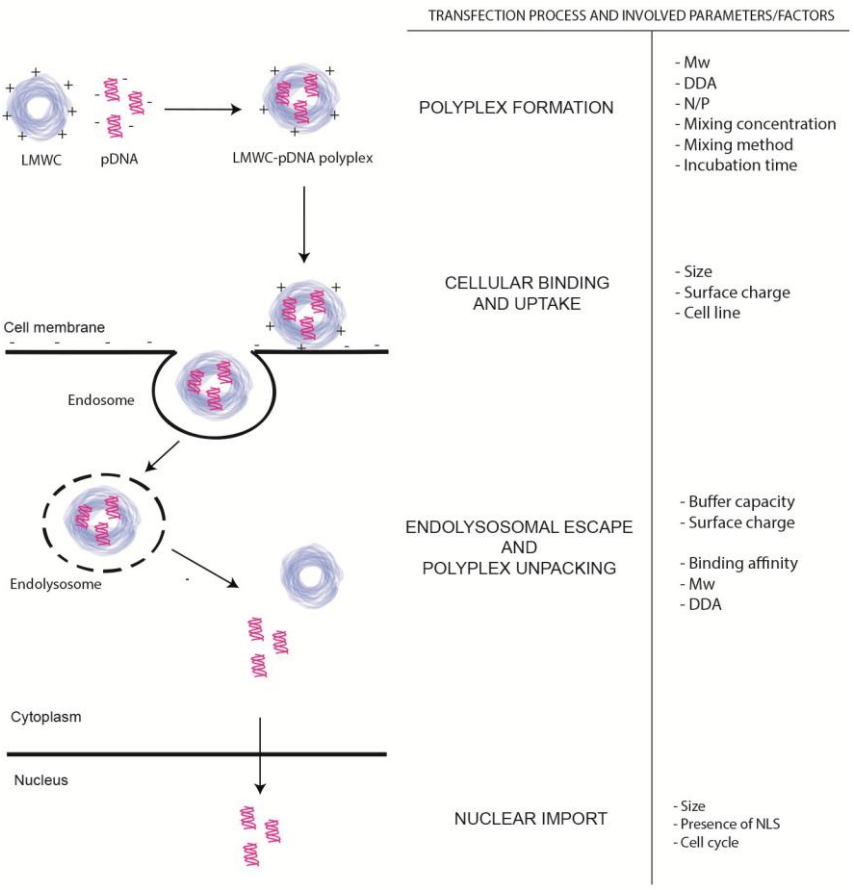

Figure 6. Schematic representation of the transfection process and the parameters/factors involved in each step.

although CvME pathway is less well understood [56]. It is described that LMWC based polyplexes could enter the cell via these two endocytic pathways; however, there is a lack of consensus in the scientific area [53,57]. The diversity that exists in the published results may be attributed to the fact that many factors

determine the uptake pathway of Ch-pDNA polyplexes. Depending on the particle size, endocytosis will occur by one endocytic route or another. It is described that small particles (around 60 nm) are internalized through CvME, whereas intermediate (120 nm) and larger (around 200 nm) particles are taken up by CME or macropinocytosis (>1 μm) [58].

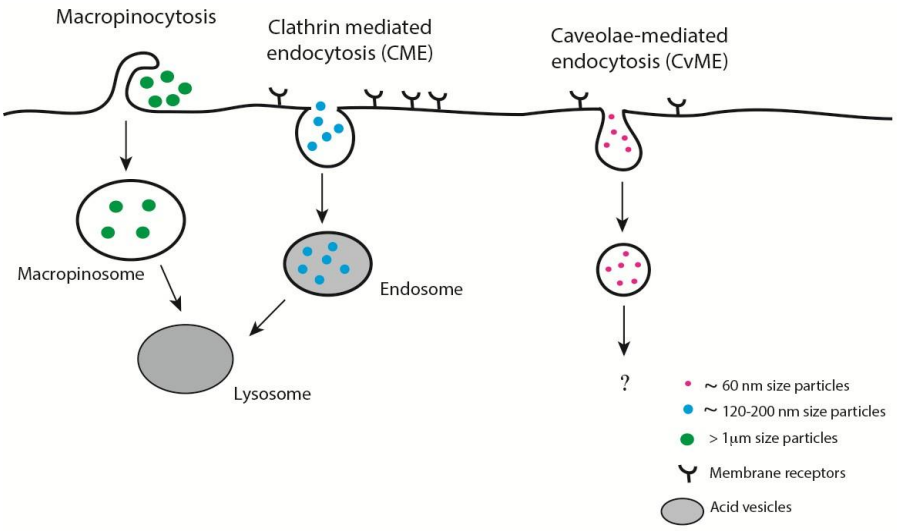

Figure 7. Schematic representation of different endocytic pathways involved in gene delivery.

The surface charge also affects the cellular uptake level of the polyplexes. Positively charged polyplexes will interact efficiently will the cell membrane and favor the internalization [54]. Finally, the endocytic pathway is cell type dependent Depending on the variations on the membrane phenotype and the types of receptor, each cell line will take up the complexes via different endocytic routes. According to Douglas *et al.* Ch polyplexes (around 150 nm) are internalized via CME and CvME in 293T cells, but only via CvME in Chinese hamster ovary (CHO) cells [59]. Although internalization of the polyplexes is the first cellular barrier, it

has been shown not to be the unique key step in the transfection process. Several authors have reported no correlation between polyplex uptake and transfection efficiency [18,30,53] indicating that internalized polyplexes must overcome other important barriers previous to gene expression.

3.2 Endolysosomal Escape and Polyplex Dissociation

The endocytic pathway whereby the polyplexes are internalized will determine the intracellular route of the corresponding non-viral vector and therefore the transfection efficiency. Regardless of the mechanism of internalization, polyplexes must escape from the endosome before being degraded in the lysosome. Polyplexes that enter the cell via CME are confined within endosomes that will suffer a maturation process involving the compartment acidification resulting in late endosomes and finally, lysosomes [56]. The release of the pDNA cargo from these vesicles seems to be the bottleneck in the transfection process [57], since the acidic environment inside lysosome may lead to the degradation of the pDNA if it does not escape from the endosome on time. However, routing through endolysosomal pathway might be essential for endosomal escape of Ch based vectors. It has been described that the endolysosomal escape of LMWC based polyplexes occurs by the "proton sponge" mechanism (described in Section 2.2.5), which is only possible when the vectors are internalized through acidic vesicles, as it is the case of CME. Another way for endosomal escape of Ch based vectors is membrane destabilization. Acidic environment of the endolysosome increases the charge of the polyplex causing anionic phospholipids in the endosomal membrane to flip from the cytoplasmatic face to the intra-endosomal face. This provokes a membrane disorganization allowing the escape of the polyplexes [60]. Non-endosomal pathways, such as CvME, deprive the opportunity for pH-responsive polymers, like Ch, to promote membrane destabilization [61] or endosomolysis by proton sponge effect. Thus, the polyplexes are entrapped inside the

vesicle, preventing the release of the pDNA and leading to its degradation [62].

DNA unpacking is an important rate-limiting step in the transfection process of Ch based polyplexes. Thibault *et al.* revealed that the kinetics of polyplex dissociation is the most critical formulation-dependent intracellular process, indicating the relationship between Ch physicochemical characteristics (M_W and DDA) and transfection efficiency [53]. A high affinity between Ch and pDNA may be a limiting step in the successful dissociation of the plasmid from the carrier. At this point, LMWC offer an advantage over HMWC, since the polyplexes formed with the lower M_W polymers tend to form less stable polyplex leading to an easier unpacking of the DNA.

3.3. Tools for the Study of Uptake Pathways

The study of the uptake mechanisms is a mandatory issue for the design of an adequate non-viral vector, as it will determine their intracellular fate and thus, they final gene expression level. Nowadays, several tools are available for the study of the uptake pathways of a determinate pDNA carrier.

The use of endocytosis inhibitors is an effective way to determine if a specific endocytic pathway plays an important role in the uptake of the polyplex to be examined. Firstly, endocytosis must be distinguished from non-endocytic pathways. The most direct way to determine if the complexes are being endocyted is to use the energy depletion method (lowering the incubation temperature of the cells to 4 °C), since most endocytic pathways are energy dependent [57,63]. To distinguish the phagocytic and macropinocytic pathways from CME and CvME, the most commonly used inhibitors are amiloride (inhibitors of sodium-proton spongue) and wortmannin (inhibitor of phosphoinositide). CME could be inhibited by using hypertonic medium, cytosol acidification or chlorpromazine drug. According to CvME, genestein, nystatin and filipin are the most commonly used specific inhibitors [57,63,64].

Inhibitors for the study of intracellular fates of complexes are also available. Monensin, bafilomycin A prevent the maturation and fusion of endosomes into lysosomes by inhibiting the acidification of the endosome. Moreover, cloroquine can be used to swell and disrupt endocytic vesicle by osmotic effects [65].

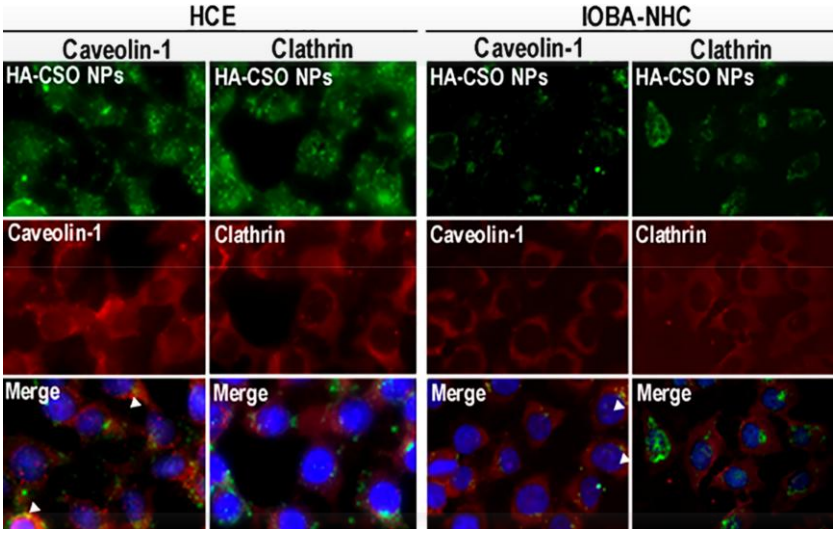

Figure 8. Caveolin-1 and clathrin immunofluorescence in HCE and IOBA-NHC cells after incubation with hyaluronic acid-chitosan oligomer-based nanoparticles (HA-CSO NP). Merged images showed co-localization of HA-CSO NP with caveolin (staining at arrowheads). Reproduced from [64]. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium.

Molecular probes, markers and dyes can also be used to corroborate the information obtained with the cellular uptake pathways inhibitors. Transferrin is commonly used as a probe of CME and so is cholera toxin beta subunit for CvME. These can be combined with fluorophores in order to visualize them under a fluorescent microscope. The colocalization of these probes with the fluorescently labelled polyplexes can provide information about the internalization route of the corresponding vector (Figure 8). Organelle specific dyes such as

Lysotracker and Lyso Sensor (lysosomal dyes) and Cell light (dyes for early endosomes) are other ideal tools for the detection and colocalization of the intracellular trafficking of the non-viral vectors.

3.4 Nuclear Import

The last barrier before gene expression is the internalization of the pDNA across the nuclear membrane. The nuclear membrane is composed of a double lipid bilayer with tightly regulated aqueous channels forming the nuclear pore complex (NPC). The NPC has 10 nm of diameter in its relaxed state, which allows the passive diffusion of small molecules. However, active transport of larger molecules is possible thanks to the recognition of nuclear localization signals (NLS). Particle size of LMWC based polyplexes is generally >100 nm, therefore, it is unlikely that they diffuse through the nuclear membrane. Therefore, the incorporation of NLS to the polyplex formulation is a strategy that can be followed in order to enhance the internalization of the pDNA to the nucleus.

However, nuclear delivery can still take place during the cell division process. It is well described that the transfection efficiency correlates with cell division and cell growth [66]. Thus, the transfection efficiency in proliferating cells is supposed to be higher than in cells that do not undergo mitosis regularly. At this point, the nuclear import is a poorly characterized step in the transfection process, which should be further investigated in order to better understand the exact mechanisms whereby LMWC-pDNA polyplexes are internalized.

3.5 Factors Involved in the Transfection Efficiency of LMWC based Polyplexes

Transfection efficiency of highly deacetylated LMWC based vectors is influenced by several formulation parameters such as, serum content, pH of the transfection medium, N/P ratio, pDNA concentration, and also by the employed cell type. These parameters will define the physico-chemical characteristics of the polyplexes (described in Section 3.3) and

thus, the transfection efficiency.

There is no consensus in the scientific community regarding the influence of serum in the transfection efficiency of LMWC based polyplex. According to some authors, the presence of serum enhances the transfection efficiency of Ch based polyplexes [41,67,68]. The reason for the increment of transfection in the presence of serum is not clear. Some authors argue that serum content might improve the cell function including cell division and endocytosis. Nimesh *et al.* found an increment in the cellular uptake when cells where expose to polyplexes with serum content, probably because the Ch forms small hydrodynamic diameter complexes with the negatively charged serum proteins which can be efficiently uptaken [41]. Although several studies have demonstrated the advantage of chitosan based vectors for transfecting cells in serum containing medium, there are still some authors that preferred to use serum free Opti-MEM transfection medium, making clear the controversy that exists about the influence of the serum content on the transfection process [28,29].

Another important parameter to consider is the pH of the transfection medium. Ch is a pH responsive polymer, which means that its charge density depends on the pH value of the solutions. It is well described in the scientific literature that medium acidification enhanced the cellular uptake and the transfection efficiency of LMWC based polyplexes [30,41]. Our research group has recently reported that medium acidification from 7.4 to 6.5 increases the zeta potential and decreases the size of ultrapure oligochitosan bases polyplexes. Positively charged complexes can efficiently bind to negatively charged cell membranes facilitating the cellular uptake and improving the transfection efficiency. These results suggest the possible application of this kind of vectors in cells with an acidic environment, such as tumor cells. The N/P ratio used to elaborate the polyplex also influences the transfection efficiency. The polyplexes must be stable enough to retain the pDNA, but they must be able to release the cargo once inside the

cell. The required N/P ratio to obtain this balance between DNA packing and release will depend on the Mw and DDA of the used Ch. Lavertu *et al.* reported that LMWC would require a higher N/P ratio to completely condense DNA [69].

4. Chemical Modifications of Ch to Overcome Transfection Barriers

In order to ensure an efficient transfection process, the elaborated non-viral vectors must overcome all the biological barriers described above: cellular binding and uptake, endolysosomal escape and nuclear import. Apart from the biological difficulties, the enhancement of the complexes colloidal stability is a key issue for high transfection efficiencies.

With the aim of addressing the obstacles previously mentioned, LMWCs could be modified by several ways. Among the modification reactions, chemical grafting of molecules or polymers on the C2 amine and the C6 hydroxyl are the most usual. Although the C6 hydroxyl is reactive and easy to be functionalized, modifications on the C2 amine are normally employed in a large number of research works.

The principal drawback in the use of chitosan's amine functional groups for modification is that this chemical reaction may generate steric hindrance and decrease the number of ionisable amines that bind to pDNAs. Thus, the nonbonding pair of electrons on the primary amine of the chitosan is nonetheless a good candidate for nucleophilic attack and it allows several reactions. Thus, quaternization, amidation and reductive *N*-alkylation are the main chemical reactions used to overcome the barriers in the transfection process.

Quaternization of chitosan has been performed to improve solubility at physiological pH. For this purpose two methods of quaternization have been performed: N-trialkylation usually with CH3I [51,70]. Another technique based on glycidyltrimethylammonium chloride reaction is also used for Ch quaternization [71].

Amidation is another widely employed technique to graft molecules and

polymers to the amine group of chitosan. It can be achieved using carboxylic compounds with carbodiimides such as EDC (1- ethyl- (3- dimethylaminopropyl) carbodiimide hydrochloride) or DCC (dicyclohexyl carbodiimide) coupled with NHS (*N*-hydroxysulfosuccinimide) to accelerate the reaction [72]. Amidation of chitosan could overcome several drawbacks related to the transfection process, depending on the chosen molecule. In this respect, the grafting of molecules such as urocanic acid, imidazole-4-acetic acid and histidine [73–75] could improve the endosomal escape of Ch based vectors. Moreover, grafting Ch with arginine has been proven to increase cell uptake and PEG-succinimidyl ester or Folate–PEG–COOH molecules could enhance pharmacokinetics [76]

Finally, the reductive *N*-alkylation method serves to graft an aldehyde derivative on the amine of Ch. Grafted species include fluorescent probes as 9-anthraldehyde [77], phosphorylcholine [78,79], oligosaccharides [18,46,52,80] and dextran like polymers [81]. Normally, reductive *N*-alkylation is directed to reduce cytotoxicity and increase transfection efficiency. In the case of dextran molecule, it is used with the aim of improving complexes stability. Succinoyl groups have also been grafted to Ch for gene delivery applications [82].

5. Freeze-Drying of Polyplexes

The poor long-term stability of aqueous suspensions [31] of polyplexes is one of the major limitations for their wide scale clinical application. Aggregation of polyplexes over time provokes a loss of transfection, and thus a fresh preparation of the polyplexes prior to their administration is required. However, day-to-day preparation of the vectors leads to batch-to-batch variations in the product quality, safety and transfection rates [83]. Thus, the development of stable and transfection competent polyplexes is an important step from a promising technological formulation to its clinical application.

Freeze-drying or lyophilization is the most common method for preparing dry formulations, which ensures a long-term

conservation of the preformed complexes [32,84,85]. Freeze-drying process can be divided in three steps: freezing, primary drying (ice sublimation) and secondary drying (desorption of unfrozen water). These are stress factors that could cause damage either in the polymer or in the DNA. The final lyophilized product should preserve the original chemical and physical characteristics and be stable over time. In order to avoid the possible damages caused by the freezing or drying processes, cryo/lyo protective agents are generally added to the complexes. The most popular cryoprotectants reported in the literature are sugars such as, trehalose, sucrose, glucose and mannitol, which are also called stabilizers [86].

Compared to *in vitro* studies, clinical trials and *in vitro* experiments require higher doses of DNA, formulated in a volume suitable for each administration. It is well know that the mixing conditions of the polyplex components play an important role in the final characteristics of the non-viral vector, and is very complicated to elaborate formulations at high concentrations with the conventional preparation methods of polyplexes. The use of polyplexes *in vivo*, and of course in clinical trials can be hampered if the dose needed to obtain a therapeutic effect is very high, due to the low concentration of the polyplexes suspension obtained from a diluted polymer and plasmid solutions. Thus, polyplex suspensions need to be concentrated. At this point, lyophilization could be considered as an advantageous method to concentrate vectors suspensions and thus obtain higher pDNA concentrations, as the final dried formulation can be reconstituted in reduced volumes of the desired diluent [87,88]. Up to now there are not many works related to the lyophilization of LMWC-pDNA polyplexes, thus, according to the positive results obtained with other complexes, the possibility of freeze-drying LMWC based vectors should be considered.

6. *In Vivo* Applications of LMWC based Vectors for pDNA Delivery

Once the polyplexes are elaborated, characterized and their transfection efficiency is proven *in vitro*, the next step

is to determine the efficiency and toxicity of the vectors *in vivo*. Due to its well-characterized biocompatibility and biodegradability, LMWC has been widely used for *in vivo* gene therapy purposes (Table 2). In addition, Ch is a highly mucoadhesive polymer, which makes it an excellent candidate for ocular and lung delivery of nucleic acids [89,90].

6.1 Ocular Delivery

The ocular system is a privilege organ for the localized delivery of non-viral vectors to specific ocular tissues, and it is affected by many well understood genetic-based diseases. Several research groups have investigated the potential use of LMWC based vectors for the delivery of pDNA to different ocular tissues.

Novafect O15 and O25 oligochitosans have been used to transfect corneal and retinal cells *in vivo*. Klausner *et al*. injected Ch-DNA nanoparticles, elaborated with various plasmids with different characteristics, to the corneal stroma. They found that these LMWC based polyplexes were able to transport pCpG-GFP plasmid to keratocytes in the cornea, and that the cells could efficiently express high levels of GFP [28,34]. Our research group has also used Novafect O15 and O25 for the delivery of pDNA to the retina. The study showed for the first time that polyplexes based on these LMWC at N/P ratio of 10 are able to transfect different cells of the retina rats depending on the administration route. After subretinal injection of the polyplexes gene expression was observed mainly in the cells of the retinal-pigmented epithelium (RPE). Polyplexes were also administered by intravitreal injections, obtaining GFP expression in the ganglion cell layer and in the inner layers of the rats' retina (Figure 9) [29,33]. Bioadhesive hyaluron-Ch nanoparticles loaded with a reporter plasmid encoding the enhanced green fluorescent protein (pEGFP) were topically administrated to the cul-de sac of rabbits. Positive transfection results were observed in the corneal epithelium for up to 7 days [91].

6.2 Lung Delivery

Non-viral gene delivery to the lung holds therapeutic potential for the

treatment of diseases such as cystic fibrosis and lung cancer. The mucoahdesive properties of Ch offer advantages for the delivery of pDNA to this target tissue. One of the most non-invasive approaches to target the lung is via inhalation. Mohammadi *et al.* nebulized Ch-DNA nanoparticles attached to Fibronectin Attachment Protein of Mycobacterium bovis (FAP-B) for the delivery of pGL3-control plasmid encoding lucifrase to mice lung. They reported high levels of luciferase gene expression in the dissected lungs after 48 h [92].

Intratracheal administration, a more invasive route, has also been used to target the lung. According to Kopping-Hoggard *et al.* LMWC based polyplexes showed a 10–20 fold higher *in vivo* transfection efficiency compared to HMWC based vectors, after intratracheal administration. This occurs as a result of the weaker association of pDNA to Ch and the retained capacity of the polymer to protect the plasmid against DNase degradation, enabling an efficient release and delivery of the intact transgene [22]. Trisaccharide-sustituted Ch oligomers have been also administered via the trachea, observing luciferase gene expression in the mouse lung 24 hours post administration [52].

6.3 Other Delivery Routes

The nasal delivery has attracted researcher's attention since it has been established that nasally administered DNA vaccines can induce effective humoral and cellular responses. In addition, it is a non-painful administration route, which could increase patient compliance. Yang *et al.* investigated the effect of Ch *M*w on *in vivo* delivery efficiency of the plasmid encoding the Human cholesteryl ester transfer protein (CETP). They found that nasal vaccination of LMWC/pDNA polyplexes elicited specific anti-CET IgG antibodies whose presence lasted for more than 21 weeks [93]. More recently, mannosylated Ch nanoparticles have been administered by the nasal mucosa route to elicit serum anti-GFP IgG anitibodies and inhibit the growth of gastrin-releasing peptide (GRP) dependent tumor cells. The results confirmed that these Ch based carriers are

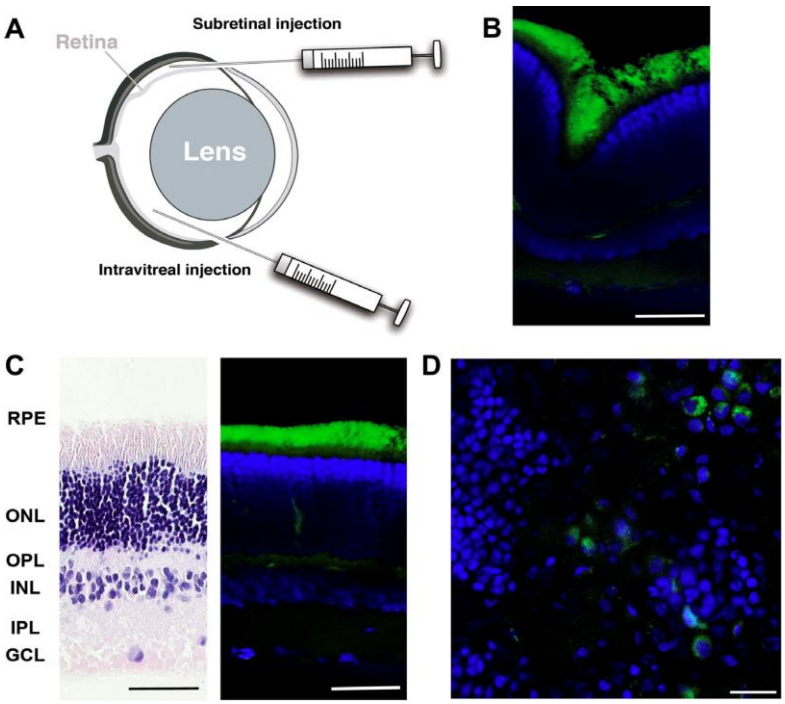

Figure 9. *In vivo* gene expression of GFP after administration of oligochitosan/DNA polyplexes at N/P ratio 10 to rats. (**A**) Schematic drawing of the subretinal and intravitreal injection. (**B**) Cross-section of a treated retina close to the place of the subretinal injection. Enhanced green fluorescent protein (EGFP) expression with Hoechst 33342 staining for cell nuclei. Scae bar = 50 µm. (**C**) Hematoxylin-eosin rat retina cross-section, showing the different layers of the retina. RPE (Retinal Pigment Epithelium layer), ONL (Outer nuclear layer), OPL (Outer plexiform layer), INL (Inner nuclear layer), GCL (Ganglion cell layer) and fluorescent microscopy image of a 5-µm treated retina cross-section. Scale bar = 50 µm. (**D**) Whole-mount views of several sections of the retina focused at the RPE layer. Scale bar = 100 µm. Reproduce from [33] with permission from Elsevier.

feasible in DNA vaccine delivery and they offer a possibility for efficient tumor immunotherapy [94].

Other administration routes such as the subcutaneous (SC) and intramuscular (IM) have also been used for the delivery

of pDNA. Jean *et al.* found that SC administration of Ch/pVax1-4sFGF-2 or Ch/pVax1-PDGF-bb complexes lead to the expression and distribution of high levels of FGF-2 and PDGF-BB recombinant proteins in serum, and the IM administration induced a rapid production of specific antibodies, which is promising for the development of prophylactic or therapeutic vaccines [95]. The same research group described the ability of Ch based complexes to deliver the pVax1-GLP-1 plasmid encoding the native recombinant GLP-1 protein in a diabetic rat model [96].

In relation to cancer gene therapy, the most direct approach to achieve the expression of genes in tumor tissues is the injection of polyplex solution into the tumor foci. Ch/pCMV-Luc polyplexes have been intratumorally administered to subcutaneously generated tumors, resulting in acceptable transfection efficiencies [97]. Finally, transfection efficiency of LMWC based polyplexes has also been investigated after their systemic administration. This route allows the possibility of targeting concrete organs and and tissues that may not be reached via a local administration. Zhang *et al.* elaborated hydrophobically modified LMWC vectors for the delivery of pCMV-Luc reporter gene. After 3 days of the systemic administration in the tail vein, a very strong expression of the gene was observed in the kidney of the treated mice (Figure 10) [98].

LMWC functionalized with bPEI and conjugated with tuftsin for macrophage targeting has also been proven to be an adequate carrier for the systemic administration of pDNA. An increased gene expression was observed in mice spleen, heart and brain 7 days post administration of the polyplexes, compared to HMWC based counterparts [99].

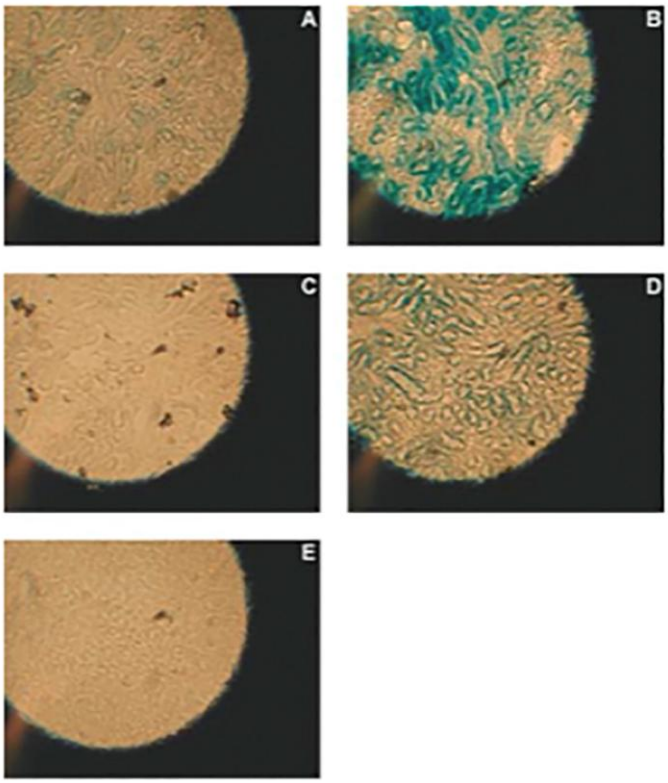

Figure 10. Gene expression in mice kidneys after systemic injection LMWC/pDNA complexes. Complexes of pUC 19 DNA with (**A**) LMWC; (**B**) 3% hydrophobically modified LMWC; (**C**) 18% hydrophobically modified LMWC and (**D**) branched PEI at N/P 5 were injected into the tail vein of the mice. The kidneys were harvested 3 days after administration. (**E**) Naked plasmid DNA injected as a control. Magnification, ×200. Reproduced from [98] with permission from Wiley.

Table 2. Selected studies using LMWC based polyplexes for pDNA delivery.

Route of administration	Animal model	Chitosan	pDNA	Comments	Objective	Reference
Corneal injection	Sprague-Dawley rats	Novavect O15 (5.7 kDa, DDA 99%) Novafect O25 (7.3 kDa, DDA 99%)	gWiz-Luc (luciferase encoding reporter gene) 1.5 µg gWiz-GFP (GFP encoding reporter gene) 1.5 µg		Treatment of acquired and inherited corneal disorders	[27]
Corneal injection	Sprague-Dawley rats	Novafect O15 (5.7 kDa, DDA 99%)	gWiz,-Luc, pCpG-Luc, pEPI-CMV, pEPI-UbC (encoding for luciferase) 1.5 µg gWiz-GFP, pCpG-GFP (encoding for GFP) 1.5 µg		Treatment of acquired and inherited corneal disorders	[33]
Subretinal, intravitreous injection	Sprague-Dawley rats	Novafect O15 (5.7 kDa, DDA 99%)	pCMS-EGFP reporter gene (100ng)		Treatment of retinal disorders	[28]
Subretinal, intravitreous injection	Sprague-Dawley rats	Novafect O25 (7.3 kDa, DDA 99%)	pCMS-EGFP reporter gene (100ng)		Treatment of retinal disorders	[32]

Topic administration	Rabbits	Ultrapure Ch hydrochloride salt (113 kDa).	pEGFP reporter gene (25μg, 50μg, 100 μg)	Ch was mixed with HA salt, and NPs were prepared by ionotropic gelification	Treatment of ocular diseases	[89]
Aerosol	Balb/c mice	Ch Chitoclear (126 kDa, DDA 98%)	pGL3-control plasmid encoding luciferase	Electrostatially formed polyplexes were conjugated with FAP-B	Lung targeting	[90]
Intratraqueal administration	Balb/c mice	UPC, Protasan UPG 210	gWiz-Luc, pCMV-Luc (luciferase encoding reporter genes) 5μg, 10μg, 25 μg	Fully deacetylated Ch was depolymerized to obtain oligomers with number average DPn 25 and 18	Lung targeting	[21]
Intratraqueal administration	Balb/c mice	Fully de-N-acetylated Ch (3.6-7 kDa)	gWiz-Luc, pCMV-Luc (luciferase encoding reporter genes	Ch oligomers were substituted with trisaccharides, obtaining oligormes with 7, 23, 40% of substituted amines	Lung targeting	[50]
Intranasal administration	Sprague-Dawley rats	Ch Mw: 5, 173 kDa	pEGFP-C3 encoding GFP; pDNA encoding CETP-C		Immunotherapeutic DNA vaccine for atherosclerosis treatment	[93]

Intranasal administration	C57BL/6 mice	Ch Mw: 115 kDa, DDA 95%	pGRP (0.5mg)	Ch was conjugated with D-mannose	Production of anti-GRP IgG and inhibition of tumor growth	[94]
Intramuscular and subcutaneous administration	Balb/c mice	Depolymerized Ch 92-10 and 80-80 (Mw-DDA)	pVax1-4sFGF-2 and pVax1-PDGF-BB (encoding for FGF-2 and PDGF recombinant proteins)		Enhancing the repais of cartilage lesions or enhancing bone defect fill	[95]
Intramuscular and subcutaneous administration	Zucker Diabetic Fatty rats	Depolymerized Ch 92-10 and 80-80 (Mw-DDA)	pVax1-GLP1 encoding for the recombinant GLP-1 (165 μg)		Type 2 diabetes treatment	[96]
Intratumoral administration	C.B-17/Icr-scid-bg mice	Ch Mw 15.5 kDa, DDA 75-85%	pAcEGFP1-C1 and Luc reporter plasmids encoding GFP and luciferase (100 μg)		Cancer treatment	[97]
Intravenous administration	Mice		pUC 19 encoding β-galactosidase reporter gene	Hydrophobically modified LMWC		[98]
Intravenous administration	Balb/c mice	Depolimerized Ch (7 kDa and 10 kDa)	pGL3 luciferase reporting gene (25 μg)	Ch was conjugated with LMWP and further with tuftsin		[99]

7. Future Prospects

Although LMWC have been investigated and developed for the delivery of DNAs for fifteen years, the efficient expression of the desired genes into the target cells is not as close as we wish. The resolution of many of the challenges associated with the production of the non-viral vectors, and their accurate analyses, has been the key for the use of LMWC based carriers in biomedical applications and particularly, for gene delivery purposes [100,101].

The physicochemical and biological basis for the successful *in vitro* function of these systems have also been partly elucidated, resulting in the improvement of the susceptibility to degradation of DNA by DNAses, the low cellular membrane permeability and the low solubility and stability at physiological pH [22,29]. Nevertheless, polyplexes elaborated with LMWC face multiple obstacles that still remain unsolved for its *in vivo* success. In order to increase the translation from the raw material to the clinic, efforts are focusing on the chemical and biological modifications of Ch. Specific ligands attached to the LMWC-based nanocomplexes should enhance their *in vivo* targeting toward the desired tissue after systemic administration. Nevertheless, aggregation of the polyplexes in the presence of biological polyanions, due to their high superficial charge, represents the bottleneck in the development of effective polymeric non-viral vectors for systemic administration. Against this major problem, LMWC grafted with neutral polymers have been extensively studied [51].

Due to the drawbacks associated with intravenously administered non-viral vectors, in our opinion, future clinical trials will be focused in the direct administration of the carriers into the target tissue. However, we should point out that with a systemic administration a wider dissemination of the plasmid would be achieved, which is beneficial for the treatment of several pathologies like multi-compartment localized tumors or tumors with metastasis.

In conclusion, pending challenges to

obtain successful treatments for human genetic diseases include the production of adequate quantities of highly characterized nanoplexes with features that satisfy regulatory agencies, and the matching of current capabilities in protein expression, targeting and safety profile to specific clinical indications.

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Author Contributions

All authors contributed extensively to the work presented in this review. Mireia Agirre wrote the main manuscript. Edilberto Ojeda provided general advice in relation to the cellular uptake and intracellular trafficking of the polyplexes. Jacques Desbrieres provided data and gave general advice related to chitosan modifications for the review. Gustavo Puras gave general advice related to the *in vivo* administrations of the polyplexes and corrected the final manuscript. Jon Zarate designed coordinated, gave general advice and corrected the manuscript. Jose Luis Pedraz supervised and managed the whole work as the director of the research group.

Conflicts of Interest

The authors declare no conflict of interest.

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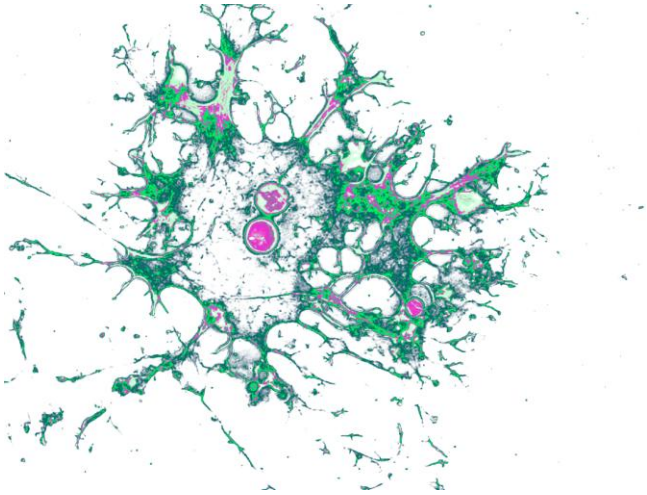


Helburuak
Objetivos
Objectives

1995. urtean Mumper eta kideek kitosanoa geneen garraiorako sistema bezala aurkeztu zutenetik, hainbat izan dira azido nukleikoak eramateko polimero natural honek dituen ezaugarri oparoak deskribatu dituzten lanak. Kitosanoz eraturiko poliplexoen transfekzio efizientzia hobetzeko, oso garrantzitsua da eragina izan dezaketen parametro fisikokimikoak eta transfekzio prozesua ondo ezagutzea.

Doktorego-tesi honen helburu nagusia, pisu molekular baxuko kitosanoz osaturiko poliplexoak transfekzio ez-biralerako sistema gisa *in vitro* aztertzea da. Xede hau lortzeko hiru helburu espezifiko ezarri dira:

1. Pisu molekular baxuko kitosanoekin eraturiko poliplexoen transfekzio prozesuan eragina duten faktore fisikomikoak, pHa bereziki, *in vitro* ebaluatzea.

2. Oligokitosanoek tamaina handiko plasmidoak garraiatu eta zelula tumoralak transfektatzeko duten gaitasuna *in vitro* aztertzea.

3. Nerbio-sistema zentraleko gaixotasunetarako ex vivo terapia genikorako plataforma egokia izan daitezkeen giza zelula neuronal aintzindarietan, oligokitosanoz eraturiko poliplexoen transfekzio prozesua ezagutzea, izaera lipidikoa duten beste bektore ez-biralekin alderatuz.

Desde que Mumper et al describieran en 1995 el uso de quitosano como sistema para el transporte de genes, han sido numerosos los trabajos que han detallado las prometedoras características de este polímero natural como vector no-viral para la vehiculización de ácidos nucleicos. Dado que la eficiencia de transfección de los poliplexos basados en quitosano es relativamente baja, es necesario conocer los parámetros fisicoquímicos y moleculares que puedan tener alguna influencia en la eficiencia de transfección con el fín de caracterizar y conocer el proceso de transfección para poder mejorar el diseño y desarrollo de vectores no-virales basados en quitosano.

En base a estas consideraciones, el objetivo principal de esta Tesis Doctoral es la elaboración, caracterización y evaluación *in vitro* de polyplexos basados en quitosanos de bajo peso molecular como sistemas de transfección no-viral. Para la consecución de este objetivo se han establecido tres objetivos específicos:

1. Evaluar *in vitro* la influencia de factores fisicoquímicos, especialmente el pH, en el proceso de transfección de polyplexos basados en quitosanos de bajo peso molecular.

2. Analizar *in vitro* la capacidad de los oligoquitosanos para transportar plásmidos de gran tamaño y transfectar células tumorales de pulmón.

3. Conocer el proceso de transfección de los polyplexoes elaborados con oligoquitosanos en células precursoras neuronales, comparándolos con otros tipos de vectores no-virales de composición lipídica.

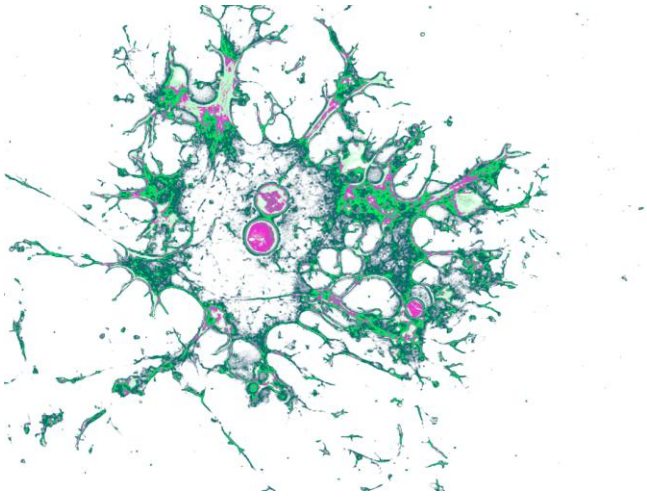
Since Mumper et al. reported in 1995 the use of chitosan as a gene delivery system numerous works have reported the promising characteristics of this natural polymer as non-viral vector for nucleic acid delivery. As the transfection efficiency of chitosan based polyplexes is relatively low, there is a necessity to gain knowledge about the physicochemical and molecular factors that may influence the transfection efficiency in order to characterize and understand the whole transfection process itself, with the aim of improving the design and development of chitosan based non-viral gene delivery vectors.

In view of these considerations, the main objective of the present work is the elaboration, characterization and *in vitro* evaluation of polyplexes based on low molecular weight chitosan, as non-viral gene delivery systems. To accomplish this purpose, three specific objectives are considered:

1. To evaluate *in vitro* the influence of physicochemical factors, especially the pH, in the transfection process of polyplexes based on low molecular weight chitosan.

2. To analyze *in vitro* the capacity of oligochitosans to deliver large plasmids and transfect lung tumor cells.

3. To understand the transfection process of polyplexes based on oligochitosans in human neuronal precursor cells, by their comparison with lipid based non-viral vectors.



Diseinu esperimentala
Diseño experimental
Experimental design

Chapter 1

Improving transfection efficiency of ultrapure oligochitosan/DNA polyplexes by medium acidification

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Puruntasun handiko oligokitosanoak (UOC) korneara eta erretinara geneak modu eraginkorrean garraiatzeko gai izan dira. Hala ere, pHa bezalako zenbait faktore fisiko-kimikok transfekzio efizientzian duten eragina oraindik erabat argitzeke dago. Zehatzago, faktore horien in vitro azterketak informazio oso baliagarria eman diezaguke etorkizuneko aplikazio klinikoetarako.Ikerketa honen helburu nagusia da HEK293 eta ARPE19 zeluletan pHaren jaitsierak UOC/pDNA poliplexoen transfekzio gaitasunean duen eragina deskribatzea.UOC/pCMS-EGFP poliplexoak eratu genituen berezko elkarketaren metodoaren bitartez. Partikulen tamainan eta zeta potentzialean faktore fisiko-kimiko garrantzitsuenen eragina diseinu experimental ortogonalaren bitartez aztertu ziren. Gainera, in vitro zelulan barneratzea eta transfekzio efizientzia zitometria bidez aztertu ziren, eta CCK-8 entseguaren bitartez zitotoxizitatea.Transfekzio ingurunearen pHak eragin handia dauka poliplexoen ezaugarri fisiko-kimikoetan, eta pHa moldatuz nanopartikulen gainazal karga kontrolatzea posible da. pHa jaistean eta ingurunea azidotzean UOC bidez garraiatutako plasmidoa zelulan gehiago barneratu zen eta transfekzio efizientzia handiagoa izan zen. Frogatutako UOC/pCMS-EGFP poliplexoetatik batek ere ez zuen zitotoxizitaterik eragin; aldiz, Lipofectamine 2000 liposomekin transfektatutako zelulen bideragarritasuna baxuagoa izan zen.Era honetako UOC bektoreak ingurune azidoan dauden zelulak transfektatzeko erabilgarriak izan daitezke, tumore zelulak kasu. Dena den, etorkizunean terapia geniko ez-biralerako eraginkorra eta segurua den medikamendua lortu ahal izateko, in vivo ikerketa gehigarriak beharrezkoak dira.

Recientemente se ha descrito la eficiencia de los oligoquitosanos ultrapuros (UOC) como vectores para el transporte de genes a cornea y retina. Sin embargo, la influencia que ejercen diversos factores fisicoquímicos, como el pH, en la eficiencia de transfección aún es desconocida. Un estudio in vitro más profundo de estos factores podría proporcionar información valiosa para las futuras aplicaciones clínicas. El objetivo de este trabajo es determinar la influencia de la disminución del pH en la eficiencia de transfección de poliplexos UOC/pCMS-EGFP en células HEK293 y ARPE19. Se elaboraron los poliplexos UOC/pCMS-EGFP por el método de auto ensamblaje. Mediante un diseño experimental ortogonal se estudió la influencia de varios factores en el tamaño y carga de dichos poliplexos. Se evaluó in vitro la captación celular y eficiencia de transfección mediante citometría de flujo, y la citotoxicidad de los vectores mediante un ensayo CCK-8. El pH del medio ejerce una fuerte influencia en las propiedades fisicoquímicas de los poliplexos, y mediante la modulación del valor del pH es posible controlar la carga superficial de los vectores. La acidificación del medio de transfección provocó un aumento en la captación celular y eficiencia de transfección de los UOC/pCMS-EGFP. Ninguno de los vectores estudiados causó citotoxicidad alguna, sin embargo, las células tratadas con el reactivo Lipofectamine 2000 sufrieron un descenso en su viabilidad. Este tipo de vectores podrían ser útiles para transfectar células que se encuentran en un ambiente ácido, como pueden ser las células tumorales. Sin embargo, son necesarios estudios in vivo adicionales para poder obtenerun medicamento efectivo y seguro para terapia génica no-viral.

Improving transfection efficiency of ultrapure oligochitosan/DNA polyplexes by medium acidification

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ABSTRACT

Context: Ultrapure oligochitosans (UOCs) have recently been reported as efficient nonviral vectors for corneal and retinal gene delivery. However, the influence of some physicochemical factors on the transfection efficiency, such as the pH, remains unclear. Deeper *in vitro* research of these factors could provide valuable information for future clinical applications.

Objective: The aim of this study is to determine the influence of the pH decrease on the transfection efficiency of UOC/pDNA polyplexes in HEK293 and ARPE19 cells.

Materials and methods: We elaborated self-assembled UOC/pCMS-EGFP polyplexes. The influence of the most important factors on the particle size and the zeta potential was studied by an orthogonal experimental design. We evaluated, *in vitro,* the cellular uptake and the transfection efficiency by flow cytometry, and the cytotoxicity of the vectors by CCK-8 assay.

Results and discussion: The pH of the medium strongly influences the physicochemical properties of the polyplexes, and by its modulation we are able to control their superficial charge. A significant increase on the cellular uptake and transfection efficiency of UOCs was obtained when the pH was acidified. Neither of our UOC/pCMS-EGFP polyplexes caused cytotoxicity; however, cells treated with Lipofectamine 2000™ showed decreased cell viability.

Conclusion: This kind of UOC vectors could be useful to transfect cells that are in an acidic environment, such as tumor cells. However, additional *in vivo* studies may be required in order to obtain an effective and safe medicine for nonviral gene therapy purpose

Key words

Gene therapy, nonviral vectors, oligomeric chitosan, pCMS-EGFP, pH influence

1. Introduction

One of the essential prerequisites for gene therapy success relies on the development of safe and effective vectors. Nowadays, vectors can be classified into two main groups: viral and nonviral. Viral gene delivery systems are the most effective ones to transfect cells due to their natural properties to infect cells and deliver genetic material with high transfection efficiency. However, the use of viral vectors has many safety concerns, such as immunogenicity, mutagenicity, oncogenic effects and toxicity (Glover et al., 2005; Wang et al., 2011). Therefore, nonviral vectors, such as chitosan, have been raising demanding attention as a safer alternative for human gene therapy.

Since Mumper et al. reported in 1995 the use of chitosan as a gene delivery system (Mumper et al., 1995), numerous authors have studied this polymer as a nonviral vector for nucleic acid (NA) delivery (Mao et al., 2010). Compared to other cationic polymers, chitosan has favorable characteristics, such as biocompatibility, biodegradability, low immunogenicity and reduced cytotoxicity (Borchard, 2001). Moreover, thanks to the amine groups, chitosans are positively charged, which allows the formation of nanoscale complexes by electrostatic interaction with anionic NAs, named polyplexes (Layman et al., 2009; Mao et al., 2010).

According to the physicochemical characteristics of the chitosan, *in vitro* transfection efficiency of chitosan/pDNA polyplexes highly depends on the molecular weight (MW) and the degree of deacetylation (DDA) of the polymer. Polyplexes elaborated with high-MW chitosans are very stable and excellent extracellular DNA protectors, but they show, at the same time, low buffer capacity and transfection efficiency (Lu et al., 2009). According to the DDA, high-DDA chitosan vectors have shown better DNA-binding capacity and more efficient transfection than complexes formed with low-DDA chitosan (Kim et al., 2007). For this reason, we have used Novafect O15 and O25 ultrapure oligochitosans (UOCs), which have been previously reported as efficient nonviral

vectors for gene delivery *in vitro* and *in vivo* (Klausner et al., 2010; Puras et al., 2013a,b).

In addition to DDA and MW, the *in vitro* transfection efficiency of chitosan/pDNA polyplexes is highly sensitive to the pH of the culture medium. According to Zhao *et al*. (2006), the optimum pH for the transfection of chondrocytes, using chitosan/pDNA polyplexes, oscillates between 6.8 and 7.0. While pH values lower than 6.5 increase the cytotoxicity, pH values higher than 7.2 provoke the dissociation of free plasmid from the polyplex, reducing the transfection efficiency (Ishii et al., 2001). Recently, extensive work has been carried out to explore the influence of medium acidification on the *in vitro* transfection capacity of chitosan- based vectors (Lavertu et al., 2006; Nimesh et al., 2010). However, no research has been conducted with polyplexes elaborated by oligomeric chitosan molecules (<10 kDa).

Therefore, the main objective of this work was to evaluate the influence of the pH decrease, to nontoxic limit value (6.5), on the *in vitro* cellular uptake and transfection efficiency of UOC/pDNA polyplexes in HEK293 and ARPE19 cells. For this purpose, several *in vitro* assays were performed with self- assembled UOC/pCMS-EGFP polyplexes. First, an orthogonal (2^3) experimental design was performed to characterize the transfection system in terms of size and zeta potential. Second, a study related to the buffer capacity of the polymers was conducted. Third, the optimal formulation for next experiments was determined by measuring the transfection efficiency and cell viability of the vectors at different N/P ratios (the molar ratios between all protonable amino groups of chitosan and the phosphate groups of pDNA); finally, the influence of the pH decrease on both cellular uptake and transfection efficiency was evaluated at the optimum N/P ratio.

2. Materials and Methods

2.1 Materials

UOC O15 and O25 (MWs of 5.7 and 7.3 kDa, respectively, DDA $\geq$ 97% and endotoxin levels $\leq$ 0.05 EU/mg) and polymeric water-soluble high-MW glutamate

salt chitosan G214 (MW of 340 KDa and DDA ≥ 90%) were purchased from NovaMatrix/FMC (Sandvika, Norway). HEK293 cells, ARPE19 cells and Eagles's Minimal Essential medium with Earle's BSS and 2 mM 1-glutamine (EMEM) were bought from the American Type Culture Collection (ATCC, Teddington, UK). Dulbecco's Modified Eagle's Medium Han's Nutrient Mixture F-12 (1:1) medium was purchased from GIBCO (San Diego, CA). Opti-MEM® I reduced medium, antibiotic/antimicotic solution and Lipofectamine™ 2000 were purchased from Invitrogen (Life Technologies, Paisley, UK). The plasmid pCMS-EGFP, which encodes the EGFP, was purchased from BD Biosciences Clontech (Palo Alto, CA) and amplified by Dro Biosystems S.L. (San Sebastian, Spain). For cellular uptake experiments, pCMS- EGFP plasmid was labeled with fluorescein isothiocyanate (FITC) by Darebio S.L (Alicante, Spain). Phosphate buffer saline (PBS), MES and Cell Counting Kit-8 (CCK-8) were purchased from Sigma-Aldrich (Madrid, Spain). Dapi Fluoromont-G was obtained from SouthernBiotech, and Alexa Fluor 488 Phalloidin was bought from Life Technologies (Paisley, UK).

2.2 Preparation of UOC/pCMS-EGFP polyplexes

UOC/pCMS-EGFP polyplexes were prepared by the self-assembly method with a final DNA concentration of 13.2 μg/ml. Previous to the polyplex formation, pCMS-EGFP and UOC stock solutions were diluted with ultrapure water to obtain final concentrations of 0.5 and 2 mg/ml, respectively. Fixed volumes of UOC solution were added to the DNA solution under vortex mixing (1200 rpm) for 15 s and were allowed to stabilize at room temperature for 30 min (Puras et al., 2013a,b). This incubation period was chosen in order to avoid the aggregation of the polyplexes, since it is reported that the physical stability of this kind of nonviral vectors is time dependent (Anchordoquy & Koe, 2000).

2.3. Fractional factorial experiment

In order to evaluate the influence of the pH of the solution (pH=X1), salt concentration of the solution ([NaCl] = X2)

and N/P ratio (N/P ratio = X3) on the size and zeta potential of the system (UOC/pCMS-EGFP polyplexes), a two-level (low level and high level) three-variable fractional factorial experiment was designed, also known as orthogonal experimental design. To analyze the factorial design, the original measurement units for the experimental variables (uncoded units) were transformed into code units, -1 (low level) and +1 (high level) (Table 1).

Table 1. Experimental trials and interactions transformed into code units.

X_1	X_2	X_3	X_1X_2	X_1X_3	X_2X_3	$X_1X_2X_3$	Response
-1	-1	-1	+1	+1	+1	-1	
+1	-1	-1	-1	-1	+1	+1	
-1	+1	-1	-1	+1	-1	+1	
+1	+1	-1	+1	-1	-1	-1	
-1	-1	+1	+1	-1	-1	+1	
+1	-1	+1	-1	+1	-1	-1	
-1	+1	+1	-1	-1	+1	-1	
+1	+1	+1	+1	+1	+1	+1	

The number of experimental runs that had to be carried out for an orthogonal experimental design is 8 (2^3). The responses, particle size and zeta potential, for each experimental combination, were expressed as nm and mV, respectively (Table 2). The effects of the variables upon the responses could depend on the other studied variables. This phenomenon is called *interaction*, which is included in the table of experimental trials as an additional column (Table 3). In the orthogonal experimental design, we can find these three interactions: X_1X_2, X_1X_3, and X2X3. The effect of each variable and each interaction (Table 3) was calculated by the following equation:

Effect or Interaction = (∑ Responses with positive sign - ∑ Responses with negative sign) / 4

2.4. Measurements of size and zeta potential

The measurements of size and zeta potential were carried out as previously described by Puras et al. (2013a,b) using a Zetasizer NanoZS (Malvern Instruments, UK). All measure- ments were carried out in triplicate. The particle size reported as hydrodynamic diameter was obtained by cumulative analysis. Only data that met the quality criteria according to the software program DTS 5.0 (Zetasizer Nano system, Malvern Instruments, UK) were included in the study.

Table 2: Experimental trials and response in terms of size and zeta potential for O15 and O25 UOC.

				Size (nm)		Zeta (mV)	
Trial	X_1 pH	X_2 N/P	X_3 [NaCl] (mM)	O15	O25	O15	O25
1	6.2	10	10	89.43	337.8	19.5	29.7
2	7.2	10	10	555.9	204.6	3.99	9.2
3	6.2	60	10	135.1	101.7	21.8	21.6
4	7.2	60	10	144.5	159.2	10.6	10.8
5	6.2	10	150	182.6	88.76	14.9	15.1
6	7.2	10	150	333.1	369.4	0.174	6.28
7	6.2	60	150	113.2	244.2	15.8	22.4
8	7.2	60	150	208.3	154.2	5.8	2.87

Table 3: Effects and interactions on size and zeta potential of the UOC/pCMS-EGFP polyplexes.

	Size (nm)		Zeta (mV)	
Effects and Interactions	O15	O25	O15	O25
X_1 e	180.37	28.73	-12.86	-14.91
X_2e	-139.98	-85.31	3.86	-0.65
X_3e	-21.93	13.31	-4.80	-6.16
X_1X_2i	-128.12	-44.98	2.26	-0.25
X_1X_3i	-57.57	66.58	0.50	0.73
X_2X_3i	42.88	55.43	-0.60	2.60

2.5 Buffereing capcity

The buffer capacity of our cationic polymers was determined by the acid–base titration assay. The samples were prepared as follows: 0.1 mg/ml of each chitosan solution was prepared in 10 ml of 150 mM NaCl, and each sample solution was titrated by 0.1 M NaOH to a pH 10. Then, samples were titrated again with 0.1 M HCl solution, in order to obtain mixtures with different pH values. A Crison pH-Meter GLP 21 was used for the measurements.

2.6. In vitro transfection assays

For transfection studies, HEK293 and ARPE19 cells were seeded without antibiotic/antimicotic on 24-well plates at a density of 150,000 and 100,000 cells per well, respectively, and allowed them to adhere to reach 70–90% of confluence at the time of transfection. In order to determine the optimal formulation for the following *in vitro* assays, we studied the transfection efficiency of the polyplexes at N/P ratios ranging from 10 to 60 in HEK293 cells. For

this purpose, formulations were prepared after mixing 1:1 polyplexes (containing 1.65 μg of the pCMS-EGFP) and hypertonic transfection medium (Opti- MEM® I, containing 580 mM of mannitol) at pH 7.4. After 4 h of incubation with the vectors at 37 ºC, the transfection medium was removed and replaced with complete medium. Cells were allowed to grow 72 h before processing them for fluorescent microscopy and flow cytometry analysis. Experimentswith Lipofectamine™ 2000/pCMS-EGFP complexes were prepared at a 2/1 ratio (w/w) according to the manufacturer's protocol and were used as our positive controls.

Next transfection assays were designed to evaluate the pH influence on the transfection efficiency of the selected formulations in HEK293 and ARPE19. In this case, the transfection assay was carried out following the procedure described in the preceding paragraph, but including also transfection at pH 6.5.

2.7. Analysis of EGFP expression

Qualitative expression of EGFP was analyzed using an inverted microscope equipped with an attachment for fluorescent observation (model EclipseTE2000-S, Nikon). Flow cytometry analysis was conducted using a FACSCalibur system flow cytometer (Becton Dickinson Biosciences, San Jose, CA). By the end of the incubation period, cells were detached with 300 μl of 0.05% trypsin/EDTA. After detachment, complete medium was added to inhibit trypsin activity. Then, cells were centrifuged at 1500 x*g*, and the supernatant was discarded. Cells were resuspended in cold PBS, diluted in FACSFlow liquid and transferred to specific flow cytometer tubes to quantify EGFP expression in live cells. For each sample 10,000 events were collected. Transfection efficiency was quantified by measuring the fluorescence of EGFP at 525 nm (FL1). Control samples (nontransfected cells) were displayed on a dot plot of forward scatter against side scatter to establish a collection gate and exclude cells debris and dead cells. The samples containing Lipofectamine-transfected cells were used as a control to compensate FL2 signal in FL1 channel.

Transfection data were normalized using Lipofectamine™ 2000 (mean ± SD; *n=3*).

2.8. Cell uptake studies

For cell uptake studies, HEK293 and ARPE19 cells were manipulated as described in the section ‘‘*In vitro transfection assays*’’ for transfection experiments. Then, the regular growth media was removed from the well and the cells were exposed to polyplexes, containing 1.65 μg of the pCMS- EGFP labeled with fluorescein isothiocyanate (FITC-pCMS-EGFP), at both pH. After 4 h of incubation with the vectors at 37 ^{o}C, the transfection medium was removed and cells were washed with PBS and fixed with formaldehyde 4% for the quantitative and qualitative analysis, by flow cytometry (FACSCalibur, Becton Dickinson Biosciences, San Jose, CA) and fluorescence microscopy (Eclipse TE200-S, Nikon Instruments Europe B.V., Amstelveen, The Netherlands), respectively. Each formulation was analyzed by triplicate.

To obtain cellular uptake quantitative data, cells were analyzed after 4 h of incubation by flow cytometry at 525 nm (FL1) after detachment from the wells. Ten thousand events were collected for each sample. Cellular uptake data were expressed as the percentage of FITC-positive cells. For the qualitative study, cells were seeded in coverslips containing plates and treated with the vectors. After 4 h of incubation, preparations were mounted on Dapi Fluoromount- G for their posterior analysis. Images were examined under the microscope.

2.9. Cell viability and cytotoxicity

Potential cytotoxicity of selected UOC/pDNA polyplexes was determined quantitatively by BD Via-Probe kit and CCK-8 assays and qualitatively by analyzing cell morphology under microscope observation after phalloiding staining.

In order to determine the optimal formulation for the following *in vitro* assays, BD Via-Probe kit assay was performed. The measurements were performed at 72 h on the same selected samples as those used in the transfection

efficiency studies (see section ‘‘*In vitro* transfection assays’’). The BD Via-Probe kit reagent (5 μl) was added to each sample, and after 10 min of incubation, fluorescence of dead cells was measured at 650 nm (FL3) on a FACSCalibur system flow cytometer. Control samples (cells incubated at pH 7.4 without polyplexes) were used as controls to compensate FL2 signal in FL3 channel.

Once the optimal formulation was determined, the cytotoxicity of the selected polyplexes was evaluated by the commercially available CCK-8, which measures cellular dehydrogenase activity. Briefly, HEK293 and ARPE19 cells were seeded onto 96-well plates at a density of 25,000 and 10,000 cells per well, respectively, and allowed to adhere to reach 70–90% of confluence. Then, 50 μl of different formulations were added to the cells. After 4 h of incubation at 37 ºC, the medium containing the formulations was replaced with 100 μl of complete medium and cells were further incubated for 72 h under the same conditions. Then, 10 μl of the CCK-8 solution reagent was added to each well and the plate was incubated at 37 ºC for 3 h. The absorbance was measured at 450 nm using a microplate reader (Multiscan EX, Labsystems, Helsinki, Finland). Results were expressed as relative cell viability (%) compared to control cells (untreated cells): $[Fluorescence]_{sample}/[Fluorescence]_{control}$ x 100.

For cell morphology analysis, HEK293 and ARPE19 cells were stained with Alexa Fluor 488 phalloidin following the manufacturer’s protocol. Briefly, after 4 h of incubation with polyplexes, cells were washed with PBS and fixed with 4% formaldehyde. Then, Alexa fluor 488 phalloidin stock solution was added and cells were incubated for 40 min at room temperature. Afterward, they were washed again and the preparations were mounted on Dapi Fluoromount-G for their posterior analysis under the microscope.

3. Results and discussion

3.1 Effects and interactions of medium pH, salt concentration and chitosan/DNA N/P ratio.

The most important physicochemical properties that affect the *in vitro* transfection efficiency of chitosan/pDNA polyplexes are the size and the zeta potential. However, the exact influence of factors like medium pH and salt concentration and N/P ratio on these properties remains unclear (Gao et al., 2009). For this reason, an orthogonal experimental design was chosen to address the effect of these three factors. The design matrix of uncoded values for the factors and the responses, in terms of size and zeta potential, are shown in Table 2. Moreover, the effects and the interactions of the factors are shown in Table 3. As observed in Table 3, the interaction values for the particle size are high, explaining that the effect of each factor on the particle size depends on the rest of the analyzed factors. The clearest data showing dependence between two factors could be observed in the X_1X_2 interaction for the particle size of O15 polyplexes, whose value was -128.12. This means that depending on whether the pH of the medium is acidic or basic, the effect of N/P ratio on the particle size of O15 polyplexes is just the opposite. For example, when the osmolarity value is low, the increment of the N/P ratio from 10 to 60 at pH value of 6.2 increases the size, while at pH 7.2 it decreases. As observed in Table 3, the independent effects of each factor in the particle size indicate that the increase of the pH from 6.5 to 7.4 increases the size of the polyplexes (+180.37 nm for O15 and +28.73 nm for O25); the increase of the N/P ratio from 10 to 60 reduces the size (-139.98 nm for O15 and -85.31 nm for O25) and the increase of salt concentration from 10 to 150 mM hardly affects the particle size (-21.93 nm for O15 and +13.31 nm for O25). However, there are certain results in conflict with the above-mentioned independent effects. For example, there is an increase in the particle size when the N/P ratio is raised from 10 to 60, as it is the case of trials 1 and 3 for O15, and trials 5 and 7 for O25 (Table 2). Therefore, due to the strong interactions that exist between the three factors, it is essential to do an overall analysis of the variables in order to describe the exact behavior of the system.

Some authors suggest that the transfection efficiency of chitosan/pDNA

nanoparticles does not depend on the size in the range of 250–1300 nm (Duceppe & Trabrizian, 2010). On the contrary, Lavertu et al. (2006) showed that polyplexes around 700 nm in diameter were able to produce higher EGFP expression in HEK293 cells comparing with smaller ones (280 nm). In addition, other authors have demonstrated that the final size of the polyplex can affect transfection, as the endocytic pathways differ with regard to the size of the endocytic vesicle (Conner & Schmid, 2003). Due to these conflicting results, most of the research groups support the theory that the final size of the polyplex is not as important as the zeta potential on the stability of suspensions, on the adhesion of particle systems onto biological surfaces and therefore on the transfection efficiency. Consequently, we suggest that the investigation of the zeta potential is the main issue in the design of efficient nonviral vectors for gene delivery purposes, being necessary a positive zeta potential for an efficient *in vitro* transfection (Lee et al., 2004; Duceppe& Trabrizian, 2010).

As observed in Table 2, UOC/pCMS-EGFP polyplexes have a positive surface charge. This is explained by the residual protonated amine groups of UOC that do not take part in the neutralization with the negatively charged phosphate groups of the pDNA. Furthermore, it should be stressed out the absence of interactions for the zeta potential, which explains that the effect of each factor on the zeta potential of the polyplexes is independent. Regarding the effect of the pH on the zeta potential, it is clearly observed from Table 3 that an increase in the pH value from 6.2 to 7.2 results in a decrease of the zeta potential of 12.86 and 14.91 mV for both UOC O15 and O25, respectively. Moreover, although it is a weaker effect, an increase in the suspension buffer salt concentration decreases the superficial charge of the polyplexes. Regarding the N/P ratio, it does not significantly affect the zeta potential. This could be due to the selected low N/P ratio (N/P 10), which might be too high to observe differences in zeta potential results. However, we should take into consideration that the behavior of the polyplexes elaborated with UOC could be different

from what it has been observed with other higher MW chitosans. Accordingly, as it has been described for Novafect O15 and O25 chitosans, no significant differences have been observed in the zeta potential values when the N/P ratio raises from 10 to 60 (Klausner et al., 2012; Puras et al., 2013a,b).

This dependence of the zeta potential on the pH and the salt concentration is consistent with the results obtained in trials 6 and 8, where almost electrostatically neutral polyplexes were found at pH value of 7.2 and [NaCl] of 150 mM, while superficial charge became highly positive at pH 6.2 and [NaCl] 10 mM. Similarly, other authors have also concluded that the charge of chitosan/pDNA complexes strongly depends on the pH and salt content of the suspension medium (Lee et al., 2004; Nimesh et al., 2010).

3.2 Buffer capacity of UOCs

Endosomal release of the pDNA is one of the most important steps that must be overcome to avoid lysosomal degradation. It has been reported that the capacity to escape from the endosome depends on the intrinsic buffer capacity of the vector system (Tripathi et al., 2012). As reported by the literature, chitosans have a modest buffering capacity comparing with other cationic synthetic polymers, such as chitosan-based oligoamine polymers and polyethylenimine, which reduces its biomedical applications (Lu et al., 2009).

Despite the poor buffering capacity of chitosans, we hypothesized that this quality could be improved with the use of oligomeric chitosans (<10 kDa), such as O15 and O25. Accordingly, as observed in Figure 1, our data indicate that UOCs have significantly higher buffer capacity compared to the high-MW chitosan (G214), which is more significant in the pH interval between 9 and 3. This is in agreement with a study that showed that the buffer capacity of chitosan copolymers decreased when the MW of chitosan was increased (Lu et al., 2009).

3.3 Selection of the optimal formulation by transfection and viability assays in HEK293 cells

In order to establish the best

formulations for the following studies, HEK293 cells were transfected by both UOC polyplexes at N/P ratios from 10 to 60. As reported by other authors, the relative percentage of transfected cells strongly depends on the N/P ratio of chitosan polyplexes (de Martimprey et al., 2009; Strand et al., 2010). In our work, the maximum relative percentage of EGFP-positive cells was obtained at N/P ratios of 10, 20 and 30, while the lowest transfection level was at N/P of 60 (Figure 2).

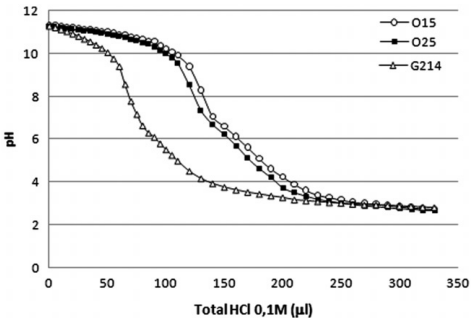

Figure 1 Acid–base titration profiles of O15 UOC, O25 UOC and G214 chitosan in 150 mM NaCl solution.

According to the results obtained by BD Via-Probe kit assay, the increment of N/P ratio seems to affect negatively the viability of the cells. Therefore, taking into account the transfection and viability results, formulations elaborated at N/P ratio of 20 were selected as the most suitable for next *in vitro* assays.

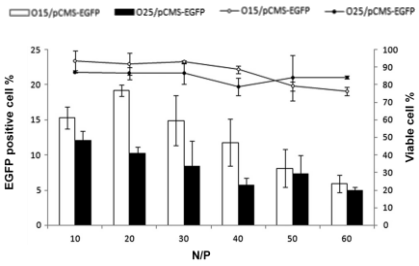

Figure 2. EGFP expression and cell viability in HEK293 cells after the transfection with O15 (white) and O25 (black) UOC/pCMS-EGFP polyplexes at N/P ratios from 10 to 60. Transfection data were normalized to Lipofectamine 2000™.

The hydrodynamic diameter and superficial charge of the polyplexes were determined in the selected formulations. As it is observed in Figure 3, medium acidification from 7.4 to 6.5 reduces the particle size of all the polyplexes, especially the ones elaborated with O15 and O25 UOC. In relation to the superficial charge, polyplexes elaborated at pH 6.5 have a significantly higher zeta potential, compared to those formed at pH 7.4. Comparing the

polyplexes elaborated with O15 and O25 UOC with those formed with G214 chitosan, it is clearly observed that when the MW of the chitosan is higher, the particle size and zeta potential of the formed vectors increase. These results are in accordance with those previously published (Huang et al., 2004).

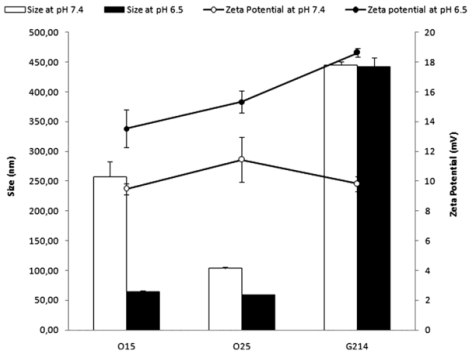

Figure 3. Size and zeta potential of O15 and O25 UOQ/pCMS-EGFP and G214/pCMS-EGFP polyplexes (N/P ratio of 20) at pH 7.4 (white) and 6.5 (black)

3.4 Cellular uptake of the FITC-pCMS-EGFP

The cell uptake is the first cellular barrier that influences the transfection efficiency of polyplex (Yue et al., 2011). To evaluate the internalization of FITC-pCMS-EGFP in HEK293 and ARPE19 cell lines, the cellular uptake experiments were carried out with three different formulations based on O15, O25 and G214 at pH 7.4 and 6.5 and N/P of 20. According to our results, cell uptake of FITC-pCMS-EGFP by both cell types strongly depends on the pH value, being higher at pH 6.5 in both cell lines (Figure 4). The higher cellular uptake at pH 6.5 can be explained by the strong increase of the zeta potential after medium acidification, as observed in Figure 3. In accordance with other authors, we have concluded that the positive superficial charge of the polyplexes allows the electrostatic interaction with the negatively charged cell membrane, increasing the percentage of the cellular uptake (Nimesh et al., 2010; Yue et al., 2011; Kang et al., 2012).

Furthermore, as it can be observed in Figure 3, polyplexes elaborated at pH 6.5 present a smaller particle size compared to those formed at pH 7.4, especially with O15 and O25 UOCs. This reduction of the particle size could also explain the higher cellular uptake achieved at pH 6.5. Surprisingly, in ARPE19 cells, polyplexes

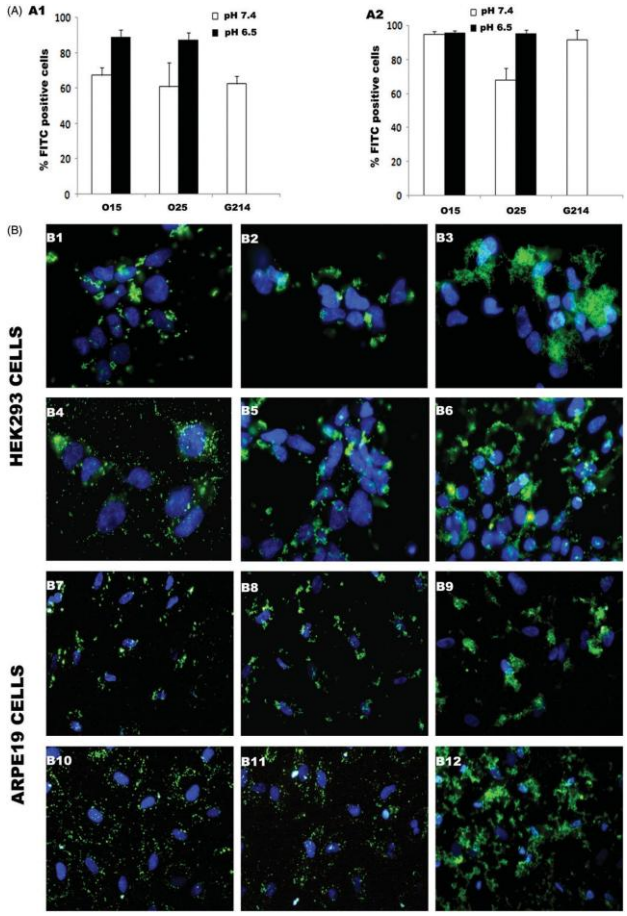

Figure 4. Cellular uptake of FITC-labeled pCMS-EGFP delivered by UOC polyplexes (N/P = 20). Percentage of FITC-positive cells treated with O15 and O25 UOC and G214 chitosan at pH 6.5 and 7.4 in HEK293 (A1) and ARPE19 (A2) cell lines. Fluorescent images of FITC-pCMS-EGFP uptake: O15 UOC/FITC-pCMS-EGFP at pH 7.4 in HEK293 (B1); O25 UOC/FITC-pCMS-EGFP at pH 7.4 in HEK293 (B2); G214/FITC-pCMS-EGFP at pH 7.4 in HEK293 (B3); O15 UOC/FITC-pCMS-EGFP at pH 6.5 in HEK293 (B4); O25 UOC/FITC-pCMS-EGFP at pH 6.5 in HEK293 (B5); G214/FITC-pCMS-EGFP at pH 6.5 in HEk293 (B6); O15 UOC/FITC-pCMS-EGFP at pH 7.4 in ARPE19 (B7); O25 UOC/FITC-pCMS-EGFP at pH 7.4 in ARPE19 (B8); G214/FITC-pCMS-EGFP at pH 7.4 in ARPE19 (B9); O15 UOC/FITC-pCMS-EGFP at pH 6.5 in ARPE19 (B10); O25 UOC/FITC- pCMS-EGFP at pH 6.5 in ARPE19 (B11); G214/FITC-pCMS-EGFP at pH 6.5 in ARPE19 (B12).

elaborated with O15 UOC at pH 7.4 shown a similar cellular uptake to those at pH 6.5.

These polyplexes elaborated at pH 7.4 have a particle size around 250 nm, which is significantly higher than the rest of the UOC-based polyplexes. In addition, polyplexes formed with G214 chitosan, which particle size is above 400 nm, shown around 90% of cellular uptake. These results suggest that the particle size is not a critical factor for the cellular uptake process in ARPE19 cells. Thus, the influence of the particle size may be different depending on the tested cell line as Conner & Schmid (2003) concluded. In addition, it should be highlighted the lower cellular uptake obtained in HEK293 cells compared to ARPE19 cells. Based on these results, we suggest the possibility of a large- cell-type-dependet internalization of the polyplexes. However, for a better understanding of this issue, more specific assays about the internalization procedure of the polyplexes line are needed.

Huang *et al*. (2004) reported that the MW of the chitosan also influences the cellular uptake. According to them, the uptake of chitosan nanoparticles by A549 cells fell by 26% when chitosan MW decreased from 213 to 10 kDa. Contrary to these results, we observed that the polyplexes elaborated with oligomeric chitosans (O15 and O25) presented a similar cellular uptake compared to those elaborated with high MW G214, especially in HEK293. Despite the apparent importance of the MW on the cellular uptake, these authors claimed that chitosan DDA has a higher influence, describing a 41% decrease when DDA was lowered from 88 to 46%. This could explain why the cellular uptake for G214 (DDA of 92%) was not higher than that obtained with UOCs (DDA 99%). Moreover, other authors have concluded that the MW of the cationic polymer does not influence on the cellular uptake of the polyplexes (Layman et al., 2009), which reinforce the higher importance of chitosan DDA on this process.

3.5. Influence of pH decrease on the transfection efficiency of UOC/pCMS-EGFP polyplexes.

According to literature, the transfection efficiency presented by chitosan-based gene carriers is not sufficient for practical applications, and, therefore, its improvement has become the main goal in many research works (Wang et al., 2011). In addition, the determination of the best formulation conditions for an efficient *in vitro* transfection is a critical step in the development of clinically relevant chitosan-based nonviral gene carriers (Strand et al., 2005). For this reason, we measured the relationship between the experimental parameters and the transfection capacity of UOC/pCMS-EGFP polyplexes in HEK293 and ARPE19 cells.

The efficacy of gene delivery is strictly controlled by the ability of the polymer to bind, protect and release its pDNA cargo (Strand et al., 2010). An accurate balance between DNA- binding capacity and DNA release must be achieved. Transfection efficiency is hampered by great binding strengths between chitosan carrier and its pDNA cargo (Alatorre-Meda et al., 2011). We have recently described that after agarose gel electrophoresis assay, cationic UOCs are able to complex, release and protect the pDNA in a N/P ratio range from 10 to 30 (Ishii et al., 2001; Puras et al., 2013a,b).

We have recently demonstrated that the transfection efficiency of UOC/pDNA polyplexes drastically increases, in HEK293 cells, when pH slightly decreases from 7.4 to 7.1. However, O25 UOC/pDNA polyplexes were not able to transfect ARPE19 cells, neither at pH 7.4 nor at pH 7.1 (Puras et al., 2013a,b). For this reason, we decided to further decrease the pH, to nontoxic limit value (6.5), with the aim of improving the *in vitro* results obtained at pH 7.1.

Surprisingly, the chitosan/plasmid complexes elaborated by Ishii et al. were unable to transfect CHO cells at pH 6.5, while at pH 7 the transfection efficiency was optimal. In, contrary, according to our results, a significant increment of EGFP-positive cells occurs when the pH is acidified from 7.4 to 6.5, with both UOCs and in both cell lines (Figure 5). This is in

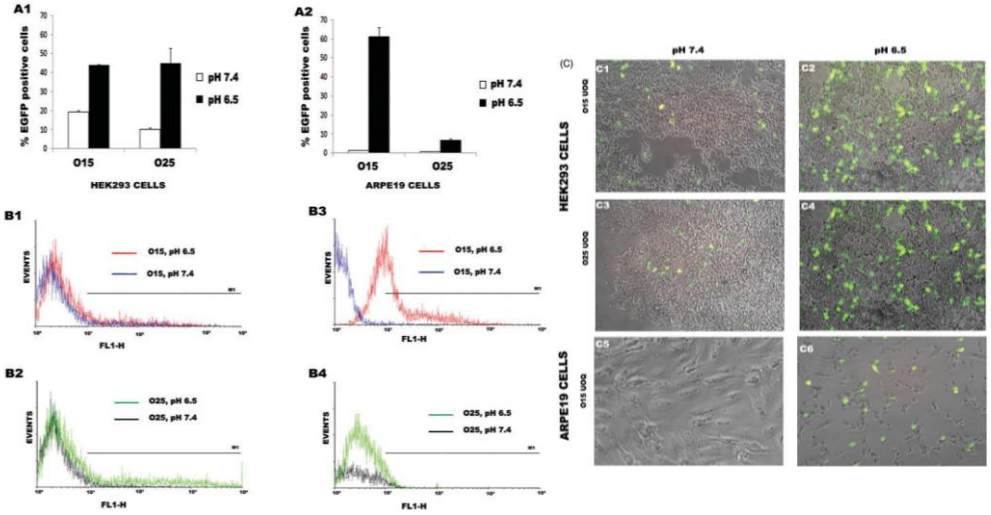

Figure 5. Transfection efficiency of O15 and O25 UOC/pCMS-EGFP polyplexes (N/P ¼ 20) at different pH values in HEK293 (A1) and ARPE19 cells (A2). Transfection data were normalized to Lipofectamine 2000™. Flow cytometry histograms (FL1/events) comparing transfection efficiencies at pH values of 6.5 and 7.4: HEK293 cells transfected with O15 UOC/pCMS-EGFP polyplexes (B1); HEK293 cells transfected with O25 UOC/pCMS-EGP polyplexes (B2); ARPE19 cells transfected with O15 UOC/pCMS-EGFP polyplexes (B3); ARPE19 cells transfected with O25 UOC/pCMS-EGFP polyplexes (B4). Overlay of phase contrast image with fluorescent illumination (GFP channel) to evaluate transfection efficiency: HEK293 cells transfected with O15 UOC vectors at pH 7.4 (C1); HEK293 cells transfected with O15 UOC vectors at pH 6.5 (C2); HEK293 cells trasnfected with O25 UOC vectors at pH 7.4 (C3); HEK293 cells transfected with O25 UOC vectors at pH 6.5 (C4); ARPE19 cells transfected with O15 UOC vectors at pH 7.4 (C5); ARPE19 cells trasnfected with O15 UOC vectors at pH 6.5 (C6).

agreement with previously reported results in HEK293 and ARPE19 cells (Sato et al., 2001; Nimesh et al., 2010). Nevertheless, polyplexes based on high-MW G214 chitosan did not show transfection in any of the conditions (data not shown), although they present high cellular uptake levels. This could be explained in part due

to the low capacity of these high-MW chitosan to release the DNA once inside the cell, as it has been reported it in the literature (Klausner et al., 2012). Furthermore, it must be highlighted that the significant increase in transfection efficiency is observed in ARPE19 cells using UOCs at pH 6.5, since this cell line was not transfected at pH 7.4.

As confirmed with zeta measurements, at a lower pH value UOC/pCMS-EGFP polyplexes have more positive surface charge (Figure 3), which favors the binding with the negatively charged cell membrane and the penetration into the cell (Figure 4), resulting in a better transfection efficiency (Figure 5).

It has been described that highly protonated chitosan complexes (+21 mV) cannot release from the endosome, which hampers the gene expression (Ishii et al., 2001). Despite the positive superficial charge (around +15 mV) of our UOC-based carriers, we suggest that they have enough ability for proton absorption, facilitating the rupture of the endosomal vesicles and the release of the polyplex into the cytoplasm. The lack of transfection observed with G214 polyplexes could be explained by the low buffer ability of G214 chitosan, which enables endosomal escape (Panyam & Labhasetwar, 2003; Lu et al., 2009). Furthermore, O15 UOC polyplexes showed higher relative percentage of EGFP-positive cells compared to O25 UOC in ARPE19 cells at pH 6.5. This significant increase in transfection did not correspond to cell uptake results. Some authors have reported that differences in the intracellular trafficking and nuclear transport of the delivered pCMS-EGFP could explain the absence of correlation between the amount of internalized pCMS-EGFP and the level of transgene expression (Strand et al., 2010). However, this hypothesis should be verified in future experiments by tracing the localization of endosomes and DNA with fluorescent probes.

These *in vitro* transfection results support the potential use of UOC-based polyplexes for delivering genetic materials into cells, which are in an acidic environment. For instance, one of the most

direct application of these polyplexes could be their local administration in tumor tissues, where the pH is more acidic than the physiological value (Tannock & Rotin, 1989; Han et al., 2010; Noh et al., 2010; Salva et al., 2010; Huang et al., 2012). In addition, considering the lower pH value of the vitreous humor, these polyplexes could be administered by intravitreous injection, achieving considerable gene expression levels (Bassnett & Duncan, 1985; Puras et al., 2013a,b).

3.6. Cell viability and cytotoxicity

To assess the potential citotoxicity of the UOC/pCMS-EGFP polyplexes and the influence of pH decrease on cell viability, CCK-8 assay was performed. It revealed the low cytotoxicity of UOC/pCMS-EGFP polyplexes after 72 h (Figure 6A). Slightly lower cell viability was observed in cells treated with polyplexes at pH 6.5, in both HEK293 and ARPE19 cell lines. This decreased viability could be explained by the increment of the superficial charge of polyplexes at pH 6.5. It has been demonstrated that the cytotoxicity is related to the interaction between polyplexes and cell membranes, which is higher with positively charged polyplexes (Nimesh et al., 2010). Cells incubated with Lipofectamine™ 2000 transfection reagents showed a considerable lower cellular dehydrogenase activity in comparison to cells transfected with polyplexes, which confirms the cytotoxic effect of Lipofectamine 2000™ on HEK293 and ARPE19 cells.

Actin–phalloiding staining did not show morphological disorders of cells transfected with UOC/pCMS-EGFP at pH 7.4 (Figures 6B2 and B5). When the experiments were carried out at pH 6.5 values, cell morphology was slightly affected, especially in ARPE19 cells (Figures 6B1 and B4), although cell viability assays did not demonstrate cytotoxicity. As it can be seen in Figures 6(B3 and B6), after the treatment with Lipofectamine 2000™, the morphology of HEK293 and ARPE19 cells completely changed, which indicates the higher toxicity of this lipidic vectors compared to UOC-based vectors.

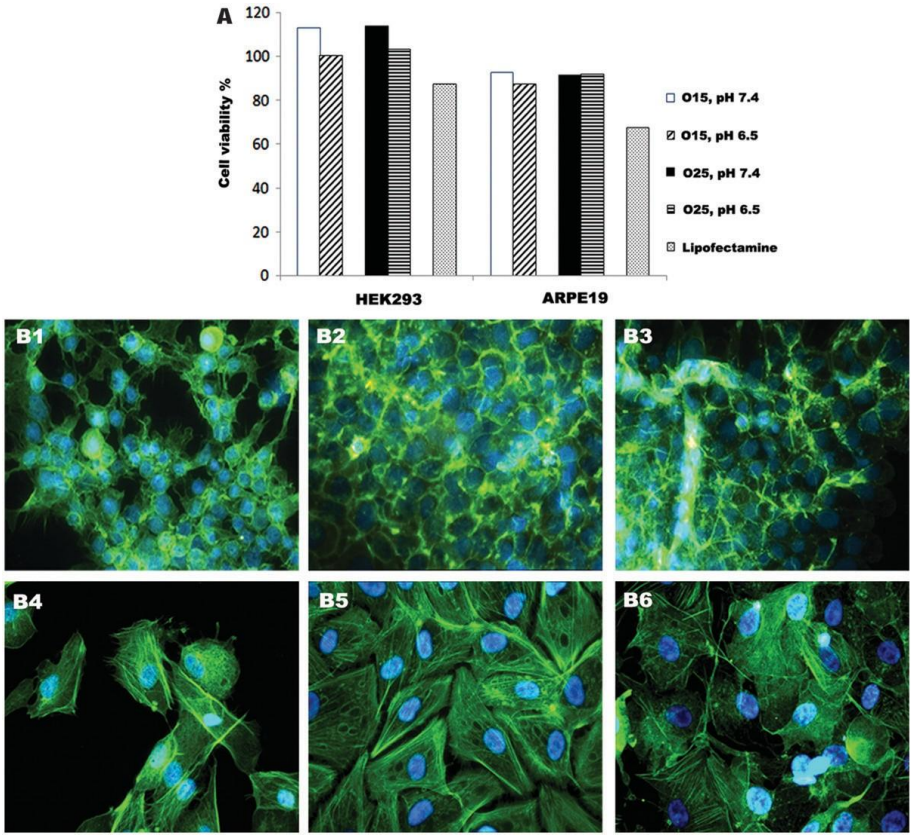

Figure 6. Cell viability and morphology of HEK293 and ARPE19 cells after transfection with O15 and O25 UOC vectors (N/P =20) and Lipofectamine 2000™. Influence of pH on cell viability of HEK293 and ARPE19 cells by CCK-8 assay (A). Phalloidin and DAPI staining of HEK293 and ARPE19 cells after 4 h transfection; HEK293 cells transfected with O25 UOC vectors at pH value of 6.5 (B1); HEK293 cells tranfected with O25 UOC vectors at pH value of 7.4 (B2); HEK293 cells transfected with Lipofectamine 2000™ (B3); ARPE19 cells transfected with O25 UOC vectors at pH value of 6.5 (B4); ARPE19 cells transfected with O25 UOC vectors at pH value of 7.4 (B5); ARPE 19 cells transfected with Lipofectamine 2000™ (B6).

In resume, our results indicate a very low toxic effect of the UOC/pCMS-EGFP polyplexes on these cells, regardless of the pH of the incubation medium.

4. Conclusion

In this study we found that the pH value of the medium clearly affects the physicochemical properties of our UOC/ pCMS-EGFP polyplexes, turning them more positively charged when we acidified the medium. The strong increase of the zeta potential enhances the electrostatic interaction between the vectors and cell membrane, improving the cellular uptake and the transfection efficiency in both HEK293 and ARPE19 cells. Buffer capacity of UOCs contributes to an efficient intracellular trafficking compared to high-MW counterparts. Neither of our UOC/pCMS-EGFP vectors caused toxicity in our experimental conditions. However, cells treated with Lipofectamine 2000™ showed decreased cell viability. These results demonstrated the relevance of understanding the physicochemical behavior of UOC-based vectors for its biomedical application. Accordingly, this kind of vectors could be useful to transfect cells that are in an acidic environment, such as tumor cells. However, additional *in vivo* studies may be required in order to obtain an effective and safe medicine for nonviral gene therapy purpose.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Chapter 2

Delivery of an adenovirus vector plasmid by ultrapure oligochitosan based polyplexes

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Purutasun handiko oligokitosanoz (UOC) eraturiko bektore ez-biralekin posible izan da korneara eta erretinara pCMS-EGFP plasmidoa (5,5kbp) garraiatzea eta transfektatzea. Hala ere, orain arte ez du inork frogatu UOCekin eraturiko poliplexoak tamaina askoz handiagoko plasmidoak transfektatzeko gai direnik. Izan ere, adenobirus onkolotikoa kodifikatzen duten plasmidoak (40kbp) transfektatzea, tamaina txikiko plasmidoekin alderatuz, erronka zailagoa da. Lan honetan, O15 eta O25 UOC/pAdTLRGD poliplexoak eratu genituen berezko elkarketaren metodoaren bitartez, eta diseinu experimental ortogonalarekin N/P ratioak, transfekzio ingurunearen pHak eta gatzen kontzentrazioak partikula tamainan eta zeta potentzialean duten eragina aztertu genuen. Prestatutako poliplexo guztiek partikula tamaina 200nm baino txikiagoa eta zeta potentzial positiboa zeukaten. Bi parametro hauek argi eta garbi N/P ratioaren, transfekzio ingurunearen pHaren eta gatzen kontzentrazioaren eraginpean zeudela ikusi zen. Era berean, ezaugarri honak medio aukeratutako poliplexo guztiak izan ziren gai plasmidoa lotu, askatu eta DNAasen degradaziotik babesteko. Gainera, HEK293 eta A549 zeluletan eginiko transfekzio saioek demostratu zuten UOC/pAdTLRGD poliplexoak gai direla plasmidoa egoki garraiatu eta bi zelula motak transfektatzeko. Beraz, emaitza hauen arabera, O15 eta O25 OUCekin eratutako poliplexoak etorkizuneko minbiziaren kontrako terapia geniko onkolitikoaren *in vivo* aplikazioetan erabilgarriak direla iradoki daiteke.

Recientemente se ha descrito que los oligoquitosanos ultrapuros (UOC) son vectores eficientes para el transporte del pCMS-EGFP (5,5 Kbp) a cornea y retina. Sin embargo, la vehiculización de plásmidos oncolíticos representa un reto único ya que, hasta el momento no se ha probado la capacidad de estos quitosanos para la trasportar plásmidos de 40 Kbp. En este trabajo, elaboramos poliplexos basados en UOC O15 y O25 auto ensanblados con el plásmido pAdTLRGD, y estudiamos la influencia del ratio N/P, el pH del medio de transfección y la concentración de sales en el tamaño y potencial zeta de dichos vectores, mediante un diseño experimental ortogonal. Todos los poliplexos mostraron un tamaño menor a 200 nm y un potencial zeta positivo. Estos parámetros se vieron afectadas por el ratio N/P, el pH y la concentración de sales. Los poliplexos seleccionados fueron capaces de retener, liberar y proteger el plásmido de la degradación enzimática por DNasas. Los experimentos de transfección llevados a cabo en células HEK293 y A549 demostraron que estos poliplexos son capaces de tranportar el plásmido y transfectar ambas líneas celulares. Estos resultados sugieren que los poliplexos basados en oligoquitosanos O15 y O25 podrían ser apropiados para futuras aplicaciones *in vivo*.

Delivery of an adevirus vector plasmid by ultrapure oligochitosan based polyplexes

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ABSTRACT

Ultrapure oligochitosans have been recently reported as efficient non-viral vectors for the delivery of pCMS-EGFP plasmid (5.5 kbp) to the cornea and retina. However, the delivery of oncolytic adenoviral plasmids (40 kbp) represents a unique challenge. In this work, we elaborated self assembled O15 and O25 UOC/pAdTLRGD polyplexes, and we studied the influence of the N/P ratio, the pH of the transfection medium and the salt concentration on the particle size and zeta potential by an orthogonal experimental design. All polyplexes showed a particle size lower than 200 nm and a positive zeta potential. These parameters were influenced by the N/P ratio, salt concentration, and pH of the transfection medium. The selected polyplexes were able to bind, release, and protect the plasmid from DNase degradation. Transfection experiments in HEK293 and A549 cell lines demonstrated that UOC/pAdTLRGD polyplexes were able to deliver the plasmid and transfect both cell lines. These results suggest that O15 and O25 UOC based polyplexes are suitable for future *in vivo* applications.

Key words

Chitosan, transfection efficiency, non-viral vectors, adenovirus vector plasmid, EGFP

1. Introduction

Cancer gene therapy, along with immunotherapy, represents a challenging approach to circumvent some of the limitations associated to conventional cancer treatment by chemotherapy, radiotherapy or surgery (World Health Organization (WHO) 2014). In fact, more than 63% of all gene therapy clinical trials focus on cancer. As adenovirus is a suitable vector for transient cancer cell modification, adenoviral vectors are the most frequently used in cancer gene therapy (Gene Therapy Clinical Trials Worldwide, 2014).

An emerging approach that raised interest for cancer treatment is the use of oncolytic adenoviruses (Buonaguro et al., 2012; Zhou and Tang, 2014). Replication-competent oncolytic adenoviruses are able to replicate selectively in tumor cells and, moreover, the progeny adenovirus has the capability to spread through the tumor tissue infecting other cancer cells. In addition, to potentiate their therapeutic efficiency, oncolytic adenoviruses have been armed with therapeutic genes or nucleic acid inhibitors (Alemany, 2012) However, the interaction of the adenovirus capsid with blood factors causes difficulty for the amount of virus that reaches tumors systemically. Due to the theoretically unlimited packing capacity of non-viral vectors (Charbel Issa and MacLaren, 2012), these may be used as delivery vehicles of highly infectious adenovirus plasmids (Kwon et al., 2011; Kim et al., 2012) that could solve the numerous limitations of injecting virus particles in the bloodstream by avoiding interactions with Kupffer liver cells, platelets, erythrocytes, complement, clotting factors and neutral- izing antibodies (Coughlan et al., 2010).

Among non-viral carriers, chitosan, a linear polysaccharide of β-1–4 linked *N*-acetyl-D-glucosamine and D-glucosamine subunits connected by a (1–4) glycosidic bond, presents favorable characteristics as a non-viral gene vector: it is biocompatible, biodegradable, low immunogenic, and non-toxic (Kean and Thanou, 2010; Strand et al., 2001; Filion et al., 2007). Considering the favorable properties of chitosan for gene delivery purposes (Mao et al., 2010; Buschmann et al., 2013), its inherent

anticancer activity (Tan et al., 2009; Noh et al., 2010; Huang et al., 2012; Liu et al., 2014; Ki et al., 2014), and our previous results that support the use of ultrapure oligochtiosan (UOC) as efficient delivery system for pCMS-EGFP reporter plasmid (5.5 kbp) in slightly acidic environments, such as tumor tissues (Agirre et al., 2015), we propose the delivery of large oncolytic adenoviral plasmids mediated by UOC. As a first step for a future efficient oncolytic adenoviral gene therapy, we have evaluated the capacity of O15 and O25 UOC based polyplexes to deliver and transfect the replication incompetent adenoviral plasmid pAdTLRGD (40 kbp) expressing the EGFP and luciferase reporter genes, in human embryonic kidney cells (HEK293) and in a human lung adenocarcinoma epithelial cell line (A549). For this purpose, the polyplexes were elaborated by the self-assembly method and an orthogonal (2^3) experimental design was performed to evaluate the influence of factors such as N/P ratio, pH, and salt concentration on the size and zeta potential of the polyplexes. The binding, release and protection capacity of the polyplexes was evaluated by electrophoresis in agarose gels and finally, the expression of the reporter gene (EGFP) was evaluated in HEK293 and A549 cells.

2. Material and methods

2.1. Materials

O15 and O25 UOCs (MWs of 5.7 kDa and 7.3 kDa, respectively, DDA ≥ 97% and endotoxin levels ≤ 0.05 EU/mg) were purchased from NovaMatrix/FMC (Sandvika, Norway). HEK293 cells, A549 cells and Eagles's minimal essential medium with Earle's BSS and 2 mM l-glutamine (EMEM) were bought from the American type culture collection (ATCC). Dulbecco's modified Eagle's medium (DMEM) Han's nutrient mixture F-12 (1:1) medium was purchased from GIBCO (San Diego, California, US). Opti-MEM® I reduced medium and Lipofectamine™ 2000 were purchased from Invitrogen (Life Technologies, US). Phosphate buffer saline (PBS), Agarose D5 and MES were purchased from Sigma–Aldrich (Madrid, Spain). pAdTLRGD has been previously

described (Bayo-Puxan et al., 2006). This plasmid was grown in *E. coli* DH5alpha strain using Luria–Bertani broth with ampicillin at 100 μg/ml and purified using the Invitrogen PureLink HiPure Plasmid Maxiprep kit (Invitrogen, Carlsbad, CA).

2.2. Preparation of UOC/pAdTLRGD polyplexes

UOC/pAdTLRGD polyplexes were prepared by the self-assembly method with a final DNA concentration of 13.2 μg/ml. Different volumes of UOC solution (2 mg/ml) were added to the DNA solution (0.5 mg/ml) under vortex mixing (1200 rpm) for 15 s and were allowed to stabilize at room temperature for 30 min (Puras et al., 2013b). This incubation period was chosen in order to avoid the aggregation of the polyplexes, since it is reported that the physical stability of this kind of non-viral vectors is time- dependent (Anchordoquy and Koe, 2000).

2.3. Fractional factorial experiment

The influence of the pH of the solution (pH =X_1), salt concentration of the solution ([NaCl] = X_2) and chitosan/DNA N/P ratio (N/P ratio = X_3) on the size and zeta potential of UOC/ pAdTLRGD polyplexes was evaluated at two levels (low level and high level) and three variables fractional factorial experiment, also known as orthogonal experimental design. The original measurement units for the experimental variables (uncoded units) were transformed into code units, -1 (low level) and +1 (high level) (Table 1), for the analysis of the factorial design.

Table 1: Table of experimental trials and interactions transformed into code units

X_1	X_2	X_3	X_1X_2	X_1X_3	X_2X_3	$X_1X_2X_3$	Res-ponse
-1	-1	-1	+1	+1	+1	-1	
+1	-1	-1	-1	-1	+1	+1	
-1	+1	-1	-1	+1	-1	+1	
+1	+1	-1	+1	-1	-1	-1	
-1	-1	+1	+1	-1	-1	+1	
+1	-1	+1	-1	+1	-1	-1	
-1	+1	+1	-1	-1	+1	-1	
+1	+1	+1	+1	+1	+1	+1	

The number of experimental runs that had to be done to carry out an orthogonal experimental design is 8 (2^3). The responses, particle size and zeta potential, for each experimental combination were expressed as nm and mV, respectively (Table 2). The effects of the variables on the responses

could depend on the other studied variables. This phenomenon is called interaction. The effect of each variable and the corresponding interactions are included in the table of experimental trials (Table 3). Three different interactions can be found in the orthogonal experimental design: X_1X_2, X_1X_3 and X_2X_3. The effect of each variable and each interaction (Table 3) was calculated by the following equation:

Effect or Interaction = (∑ Responses with positive sign - ∑ Responses with negative sign) / 4

2.4. Size and zeta potential measurements

The measurements of size and zeta potential were carried out as previously described by Puras *et al*. (2013a) using a Zetasizer NanoZS (Malvern Instruments, UK). Briefly, 100 ml of the polyplexes were resuspended in 900 ml of the corresponding solution with a determinate pH value and salt concentration. The particle size reported as hydrodynamic diameter was obtained by dynamic light scattering, and the zeta potential was obtained by laser Doppler velocimetry. All measurements were carried out in triplicate.

2.5. Cryo-TEM microscopy

The morphology of polyplexes was observed by cryo-TEM microscopy. Briefly, one drop of the sample solution was vitrified by rapid freezing in liquid ethane using a Vitrobot Markt IV (FEI). This vitrified sample grid was transferred through 655 turbo pumping station (Gatan) to a 626 DH single tilt liquid nitrogen cryo-holder (Gatan), where it was maintained at -180 o C. Copper grid (300 mesh Quantifoils) was hydrophilized by glow-discharge treatment. The sample was examined in a TEM, TECNAI G2 20 TWIN (FEI), operating at an accelerating voltage of 200 keV in a bright-field and low-dose image mode.

2.6. Gel retardation assay

The capacity of O15 and O25 UOCs to complex, release and protect the DNA from DNase I enzymatic digestion was estimated by agarose gel electrophoresis assays. Naked DNA or polyplexes samples at N/P ratios of 1, 2, 5, and 10 (containing

Table 2: Table of experimental trials and response in terms of size and zeta potential for O15 and O25 UOC/pAdTLRGD

				Size (nm)		Zeta (mV)	
Trial	X_1 N/P	X_2 [NaCl] (mM)	X_3 pH	O15	O25	O15	O25
1	10	5	Acid	102.9	110.3	17.5	43
2	60	5	Acid	84.78	92.6	16.9	47.4
3	10	150	Acid	114.7	99.02	62.5	40.8
4	60	150	Acid	117	123.3	50	45.3
5	10	5	Neutral	145.2	120.6	-9.75	11
6	60	5	Neutral	84.82	105.4	12.9	12.4
7	10	150	Neutral	153.7	128.5	1.68	8.75
8	60	150	Neutral	161.5	94.21	6.75	10

Table 3: Table of effects and interactions on size and zeta potential of the UOC/pAdTLRGD polyplexes. X_1e: N/P effect, X_2e: [NaCl] effect, X_3e: pH effect, X_1X_2i: N/P-[NaCl] interaction, X_1X_3i: N/P-pH interaction, X_2X_3i: [NaCl]-pH interaction.

	Size (nm)		Zeta (mV)	
Effects and Interactions	O15	O25	O15	O25
X_1 e	-17.1	-10.7275	3.6575	2.8875
X_2e	32.3	4.0325	20.8475	-2.2375
X_3e	-31.46	5.8725	-33.8325	-33.5875
X_1X_2i	22.15	5.7225	-7.3725	-0.0125
X_1X_3i	-9.19	-14.0175	10.2025	-1.5625
X_2X_3i	10.29	-5.6775	-18.2025	-0.0875

132 ng DNA) were loaded to a 0.3% agarose gel and exposed for 3 h to 70 V. DNA bands were stained with GelRedTM (Biotium, Hayward, California, USA) and images were observed with a ChemiDocTM MP Imaging System (BioRad Madrid, Spain). The DNA protection capacity against enzymatic digestion was analyzed by adding a DNase I solution to UOC/pATLRGD vectors and incubating the mixture at 37 ºC for 30 min. Afterwards, a 2% sodium dodecyl sulphate (SDS) solution was added to the samples in order to release the DNA from the complexes. The integrity of the DNA in each sample was compared to untreated DNA.

2.7. Cell culture and in vitro transfection

HEK293 and A549 cells were seeded in 24 well plates at an initial density of 15

x 10^4 and 12 x 10^4 cells in 300 μl of EMEM and D-MEM/F-12 containing 10% fetal bovine serum, respectively. Cells were incubated overnight at 37 ºC and 5% CO2 to reach 70–80% of confluence at the time of transfection. Then, the regular growth medium was removed and the cells were exposed to polyplexes containing 1.65 μg of DNA and adequate amounts of UOC solution depending on the N/P ratio. Formulations for transfection were prepared after mixing the polyplexes and the hypertonic serum free Opti-MEM® transfection medium (pH 7.1) at 1:1 ratio in order to obtain a final isotonic medium. Each formulation was tested in triplicate. After 4 h of incubation at 37 ºC, the polyplexes were replaced by 300 μl of regular growth medium. A549 and HEK293 cells were allowed to grow for 24 and 72 h, respectively. Following the manufacturer's protocol, Lipofectamine™ 2000 was used in combination with pAdTLRGD as transfection positive control and cells transfected with the naked pAdTLRGD and non- treated cells were used as negative controls.

2.8. Analysis of EGFP expression

Qualitative expression of EGFP was analyzed using an inverted microscope equipped with an attachment for fluorescent observation (model EclipseTE2000-S, Nikon). Flow cytometry analysis was conducted using a FACSCalibur system flow cytometer (Becton Dickinson Biosciences, San Jose, USA), in order to quantify the % of EGFP positive cells and the mean fluorescent intensity (MFI). At the end of the incubation period, cells were detached with 200 μl of trypsin/EDTA and 400 μl of complete medium were added to inhibit trypsin activity. Then, the cells were centrifuged at 1500 x *g* and the supernatant was discarded. Cells were resuspended in cold PBS, diluted in FACSFlow liquid and transferred to specific flow cytometer tubes to quantify EGFP expression. For cell viability measurements, BD-via probe reagent was added to each sample to exclude dead cells from the analysis. Transfection efficiency was expressed as the percentage of EGFP positive cells at 525 nm (FL1). Control samples (non-

transfected cells) were displayed on a dot plot of forward scatter against side scatter to establish a collection gate excluding cells debris and dead cells. Cell transfected with Lipofectamine and non-transfected cells with BD-Via Probe reagent were used as controls to compensate FL2 signal in FL1 and FL3 channels. For each sample 10,000 events were collected.

2.9. Statistical analysis

All data are expressed as mean ± SD, n = 3. The Kruskal–Wallis Test and Dunn's Multiple Comparison post-test were applied with InStat programme (GraphPad Software, San Diego, CA, USA) in order to make statistical comparisons. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Effects and interactions of N/P ratio, salt concentration and pH on size and zeta potential

An orthogonal experimental design was chosen to address the effect of the N/P ratio, the salt concentration, and the pH of the transfection medium on size and zeta potential of O15 and O25 UOC/pAdTLRGD polyplexes. Table 2 shows the design matrix of uncoded values for the factors and responses. The effect of each factor and the interactions can be observed in Table 3. Regarding the size, all polyplexes had a particle size lower than 200 nm (Table 2). Particle size decreased when the N/P ratio was raised from 10 to 60 (17.1 nm for O15 and 10.73 nm for O25), however, only the size of polyplexes formed with O15 UOC was affected by the salt concentration and the pH (Table 3). Increasing [NaCl] from 5 mM to 150 mM the size increased 32.3 nm; and, when the pH was raised to neutral values, around 7.4, the size decreased 31.46 nm (Table 3). Regarding the interactions, the X_1X_2 showed a relevant interaction with a value of 22.15 in the particle size of O15 UOC based polyplexes (Table 3). Another significant interaction (-14.075) between N/P ratio and pH was also observed in the case of O25 based polyplexes size (Table 3). There was no other significant interaction concerning the size of the polyplexes.

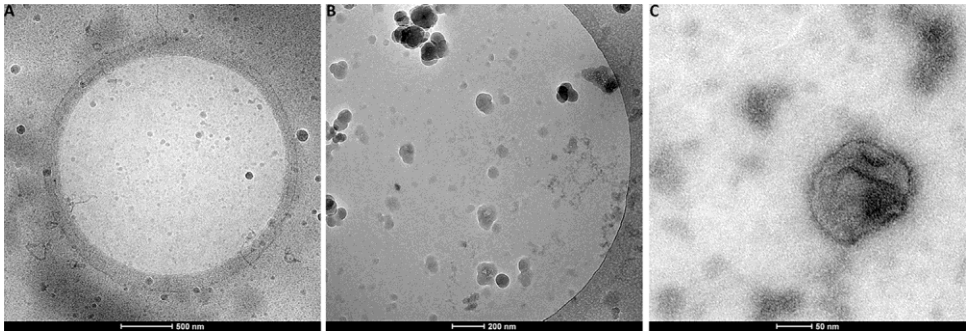

Figure 1. Cryo-TEM images of UOC/pAdTLRGD polyplexes: (A) O25 UOC/pAdTLRGD polyplexes at N/P ratio 60; (B) O15 UOC/pAdTLRGD polyplexes at N/P ratio 10; (C) O15 UOC/ pAdTLRGD polyplexes at N/P ratio 60.

According to the zeta potential data, the charge of the particles decreased around 33 mV when the pH was raised from acid to neutral values. The N/P ratio and salt concentration did not significantly affect the zeta potential of UOC/pAdTLRGD polyplexes, except for O15 polyplexes, which charge increased 20.84 mV when the salt concentration was raised to 150 mM. Regarding the interactions, two relevant interactions were observed in the case of O15 polyplexes, X_1X_3 interaction (10.2025) and X_2X_3 interaction (-18.2015) (Table 3).

3.2. Cryo-TEM characterization

The morphology and size of UOC/pAdTLRGD polyplexes was assessed by cryo-TEM microscopy. Fig. 1 shows polyplexes with a slightly spherical nature and with a particle diameter inferior to 200 nm, in agreement with the size measurements obtained by DLS.

3.3. Agarose gel electrophoresis assays

Fig. 2 shows the results obtained in the agarose gel electrophoresis at different N/P ratios, where the binding, the release efficiency and the protection capacity of O15 and O25 UOC based polyplexes were studied. In both Fig. 2A and B, lines 1–3 correspond to free DNA (untreated DNA, DNA treated with SDS and DNA incubated with DNase I and

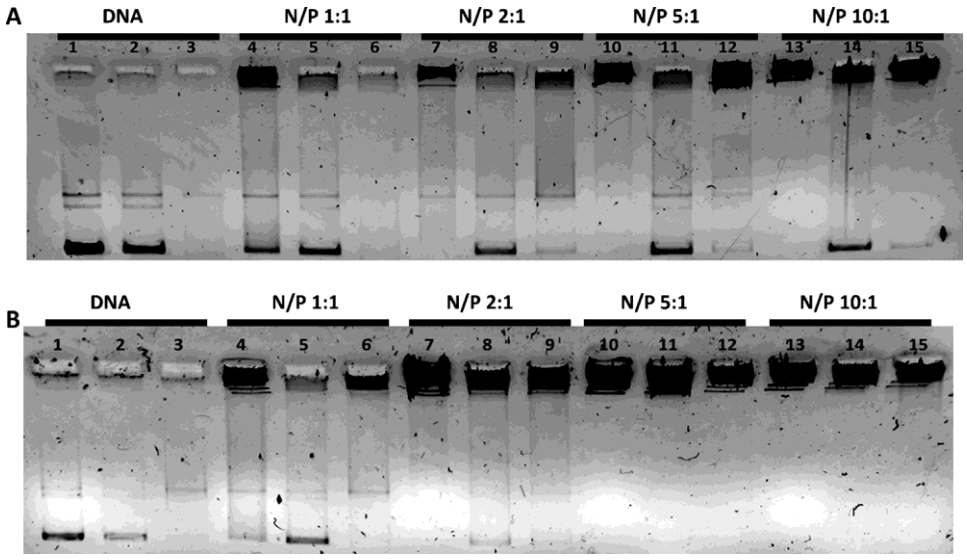

Figure 2. Binding, release and protection capacity of (A) O15 and (B) O25 UOC based polyplexes at different N/P ratios visualized by agarose gel electrophoresis. Polyplexes were treated with SDS (lanes 2 5, 8, 11 and 14) and SDS + DNase I (lanes 3, 6, 9 and 15).

SDS, respectively). In lanes 4, 7, 10 and 13 polyplexes were formulated at different N/P ratios and their binding affinity was evaluated. Polyplexes formed with O15 UOC needed an N/P ratio of 5 to completely bind the plasmid (Fig. 2A, lane 10). However, polyplexes formed with O25, were able to completely bind the DNA at a lower N/P ratio (N/P 2:1) (Fig. 2B, lane 7). Lanes 5, 8, 11 and 14 represent the bands of those polyplexes treated with SDS in order to release the DNA from the vectors. It is clearly observed that at N/P ratios of 5:1 and 10:1, O15/pAdTLRGD polyplexes were able to release the plasmid (Fig. 2A, lanes 11, 14) but it did not occur in the case of O25/pAdTLRGD polyplexes, where polyplexes were able to release the DNA at an N/ P ratio below 2 (Fig. 2B). Finally, for lanes 6, 9, 12 and 15

polyplexes were incubated with DNase I and treated with SDS. The absence of band in lane 3 suggests that the DNase I enzyme worked properly. The lower N/P ratio tested (1:1) was not able to protect the DNA in the case of O15/pAdTLRGD polyplexes (Fig. 2A, lane 6). O25 UOC was able to protect the DNA in all the tested proportions as observed on Fig. 2B, lanes 6, 9, 12 and 15.

3.4. Transfection efficiency in HEK293 and A549 cells

Transfection efficiency of UOC/pAdTLRGD polyplexes was evaluated in HEK293 and A549 cells, 72 h and 24 h post transfection (time to reach confluency), respectively. As it can be observed in Fig. 3 the % of EGFP positive HEK293 cells depended on the N/P ratio, in the case of O15 (Fig. 3A1) and O25 (Fig. 3A2) UOCs. Significantly higher transfection efficiencies (around 6%) were obtained at N/P ratio 5 with both UOCs ($P < 0.05$) and the percentage of transfected cells decreased as the N/P ratio increased. No transfection was observed when the naked pAdTLRGD plasmid was added to the wells, obtaining similar results to non-treated control cells (data not shown). The qualitative analysis obtained by fluorescent microscopy supported our cytometry results (Fig. 3B1–B3). However, significantly higher transfection, around 22% ($P < 0.01$), was observed by flow cytometry and microscopy when cells were treated with Lipofectamine (positive control) (Fig. 3). Regarding cell viability, all the tested formulations presented viability values higher than 80% and no differences were observed between the tested formulations. In order to study the relation between the percentage of transfected cells and the level of protein expression, MFI was analyzed (Fig. 4). The results showed that the MFI depended on the N/P ratio and the type of used UOC, obtaining significantly higher intensity values at N/P 5:1 with both formulations. Comparing both UOCs, MFI values seem to be higher in the case of O15; however, no statistically significant differences were observed.

In the lung adenocarcinoma A549 cell line, the percentage of EGFP positive cells

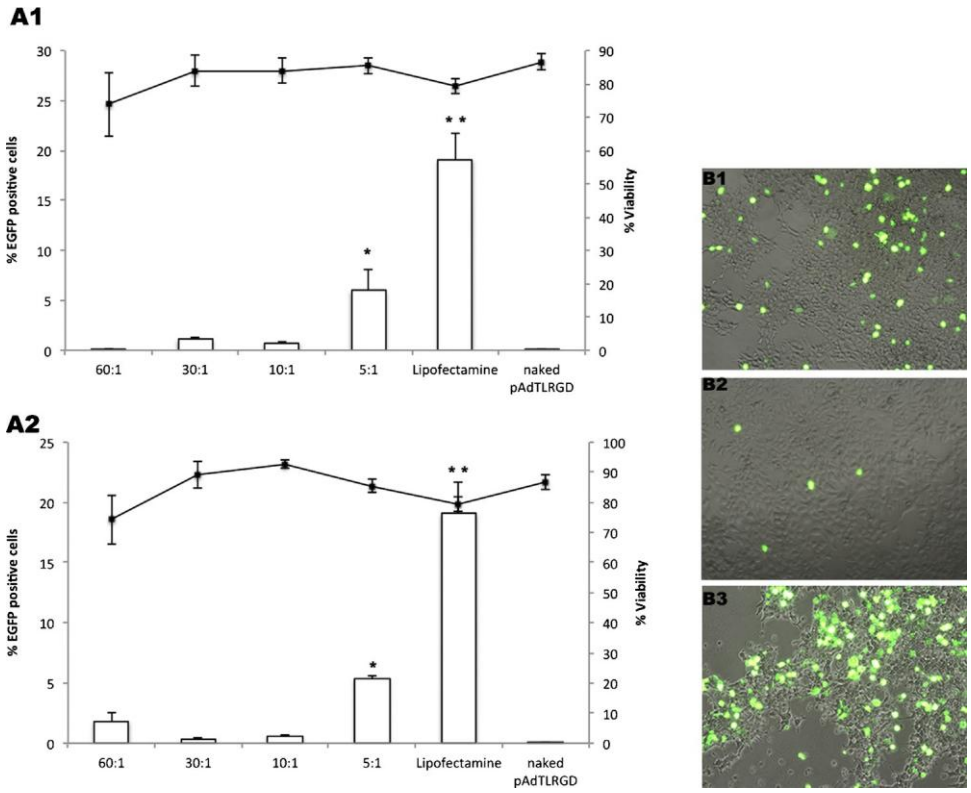

Fig. 3. Transfection efficiency of UOC/pVK503TL polyplexes in HEK293 cells at different N/P ratios. (A) % of EGFP positive cells (bars) and cell viability (lines) after the treatment with (A1) O15 UOC/pAdTLRGD polyplexes and (A2) O25 UOC/pAdTLRGD polyplexes; (B) Overlay of phase contrast image with fluorescent illumination (GFP channel) of cells transfected with (B1) O25 UOC/pAdTLRGD polyplexes at N/P ratio 5 and (B2) O25 UOC/pAdTLRGD polyplexes at N/P ratio 10; (B3) Cells transfected with Lipofectamine 2000. Error bars represent ±SD (n = 3). *$P < 0.05$ compared to naked pAdTLRGD. **$P < 0.01$ compared to naked pAdTLRGD.

was lower compared to the percentage obtained in HEK293 cells (data not shown). However, as it can be observed in the microscope photographs (Fig. 5A1 and A2) and in the dot plots cytometry data (Fig. 5B1 and B2), the UOC based

polyplexes, at N/P ratio of 5, were able to deliver the plasmid for the subsequent production of EGFP. No transfection was observed at higher N/P ratios (data not shown). A549 cells treated with Lipofectamine showed the highest level of transfection (Fig 5A3 and B3). On the other hand, there was not EGFP expression when the naked plasmid was used (Fig 5A4 and B4).

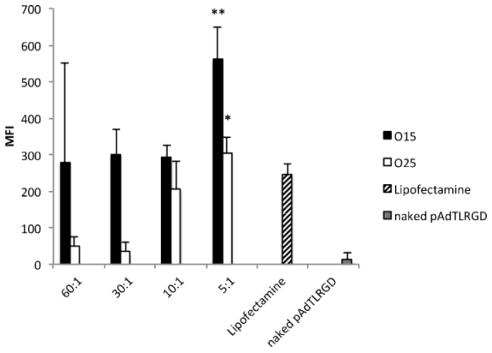

Figure 4. Mean fluorescent intensity (MFI) of HEK293 cells transfected with O15 (dark bars) and O25 (white bars) UOC/pAdTLRGD polyplexes at different N/P ratios. Error bars represent ±SD (n=3).). *P < 0.05 compared to naked pAdTLRGD.**P < 0.01 compared to naked pAdTLRGD.

4. Discussion

The current study was designed to evaluate the ability of UOC O15 and O25 to deliver pAdTLRGD, a replication-incompetent adenoviral plasmid (40 kbp), as a first step towards developing hybrid delivery systems composed of non-viral vectors and oncolytic adenoviral genomes.

pAdTLRGD was complexed directly after purification in a supercoiled form. The use of covalently closed circular DNA is preferred compared to linearized DNA because it avoids the exposure of DNA terminus to exonucleases and subsequently obtaining an increase in the transfection efficiency (Stanton et al., 2008). Self-excising plasmids containing the genome of oncolytic adenoviruses would allow the delivery of such viruses in a circular DNA form using non-viral vehicles.

Our research group has recently reported that factors like N/P ratio, the pH of the transfection medium and the salt concentration have an influence on size and zeta potential of O15 and O25 UOC/ pCMS-EGFP polyplexes (Agirre et al., 2015). However, it is well known that the characteristics of the plasmid influence the properties of the polyplexes and consequently their transfection efficiency.

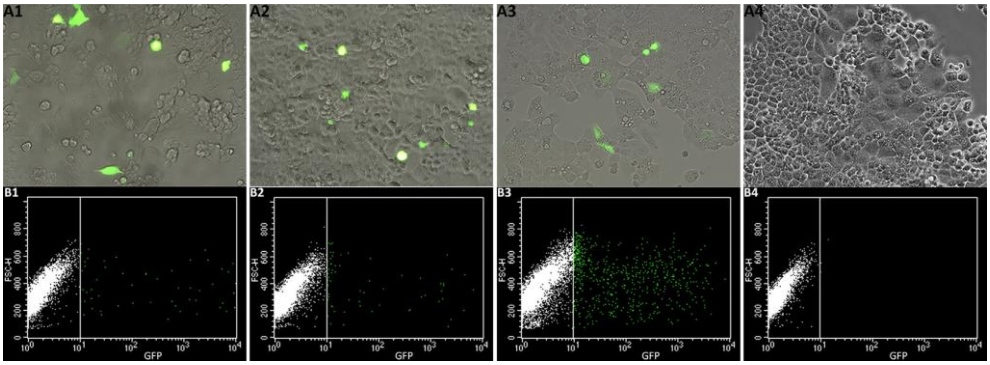

Figure 5. Transfection efficiency of UOC/pAdTLRGD polyplexes in A549 cell line. (A) Overlay of phase contrast image with fluorescent illumination (GFP channel) of cells transfected with (A1) O15 UOC/pAdTLRGD at N/P ratio 5; (A2) O25 UOC/pAdTLRGD at N/P ratio 5; (A3) Lipofectamine 2000; (A4) naked pAdTLRGD plasmid. (B) Flow citometry dot plots (GFP vs FSC) of cells transfected with (B1) O15 UOC/pAdTLRGD at N/P ratio 5; (B2) O25 UOC/pAdTLRGD at N/P ratio 5; (B3) Lipofectamine 2000; (B4) naked pAdTLRGD plasmid.

For this reason, we carried out a two levels three variables fractional factorial experiment to characterize UOC/ pAdTLRGD polyplexes.

All the formed polyplexes were in the nano-range, around 200 nm, which has been reported to be beneficial for a higher cellular uptake compared to microparticles (Bivas-Benita et al., 2004). In addition, particle size has a major role in determining the localization of nanoparticles in specific organs, and it has been described that after intravenous administration, positively charged particles smaller than 200 nm are trapped in the lung capillaries (Schroeder et al., 2011). The reduction of the particle size at high N/P ratios (Table 3) can be attributed to the ability of the UOC to precondense DNA (Puras et al., 2013b). Known *et al*. (2011) also reported similar particle sizes when they elaborated lipoplexes with viral genome DNA. Moreover, Kim et al. (2012) also described polyplexes of 70–80 nm

using bioreducible polymers for the delivery of linearized oncolytic adenoviral plasmid DNA.

The interactions observed between the salt concentration and the N/P ratio for O15 UOC polyplexes, and between the N/P ratio and the pH for O25 UOC based polyplexes, showed that there is a dependency between the studied factors. The effect of the N/P ratio on the particle size of the polyplexes can change depending on the value of the salt concentration and the pH. Similar interactions were obtained in our previous works for O15/pCMS-EGFP polyplexes (Agirre et al., 2015). Some authors suggest that the transfection efficiency of chitosan/pDNA nanoparticles does not depend on the size when it the ranges between 250 and 1300 nm (Duceppe and Trabrizian, 2010). However, other works have demonstrated that the final size can affect the transfection efficiency, as the endocytic pathways differ regarding the size of the endocytic vesicle (Conner and Schmid, 2003). Therefore, due to the controversy regarding the particle size, the investigation of other physicochemical properties, such as the zeta potential, could be helpful to improve *in vitro* transfections (Lee et al., 2004; Duceppe and Trabrizian, 2010). Positively charged particles interact with the negatively charged cell membrane facilitating the cellular uptake and thus enabling the transfection efficiency (Yue et al., 2011; Kang et al., 2012). UOC/pAdTLRGD polyplexes have a positive surface charge that can be modified depending on the pH (Nimesh et al., 2010). The observed interactions indicated that the effect of the pH in the zeta potential of our polyplexes depended on the N/P ratio and salt concentration. Regarding the orthogonal experimental design, we suggest that with high N/P ratios, in acidic and low salt concentration conditions we will obtain the desired small and positively charged particles.

Interestingly, we did not find significant differences in the physicochemical properties (size and zeta potential) of the polyplexes elaborated with pAdTLRGD plasmid, and polyplexes based on pCMS-EGFP plasmid, which is

10 times smaller (Agirre et al., 2015). Polyplexes were in the nanorange (<500 nm) and positively charged. Regarding polyplex morpholgy, we observed similar morphology than those reported previously with other plasmids such as pCMS-EGFP (Puras et al., 2013b). Other authors have also reported no differences in the structure and size of the lipoplexes when the size of the used pDNA is around 52.5 kbp compared to smaller plasmids (Kreiss et al., 1999).

In order to achieve an efficient transfection, the carrier material must be able to condense, release and protect the DNA against enzymatic degradation (Strand et al., 2010). In addition, a delicate balance between DNA binding capacity and DNA release must be achieved, since a strong binding between chitosan and plasmid could hamper the transfection process (Alatorre-Meda et al., 2011). It has been previously reported the ability of O15 and O25 oligochitosans to condense, release and protect a wide range of small plasmids (9 kbp) (Klausner et al., 2012). The agarose gel electrophoresis assay revealed that the ability of UOC based polyplexes to bind, release and protect the DNA depended on the N/P ratio. The slightly lower MW of O15 UOC explains the need of a higher N/P ratio for completely binding the DNA, and it also explains the absence of DNA protection at low N/P ratios (Agirre et al., 2015). Polyplexes elaborated with high MW chitosan are very stable and excellent DNA protectors, however, the release of the DNA cargo could be restricted due to the high stability of the polyplexes (Huang et al., 2005; Lu et al., 2009). Regarding the influence of the plasmid size, the agarose gel reveals that, despite the high size of pAdTLRGD, UOC based polyplexes are able to completely condense, release, and protect the nucleic acid depending on the N/P ratio. This is in agreement with other works where DOTAP/DOPE lipoplexes were able to condense and protect adenoviral genome DNA at a specific weight ratio (Kwon et al., 2011). However, compared to polyplexes elaborated with smaller plasmids, lower N/P ratios are required to completely condense the DNA

when a 40 Kbp plasmid is used (Puras et al., 2013a,b).

Once we studied that our formulations were biotechnologically suitable and had the desired characteristics for the delivery of big size plasmids, we evaluated the *in vitro* transfection efficiency of UOC/pAdTLRGD polyplexes in HEK293, a cell model for transfection studies, and in A549, a lung adenocarcinome cell line, both also commonly used in oncolytic adenovirus studies. Transfection studies were carried out in a slightly acidic pH, 7.1, as it is well reported that the microenvironment in tumors is generally more acidic than in normal tissues (Tannock and Rotin, 1989). In addition, chitosan mediated transfection efficiency strongly depends on slight changes in the pH value of the transfection medium (Nimesh et al., 2010) and, as it is described in the experimental design, the acidic environment do not deteriorate the characteristics of our polyplexes.

In HEK293 cells, the highest percentages of EGFP positive cells and MFI values were obtained at N/P 5 (Fig. 3 and 4). The low transfection efficiency and EGFP expression at high N/P ratios could be explained by the strong interactions between the polymer and the plasmid, which could hamper the release of the DNA from the vector (Strand et al., 2010). Comparing O15 and O25 UOCs, the expression of EGFP tended to be higher with O15 than O25 UOC (Fig. 4), which could be due to the lower MW of O15 UOC (Klausner et al., 2010). Regarding cell viability, the low cytotoxicity of chitosan based particles has been widely reported in the literature (Aam et al., 2010).

In A549 the percentage of transfected cells (data not shown) was lower than the obtained in HEK293 cells, which could be due to the differences in the cell internalization process (Gu et al., 2014). The cell line dependent transfection efficiency has been also described in other research works with a variety of non-viral vectors (Douglas et al., 2008; Izumisawa et al., 2011; Wang et al., 2012).

The results showed that despite the big size of the pAdTLRGD plasmid (40

kbp), the formed polyplexes were able to cross the cell membrane and translocate into the nucleus to efficiently transfect the cells. The percentage of EGFP positive cells was significantly lower than the percentage obtained with smaller plasmids when they were delivered by this UOC based formulations (Klausner et al., 2012). Some authors have reported that the uptake and expression of plasmid DNA is not influenced by the size of the plasmid, at least up to 16 kbp (Wells et al., 1998). However, other authors have reported that the transfection efficiency of large size plasmid is lower than the one obtained with smaller plasmids (Walker et al., 2004; Yin et al., 2005). This is in accordance with our results, as higher transfection efficiencies were obtained with the smaller plasmid (pCMS-EGFP) using the same UOCs as carriers (Klausner et al., 2012; Puras et al., 2013a).

The advantage of non-viral vectors over viral vectors regarding the packing capacity has been proven, as the UOC based polyplexes are able to deliver plasmids of 40 Kbp and produce EGFP protein. Despite the low transfection efficiency, the property of replication-competent oncolytic adenoviruses to amplify themselves inside tumors would offer the opportunity to use our non viral vectors for *in vivo* gene delivery.

6. Conclusion

In the present study, we designed and characterized polyplexes based on O15 and O25 UOCs for the delivery of pAdTLRGD, which is a replication incompetent Ad plasmid of 40 kbp. The polyplexes were in the desirable nanorange, and their size and zeta potential varied depending on the formulation conditions, as it occurs with smaller plasmids. According to the agarose gel results and even though the large size of the plasmid, the formulations were able to condense, release, and protect the nucleic acids against DNase I. The transfection efficiencies in HEK293 and A549 showed that the transfection is cell line dependent, and vary considerably depending on the used N/P ratio. The obtained transfection percentages were lower than previously reported data with other reporter plasmids

like pCMS-EGFP; however, considering the possible applications of these formulations, which is the case of the delivery of replication competent adenoviral genomes, the transfection of some of the tumor cells may be enough to trigger the infection of the rest of the tumor tissue. These preliminary results suggest that O15 and O25 UOC based polyplexes could be used as possible vectors for the delivery of oncolytic Ad plasmids. At this point, further research is required in order to progress in the design of an effective hybrid delivery system composed of oncolytic viral genomes and non-viral carriers, to evaluate the *in vivo* application of a promising therapy applied to cancer therapy.

Conflict of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article.

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Chapter 3

New insights into gene delivery to human neuronal precursor NT2 cells: a comparative study between lipoplexes, nioplexes and polyplexes

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Giza NTera2/D1 teratokartzinomatik eratorritako zelulen transfekzioa etorkizun handiko estrategia da nerbio sistema zentralera (NSZ) proteina edo agente biologiko exogenoak garraiatzeko. Transfekzio efizientzia altua duten bektore ez-biral egokiak garatzeko, ezinbestekoa da transfekzio prozesu osoa ondo ezagutzea. Lan honetan, hiru bektore ez-biral garatu genituen eta beraien tamaina eta karga karakterizatu: lipoplexoak (144 nm; -29,13 mV), nioplexoak (142,5 nm; + 35,4 mV) eta poliplexoak (294,8 nm; +15,1 mV). Ondoren, bektoreen transfekzio efizientzia, zelulan barneratzeko gaitasuna eta zelula barneko trafikoa aztertu genituen. Lipoplexoekin eta nioplexoekin EGFP portzentai altuenak eta baxuenak lortu genituen, hurrenez hurren. Zelulan barneratze emaitzak transfekzio portzentajeekin korrelazio argia zutela ikusi genuen. Endozitosi mekanismoari dagokionez, lipoplexoek klatrina bidezko endozitosia (CME) erabiltzen dute, poliplexoek aldiz, kabeola bidezko endozitosia (CvME). Endosoma baten konpartimentua antzeratuz, behin endosoma barnean daudela, bektoreek DNA askatzeko gaitasuna zutela frogatu genuen. 6 ordutara, poliplexoek garraiatutako material genetikoa endosoma konpartimentuan aurkitzen zela ikusi genuen, lipoplexoek garraiatutakoa aldiz, nuklean aurkitu genuen. Honek, gure bektore lipidikoen zelula barneko garraioa kitosanoekin eratutako bektoreena baino azkarragoa dela adierazi zigun. Guztira, gure lanak, bektore ez-biral desberdinek NT2 zelulak transfektzatzeko duten gaitasunari buruzko ezagutza berria aurkezten du, ex vivo terapia genikoraro plataforma baten garapenaren lehen pasu giza.

La transfección de las células NT2era2/D1 derivadas de teratocarcinoma humano (o células NT2) representa una estrategia prometedora para la liberación de proteínas o diversos agentes biológicos exógenos al sistema nervioso central (SNC). El desarrollo de adecuados vectores no-virales con altas eficiencias de transfección requiere un conocimiento profundo del propio proceso de transfección. En este trabajo, elaboramos y caracterizamos, en base a su tamaño y potencial zeta, tres vectores no-virales diferentes: lipoplexos (144 nm; -29.13 mV), nioplexos (142.5 nm; +35.4 mV) y poliplexos (294.8 nm; +15.1 mV). Además, comparamos la eficiencia de transfección, captación celular y tráfico intracelular de los tres tipos de vectores en la línea celular NT2. Los lipoplexes mostraron altas eficiencias de transfección, mientras que con los nioplexes las eficiencias fueron muy bajas. Los resultados de captación celular se correlacionaron claramente con los respectivos datos de transfección. En cuanto a los mecanismos de endocitosis, la vía principal de entrada de los lipoplexes resulto ser la mediada por clatrinas, mientras que la de los poliplexos fue la mediada por caveolas (CvME). Mediante la simulación de endosomas artificiales demostramos que los vectores eras capaces de liberar el ADN una vez dentro del endosoma tardío. Los resultados mostraron que a las 6 horas, el material genético transportado mediante los poliplexos estaba localizado en el endosoma tardío, en cambio, el ADN transportado por los lipoplexos se encontraba localizado en el núcleo, lo que nos indica que el tráfico intracelular de los lipoplexes es más rápido que el de los poliplexos. En conjunto, este trabajo aporta nuevo conocimiento sobre el proceso de transfección mediado por diferentes vectores no-virales en la línea celular NT2, como un primer paso para el desarrollo de una terapia génica ex vivo.

New insights into gene delivery to human neuronal precursor NT2 cells: a comparative study between lipoplexes, nioplexes and polyplexes

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ABSTRACT

The transfection of human NTera2/D1 teratocarcinoma-deriverd cell line (or NT2 cells) represents a promising strategy for the delivery of exogenous proteins or biological agents into the central nervous system (CNS). The development of suitable non-viral vectors with high transfection efficiencies requires a profound knowledge of the whole transfection process. In this work, we elaborated and characterized in terms of size and zeta potential three different non-viral vectors: lipoplexes (144 nm; -29.13 mV), nioplexes (142.5 nm; +35.4 mV) and polyplexes (294.8 nm; +15.1 mV). We compared the transfection efficiency, cellular uptake and intracellular trafficking of the three vectors in NT2 cells line. Lipoplexes and nioplexes exhibited the highest and the lowest percentages of EGFP positive cells, respectively. Cellular uptake results had a clear correlation respect to the corresponding transfection efficiencies. Regarding the endocytosis mechanism, lipoplexes enter in the cell, mainly, via clathrin-mediated endocytosis (CME) while polyplexes via caveolae-mediated endocytosis (CvME). By simulating an artificial endosome, we demonstrated that the vectors were able to release the DNA cargo once inside the late endosome. The data collected from this assay showed that at 6h, the genetic material carried by polyplexes was still located in the late endosome, while DNA carried by lipoplexes was already in the nucleus, indicating a faster intracellular traffic of the lipid based vectors. Overall, our work gives new insights into the transfection process of NT2 cells by different non-viral vectors as a first step in the development of *ex-vivo* gene therapy platform.

Key words

Non-viral vectors, transfection, endocytosis, pCMS-EGFP, NTera2/D1 cells

1. Introduction

Cell therapy is a field that moves rapidly with new cell lines and tissue-engineered constructs developed globally every year. The human NTera2/D1 teratocarcinoma-derived cell line (or NT2 cells) can be differentiated into well-characterized populations of neuron-like cells (or NT2-N) that engraft and mature when are transplanted into the adult central nervous system (CNS) of rodents and humans [1-7]. Moreover, human transplantation studies in the brain of stroke patients demonstrated a lack of tumorigenicity of these cells [6,8]. On the other hand, the NT2 cell line can be used as derive cellular vehicles with glioma tropism [9], adding a human cell-based delivery vehicle for clinical glioblastoma therapy, as it was similarly demonstrated for neural stem cells in animal models [10,11]. Although these features suggest that NT2 cells would be an excellent platform for *ex vivo* gene therapy in the CNS [3,7,9,12,13], the use of non-viral strategies for gene transfer has been scarce [14,15].

In the last decade, cationic lipid-mediated [16] and cationic polymer-mediated non-viral gene delivery [17] methods have become useful tools for cellular transfection. Although gene therapy is still dominated by viral vectors, which present higher transfection efficiencies compared to non-viral gene delivery systems, safety issues such as immunogenicity and mutagenicity hinder their clinical applications [18-20]. At this point, non-viral gene delivery has been the subject of increasing attention due to its relative safety, simple use and easy handling compared to viral vectors [21,22]. Among lipid based non-viral vectors, liposomes are the most widely used carriers for *in vitro* and *in vivo* gene delivery [23]. Lipofectamine 2000 is a commercialized cationic liposome based reagent, which has been extensively used for *in vitro* gene delivery purposes due to its high transfection efficiency [24]. In addition to liposomes, niosomes (non-ionic surfactant vesicles) have also gained interest as an alternative to liposomes due to their similar structure. Although the use of these structures has been

mainly restricted to drug delivery, our research group has recently obtained some promising results that suggests the possible use of cationic niosomes as non-viral vectors for gene delivery [25,26]. Finally, among cationic polymers, chitosan is an appropriate candidate for non-viral gene delivery due to its biocompatibility, biodegradability and low cost [27]. Our research group has been working with ultrapure oligochitosans (UOC) obtaining promising results [28,29]. The non-viral carriers (liposomes, niosomes or polymers) are typically cationic in nature, and electrostatically interact with negatively charged DNA molecules forming the so-called lipoplexes nioplexes and polyplexes, respectively.

Regardless the gene carrier, it is well known that the final gene expression will be highly influenced by the uptake of the non-viral vector, the internalization pathway and their ability to escape from the endosomes and import the genetic material into the nucleus [30]. At the same time, the internalization route of the vectors will depend on their composition and physicochemical characteristics, such as size, polydispersity, surface charge and morphology [31]. Thus, it is essential to study, specifically, the uptake and intracellular trafficking of each selected non-viral vector, in order to understand their possible limitations and improve their design.

Therefore, this work was intended to carry out a comparative study of three types of non-viral vectors in human neuronal precursor NT2 cell line. Specifically, we compared lipoplexes based on the commercialized Lipofectamine 2000; nioplexes based on a previously described novel noisome formulation; and polyplexes based on UOCs. For this purpose, the vectors were characterized in terms of size, polydispersity index (PDI) and surface charge. The percentage of transfected cells, mean fluorescent intensity (MFI) and cell viability were analysed by flow citometry. Additionally, the expression of the enhanced green fluorescent protein (EGFP) was analysed by Western Blot. To further explain the differences in the transfection efficiencies that we found among the three formulations, we performed qualitative and quantitative cellular uptake studies, by confocal

microscopy and flow citometry, respectively, using Cy3 or FITC labeled plasmids. The endocytosis pathways of the studied vectors were determined by colocalization assays and quantified by the Mander´s overlap coefficient. Finally, the endosomal escape was analysed by simulating the interaction of the vectors with anionic micelles, analogues of endosomal compartment.

2. Material and methods

2.1. Propagation of pCMS-EGFP

The pCMS-EGFP reporter plasmid was propagated in *Escherichia coli DH5-α* and purified using the Qiagen endotoxin-free plasmid purification Maxi-prep kit (Qiagen, Santa Clarita, CA, USA) according to the manufacturer's instructions. The concentration of pDNA was quantified by measuring the absorbance at 260 nm using a NanoDrop® (ND-1000 spectrophotometer, Thermo Fisher Scientific Inc., Denver, USA). The purity of the plasmid was verified by agarose gel electrophoresis in Tris borate-EDTA buffer, pH 8.0 (TBE buffer). DNA bands were detected using GelRed™ (Biotium, Hayward, California, USA) and images were observed with a ChemiDoc™ MP imaging system (Bio-Rad, USA).

2.2 Niosomes elaboration

The cationic lipid 2,3-di(tetradecyloxy)propan-1-amine used for niosome elaboration was synthesized as previously describe by Grijalvo et al [32]. Niosomes were prepared by the oil in water (o/w) emulsification method [26]. 5 mg of the cationic lipid were gently ground with 20 µl of squalene. Then, 1 ml of dichloromethane was added and emulsified with 5 ml of aqueous phase containing the non-ionic surfactant polysorbate 80 (0.5 %, w/w). The emulsion was obtained by sonication (Branson Sonifier 250, Danbury) for 30 s at 50 W. The organic solvent was removed from the emulsion by evaporation under magnetic agitation for 3 h. Upon dichloromethane evaporation, a dispersion containing the nanoparticles was formed by precipitation of the cationic nanoparticles in the aqueous medium. The final concentration of the cationic lipid in the niosome formulation was 1mg/ml.

2.3. Preparation and characterization of the complexes

Lipoplexes, nioplexes and polyplexes were prepared by mixing at determinate weigh/weight (w/w) ratios Lipofectamine 2000 (Invitrogen, Life technologies, USA) solution, niosomes or UOC (Novafect O15 with a molecular weight of 5.7 KDa and 97 % degree of deacetylation, purchased from NovaMatrix/FMC, Sandvika, Norway) solution with the pCMS-EGFP plasmid. (i) Lipoplexes were prepared at a Lipofectamine/DNA w/w ratio of 2:1 by mixing gently Lipofectamine solution (1mg/ml) and pCMS-EGFP solution (0.5 mg/ml) (ii) Nioplexes were prepared at a fixed cationic lipid/DNA ratio 20:1 (w/w). (iii) For polyplexes preparation, UOC solution was dissolved in miliQ water to a final concentration of 2 mg/ml. pCMS-EGFP solution was added under vortex mixing (15 s) to the chitosan solutidon at a final UOC/DNA w/w ratio of 13:1. In all cases, the formulations were incubated for 30 minutes at room temperature to allow the correct formation of the complexes.

The complexes were characterized in terms of size and zeta potential. The measurements were carried out as previously described by Ojeda et al. using a Zetasizer NanoZS (Malvern Instruments, UK) [26]. Briefly, 50 µl of the complexes were resuspended in 950 µl of NaCl 0.1 mM. The particle size reported as hydrodynamic diameter was obtained by Dynamic Light Scattering, and the zeta potential was obtained by Laser Doppler Velocimetry. All measurements were carried out in triplicate.

2.4. Cell culture and transfection protocol

Human teratocarcinoma NTERA2/D1 (NT2) cells from the American Type Culture Collection (ATCC®, CRL-1973™) were maintained in complete medium: Dulbecco's Modified Eagle medium (DMEM®, ATCC 30-2002™), supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St Louis, MO, USA) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Gibco, Life Technologies S.A., Madrid, Spain), at 37ºC under a humidified atmosphere containing 5% CO2.

Before transfection, NT2 cells were seeded in 24 well plates at an initial density of 8×10^4 cells per well with 400 µl complete medium and allowed to grow to 70-80 % confluence. Then, the complete medium was replaced with serum-free Opti-MEM® (Gibco, Life Technologies S.A.), and the cells were exposed to the complexes at the corresponding w/w ratios (containing 1.25 µg pCMS-EGFP per well) (Table 1). In the case of polyplexes, Opti-MEM® containing 270 mM mannitol was used in order to obtain a final isotonic medium. In addition, the pH was adjusted to 7, as it has been previously described the improved transfection with this kind of formulations at slightly acidic pH values. After 4 h of incubation at 37 ºC, the complexes were removed and replaced with 380 µl complete medium, and were allowed to grow until the analysis time.

2.5 Analysis of EGFP expression and cell viability

Qualitative expression of EGFP was analysed using an inverted microscope equipped with an attachment for fluorescent observation (model EclipseTE2000-S, Nikon). Flow cytometry analysis was conducted using a FACSCalibur system flow cytometer (Becton Dickinson Biosciences, San Jose, USA), in order to quantify the percentage of EGFP positive cells and the mean fluorescent intensity (MFI). At the end of the incubation period, cells were detached with 200 µl of trypsin/EDTA and, 400 µl of complete medium were added to inhibit trypsin activity. Then, cells were transferred to specific flow cytometer tubes to quantify EGFP expression. For cell viability measurements, BD-via probe reagent (7-AAD) was added to each sample. Transfection efficiency was expressed as the percentage of EGFP positive cells at 525 nm (FL1) and cell viability was expressed as the percentage of 7-AAD negative cells at 650 nm (FL3). Control samples (non-transfected cells) were displayed on a dot plot of forward scatter against side scatter to establish a collection gate. For each sample 10,000

Table 1: Summary of the main components, w/w ratios and DNA quantities employed for the elaboration of the three formulations

Formulation	Main Component	w/w ratio	DNA quantity (µg)
Lipoplexes	Lipofectamine 2000	2:1	1.25
Nioplexes	2,3-di(tetradecyloxy)propan-1-amine cationic lipid	20:1	1.25
Polyplexes	Novafect O15 ultrapure oligochitosan	13:1	1.25

events were collected.

In addition, EGFP protein expression was analysed by Western Blot of whole cell homogenates. Thus, expression was analysed semiquantitatively at 24, 48 and 72 h after transfection with the different reagents. For each independent analysis, cells from 3 wells per condition were lysed and pooled in 300 µl lysis buffer: Tris-HCl (50 mM, pH 7.4), 150 mM NaCl, 1% Igepal (Sigma-Aldrich), 0.5% sodium deoxycholate (Sigma-Aldrich), 0.1% sodium dodecyl sulfate (SDS), 2.5 mM CHAPS (Sigma-Aldrich) and protease inhibitor cocktail (50 µl/mg protein; Sigma-Aldrich). Solubilized proteins were collected from supernatants of samples after centrifugation at 40,000 x g for 5 min at 4 ºC). Protein concentrations were determined with the BCA protein quantification kit (Abcam, Cambridge, UK; BD Transduction Laboratories, San Diego CA, USA). Increasing amounts (4, 6, 8, 10 µg) of proteins from cells 24 h after transfection with Lipofectmine 2000 and 10 µg of samples from the rest of conditions were run in parallel and resolved by electrophoresis on SDS-polyacrylamide (SDS–PAGE) 10% gels. The uppermost part of the gel containing proteins above the 75 kDa standard was separated and stained with Coomassie Blue for a protein load control. The rest of proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Bioscience, Buckinghamshire, UK). Blots were blocked in 5% non-fat dry milk/phosphate buffered saline containing 0.5% BSA and 0.2% Tween (blocking

buffer) for 1 h, and incubated with rabbit polyclonal anti-GFP antibody (Invitrogen, Ref. A11122) 1:2000 diluted in blocking buffer. After extensive washing, the PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibodies diluted to 1:10,000 in blocking buffer for 2 h at room temperature. Immunoreactive bands were detected with ECL reagent according to the manufacturer's instructions (Amersham Bioscience, Buckinghamshire, UK). EGFP-specific immunoreactive bands were acquired from four independent experiments using an ImageQuant 350 imager (GE Healthcare, Madrid, Spain) and quantified by densitometry using ImageJ image analysis software (ImageJ, NIH, Bethesda, MD, USA). Optical density (OD) values from bands corresponding to increasing protein amounts from Lipofectmine 2000-transfected at 24 h post-transfection used for linear regression analysis, thus obtaining a linear regression equation, which allowed us to calculate the fold change of EGFP immunoreactivity for each condition (transfection reagent and post-transfection time) compared to that in samples from cells at 24 h after transfection with Lipofectamine 2000.

2.6. Cellular uptake assays

NT2 cells were cultured and seeded as for transfection experiments. Then, the regular growth media was removed from the wells and the cells were exposed to the complexes containing 1.25 μg of the pCMS-EGFP labelled with fluorescein isothiocyanate (FITC) or Cy3. After 1h or 4h of at 37 ºC, the transfection medium was removed and cells were washed with PBS and fixed with formaldehyde 4% for the quantitative and qualitative analysis, by flow cytometry and confocal laser scanning microscopy (CLSM), respectively. Each formulation was analysed by triplicate.

Cells were analysed after 1h or 4h of incubation by flow cytometry at 525 nm (FL1) after detachment from the wells. 10,000 events were collected for each sample. Cellular uptake data was expressed as the % of FITC positive cells. For the qualitative study, cells were seeded in coverslips containing plates and treated

with the vectors (containing Cy3 labelled DNA). After 4h of incubation, cells were stained with Phalloidin AlexaFluor-488 and preparations were mounted on Dapi Fluoromount-G (Southern Biotech) and then, images were obtained with an Olimpues Fluoview FV500 microscope. CLSM images were captured in the General Service of Analytical Microscopy and High Resolution in Biomedicine of the University of the Basque Country (UPV/EHU, Bizkaia, Spain).

2.7. Colocalization assays

The endocytic processes involved in the internalization of the complexes were analysed by colocalization assays. NT2 cells were seeded in coverslips (as described for transfection protocol) and co-incubated with the complexes (containing the FITC labeled pCMS-EGFP) and different endocytosis fluorescent markers: AlexaFluor546-Transferrin (50 μg/ml), AlexaFluor555-Cholera toxin (10 μg/ml) or Lysotracker (140 nM), which are markers for clathrin-mediated endocytosis (CME), caveolae/raft-mediated endocytosis (CvME) and late endosomal compartment, respectively. After 3 hours of incubation with the complexes, the markers were added to the cells and they were incubated one more hour. Then, the cells were fixed with formaldehyde 4% and mounted on DAPI fluoromont G for their posterior examination by CLSM. Colocalization of green and red signal was analysed by ImageJ software and quantified by the Mander´s coefficient.

2.8. Vulnerability assay of the complexes in the late endosome

As an analogue of the endosomal compartment, anionic micelles based on phosphatidylserine (PS) were prepared, as described previously [33]. PS was dissolved in chloroform at 1.6 mM and left under magnetic stirring until the solvent was completely evaporated. Phosphate buffer was added to the dried sample and a dispersed solution was obtained by sonication (Branson Sonifier 250, Danbury). PS micelles and the complexes were incubated at a w/w ratio of 1:50 (pCMS-EGFP: PS) for 1 hour. Thereafter, the amount of the released DNA from each complex was determined by agarose gel

electrophoresis. Naked plasmid or complexes (containing 205 ng pCMS-EGFP) were loaded to a 0.8 % agarose gel and exposed for 30 min to 100 V. DNA bands were stained with GelRed™ (Biotium, Hayward, California, USA) and images were observed with a ChemiDocTM MP Imaging System (BioRad, Madrid, Spain) and analysed with Image LabTM software (Bio-Rad, Madrid, Spain).

2.9. Statistical analysis

All data are expressed as mean ± SD, n=3. The Kruskal-Wallis Test and Dunn's Multiple Comparison post-test were applied with InStat programme (GraphPad Software, San Diego, CA, USA) in order to make statistical comparisons. Differences were considered statistically significant when $P<0.05$.

3. Results

3.1. Size, PDI and zeta potential of the complexes

Fig.1 summarizes the characterization of the complexes. While lipoplexes and nioplexes presented a particle size around 140 nm with low PDI (0.21), polyplexes showed bigger size (294 nm) and PDI (0.44) (Fig. 1A). The higher polidispersity of the polyplexes can also be observed in the size distribution curve (Fig. 1B). Regarding the surface charge, it varied from negative to positive values depending on the vector. Lipoplexes were negatively charged (-29.13 mV), while nioplexes and polyplexes had a positive zeta potential, +35.4 mV and +15.1 mV, respectively (Fig. 1A).

A

	Size (nm)	pdi	zeta ptential (mV)
Lipoplexes	144.1±6.3	0.21±0.04	-29.13±1.43
Nioplexes	142.5±0.64	0.21±0.04	35.4±0.7
Polyplexes	294.8±23.36	0.44±0.04	15.1±3.05

B

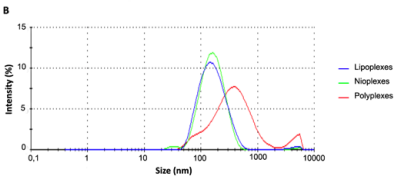

Figure 1. Characterization of the complexes. (A) Size, PDI and zeta potential values. Each value represents the mean ± standard deviation of three measurements. (B) Size distribution curve of the complexes.

3.2. NT2 cell viability and EGFP expression

The percentage of viability was

determined by flow citometry at 24, 48 and 72 h post-incubation. Cell viability was above 70 % in the three tested formulations at all analysed times (Fig. 2), showing minimal differences among the formulations in all tested times.

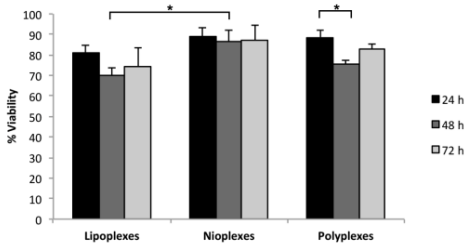

Figure 2. Cell viability of NT2 cells at 24, 48 and 72 hours after the treatment with the complexes. Error bars represent ± SD (n=3). *$P < 0.05$.

The percentage of EGFP positive live cells at 24, 48 and 72 hours post-transfection was analysed by flow citometry (Fig. 3A). No statistical differences were observed among the tested times except for lipoplexes; in this case, a significant decrease was observed at 72h (37 %) compared to 24 h (22 %). Among the three formulations, lipoplexes exhibited the highest transfection percentages (up to 35 % at 24 h), and nioplexes the lowest (5 %), while polyplexes were able to transfect almost 20 % of the cells. Microscopic photographs (Fig. 3B) corroborated these results, as almost no EGFP positive cells were observed after their transfection with nioplexes (Fig. 3 B6). A well defined cell population can be observed in flow citometry dot plots (FSC vs SSC) in all cases (Fig. 3 C1, C3, C5 and C7). In FL1 (GFP) vs FL3 (7-AAD) dots plots, the higher transfection efficiency of lipoplexes (Fig.3 C4) and polyplexes (Fig. 3 C8) compared to nioplexes (Fig. 3 C6) and the non-treated cells (Fig. 3 C2) is clearly represented.

In order to show the production of EGFP in the cells, MFI of the transfected cells was calculated (Fig. 4A) and Western Blot analysis of EGFP expression was performed (Fig. 4B). Both, MFI measurements and densitometric analysis of EGFP immunostaining revealed that lipoplexes and nioplexes exhibited the highest and the lowest MFI values, respectively. Interestingly, lipoplexes

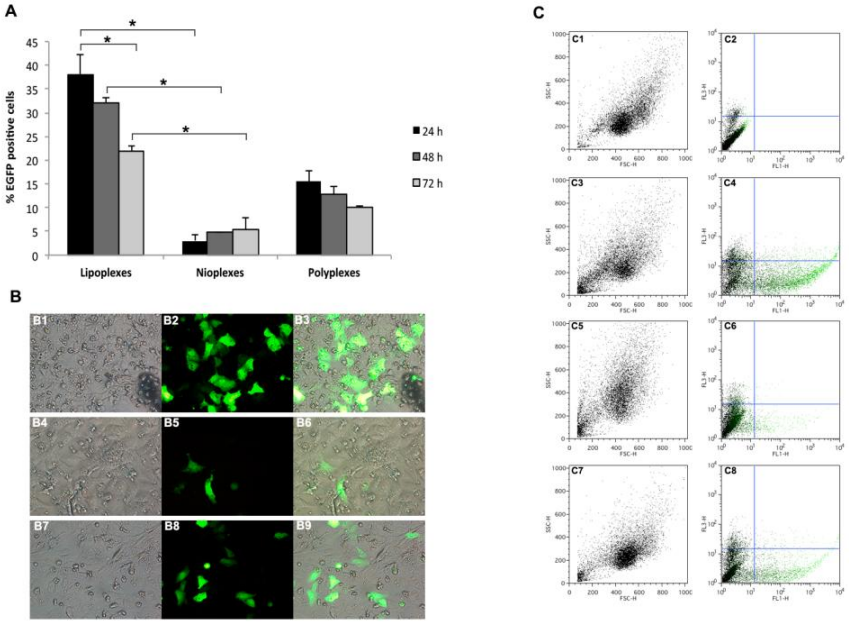

Figure 3. Transfection efficiency of the three tested complexes. (A) Bar graphs showing percentage of EGFP positive live cells at 24, 48 and 72 hours post-transfection. Error bars represent ± SD (n=3) **P* < 0.05. (B) Microscope photographs of NT2 cells at 48 h post-transfection. (B1, B4, B7) phase contrast images, (B2, B5, B8) fluorescent images and, (B3, B6, B9) overlay images of cells transfected with (B1, B2, B3) lipoplexes, (B4, B5, B6) nioplexes and (B7, B8, B9) polyplexes. (C) Flow cytometry dot plots representing (C1, C2, C3, C5, C7) FSC *vs* SSC and (C2, C3, C4, C6, C8) FL1 *vs* FL3 of NT2 cells treated with (C1, C2) negative control, (C3, C4) lipoplexes, (C5, C6) nioplexes and (C7, C8) polyplexes. FL1 channel corresponds to GFP and FL3 channel corresponds to 7-AAD.

exhibited the highest MFI values, while nioplexes the lowest. Kruskal-Wallis test followed by Dunn's multiple comparison revealed no statistical differences in MFI among the tested times except for polyplexes. In this case, a significant increase is observed at 48 hours compared to the previous studied time. In contrast, significant differences were observed between the different formulations.

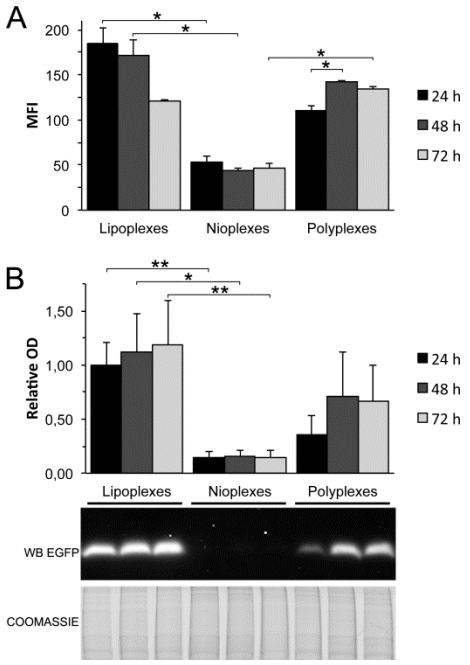

Figure 4. Expression of EGFP protein at 24, 48 and 72 hours post-transfection. (A) Bar graph showing MFI values for cells transfected with the different complexes. Error bars represent mean ± SD (n=3). (B) Bar graph depicts results of semiquantitative analysis of EGFP protein expression by Western Blot. A representative Western Blot is shown below the bar graph. Error bars represent mean ± SD (n=4). Kruskal-Wallis test followed by Dunn's multiple comparison post-test. **P* < 0.05, ***P* < 0.01.

Specifically, 24 and 48 hours after transfection, MFI values were significantly higher in lipoplexe- than in nioplexe-transfected cells and significant differences were also detected at 72 hours between nioplexe- and polyplexe-transfected cells (Fig. 4. A). In addition, Western blot analysis corroborated these results. As seen in Fig. 4B, barely detectable EGFP was produced after transfection with nioplexes. By contrast, a well-defined band appeared with lipoplexes and polyplexes, at all tested points. In agreement with MFI values, a more intense EGFP band was obtained with lipoplexes.

3.3. Cellular uptake

The flow citometry analysis carried out with FITC-labelled pDNA complexes indicated that cellular uptake was clearly time dependent. The percentage of FITC positive cells increased along the time, from 50 % (lipoplexes), almost no uptake (nioplexes) and 20 % (polyplexes) after 1 h of incubation, to 70 % (lipoplexes), 10 % (nioplexes) and 30 % (polyplexes) after 4 h of incubation with the complexes (Fig. 5A). However, no statistically significant differences were observed. In the flow citometry histogram (Fig. 5B) the uptake of the complexes after 4 h of incubation is observed: lipoplexes > polyplexes >

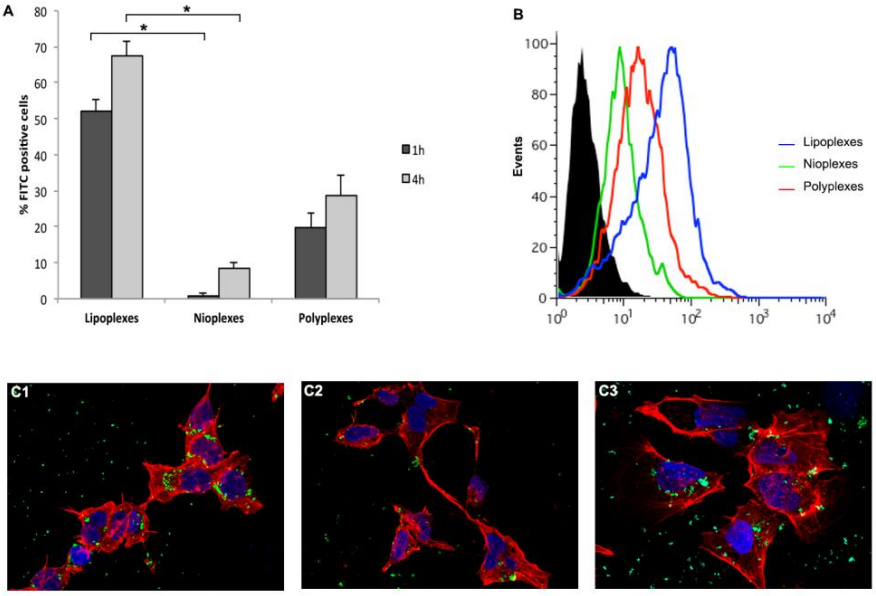

Figure 5. Cellular uptake of the complexes. (A) Bar graphs showing the percentage of FITC positive NT2 cells after the incubation for 1 hour or 4 hours with the complexes. Error bars represent ± SD (n=3). *P < 0.05. (B) Flow cytometry fluorescent histograms representing the cellular uptake of the complexes (formed with FITC labelled plasmid DNA) after 4 h of incubation. Black filled curve represents untreated cells. (C) Merged images of NT2 cells 4 hours after the addition of (C1) lipoplexes, (C2) nipolexes and (C3) polyplexes. Images are at 63X magnification. Blue colouring shows cell nuclei stained with DAPI; red colouring shows F-actin stained with Phalloidin; green colouring shows Cy3 labelled plasmid DNA complexed with the corresponding non-viral vector.

nioplexes. Confocal microscope images supported the quantitative results (Fig. 5 C1, C2, and C3).

3.4. Cell uptake mechanisms

The presence of CME and CvME pathways in NT2 cells was determined with specific markers of endocytic routes, Trasnferrin-Alexa Fluor and Cholera Toxin-Alexa Fluor, respectively. Flow citometry histograms (Fig. 6 A1 and A2) illustrated that almost 100 % of NT2 cells were properly stained after the treatment with fluorescent markers. This indicated that both CME and CvME are present in the studied cell line.

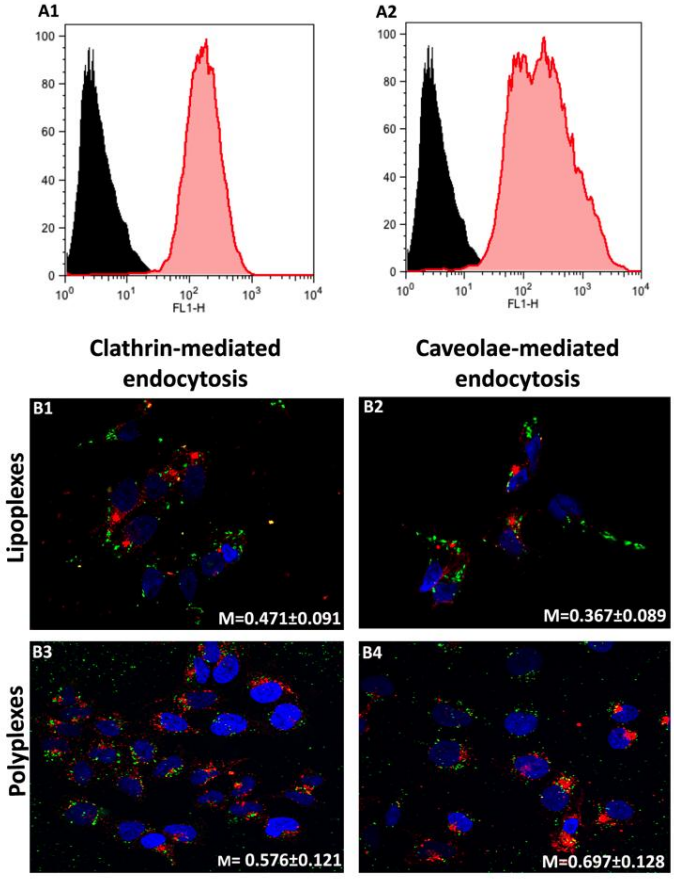

Figure 6. Endocytosis pathway of the complexes. (A) Flow citometry fluorescent histograms of cells treated with (A1) Transferrin-Alexa Fluor 488 at 50 μg/ml and (A2) Cholera toxin-Alexa Fluor 488 at 10 μg/ml. Black filled curve represents untreated cells. (B) Colocalization of test complexes (formed with FITC labelled plasmid DNA) (green) with the specific markers of endocytic pathways (red): clathrin-mediated endocytosis (Alexa Fluor 546-Transferrin at 50 μg/ml) and caveolae-mediated endocytosis (Alexa Fluor 555-cholera toxin at 10 μg/ml). The images were taken after 4 hours of incubation with the test complexes. Images are at 63x magnification. Nuclei are stained with DAPI (blue). Presence of yellow colour represents the overlay of the endocytic pathway marker and the vectors. The colocalization values are given as the fraction of complexes colocalizing with fluorescently labelled endocytic structure, represented by Mander´s (M1) coefficient.

Fig. 6B illustrates the confocal images 4 h after the co-treatment with the endocytosis markers (red) and lipoplexes /polyplexes (green). Due to the low cellular uptake of nioplexes, this formulation was discarded for uptake mechanism studies. Colocalization of red and green fluorescence showed visible yellow/orange punctuates structures (Fig. 6B). In addition, we performed a quantitative analysis; the colocalization values were represented as the fraction of complexes colocalized with the fluorescently labelled endocytic structures, represented by the Mander´s (M1) coefficient. The colocalization value of lipoplexes with Trasnferrin-Alexa Fluor (marker of CME pathway) was higher than the colocalization with Cholera toxin-AlexaFluor (marker of CvME pathway): Mander´s overlap coefficients were 0.471 and 0.367, respectively (Fig.6 B1 and B2). The overlap coefficients were superior for polyplexes, 0.576 with CME marker and 0.697 with CvME marker (Fig.6 B3 and B4).

3.5. Intracellular trafficking and endosomal escape

The colocalization of the complexes with the late endosomes at 4 and 6 hours was visualized and quantified by labelling the endosomal compartment with a specific marker: Lysotracker (red colour). Colocalization of red and green fluorescence gave rise to visible yellow/orange punctuate structures (Fig. 7A). The quantitative value was given as the Mander´s overlap coefficient. The results showed that polyplexes were located mainly in the late endosome after 4 hours (M= 0.735), and remained at least until 6 h (M=0.764). The values with lipoplexes were lower at both times (0.543 at 4h and 0.489 at 6h). In Fig. 7 A3 we observed that at 6 hours, the DNA carried by lipoplexes was located in the nucleus.

In order to evaluate whether the complexed DNA can be released from the endosomes, we examined the amount of released DNA after mixing the complexes with the previously elaborated PS micelles. The vesicles showed sizes of 170 nm and zeta potential of -65 mV. As observed in the agarose gel (Fig. 7C), DNA was released from all the complexes after their

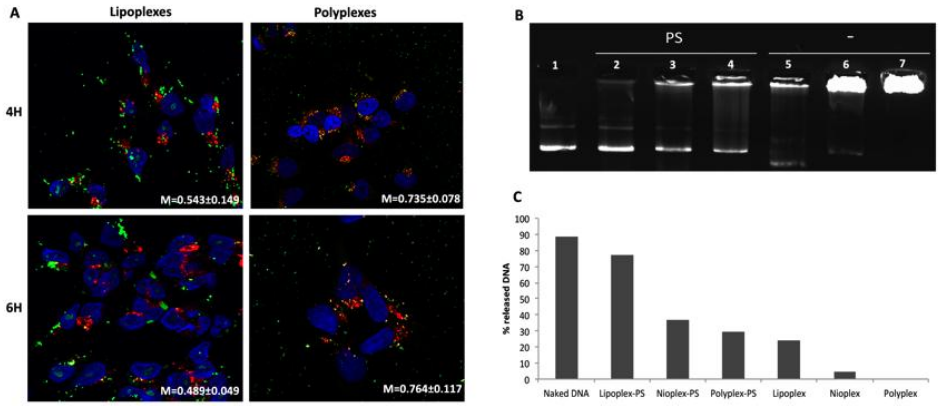

Figure 7. (A) Colocalization of test complexes (formed with FITC labelled plasmid DNA) (green) with Lysotracker Red-DND-99 that is a specific marker of the late endosome (red). The images were taken after 4 and 6 hours of incubation with the test complexes. Markers were added 1 hour prior to the end of the incubation time. Images are at 63 x magnification. Nuclei are stained with DAPI (blue). Presence of yellow colour represents the overlay of the endocytic pathway marker and the vectors. The colocalization values are given as the fraction of complexes colocalizing with fluorescently labelled endocytic structure, represented by Mander´s (M1) coefficient. (B) DNA release profiles measured with gel electrophoresis. Lane 1: naked DNA; lane 2: lipoplexes incubated with PS; lane 3: nioplexes incubated with PS; lane 4: polyplexes incubated with PS; lane 5: lipoplexes; lane 6: nioplexes; lane 7: polyplexes. PS refers to phosphatidyl serine micelles. (C) The percentage of released DNA quantified from the agarose gel using the Image Lab ™ software.

incubation with PS micelles (Fig. 7B). Almost 80 % of the total DNA was released from lipoplexes (lane 2); 40 % from nioplexes (lane 3) and 30 % from polyplexes (4). The release of the DNA was lower without the previous incubation with the anionic micelles: 30 %, 5% and 0 % with lipoplexes (lane 5), nioplexes (lane 6) and polyplexes (lane 7), respectively.

4. Discussion

The transfection of the, well-established, human NT2 or NT2-N cells is widely employed to obtain suitable platforms for the delivery of exogenous proteins into the CNS [3,7,12,13]. Indeed, the glioma trophism of NT2 cells [9] makes this undifferentiated cell line an interesting tool to derive new cellular

vehicles for clinical glioblastoma therapy. Until now, most of the studies based on genetically engineered NT2 cells rely on the use of viral vectors. However, the development of non-viral transfection strategies to produce stable or transient population of NT2 cells expressing exogenous gene products has obvious advantages, especially if they are subsequently grafted into animals´ models of human nervous system diseases.

The successful design of non-viral carriers for gene delivery requires a profound understanding of the transfection process, which is a complex multi-step process influenced by several factors. The vector is first bound to the cell membrane followed by internalization through an endocytic route. In order to avoid lysosomal degradation, the genetic material has to be released from the endosomes into the cytoplasm and target the nucleus to start the transcription process and protein synthesis. [30]. In recent years, many non-viral vectors have been developed and their efficient transfection has been demonstrated. However, less attention has been paid to the transfection process itself, starting from the binding to the cell membrane until the DNA reaches the nucleus. Regarding the gene delivery to NT2 cells, no works concerning the whole transfection process mediated by lipoplexes, nioplexes or polyplexes have been previously reported. The understanding of how the physicochemical characteristics of the vectors influence their intracellular traffic could lead to the design of new formulations with specific characteristics that could favour an appropriate intracellular pathway to obtain high transfection efficiencies. Thus, we have compared in neuronal precursor NT2 cells the transfection efficiency and uptake mechanisms of three different non-viral vectors.

The physicochemical characteristics and the composition of the non-viral vectors influence their interaction with the cell membrane and their intracellular uptake [31,34]. Thus, the characterization of the complexes is essential to better understand their behaviour when they are in contact with the cells. The data obtained

from lipoplexes based on Lipofectamine (size of 144.1 nm and zeta potential of -29.13 mV) (Fig. 1) is corroborated by other works, where lipoplexes elaborated with Lipofectamine (w/w ratio 4:1) had similar size and zeta potential values (150 nm and -0.4 mV) [35]. The lower zeta potential that we obtained could be explained due to the lower w/w (2:1) that we used to elaborate the lipoplexes. Concerning nioplexes and polyplexes our previous works supported our current results [25,28].

The transfection efficiency of the three vectors and the EGFP production was analysed along the time. Our results revealed that lipoplexes exhibited the highest transfection efficiencies and protein expression levels, which decreased along the time (Fig. 3 and Fig. 4). Lipofectamine is a well-known transfection agent with high transfection efficiency [35]. Although we did not show significant toxicity signs with our *in vitro* viability assay (Fig. 2), the *in vivo* application of this reagent has been limited due to its high toxicity compared to other compounds [36]. Our group has previously reported positive results obtained with nioplexes *in vitro*, ARPE 19 cells, and *in vivo* [25]. Surprisingly, nioplexes did not exhibit the expected transfection efficiencies in NT2 cells. So, we must consider that transfection efficiency is cell line dependent [37]. As an alternative to lipid based vectors, low molecular weight and highly deacetylated chitosan have showed promising results [28,29,38]. Against all predictions, our results indicated that polyplexes were more efficient for the transfection of NT2 cells than nioplexes. Although the transfection efficiency values were lower than the ones obtained with lipoplexes (15 % versus 35 % at 24 h shown in Fig. 3), the low toxicity of this polymer enhances its possible clinical applications. In addition, we should consider that the transfection efficiency of chitosan could be enhanced by its conjugation with several compounds [39].

In order to understand the differences in the transfection efficiency observed between the vectors, the cellular uptake of the carriers was determined after 1 and 4 hours of incubation (Fig. 5). The time dependent cellular uptake was already described before

by Apaolaza et al. They reported that the cellular uptake of solid lipid nanoparticles in ARPE19 and HEK293 increased along the time, obtaining the highest results after 8h of incubation [40]. Douglas et al. described that the cellular uptake of alginate-chitosan nanoparticles in 293T, COS7 and CHO cells reached a plateau by 2 h post-treatment [37]. As our transfection experiments were stopped after 4 h of incubation, we decided not to prolong the uptake studies more than 4h since our aim was only to compare the cellular uptake of the three formulations. The cellular uptake was clearly influenced by the carrier type. Lipoplexes (negatively charged) entered in almost 70 % of the cells at 4h and polyplexes (positively charged) in 30 % of the cells. Although the uptake of positively charged nanoparticles is presumably favored, the evidence of uptake of negatively charged nanoparticles has been reported. Despite the expected unfavorable interaction between the particles and the negatively charged cell membrane [41], recent work by Gainza et al. has revealed high uptake levels of lipid carriers (-34 mV) in several cell lines [42]. The uptake of negatively charged lipoplexes could be explained due to the interactions that may exist between the cationic lipids present in lipoplexes formulation and the lipid compounds in the cell membrane. Despite the desirable physicochemical characteristics observed in the nioplexes, (140 nm and +35 mV), we unexpectedly found that the cellular uptake of these vectors in NT2 cells was very low (10 % at 4h). Mochizuki et al. also reported low cellular uptake results with complexes based on cationic lipids and FITC labeled DNA [33]. By contrast, our research group has preliminary results that indicate that the cellular uptake of these nioplexes is almost 100 % in ARPE19 cells. All these data suggest that it is necessary to investigate the behavior of the formulations in every selected cell lines. Overall, the cellular uptake results showed a clear correlation respect to the corresponding transfection efficiency.

In order to obtain further information about the transfection process of our carriers and considering the different physicochemical characteristic of the studied vectors, we performed colocalization studies to elucidate if the characteristics of the vectors could influence their uptake

mechanism and intracellular traffic. Due to the low cellular uptake of nioplexes, these were discarded for the following experiments. Endocytosis has been postulated as the main mechanism of entry for non-viral vectors. Regarding nanoparticle uptake, CME and CvME are the most common and studied endocytosis routes [43]. By confocal imaging and colocalization assays (Fig. 6), we concluded that cell entry of lipoplexes (140 nm, -29 mV) occurred mainly through CME, while polyplexes (290 nm, +15 mV) entered the cell mainly by CvME. However, we did not observe radical differences, indicating that both pathways would be involved in the uptake of the two formulations. Although the data reported in the literature regarding the relationship between nanosizes and endocytic pathways is sometimes inconsistent, according to Rejman et al. internalization of microspheres with diameter < 200 nm involved clathrin-coated pits, while an increase in size provoked a shift of the mechanism that relies on caveolae-mediated endocytosis [44]. Moreover, the internalization process may be also affected by the components of the vectors. The entry of Lipofectamine based lipoplexes by the CME in a myoblast cell line was previously reported by Billiet et al. [35]. Peng et al. described that chitosan/poly(g-glutamic acid) complexes were internalized by macropynocitosis and caveolae-mediated pathway in HT1080 human fibrosarcoma cell line [45]. The major problem of analyzing the effects of the size in the trafficking process is the high polydispersity of many nanomaterials, as it occurs with our chitosan based polyplexes, where large and small particles coexist and different entry pathways may be involved. The surface characteristics of nanoparticles can also influence their internalization mechanisms. Unlike our results, according to the literature, positively charged vectors predominantly internalize through CME, while negatively charged formulations utilize CvME [46]. Nevertheless, exemptions including negatively charted PLGA nanoparticles that enter the cell through caveolae-independent pathways and PEI based cationic polyplexes that utilize CvME have also been reported in the literature [43,47]. In addition, we cannot forget that apart from CME and CvME,

clathrin- and caveolae- independent endocytosis routes may also take part in the internalization of the nanoparticles [34,48]. It is worth mentioning that depending on the cell line the internalization pathway of a specific complex could vary. Thus, our results should only be considered for the NT2 cell line.

At 4 hours, the highest colocalization for both lipoplexes and polyplexes was observed in the late endosome (Fig. 7) being superior with polyplexes, (0.735) compared to lipoplexes (0.543). Particles that enter via CME are confined within endosomes that will suffer a maturation process involving the compartment acidification that results in late endosomes and finally, lysosomes. Although some authors described the CvME as a route away from lysosomal degradation, recently, it has been postulated that the caveosomes are considered to fuse with normal acidified endosomes, allowing the transfer of material to lysosomes [49]. This hypothesis supports our results since the polyplexes that enter the cell mainly via caveolae-mediated pathways, were entrapped in the late-endosomes. The next step in the transfection process consists in the endosomal escape of the DNA in order to avoid lysosomal degradation. Several endosomal escape mechanisms are described in the literature, which vary depending on the nanoparticle [50]. A widely used approach consists of the "proton-sponge effect" which involves nanoparticles having a high buffering capacity, as it is the case of cationic polymers such us PEI. We previously described that UOC posse's higher buffering capacity that high molecular weight chitosans [51]. Another mechanism involves the electrostatic interactions between the nanoparticles and the anionic lipids of the endosome membrane, which causes the destabilization of the endosomal membrane allowing the release of the cargo. As an analogue of the endosomal compartment, we elaborated anionic micelles made from PS, according to Mochizuki´s et al. protocol [33]. As observed in Fig. 7B and 7C, the contact of the vectors with the PS micelles led to the release of the DNA from the particles. This simulated the ability of the studied formulations to release the DNA once they contact the lipid membrane of the endosome, which is a requisite for an efficient

transfection.

The velocity of the internalization process could vary depending on the carrier, especially depending on its size [44]. We observed a difference in the internalization velocity of the two studied vectors that could be related to the differences in their size. The colocalization values that we obtained with lipoplexes and the specific markers at 4 h and 6h were lower compared to the values obtained with polyplexes, which means that at the tested times most of the lipoplexes were not located within the endosomes. Moreover, 6 h after the addition of the particles to the cells, the FITC-labeled DNA carried by lipoplexes was located in the nucleus while the DNA carried by polyplexes was still located within the late endosomes (Fig.7A). This suggested that the internalization process of lipoplexes is a relatively rapid process compared to polyplexes. Rejma et al described that internalization process of latex beads with 50-100 nm diameter was more rapid than the process of 200 nm particles [44].

5. Conclusion

In summary, we have elaborated and characterized three different vectors for pDNA delivery to human neuronal precursor NT2 cells. Their transfection efficiency and cellular uptake was analysed along the time observing a correlation between both processes. In order to further understand the behavior of the complexes inside the cells, we determined the uptake mechanism of lipoplexes and polyplexes, concluding that they enter in the cells mainly via CME and CvME, respectively. In addition, at 4 hours the complexes were localized in the late endosome and were able to release the DNA. Differences in the internalization velocity were observed between lipoplexes and polyplexes. The DNA carried by lipoplexes was localized in the nucleus at 6 h, while the DNA carried by polyplexes remained in the late endosome. It is difficult to draw general conclusions about how to produce complexes with optimal transfection efficiencies. Therefore, it is essential to evaluate and design an appropriate vector for each cell line. According to our results, the cellular uptake of the complexes is a bottleneck in the

transfection process of NT2 cells. Overall, this work gives some knowledge about the main consideration that need to be taken into account for the design and development of novel non-viral vectors to efficiently transfect human neuronal precursor NT2 cells as a first step in the development of a suitable platform for the delivery of exogenous biological agents into the CNS.

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Conflicts of interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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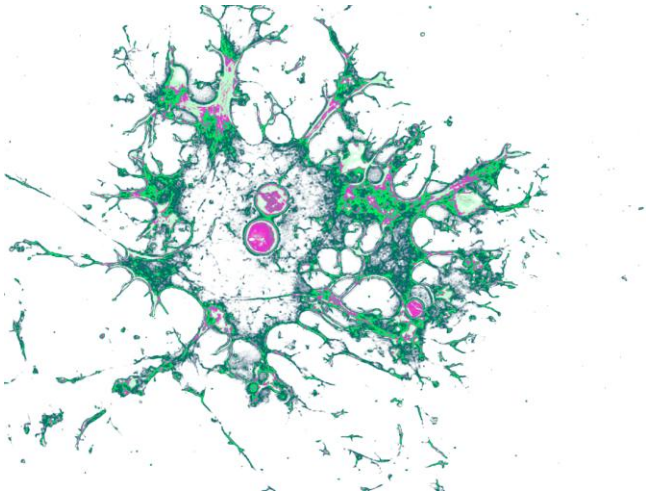


Eztabaida

Discusión

Discussion

Terapia genikoa material genetikoa zelulan barneratzea oinarri duen estrategia terapeutikoa da. Gene terapiaren bidez akats genetikoak zuzendu, terapiarako beharrezkoa den osagai biologikoaren gainespresioa eragin, edo gaitza eragiten duen genearen espresioa eten daiteke [1]. Garapenean dauden gene terapiaren aplikazioak bi motatakoak izan daitezke: *in vivo* eta *ex vivo* erakoak. *In vivo* erako estrategian genea zuzenean injektatzen zaio pazienteari, *ex vivo* aplikazioetan aldiz, lehendabizi zelulak *in vitro* genetikoki eraldatzen dira eta ondoren kaltetutako gunean txertatzen zaizkio pazienteari, beste era batera esanda, *ex vivo* aplikazioetan zelula bidezko geneen garraioa burutzen da. Gene terapiaren arrakastarako oso garrantzitsua da garatutako bektoreak genea modu selektiboan, eraginkortasunez eta toxikotasunik gabe itu-zeluletara garraiatzea [2]. Geneen garraiorako bektoreak bi taldetan sailkatu daitezke: biralak eta ez-biralak. Bektore biralak trasfektatzeko gaitasun handia dutenez oso erabiliak dira. Gene ingeniaritza arloko aurrerakuntzei esker garatu zen Glybera, Europako Medikamenduen Agentziak (EMA) 2012an baimendutako gene terapiarako lehenengo medikamendua [3], oinarrizko osagaia AAV birusak dituena eta lipoprotein lipasa entzimaren gabezia tratatzeko aholkatuta dagoena. Hala ere, bektore biralek, ez-biralekin konparatzen baditugu, segurtsun txikiagoa dute; ezin daiteke ahaztu mutagenesi eta immunogenizitate arriskua handiagoa dela [4,5]. Gainera, garraiatu dezaketen kargaren tamaina txikia (4Kb) [6] eta ekoizpen garestia bektore biralen aplikazio klinikorako muga bezala kontutan izan behar

dira. Muga hauek direla eta ikerlariak gero eta interes handiagoa jartzen ari dira bektore ez-biralen garapenean [7]. Bektore ez-biralek abantaila gisa ez dute osagai biral kutsakorrik, erantzun immunea eragiteko aukera txikiagoa da, garraiatu dezaketen azido nukleiko kantitatea handiagoa da eta eskala handiko ekoizpena errazagoa da [6]. Hala ere, *in vivo* erako gene terapian bektore ez-biralak gehiago erabiliak izan daitezen, ezinbestekoa da beraien transfekzio gaitasuna handitzeko gainditu behar diren zelula barneko eta zelula kanpoko oztopoak ondo ezagutzea [1]. Ondorioz, bektore ez-biral eraginkorrak lortu nahi badira ezinbestekoa da tentuz garatza, karakterizatzea, ebaluatzea eta optimizatzea.

Doktorego tesi honen oinarria dira geneak garraiatzeko bektore ez-biral gisa frogatu nahi diren purutasun altuko oligokitosanoak (UOC). Kitosanoa (Ch) polimero natural kationikoa da eta lotura glikosidikoaren bitartez elkartuta dauden -D-glukosamina eta -N-azetil-D-glukosamina azpiunitateez osatuta dago. Ch-ak dituen amina taldeak, beraien karga positiboari esker, gai dira DNA-a bezalako karga negatibodun molekulekin interakzio elektrostatikoaren bidez lotzeko. Lotura horretatik sortzen den molekula berriari poliplexo izena ematen zaio (Ch/AND poliplexoa) [8]. Argitaratutako emaitzen arabera, Ch-aren pisu molekularrak eta deazetilazio graduak (DDA) eragin garrantzitsua dute Ch osagai gisa duten bektore ez-biralen egonkortasun fisiko-kimikoan eta transfektatzeko gaitasunean [9]. Doktorego tesi honetan Novafect etxeko O15 eta O25 Ch-ak erabili ditugu, izan ere pisu molekular baxuko eta deazetilazio handiko oligokitosano hauen ezaugarriak oso egokiak iruditu zitzaizkigun geneen garraiorako. Nahiz eta ikerketa lan askotan deskribatu diren polimero hauen ezaugarri egokiak geneen transfekziorako, oraindik ere lan handia egin behar da Ch-etan oinarritutako poliplexoen transfekzio gaitasun baxua hobetzeko. Beraz, transfekzio gaitasunean eragiten duten faktore garrantzitsuenak ondo aztertzea eta transfekzio

prozesuaren pauso guztiak ondo ulertzea, oso garrantzitsua da Ch osagai gisa duten bektore ez-biralen diseinu eta garapenean aurrerapausoak emateko.

1. Purutasun handiko oligokitosanoekin formulatutako poliplexoen transfekzio gaitasunean faktore fisiko-kimikoek duten eragina

Ch/DNA poliplexoen transfekzio gaitasunean eragin gehien duten bi ezaugarriak tamaina eta zeta potentziala dira. Normalean, partikulak zenbat eta txikiagoak izan badirudi zelulak modu eraginkorragoan transfektatzen direla [10, 11]. Horrez gain, partikulen tamainak mugatu egin dezake bektoreek organo ezberdinetara izan dezaketen irisgarritasuna [12]. Zeta potentzialari dagokionez, poliplexoek gainazal karga handia izateak egonkortasun koloidala ematen dio formulazioari eta, normalean, karga positibo handia duten partikulen kasuan zelula barneratze handiagoa eta transfekzio eraginkorragoa lortzen da *in vitro* [13]. Hala ere, bi ezaugarri hauetan faktore fisiko-kimikoen eraginaren azterketa zehatzagoen beharra dago. Hau kontutan izanda, doktorego tesi honen lehenengo atalean ondorengo galderari eratzuna eman nahi izan diogu: nola eragiten diote UOC-ak oinarri dituzten poliplexoen tamainari eta zeta potentzialari pH-a, gatzen kontzentrazioa eta N/P ratioa (nitrogeno eta fosfato ratioa) bezalako faktoreek?

O15 eta O25 UOC/pCMS-EGFP poliplexoen partikula tamainan eta zeta potentzialean pH-ak, gatz kontzentrazioak eta N/P ratioak duten eragina eta faktore hauen arteko interakzioak, diseinu experimental ortogonalaren bitartez aztertu ziren. Faktore bakoitzak partikulen tamainan duen eragina aztertu ostean, honako ondorioak atera daitezke (1. Taula); batetik, pH-a 6,2tik 7,4ra igotzen denean poliplexoen tamaina ere igo egiten dela; bestetik, N/P ratioa 10-etik 60-ra igotzean partikulen tamaina murriztu egiten dela; eta azkenik, gatz kontzentrazioa handitzeak ez duela ia eragiten. Dena den, faktoreen arteko interakzioak nahiko

handiak dira; beraz, faktore bakoitzak partikula tamainan duen eragina ondo ulertzeko, beste bi faktoreak ere kontutan izan behar direla ondorioztatu daiteke.

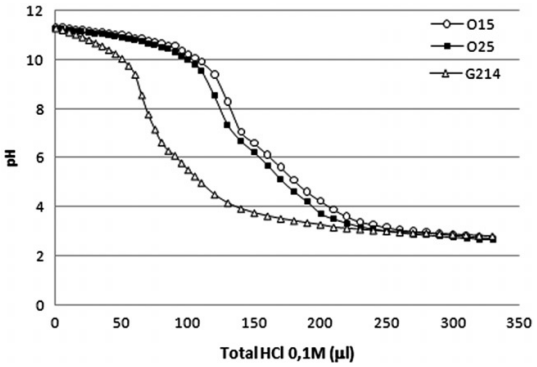
1. Taula: Efektu eta interakzioen baloreak UOC/pCMS-EGFP poliplexoen tamainan eta zeta potentzialean. X_1e: pH-aren efektua, X_2e: N/P-aren efektua, X_3e: [NaCl]-aren efektua, X_1X_2i: pH-N/P interakzioa, X_1X_3i: pH-[NaCl] interakzioa, X_2X_3i: N/P-[NaCl] interkzioak.

	Tamaina (nm)		Zeta (mV)	
Efektu eta Interakzioak	**O15**	**O25**	**O15**	**O25**
X_1 e	180.37	28.73	-12.86	-14.91
X_2e	-139.98	-85.31	3.86	-0.65
X_3e	-21.93	13.31	-4.80	-6.16
X_1 X_2i	-128.12	-44.98	2.26	-0.25
X_1 X_3i	-57.57	66.58	0.50	0.73
X_2 X_3i	42.88	55.43	-0.60	2.60

Konplexuen tamainak transfekzio gaitasunean duen eraginari buruzko orain arte argitaratutako emaitzak kontrajarriak izan dira kasu askotan [14, 15]. Bestalde, ikerketa talde gehienen ondorioak bat datoz konplexuen zeta potentzialak transfekzio gaitasunean duen eragina aztertzean, eta argi dago garrantzi handiko faktorea dela. Gure emaitzetan ikusi zen poliplexoen zeta potentzialean soluzioaren pH-ak eragin handia duela. pH-a 6,2tik 7,4ra igotzen denean UOC O15 eta O25 poliplexoen zeta potentzialak 12,86 eta 14,91 mV jaitsi ziren, hurrenez hurren (1. taula). Horrez gain, partikula tamainaren kasuan ez bezala, zeta potentzialean izan dezaketen eraginaz ari garenean, aztertutako faktoreen artean ez dela interakziorik ikusi azpimarratu beharra dago.

Geneen garraiorako polimeroekin lan egiten denean kontutan izan beharreko beste oinarrizko ezaugarri bat *buffer* gaitasuna da, ezaugarri honen araberakoa izan daitekelako poliplexoek behin zelula barnean endosomatik irteteko duten gaitasuna [16]. Nahiz eta Ch-ek orokorrean *buffer* gaitasun txikia izan [17], gure laborategian frogatuta utzi genuen UOC-ak (O15 eta O25) G214 pisu molecular altuko Ch-ak (340 kDA and › 90 DDA) baino *buffer* gaitasun handiagoa dutela (1.

irudia). Emaitza hauek are gehiago indartzen dute UOC-ei buruz geneukan hasierako hipotesia, geneen garraioarako hautagai egokiak izan daitezkela.

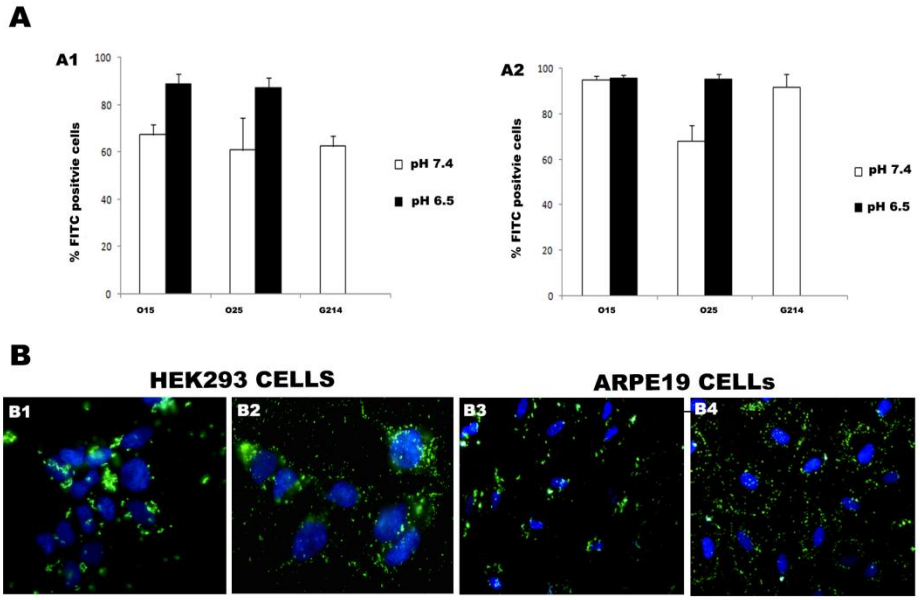


1. Irudia: O15 eta O25 UOC eta G214 kitosanoen azido-base balorazioaren perfilak NaCl 150 mM eko soluzio batean.

Formulazioaren pH-ak gure poliplexoen karga moldatu dezake, era berean, poliplexoen zeta potentzialak esekiduraren egonkortasunean eragiten duenez eta partikulak mintz biologikoetara lotzeko ezaugarri garrantzitsua denez, transfekzio gaitasunean oso kontutan izan beharreko ezaugarria da [18]. Hori dela eta, poliplexoak garraiatzeko erabiltzen den transfekziorako soluzioa azidotuz UOC-ekin burututako transfekzioaren eraginkortasuna zenbat aldatu daitekeen jakiteko nahia piztu zitzaigun.

Hipotesi hau argitzeko, lehendabizi, HEK293 eta ARPE19 zeluletan UOC/pCMS-EGFP-FITC poliplexoen barneratzean pH-aren jaitsierak duen eragina aztertu genuen. Entsegu hauetarako eta ondorengoetarako erabili beharreko N/P ratio egokiena, 20:1, aurretik egindako esperimentuetan zehaztu zen eta pisu molekular altuko Ch kontrol gisa, G214 erabili zen. Gure emaitzen arabera, 2 A1 eta A2 irudietan ikusi daitekenez (2. irudia), pH 7,4an egindako entseguarekin konparatuz, FITC positiboak diren zelulen portzentajea handiagoa da entsegua pH

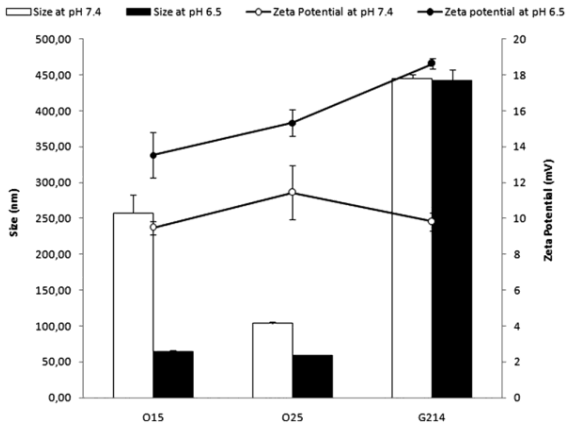
6,5-ean egiten denean. Mikroskopioko fluoreszentzia irudiek fluxu zitometroaren bidez lortutako emaitza hauek baieztatu egiten dituzte, 2B irudian ikusi daitekeen bezala.



2. Irudia: UOC-an oinarriturio kitosanoek garraiaturiko pCMS-EGFP (FITC rekin markatua)-ren zelula barneratzea (N/P= 20). pH 6,5 eta 7,4 an, UOC O15 eta O25 eta G214 ekin trataturiko FITC positivo diren HEK293 (A1) eta ARPE19 (A2) zelulen portzentaiak. FITC-pCMS-EGFP-aren barneraketaren fluoreszentzia irudiak: UOC O15/FITC-pCMS-EGFP pH 7,4an HEK293 zeluletan (B1); UOC O15/FITC-pCMS-EGFP pH 6,5ean HEK293 zeluletan (B2); UOC O15/FITC-pCMS-EGFP pH 7,4an ARPE19 zeluletan (B3); UOC O15/FITC-pCMS-EGFP pH 6,5ean ARPE19 zeluletan (B4).

Zelulan barneratze entseguan transfekziorako soluzioa azidotzean ikusitako ezberdintasunak, poliplexoen zeta potentzial positiboa handitzen delako gerta daitezke (3. irudia, lerroak). Emaitza hauetatik ondorioztatzen dugu, konplexuen karga positiboari esker zelularen mintz plasmatikoarekin interakzioa handiagoa

dela eta horrek eragin dezakeela zelulan barneratzen diren partikulen portzentajea areagotzea [19, 20]. Horrez gain, pH 6,5ean ekoiztutako UOC-ak oinarri dituzten poliplexoen partikula tamaina pH 7,4an formulatutakoena baino txikiagoa da (3. irudia, barrak). Azidotzeak eragindako tamainaren jaitsiera honek ere neurri batean azaldu dezake zelulan barneratze portzentaje handiagoa. Hala ere, ARPE19 zeluletan tamaina handiagoko partikulen (pH 7,4an ekoiztutako 250 nm-ko UOC O15 eta G214arekin formulatutako 400 nm-ko poliplexoak) barneratzea tamaina txikiagokoen antzekoa izan zen; beraz, esan genezake zelula mota honetan partikulen tamaina ez dela zelulan barneratze prozesurako ezaugarri kritikoa eta erabilitako zelula motak tamainak berak baino pisu handiagoa izan dezakeela barneratze prozesuan [21].



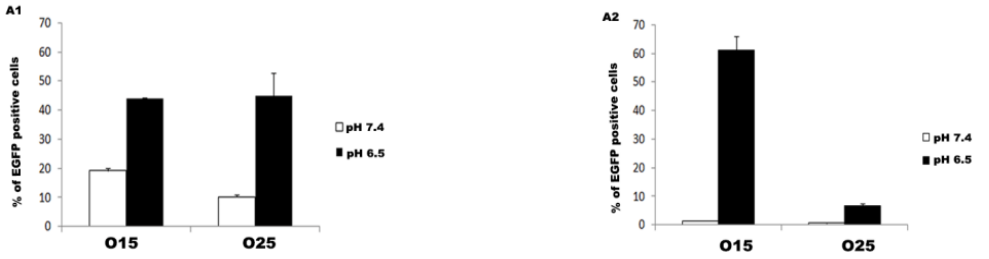
3. Irudia: O15 eta O25 UOC/pCMS-EGFP eta G214/pCMS-EGFP (N/P= 20) poliplexoen tamaina eta zeta potentziala, pH 7,4an eta 6,5ean.

pH balioek zelulan barneratze prozesuan eragin argia dutela ikusi ostean, ondorengo esperimentuetan partikulak zelulan gehiago barneratzea transfekzio eraginkortasunarekin bat zetorren aztertu genuen. Laburbilduz, ondorengo

galdera erantzutea zen gure helburua: posible al da transfekziorako disoluzioa azidotuz UOC-etan oinarritutako poliplexoen transfekzio eraginkortasuna hobetzea? EGFP positiboak ziren zelulen portzentajea fluxu zitometria bidez aztertu genuen transfekzioa martxan jarri eta 72 ordura. Orain arte argitaratutakoarekin bat etorriz, pH-a 7,4tik 6,5era jaisteak zelula EGFP positiboen portzentajearen igoera eragin zuen frogatutako UOC biekin, bai HEK293 zeluletan (4 A1 irudia) eta baita ARPE19 zeluletan (4 A2 irudia) ere [13, 22]. Entseguan erabilitako hiru Ch-en artean ezberdintasunak ikusi ziren. G214 pisu molekular handiko Ch-arekin transfekziorik ez zen lortu. Ch honek zelulan barneratu den DNA askatzeko gaitasun txikia izan daiteke ez transfektatzearen arrazoietako bat [23], era berean, frogatu zen bezala G214 Ch-ak UOC-ek baino buffer gaitasun txikiagoa izateak endosomatik ihes egitea oztopatzen du [17]. UOC-ei dagokionez, O15arekin eratutako poliplexoekin O25arekin eratutakoekin baino zelula EGFP positiboen portzentaje erlatibo handiago lortu zen, nahiz eta emaitza hau ez zetorren bat zelulan barneratze mailarekin. Zelulan barneratze mailaren eta transfekzio eraginkortasunaren arteko korrelazio falta hau beste ikertzaile batzuek ere deskribatu dutenez [24], ezinbestekoa dirudi gure bektore ez-biralen zelulabarneko prozesua ahalik eta ondoen ulertzea.

Bektore ez-biral bat eraginkorra izateaz gain ez du toxikoa izan behar. Ondorioz, garatutako poliplexoen zitotoxizitatea eta pH-aren jaitsierak zelulen bideragarritasunean izan dezakeen eragina aztertzea erabaki genuen. Lortutako emaitzen arabera esan daiteke erabilitako pH-a edozein izanik ere UOC ezberdinez egindako poliplexoak ez direla toxikoak frogatutako bi lerro zelularretan (HEK293 eta ARPE19).

Doktorego tesi honen lehenengo atal honetan, transfekzio prozesuan kontuan izan behar diren partikularen ezaugarri fisiko-kimikoetan, zehazki tamainan eta



4. Irudia: pH desberdinetan UOC O15 y O25 /pCMS-EGFP (N/P=20) poliplexoen transfekzio efizientzia HEK293 (A1) eta ARPE19 (A2) zeluletan. Transkekzio datuak Lipofektamina 2000^{TM}-rekin lorturiko datuekin normalizatu ziren.

zeta potentzialean, faktore ezberdinen eragina deskribatu dugu. Ondorio giza azpimarratu genezake, garatu ditugun poliplexoen transfektatzeko eraginkortasuna hobetzea posible dela transfekziorako erabiltzen den soluzioa apur bat azidotuz. Nahiz eta, *in vitro* lortutako emaitzak argiak izan, ezinezkoa da oraindik emaitza hauetatik aplikazio kliniko zehatz batean apustua egitea. Dena den, guk ondorengoak proposatzen ditugu: gure emaitzen arabera UOC-etan oinarritutako poliplexoak ingurune azidoan bizi diren zeluletan material genetikoa transfektatzeko proposatzen ditugu, esate baterako tumore zeluletan material genetikoa transfektatzeko [25]. Gehiago zehaztuz, *in vivo* iradokitzen dugun aplikazioa tumore ehunean zuzenean poliplexoak administratzea izango litzateke [26]. Beste aukera posible bat UOC-ekin eratutako poliplexoak *ex vivo* terapia genikoan erabiltzea da. Terapia mota honetan, *in vitro* transfekziorik eraginkorrena lortzeko baldintza egokienak zehaztea garrantzitsua da, ondoren transfektatutako zelulak pazienteari ezartzeko. Honen harira, lan honen lehenengo ondorio argia aipatutako aplikazioen egingarritasunarekin bat dator, hots, transfekzio soluzioa azidotuz UOC-ekin egindako poliplexoen transfekzioa eraginkorragoa dela orokorrean.

2. Plasmidoaren garrantzia: posible al da purutasun altuko oligokitosanoekin 40 Kbp-ko adenobirus baten bektore den plasmidoa garraiatzea?

UOC-etan oinarritutako poliplexoak tumoreetan geneak transfektatzeko baliagarriak izan daitezkela kontuan izanda, doktorego tesi honen bigarren atalean erabaki genuen UOC-ekin eraturiko poliplexoak minbiziaren aurkako terapia genikoan erabiltzeko aukera aztertzea. Arlo honetan gero eta indar handiagoa hartzen ari da adenobirus onkolitikoen (Ad) erabilera. Ad-ak modu selektiboan tumore-zeluletan erreplikatzeaz gain, erreplikazioaren ondorengoak gai dira tumore-ehunean zehar zabaldu eta gainontzeko minbizi-zelulak infektatzeko [27, 28]. Hala ere, Ad askeak zuzenean odolean injektatzen direnean zenbait arazo agertu daitezke; besteak beste, Ad-ek interakzioak pairatu ditzakete gibeleko Kupffer zelulekin, plaketekin, eritrozitoekin, konplementuarekin edota antigorputz neutralizatzaileekin [29]. Horregatik, azken aldian ikerketa ezberdinak egin dira Ad onkolitikoen plasmidoak bektore ez-biralekin garraiatu eta aipatu ditugun arazoak gainditzeko. Estrategia honetan Ad onkolitikoen eraginkortasun terapeutikoaren eta bektore ez-biralen bidezko garraio sistemikoaren arteko sinergia uztartzen dira [30, 31]. Estrategia berritzaile honen oztopo nagusia adenobirus onkolitikoak kodifikatzen dituzten plasmidoen tamaina handia da, 40 Kbp ingurukoa. Teorikoki bektore ez-biralek mugarik gabeko plasmido kantitatea garraiatu dezakete [32], horretan oinarrituta doktorego tesi honen bigarren atalean, UOC-ekin eratutako poliplexoek 40 Kpb-eko tamaina duen pAdTLRGD plasmido adenobirala garraiatu eta HEK293 eta A549 zelula-lerroak transfektatzeko duten gaitasuna frogatu nahi izan genuen. Frogarako erabili gneuen plasmidoak adenobirus onkolitikoaren sekuentzia izateaz gain EGFP proteina markatzailea espresatzeko gai da.

Lanaren lehenengo atalean UOC/pCMS-EGFP poliplexoen tamainan eta zeta potentzialean formulazioko N/P ratioaren, pH-aren eta gatz kontzentrazioaren eragina argi ikusi genuenez, jakin mina piztu zitzaigun ea pAdTLRGD bezalako tamaina handiko plasmido batean faktore horien eragina beretsua zen edo ez jakiteko. Ondorioz, bigarren diseinu esperimental ortogonala burutu genuen eta argi ikusi zen kasu honetan ere, tamaina eta zeta potentziala ezberdinak zirela poliplexoen formulazioan aipatutako faktoreetan aldaketak eginez gero (2. taula).

2. Taula: Efektu eta interakzioen baloreak UOC/pAdTLRGD poliplexoen tamainan eta zeta potentzialean. X_1e: N/P-aren efektua, X_2e: [NaCl]-aren efektua, X_3e: pH-aren efektua, X_1X_2i: N/P-[NaCl] interakzioa, X_1X_3i: N/P-pH interakzioa, X_2X_3i: [NaCl]-pH interakzioa.

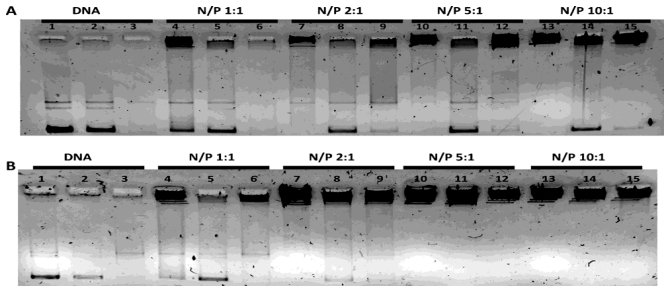
	Tamaina (nm)		Zeta (mV)	
Efektu eta Interakzioak	**O15**	**O25**	**O15**	**O25**
X_1 e	-17.1	-10.7275	3.6575	2.8875
X_2e	32.3	4.0325	20.8475	-2.2375
X_3e	-31.46	5.8725	-33.8325	-33.5875
X_1 X_2i	22.15	5.7225	-7.3725	-0.0125
X_1 X_3i	-9.19	-14.0175	10.2025	-1.5625
X_2 X_3i	10.29	-5.6775	-18.2025	-0.0875

Tamainari dagokionez, konplexu guztiak nanometrikoak ziren (200 nm inguru). N/P ratioaren kasuan, 10etik 60ra handitzean, bai O15 zein O25 poliplexoen partikula tamaina 17,1 eta 10,7 nm txikitu zen, hurrenez hurren. Gatz kontzentrazioak, aldiz, O25 poliplexoen tamainan eragina izan zuen (5etik 150 mmol-era aldatzean partikula tamaina 32,2 nm handitzen da) baina ez zuen O15 poliplexoen tamainan aldaketa nabarmenik eragin. Bestalde, pH-a handitzean O15 poliplexoen tamaina bakarrik aldatzen zen (31,5 nm) eta ez O25 poliplexoena. Zeta potentzialari dagokionez, azpimarragarria da pH-aren eragin nabarmena, izan ere pH-aren balioa apur bat handitzeak 33 mV-ko partikulen gainazal-kargaren jaitsiera eragin zuen. Hala ere, faktoreek beraien artean interakzio esanguratsuak erakutsi zituzten bi efektuentzako, batez ere tamainaren kasuan. Interakzio hauek

argi erakutsi ziguten faktore bakoitzaren eragina formulazioaren ezaugarri fisiko-kimikoetan aztertutako inguruko beste bi faktoreen menpe ere badagoela. Era berean, oso interesgarria izan zen ikustea lehenengo atalean aztertu genituen pCMS-EGFP plasmidoarekin eginiko poliplexoen eta atal honetako pAdTLRGD plasmidoarekin osatutakoen artean ezaugarri fisiko-kimikoetan ez zegoela diferentzia nabarmenik, nahiz eta pAdTLRGD plasmidoa pCMS-EGFP plasmidoa baino 10 aldiz handiagoa izan. Antzeko esperimentuetan beste ikerketa talde batzuek ikusi zuten, 52,5 Kpb-eko tamainadun plasmidoekin garatutako lipoplexoen eta pDNA txikiekin eratutako lipoplexoen egituraren eta tamainaren artean ez dagoela ezberdintasunik [33].

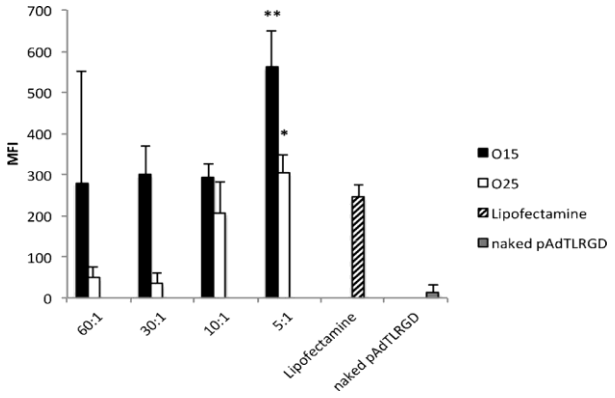
Tamainaz eta zeta potentzialaz gain, bektore ez-biralak eta pDNAk lotzeko duten afinitatea ezaugarri garrantzitsua da transfekzio eraginkorra lortzeko. Bektore ez-birala gai izan behar da plasmidoa lotzeko, entzimen digestiotik babesteko eta azkenik askatzeko, bestela ez litzateke transfekziorik emango [24]. pAdTLRGD-ren tamaina handia zela eta ondorengo zalantza geneukan: gai izango al dira UOC-ak ordura arte erabilitakoak baino hamar aldiz handiagoa den plasmidoa lotu, babestu eta askatzeko? Agarosa gelean eginiko elektroforesien emaitzen arabera, UOC-ekin eginiko poliplexoek pAdTLRGD lotzeko, babesteko eta askatzeko gaitasuna erabilitako N/P ratioaren menpe zegoen (5. irudia). Zehazki, pisu molekular txikiagoa duen O15 oligokitosanoarekin (5,7 kDa) ekoiztutako poliplexoek O25-arekin (7,3 kDa) baino polimero kantitate handiagoa behar izan zuten formulazioko pDNA guztia lotu eta babesteko (5A eta 5B irudiak). Gainera, seguraski pisu molekularrean bi polimeroen ezberdintasun txiki horren eraginez, O15 UOC-arekin eginiko poliplexoak N/P 5 eta 10 ratioak erabilita plasmidoa askatzeko gai ziren bitartean, O25-arekin eginiko plasmidoen kasuan ez genuen emaitza bera lortu. Emaitzak ikusirik ondorioztatu genuen pisu molekular handiagoko polimeroarekin eginiko poliplexoen formulazioa egonkorragoa zela,

baina formulazioaren egonkortasun horrek plasmidoaren askatzea mugatu zezakeela [9]. Orokorrean, agarosa gelean eginiko elektroforesiko emaitzen arabera, esan genezake pAdTLRGD-aren tamaina handia bada ere, erabili genituen bi UOC-ak gain izan zirela plasmidoa modu egokian lotu, entzimen digestiotik babestu eta behar zenean askatzeko.



5. Irudia: Agarosa gelean eginiko elektroforesia non, O15 (A) eta O25 (B) UOC-etan oinarritutako poliplexoek plasmidoa lotzeko, askatzeko eta babesteko duten gaitasuna erakusten den. Poliplexoak SDS-rekin (2, 5, 8, 11 eta 14. ilarak) eta SDS + DNase I-ekin (3, 6, 9 eta 15. Ilarak) tratatu ziren.

UOC/pAdTLRGD poliplexoek plasmidoen garraiorako ezaugarri fisiko-kimiko egokiak zituztela frogatu ostean, fluxu zitometria bitartez HEK293 zeluletan transfekzioaren eraginkortasuna ebaluatzea erabaki genuen. Transfekzioari dagokionez, tumoreetako mikroingurunea ehun normaletakoa baino azidoagoa dela jakinda [25] eta aurrerago UOC-ekin egindako transfekzio esperimentuetan pH-a jaitsiaz emaitza hobeak lortu genituela ikusita, bigarren atal honetako transfekzio esperimentuak pH 7,1ean egitea erabaki genuen. EGFP positiboak diren zelulen portzentaje handiena (% 6 inguru) eta fluoreszentziaren batazbesteko intentsitate (MFI) altuena 5eko N/P ratioarekin prestatutako formulazioarekin lortu genuen (6. Irudia). N/P ratio handiagoetan lortutako emaitza kaskarren arrazoietako bat izan daiteke polimeroaren eta pDNA-ren arteko lotura indartsuagoa dela eta ondorioz, pDNA ez dela bektoretik behar den bezala

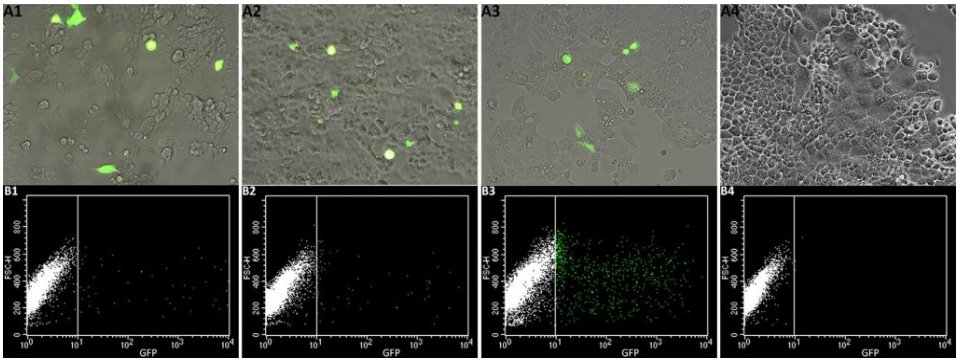


6. Irudia: N/P ratio desberdinetan, O15 (barra beltzak) eta O25 (barra zuriak) UOC/pAdTLRGD poliplexoekin transfektaturiko HEK293 zelulen fluoreszentziaren batazbesteko intentsitatea (MFI). Errore barrek desbideratze estandarra adierazten dute (n=3). *P ‹ 0.05 pAdTLRGD plasmido biluziarekin alderatuz. **P ‹ 0.01 pAdTLRGD plasmido biluziarekin alderatuz.

askatzen [24]. Erabilitako bi UOC-en konparaketa eginez gero, O15arekin estatistikoki esanguratsua den EGFP-aren MFI lortu genuen, beharbada Ch honen pisu molekularra piskat txikiagoa delako [35]. Beraz, MFI zelulak ekoiztutako EGFP-aren kantitatearen erreferentzia bezala hartuta, oligokitosano guztiek pisu molekular txikia baldin badute ere, pisu molekularraren diferentzia xumeek eragin handia izan dezakete transfekzio prozesuan.

HEK293 zeluletan transfekziorako baldintza egokienak zehaztu ostean, A549 biriketako adenokartzinoma zeluletan frogatu genuen. HEK293 zeluletan lortutako emaitzekin konparatuz EGFP positiboak ziren zelulen portzentaje baxuagoak lortu genituen. Dena den, 7. irudian ikusi daitekeen bezala, N/P 5/1 ratioarekin prestatutako poliplexoak, bai O15 zein O25-arekin, gai izan ziren 40 Kbp-eko plasmidoa A549 zeluletan transfektatu eta EGFP ekoizteko, eta nahiz eta transfekzio maila oso baxua izan, lehen aldia da ikerketa talde batek oligokitosano hauekin helburu hau lortzen duena. Gainera, HEK293 eta A549 zeluletan pAdTLRGD plasmido biluziarekin eginiko transfekzioetan ez genuen EGFP

espresiorik ikusi; hortik ondorioztatu daiteke bektore ez-biralak ezinbestekoak direla transfekzioa arrakastatsua izan dadin.



7. Irudia: UOC/pAdTLRGD poliplexoen transfekzio efizientzia A549 lerro zelularrean. (A) Zelula transfektatuen fase kontraste irudien eta fluoreszentzia irudien (GFP kanala) gainjartzea: (A1) O15 UOC/pAdTLRGD N/P 5:1 ratioan; (A2) O25 UOC/pAdTLRGD N/P 5:1 ratioan; (A3) Lipofektamina; (A4) pAdTLRGD biluzia. (B) Fluxu zitometriaren puntu diagramak (GFP *vs* FSC): (B1) O15 UOC/pAdTLRGD N/P 5:1 ratioan; (B2) O25 UOC/pAdTLRGD N/P 5:1 ratioan; (B3) Lipofektamina; (B4) pAdTLRGD biluzia.

Bigarren atal honetan tamaina handiko adenobirusdun plasmidoarekin (40Kbp) lehenengo ataleko pCMS-EGFP plasmidoarekin (5,5 Kbp) baino transfekzio portzentaje baxuagoak lortu genituen. Hala ere, nahiz eta pAdTLRGD plasmidoaren tamaina handiagatik lortutako transfekzio portzentajeak baxuak izan, garatutako poliplexoekin gai izan ginen zelularen mintzak zeharkatu, plasmidoa nukleoraino garraiatu eta EGFP proteina ekoizteko; hain zuzen ere, gure ikerketa taldea izan zen oligokitosanoekin helburu hau lortzen lenenengoa. Ikerketa lan honen bitartez bektore ez-biralek bektore biralekin alderatuz duten abantaila nagusienetakoa azaleratu genuen, izan ere bektore biralekin oso zaila edo ezinezkoa litzateke 40 Kbp-eko plasmido baten garraioa. Adenobirus onkolitikoa kodifikatuta daraman plasmidoarekin terapia konbinatua aplikagarria den jakin ahal izateko, hurrengo esperimentuak erreplikatzeko gaitasuna duten

adenobirusak kodifikatzen dituen plasmidoarekin egin beharko lirateke. Plasmidoa transfektatu ostean sortutako adenobirusek eta beraien ondorengoek tumorean zehar zabaltzeko duten gaitasunagatik, nahiz eta transfektatzeko gaitasun mugatua duten, aukera bat izan daiteke bektore ez-biralak *in vivo* erabiltzea terapia onkolitikorako. Etorkizuneko ikerketen helburu nagusienetako bat izan beharko litzateke UOC-ak adenobirusdun plasmidoekin konbinatuz lortutako poliplexoen administrazio sistemiko eraginkorra lortzea, ondoren minbizi primarioen eta metastasien tratamendu onkolitikoan arrakastarekin erabili ahal izateko. Bektore ez-biralei esker, birus onkolitikoen genoma zirkulazio sistemikoaren bitartez tumore-ehunera garraiatu eta erreplikatzea lortuko bagenu, Ad onkolitiko kutsakorrak eta hauen ondorengoak sortuko lirateke. Ad horiek tumore-zelulen lisia eragingo lukete eta aldi berean, aldameneko tumore-zelulak kutsatuko lituzkete. Baina oraindik ere lan asko egin beharra dago gure poliplexoen administrazio sistemikorako aurkitu ditugun oztopoak gainditzeko; besteak beste, *in vivo* erakutsitako ezegonkortasunak eta odoletik argitzapen azkarrak, era honetako terapia onkolitiko arrakastatsua lortzeko bide luzea egitera behartzen gaituzte. Hori dela eta, erronka zail honi aurre egiteko ezinbestekoa izango da medikamenduen askapenerako ditugun teknologietan hobekuntzak egitea.

3. Transfekzio prozesua pausuz-pausu: lipoplexoen, nioplexoen eta poliplexoen arteko azterketa konparatiboa NT2 giza-neuronen zelula prekurtsoreetan

Giza NTera2/D1 teratokartzinomatik eratorritako zelula lerroa (edo NT2 zelulak) oso erabilia da exogenoak diren proteinak nerbio-sistema zentralera (NSZ) garraiatzeko plataforma egokiaren bila dabiltzan ikertzaileen artean. Zelula hauek neuronen antzekoak diren (edo NT2-N zelulak) ondo identifikatutako zelula populazio batean ezberdindu daitezke eta ezberdindutako zelulak NSZ-an

transplantatu ostean ingurune berrira egokitzeko eta heltzeko gaitasuna dute tumorerik eragin gabe [36, 37, 38]. Horrez gain, NT2-N zelulek gliometarako tropismoa dutenez oso erabilgarriak izan daitezke glioblastomen terapia klinikorako erreferentziazko zelula-eredu gisa. Orain arte NT2 zelulak genetikoki eraldatzeko egin diren ikerketetan batez ere bektore biralak erabili dira. Hala ere, NT2 zelulek exogenoak diren geneen produktuak ekoizteko estrategia ez-biralak gutxinaka garatzen ari dira [39, 40]. Beraz, NT2 zelulen ezaugarri interesgarriak kontutan izanik, zelula hauek UOC-etan oinarritutako poliplexoekin transfektatzea posible zen planteatu genuen. Helburu hau lortuz gero, lehenengo urratsa izango litzateke NSZ-an *ex vivo* erako terapia genikorako plataforma egoki baten garapenean.

Aurreko ataletan aipatu bezala, bektore ez-biralekin lortzen den transfekzio maila, eta zehazki Ch-etan oinarritutako bektoreekin lortzen dena, nahiko baxua da; ondorioz, ahalik eta bektore eraginkorrena garatzeko ezinbestekoa da transfekzio prozesua bere osotasunean ondo ezagutzea. Genearen azken espresioa transfekzio prozesuko urrats ezberdinek mugatu dezakete. Horien artean garrantzitsuenak honako hauek dira: bektore ez-birala zelulan barneratzea, zelulan barneratzeko jarraitu duten bidea, bektoreek endosomatik ihes egiteko duten gaitasuna eta material genetikoa nukleoan sartzeko urratsa [41]. Beraz, NT2 zelulen transfekzioan erabakiorrak diren urratsak ezagutzeko, poliplexoen (Novafect O15 UOC-an oinarrituak), nioplexoen (niosoma lipidikoen formulazioak) eta lipoplexoen (Lipofektamina 2000 liposometan oinarrituak) arteko azterketa konparatiboa egitea erabaki genuen. Hiru formulazioak berezko elkarketaren metodoaren bitartez prestatu genituen eta 3. taulan laburbiltzen dira prestaketarako erabilitako osagai nagusiak, w/w ratioak eta DNA kantitateak (3. Taula).

3. Taula: Hiru formulazioen prestaketarako erabili diren oinarrizko konposatuak, w/w ratioak eta erabilitako ADN kantitateak laburbiltzen dituen taula

Formulazioa	Konposatu nagusia	w/w ratioa	DNA kantitatea (μg)
Lipoplexoak	Lipofectamine 2000	2:1	1.25
Nioplexoak	2,3-di(tetradecyloxy)propan-1-amine cationic lipidoa	20:1	1.25
Poliplexoak	Novafect O15 oligokitosano ultrapurua	13:1	1.25

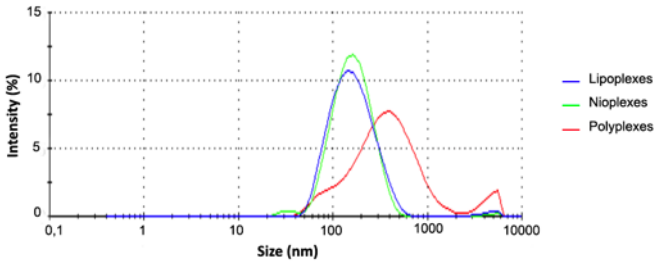
Bektore ez-biralaren ezaugarri fisiko-kimikoek eta osagaiek neurri batean zelularen mintzarekin elkarrekintza eta zelula barneko portaera baldintzatu dezakete [42]. Hori dela eta, hiru formulazioen partikula tamaina, zeta potentziala eta polidispertsio indizea (PDI) neurtu genituen (8. irudia). Partikula tamainari eta PDI-ari dagokionez, poliplexoen kasuan biak handiagoak izan ziren. Lipoplexoen eta nioplexoen partikula tamaina 140 nm ingurukoa izan zen eta PDI-a 0,21ekoa, poliplexoenaren erdia gutxi gora behera (294 nm eta 0,44, hurrenez hurren). Gainazal kargen balioak aldiz, bektorearen arabera oso ezberdinak izan ziren. Lipoplexoek -29,13 mV-ko zeta potentzial negatiboa zuten bitartean, nioplexoek eta poliplexoek gaizanal karga positiboak zituzten, +35.4 mV and +15.1 mV, hurrenez hurren (8A irudia).

Formulazioak karakterizatu eta gero, fluxu zitometria bidez transfektatzeko eraginkortasuna eta western blot bidez EGFP-ren ekoizpena neurtu genituen denbora ezberdinetan (9. irudia). Logikari kasu eginez, emaitzek argi isladatu zuten lipoplexoak transfekziorako eraginkorrenak zirela (9A irudia) eta proteina espresio maila handiena lortzen zutela (9B irudia). Izan ere, Lipofektamina transfekzio maila oso altuak ematen dituen transfekziorako erreferentziazko erreaktiboa baita. Nahiz eta formulazio honekin *in vitro* egin genituen entseguetan toxizitate zeinu argirik ez genuen ikusi, bere erabilera *in vivo* mugatua dago [43]. Orokorrean,

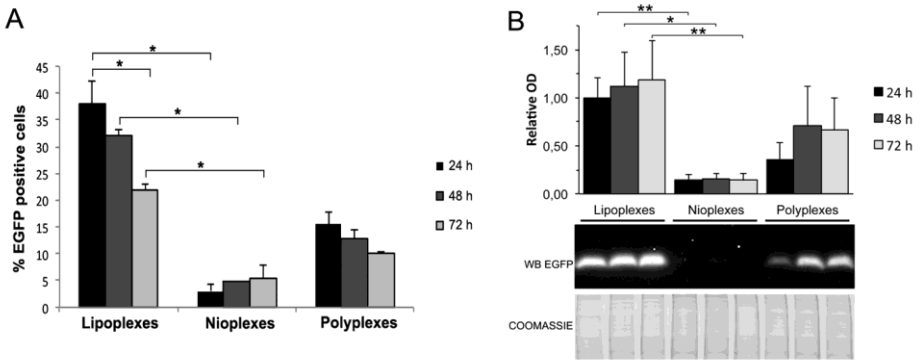
A

	Size (nm)	pdi	zeta ptential (mV)
Lipoplexes	144.1±6.3	0.21±0.04	-29.13±1.43
Nioplexes	142.5±0.64	0.21±0.04	35.4±0.7
Polyplexes	294.8±23.36	0.44±0.04	15.1±3.05

B



8. Irudia: Komplexuen karakterizazioa. (A) Tamaina, PDI eta zeta potentzialen balioak. Balore bakoitzak hiru neurriren batazbetekoa±desbideratze estandarra erakusten du.

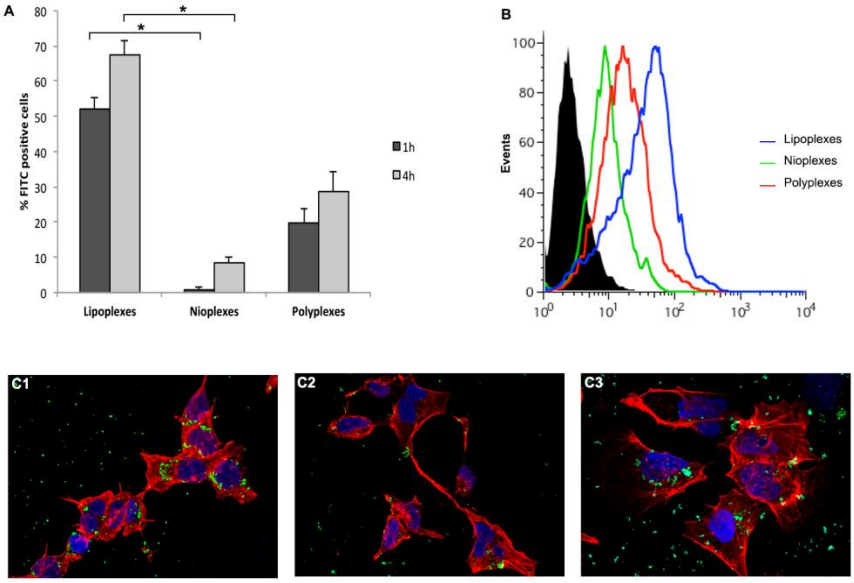


9. Irudia: Transfekzio efizientzia eta EGFP-ren espresioa, transfekzio egin eta 24, 48 eta 72 orduetara. (A) Barra grafikoa, non EGFP positibo diren zelulen portzentajeak erakusten diren. Errore barrek desbideratze estandarra adierazten dute (n=3). *P ‹ 0,05. (B) Barra grafikoa, non Western blot bidez egindako EGFP-ren analisiaren emaitza semikuantitatiboak erakusten diren. Grafikoaren azpian Western blot adierazgarri bat aurkezten da. Errore barrek desbideratze estandarra adierazten dute (n=4). *P ‹ 0,05, **P ‹ 0,01.

in vitro eta *in vivo* lortutako emaitza onengatik liposomen alternatiba oso interesgarria dira niosomak [44, 45]. Guk eginiko experimentuetan, harrigarria bada ere, nioplexoekin transfekzio portzentaje oso baxuak lortu genituen (zelula EGFP positiboak % 3) eta western blot entseguetan ez genuen EGFP-ren espresiorik ikusi (9B irudia). Formulazio polimerikoekin konparatuz formulazio lipidikoekin transfekzio eraginkorragoak lortzen direla lan askotan deskribatzen den arren [46], aurreikuspenak gaindituz, NT2 zelulak transfektatzeko UOC-etan oinarritutako poliplexoak nioplexoak baino egokiagoak direla frogatu genuen. Era berean, poliplexoekin lortutako transfekzio mailak lipoplexoekin lortutakoetatik piskat urruti badaude ere (% 15 vs % 35, transfektatu eta 24 ordura), polimero honek biobateragarritasun eta biodegradagarritasun ona eta zitotoxizitate baxua duenez, klinikan erabilia izateko aukerak asko handitzen dira. Jarraituz, kontuan izanik hiru bektoreekin lortutako EGFP-ren ekoizpena oso ezberdina izan zela, interesgarria iruditu zitzaigun hurrengo esperimentuetan jakitea diferentzia horiek eragin zituen transfekzio prozesuko zein urrats zen mugatzailea.

Zelulan barneratzea da bektore ez-biralek gainditu behar duten lehenengo oztopoa transfekzio prozesuan [47]. Barneratze prozesua aztertzeko FITC-arekin markatutako pCMS-EGFP plasmidoa erabili genuen eta zelulak ordu batez eta lau orduz inkubatu genituen. Fluxu zitometriako emaitzen arabera eta beste egile batzuek deskribatu dutenarekin bat [48], hiru formulazioek denboraren menpeko zelula barneratzea erakutsi zuten (10A irudia). Hiru formulazioen emaitzak konparatzen hasita, barren grafikoan (10A irudia) eta fluxu zitometriako histogrametan (10B irudia) ikus daitekeen bezala, lipoplexoen barneratze portzentajeak (%70 4 ordura) handiagoak dira poliplexoenak (%30 4 ordura) eta nioplexoenak (%10 4 ordura) baino. Gainera, emaitza horiek bat zetozen mikroskopia konfokaleko irudiek erakusten zutenarekin (10C irudia). Prozesua

bere osotasunean aztertuz gero, zelulan barneratze emaitzen eta transfekzio portzentajeen artean korrelazio argia ikusi genuela azpimarratu beharra dago.

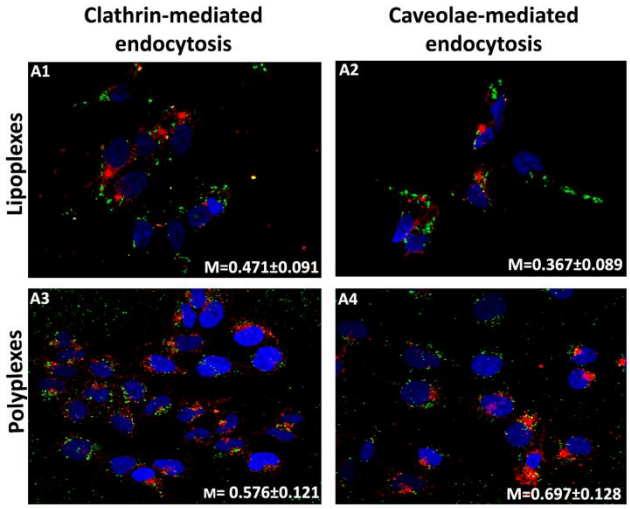


10. Irudia: Konplexuen barneratze zelularra. (A) Barra grafikoa, non FITC positibo diren NT2 zelulen portzentajea aurkezten den, 1 eta 4 ordutako inkubazioen ondoren. Errore barrek desbideratze estandarra adierazten dute (n=3). *P ‹ 0,05.(B) Konplexuen (FITC-rekin markaturiko ADN-arekin eratuak) barneratze zelularra adierazten duen fluxu zitometria histograma (4 orduko inkubazioaren ondoren). Kurba beltzak tratatu ez diren zelulak adierazten ditu. (C) NT2 zelulen irudi elkartuak (C1) lipoplexoak, (C2) nioplexoak eta (C3) poliplexoak gehitu eta 4 ordura. Irudiak 63X-eko handipenarekin hartu ziren. Kolore urdinez DAPI-rekin tindaturiko zelulen nukleoak aurkezten dira; kolore gorriz Faloidinarekin tindaturiko F-aktina aukezten da; kolore berdez bektore ez-biralekin elkarturiko eta Cy3-rekin markaturiko DNA aurkezten da.

Gure formulazioak NT2 zeluletan sartzeko gai zirela frogatu ostean, barneratze prozesu horretan jarraitutako mekanismoa aztertzea zen hurrengo urratsa. Era berean, bektoreek zituzten ezaugarri fisiko-kimikoak nahiko

ezberdinak zirela jakinda eta beste ikertzaile batzuek argitaratutakoaren arabera [49], bektoreen barneratze mekanismoa eta zelula barneko garraioa ezaugarri fisiko-kimiko horien menpe egon zitezkeela espero genuen. Nioplexoak barneratze mekanismoen eta zelula barneko garraioaren entseguetatik kanpo utzi behar izan genituen oso barneratze portzentaje baxuak izan zituztelako eta ezinezkoa zelako egoera horietan neurtzea. Nanopartikulek zelulan sartzeko mekanismoei dagokionez, klatrina bidezko endozitosia (CME) eta kabeola bidezko endozitosia (CvME) dira biderik arruntenak [50]. Mikroskopio konfokalarekin lortu genituen irudiekin eginiko kolokalizazio frogen arabera (M1 Manders-en koefizientea neurtuta), ikusi genuen lipoplexoek (140 nm, -29 mV) NT2 zeluletan sartzeko batez ere CME bidea erabiltzen zutela eta poliplexoek (290 nm, 15 mV), aldiz, CvME bidea (11. irudia). Rejman eta kideek argitaratu zuten ‹200 nm-ko tamainako mikroesferek CME barneratzeko mekanismoa erabiltzen zutela eta tamaina handiagokoak ziren heinean barneratze bidea CvME-rantz aldatzen zela [51]. Tamainaz gain, nanopartikulen gainazalaren ezaugarriek ere baldintzatu dezakete zelulan barneratze mekanismoa. Justu gure emaitzetan ikusi dugunaren kontrara, argitaratuta dago orokorrean karga positiboa duten bektoreek gehienbat CME bidea jarraitzen dutela eta negatiboki kargatutako formulazioek CvME bidea, kontutan izanik barneratze mekanismoa zelula motaren araberakoa ere izan daitekela [52]. Dena den, ez dugu ahaztu behar klatrina eta kabeola bideetaz aparte badaudela doktorego tesi honetan aztertu ez ditugun beste endozitosi mekanismo batzuk ere, eta beste bide horiek ere izan daitezkeela gure bektore ez-biralek erabiltzen dituztenak.

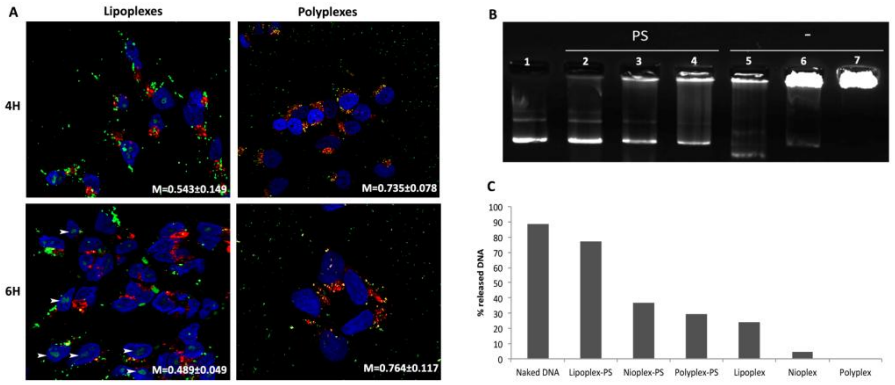
Hainbat artikulutan argitaratuta dago zelulan klatrina bidez sartzen diren partikulak endosometan pilatzen direla eta endosoma horiek heltze prozesuaren eraginez, lehenengo konpartimentuaren azidotzea jasaten dutela eta endosoma berantiar bihurtzen direla, eta ondoren lisosoma bilakatzen direla [53]. Hain zuzen



11. Irudia: Konplexu (FITC-rekin markaturiko DNA-rekin eratuak) (kolore berdez) eta endozitosi bide espezifikoen markatzaileen (kolore gorriz) arteko kolokalizazioa: klatrina bidezko endozitosia (Alexa Fluor 546-Transferrina, 50 μg/ml) eta kabeola bidezko endozitosia (Alexa Fluor 555-colera toxina, 10 μg/ml). Irudiak 4 eta 6 ordutako inkubazioen ondore atera ziren. Markatzaileak inkubazio denbora bukatu baino ordu bat lehenago gehitu ziren. Irudiak 63X-eko handipenarekin hartu ziren. Nukleoak DAPI-rekin tindatuta daude (kolore urdinez). Kolore horiaren presentziak endozitosi bidearen eta konplexuaren gainjartzea adierazten du. Kolokalizazioaren balioak, endozitosi bideen markatzaile fluoreszenteekin kolokalizatzen duen konplexuen frakzio bezala aurkezte dira, Mandersen koefizientearen bitartez (M1).

ere, gure experimentuetan ikusi genuen, gehienbat CME bidetik sartu ziren lipoplexoak zelulekin kontaktuan jarri eta lau ordura endosoma berantiarrean kokatuta zeudela (12A irudia). Duela gutxira arte, komunitate zientifikoak onartuta zuen, zelulan CvME bidetik sartzen ziren nanopartikulak lisosomen degradaziotik salbu zeudela. Hala ere, 2011an Iversen eta kideek argitaratutakoaren arabera kabeolak ohiko endosoma azidoekin fusionatu daitezke eta lisosometara pasa dezakete barruan daramaten materiala [54]. Azken hipotesi hau bat dator poliplexoekin lortu genituen emaitzekin, izan ere, batez ere CvME bidea erabiltzen

duten nanopartikulak izanik, zelulekin kontaktuan jarri eta lau ordura endosoma berantiarrean kokatuta zeudela ikusi genuen (12A irudia).



12. Irudia: (A) Konplexu (FITC-rekin markaturiko DNA-rekin eratuak) (kolore berdez) eta Lysotracker Red-DND-99 (endosoma bertiarraren markatzaile espezifikoa) (kolore gorriz) arteko kolokalizazioa. Markatzaileak inkubazio denbora bukatu baino ordu bat lehenago gehitu ziren. Irudiak 63X-eko handipenarekin hartu ziren. Nukleoak DAPI-rekin tindatuta daude (kolore urdinez). Kolore horiaren presentziak endozitosi bidearen eta konplexuaren gainjartzea adierazten du. Kolokalizazioaren balioak, endozitosi bideen markatzaile fluoreszenteekin kolokalizatzen duen konplexuen frakzio bezala aurkezte dira, Mandersen koefizientearen bitartez (M1). (B) Agarosa gelean eginiko elektroforesiaren bidez lorturiko DNA-ren askapen profilak. 1. hilara: DNA biluzia; 2. hilara: PS-rekin inkubaturiko lipoplexoak; 3. hilara: PS-rekin inkubaturiko nioplexoak; 4. hilara: PS-rekin inkubaturiko poliplexoak; 5. hilara: lipoplexoak; 6. hilara: nioplexoak; 7. hilara: poliplexoak. PS fosfatidilserinarekin eraturiko mizelei dagokio. (C) Image Lab™ softwarearekin kuantifikaturiko DNA-ren askapena.

Lisosomek DNA degradatu ez dezaten transfekzio prozesuan gainditu beharreko hurrengo pausua endosomatik ihes egitea da. Partikularen arabera, endosomatik ihes egiteko mekanismoa desberdina izan daiteke [55]. Hortaz, endosoma baten konpartimentua antzeratu nahian, fosfatidilserinazko (PS) mizela anionikoak sortu genituen eta gure formulazioek (lipoplexoak eta poliplexoak) endosomen presentzian zeramaten DNA askatzeko zuten gaitasuna aztertzeko

balio izan ziguten [56]. Experimentua egin ostean, 12B eta 12C irudiek azaltzen duten bezala, bektoreak PS mizelekin inkubatu genituenean DNA arazorik gabe askatu zen garraiatzailetik, baina ez zen gauza bera gertatu bektoreak mizelarik gabe inkubatzerakoan; DNA garraiatzaileari lotuta gelditzen zen.

Azkenik, ikusi genuen lipoplexoek eta poliplexoek zelulan sartzeko abiadura ezberdina zutela. Zehazki, markatzaile espezifikoekin inkubatzen jarri eta lau eta sei ordura lipoplexoen kolokalizazio balioak poliplexoekin lortutakoak baino baxuagoak izan ziren. Hau ikusirik, badirudi neurtutako denbora horietan behintzat, lipoplexoak ez zeudela endosoman kokatuta. Are gehiago esan genezake, NT2 zelulekin kontaktuan jarri eta 6 ordura, lipoplexoak jada nukleora helduta zeudela eta poliplexoak oraindik endosometan zeudela (12A irudia). Emaitza hauek bat datoz beste egile batzuek deskribatu dutenarekin, hau da, partikula txikiagoak (100 nm ingurukoak, gure lipoplexoak adibidez) handiagoak diren beste batzuk baino (gure poliplexoen kasua) azkarrago sartzen direla zelulan [51].

Orokorrean, ikerketa lan honek NT2 zeluletan bektore ez-biral ezberdinen transfekzio prozesuari buruzko ezagutza handiagoa ematen digu. Azpimarratu beharra dago, ezagutza hori bektore eraginkorragoak sortzeko erabili ahal izango dela. Ondorio zehatzagoetara bagoaz, argi dago UOC-etan oinarritutako poliplexoak baliagarriak direla giza neuronen prekurtsoreak diren NT2 zelulak transfektatzeko. Gainera, uste dugu UOC-ekin eginiko poliplexoen arrakastaren zati handi bat zelulan barneratze prozesuan egon daitekela. Etorkizuneko experimentuetan aurrerapausu garrantzitsua eman nahiko genuke ikerketa gai hau gaixotasun zehatz batetarantz bideratuta eta gaixotasun horretarako egokia den gene terapeutikoa erabilita. Oraindik lan asko egiteko dagoen arren, lan hau NSZ-ko gaixotasunen tratamendurako *ex vivo* terapia geniko baten garapenaren hasierako urratsa izan daiteke.

Eztabaida

Osotasunean, doktorego tesi honek, *ex vivo* zein *in vivo* terapia genikorako UOC-ak oinarri dituzten bektore ez-biralen garapenaren lehenengo urratsak deskribatzen ditu. Jakitunak gara oraindik lan asko egiteko dagoela eta gainditzeke hainbat muga daudela. Hala ere, gure ustetan, polimero honek ustiatu daitezken zenbait aukera eskaintzen ditu, pisu molekular baxuko Ch-etan oinarritutako bektore ez-biralen garapenean aurrera egiteko.

Erreferentzien zerrenda 244-248. orrialdeetan aurkitzen da.

La terapia génica es la estrategia terapéutica que consiste en introducir material genético al interior de la célula con el objetivo de lograr un efecto terapéutico mediante la corrección un defecto genético, sobreexpresión de un agente biológico necesario o supresión de la expresión de un gen indeseado [1]. Las aplicaciones de terapia génica que se están llevando a cabo pueden ser de dos tipos, *in vivo* y *ex vivo*. En las terapias *in* vivo, el material genético se inyecta directamente al paciente, mientras que en las terapias *ex vivo,* o terapia génica mediada por células, las células son previamente modificadas *in vitro* y, posteriormente, se introducen en la zona de lesión del paciente. El éxito de la terapia génica depende del desarrollo de vectores capaces de transportar de forma selectiva y eficaz el material genético a la célula diana, sin causar toxicidad alguna [2]. Los vectores que se utilizan para la vehiculización de genes se clasifican en dos tipos: virales y no-virales. Debido a sus altas eficiencias de transfección los vectores virales han sido extensamente utilizados. Gracias a los avances en la ingeniería genética se ha podido desarrollar el medicamento Glybera, aprobado en 2012 por la Agencia Europea del Medicamento (EMA) [3], el cual se basa en un virus adeno-asociado (AAV) y está indicado para el tratamiento de la deficiencia de lipoproteinlipasa. Sin embargo, no podemos olvidar los problemas relacionados con la seguridad que presentan los vectores virales, como la inmugonenicidad y mutagénesis insercional [4,5]. Además, su limitada capacidad de carga [6] y altos

costes de producción limitan sus aplicaciones clínicas. Estas limitaciones han hecho que la comunicad científica muestre interés por el desarrollo de vectores no-virales, los cuales no tienen contaminantes virales, no estimulan respuesta inmune, no presentan límite de carga de ADN y su desarrollo a gran escala es más fácil en comparación a los vectores virales [6]. No obstante, sus eficiencias de transfección son bajas, ya que el proceso está dificultado por varias barreras tanto extracelulares como intracelulares que limitan las aplicaciones de los vectores no-virales *in vivo*. Por ello, para lograr unos vectores adecuados, es imprescindible desarrollarlos, caracterizarlos, evaluarlos y optimizarlos con suma precisión.

Esta tesis doctoral se basa en el uso de oligoquitosanos ultrapuros (UOC) como vectores no-virales para el transporte de genes. El quitosano (Ch) es un polímero natural compuesto de cadenas -(1-4) D-glucosamina y N-acetil-D-glucosamina unidas por enlaces (1-4) glucosídicos. Gracias a los grupos amino del quitosano cargados positivamente es posible la unión electrostática con moléculas de carga negativa, como el ADN, dando lugar a lo que llamamos poliplexos (poliplexos Ch/ADN) [8]. Está descrito que el peso molecular y el grado de deacetilación (DDA) del quitosano juegan un papel importante en la estabilidad fisicoquímica y eficiencia de transfección de vectores basados en Ch [9]. En esta tesis doctoral hemos trabajado con quitosanos de bajo peso molecular y alto grado de deacetilación, Novafect O15 y O25 concretamente, ya que consideramos que estos Ch presentan unas características adecuadas para el transporte de genes. A pesar de que numerosos trabajos describen las prometedoras características de este polímero para su uso en terapia génica, el problema relacionado con su baja eficiencia de transfección aún persiste. Por ello, es preciso conocer los principales factores que puedan tener alguna influencia sobre la eficiencia de transfección y entender el propio proceso para poder progresar en el diseño y desarrollo de poliplexos basados en Ch.

1. Influencia de factores fisicoquímicos en la eficiencia de transfección de poliplexos basados en oligoquitosanos ultrapuros.

Las propiedades fisicoquímicas que mayor influencia ejercen sobre la eficiencia de transfección de los poliplexos Ch/ADN son su tamaño y potencial zeta. En general, parece ser que la eficiencia de transfección de partículas pequeñas es mayor [10,11]. Además, el tamaño de partícula puede determinar la habilidad de los vectores para alcanzar ciertos órganos [12]. En cuanto al potencial zeta, una alta carga superficial favorece la estabilidad coloidal de los poliplexos, y en el caso de partículas cargadas positivamente, se correlaciona con una mayor captación celular y eficiencia de transfección *in vitro* [13]. Sin embargo, la influencia que ejercen ciertos factores fisicoquímicos sobre estas propiedades no ha sido claramente descrita. Por este motivo, en la primera parte de esta tesis doctoral, nos plantemos darle respuesta a la siguiente pregunta: ¿cómo afectan factores como el pH, la concentración de sales y el ratio N/P (ratio nitrógeno fosfato) al tamaño y potencial zeta de los poliplexos basados en UOC?

Se llevo a cabo un diseños experimental ortogonal para determinar los efectos del pH, concentración de sales y el ratio N/P sobre el tamaño y potencial zeta de los poliplexos O15 y O25/pCMS-EGFP, además de las interacciones entre los distintos factores. Después de analizar la influencia de cada factor sobre el tamaño de los poliplexos las conclusiones fueron las siguientes (Tabla 1): por un lado, al aumentar el pH de 6,2 a 7,4 el tamaño de los poliplexos aumenta; por otro lado, al pasar de un ratio N/P de 10 a 60 el tamaño de partícula disminuye; finalmente, el aumento de la concentración de sales minimamente influye en el tamaño de los poliplexos. No obstante, las interacciones entre los factores son bastante altas, lo que nos indica que el efecto de cada factor es dependiente del resto de factores estudiados.

Los resultados publicados sobre la influencia del tamaño de partícula en la

eficiencia de transfección de los vectores no-virales son bastante contradictorios [14, 15]. Sin embargo, muchos grupos de investigación coinciden en que el potencial zeta de los complejos es un factor muy importante a tener en cuenta. Nuestros resultados muestran que el potencial zeta varía en función del pH de la solución. Al aumentar el pH de 6,2 a 7,4 el potencial zeta la los poliplexos UOC O15 y O25 disminuyó 12,86 mV y 14,91 mV, respectivamente (Tabla 1). Además, es destacable la ausencia de interacciones entre factores en el caso del potencial zeta, a diferencia de lo ocurrido para el tamaño de las partículas.

Tabla 1: Valor de los efectos e interacciones sobre el tamaño y potencial zeta de los poliplexos UOC/pCMS-EGFP. X_1e: efecto del pH, X_2e: efecto del N/P, X_3e: efecto de [NaCl], X_1X_2i: interacción pH-N/P, X_1X_3i: interacción ph-[NaCl], X_2X_3i: interacción N/P-[NaCl].

	Tamaño (nm)		Zeta (mV)	
Efectos e Interacciones	**O15**	**O25**	**O15**	**O25**
X_1 e	180.37	28.73	-12.86	-14.91
X_2e	-139.98	-85.31	3.86	-0.65
X_3e	-21.93	13.31	-4.80	-6.16
X_1 X_2i	-128.12	-44.98	2.26	-0.25
X_1 X_3i	-57.57	66.58	0.50	0.73
X_2 X_3i	42.88	55.43	-0.60	2.60

Otra propiedad esencial que se debe tener en cuenta a la hora de trabajar con polímeros para terapia génica es su capacidad tamponante, la cual determinará la capacidad de los poliplexos para salir del endosoma una vez dentro de la célula [16]. A pesar de la baja capacidad tamponante del Ch [17], nuestros resultados demostraron que los UOC (O15 y O25) tienen una mayor capacidad tamponante que el Ch G214 de alto peso molecular (340 KDa y › 90 DDA) (Fig. 1). Estos resultados refuerzan la idea de que los UOC pueden ser unos buenos candidatos para el transporte de genes.

Teniendo en cuenta que el pH de la solución altera la carga de nuestros poliplexos y considerando que su potencial zeta puede afectar a la estabilidad de la suspensión, adhesión de las partículas a las membranas biológicas y por lo tanto, a la eficiencia de

transfección [18], nos preguntamos si la acidificación del medio de transfección podría alterar de alguna manera la eficiencia de transfección de los poliplexos basados en UOC.

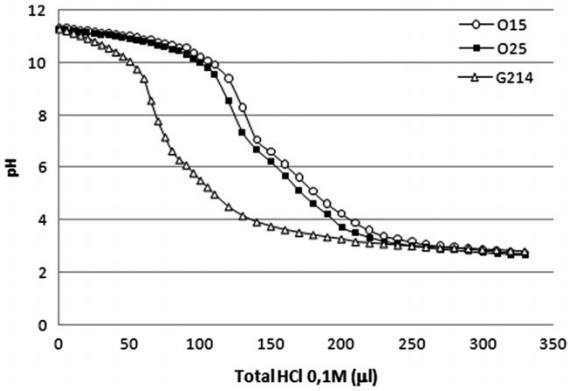


Figura 1: Perfiles de la valoración ácido-base de los UOC O15 y O25 y el Ch G214 en una solución de NaCl a 150 mM.

Con el objetivo de esclarecer esta hipótesis, en primer lugar, evaluamos la influencia del descenso del pH en la captación celular de los poliplexos UOC/pCMS-EGFP-FITC, en las células HEK293 y ARPE19. En un estudio previo, establecimos que el ratio N/P óptimo era 20:1, por lo que los posteriores experimentos se realizaron a este mismo ratio. El G214 se utilizó como un control de Ch de alto peso molecular. Como se puede ver en la figura 2, el porcentaje de células FITC positivas fue más alto a pH 6,5 que a pH 7,4, en ambas líneas celulares (Fig. 2 A1 y A2). Las imágenes de fluorescencia corroboraron los resultados de citometría, como se observa en la figura 2B (Fig. 2 B).

Las diferencias observadas podrían deberse al aumento del potencial zeta de los poliplexos al disminuir el pH del medio de transfección (Fig. 3, líneas). Concluimos que la carga positiva de los complejos favorece la interacción electrostática con la membrana celular lo que provoca un aumento en el porcentaje de captación [19, 20].

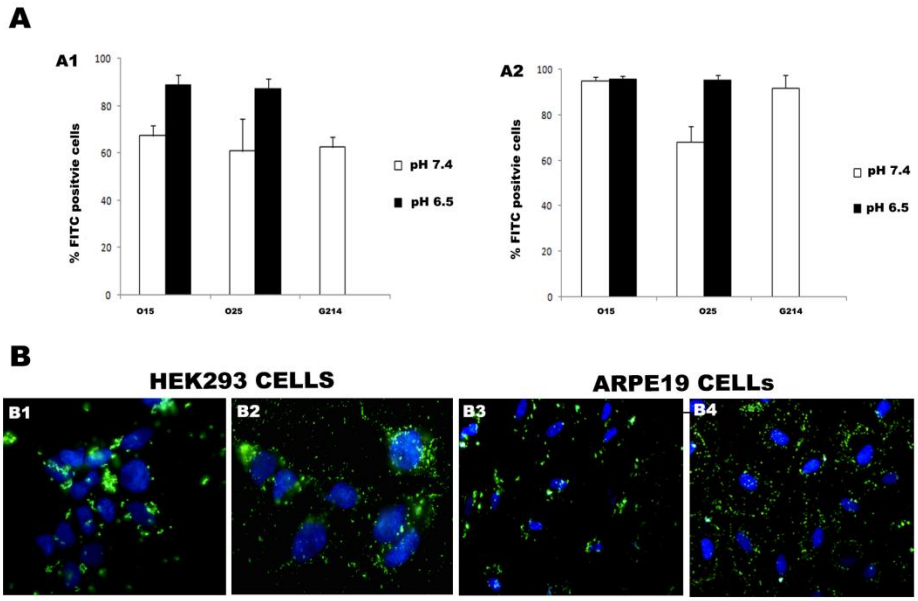


Figura 2: Captación celular del pCMS-EGFP (marcado con FITC) transportado por los poliplexos basados en UOC (ratio N/P 20). Porcentaje de células FITC positivas tratadas con UOC O15 y O25 y Ch G214 a pH 6,5 y 7,4 en las células HEK293 (A1) y ARPE19 (A2). Imágenes de fluorescencia de la captación de FITC-pCMS-EGFP: UOC O15/FITC-pCMS-EGFP a pH 7,4 en HEK293 (B1); UOC O15/FITC-pCMS-EGFP a pH 6,5 en HEK293 (B2); UOC O15/FITC-pCMS-EGFP a pH 7,4 en ARPE19 (B3); UOC O15/FITC-pCMS-EGFP a pH 6,5 en ARPE19 (B4).

Además, los poliplexos elaborados a pH 6,5 presentaron un tamaño menor que los elaborados a pH 7,4 (Fig. 3, barras). Esta disminución de tamaño observada a pH ácido, también podría explicar el mayor porcentaje de captación. No obstante, en las células ARPE19, la captación de las poliplexos de mayor tamaño (elaborados con UOC O15 y G214 a pH 7,4 con 250 nm y 400 nm, respectivamente) fue similar a la de los de menor tamaño, lo que nos indica que el tamaño de partícula no es un factor crítico en el proceso de captación en células ARPE19 y que la propia línea celular tiene una gran influencia [21].

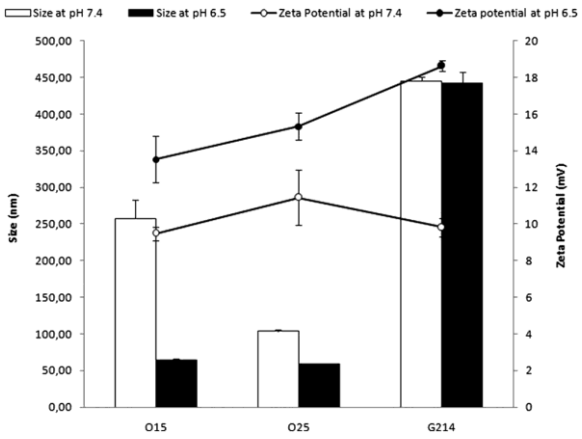


Figura 3: Tamaño y potencial zeta de los poliplexos O15 y O25 UOC/pCMS-EGFP y G214/pCMS-EGFP (ratio N/P 20) a pH 7,4 y 6,5.

Una vez que determinamos que el valor del pH ejercía una influencia clara en la captación de los poliplexos, los siguientes experimentos se diseñaron para determinar si este aumento en la captación estaba correlacionado con un aumento en la eficiencia de transfección. Resumiendo, quisimos responder la siguiente pregunta: ¿es posible mejorar la eficiencia de transfección de los poliplexos basados en UOC mediante la acidificación del medio? El porcentaje de células EGFP positivas se evaluó por citometría de flujo 72 horas post-transfección. En concordancia con otros trabajos previamente publicados, la disminución del valor del pH de 7,4 a 6,5 provocó un aumento en el porcentaje de células, tanto HEK293 (Fig. 4 A1) como ARPE19 (Fig. 4 A2), transfectadas con ambos UOC [13, 22]. Se observaron diferencias entre los tres tipos de Ch utilizados; no se obtuvo transfección con el Ch de alto peso molecular G214, lo que puede ser debido a la baja capacidad de este Ch de liberar el ADN una vez dentro de la célula [23]. También podría deberse a su baja capacidad tamponante que dificultaría su liberación del endosoma [17]. En cuanto a los UOC, con los poliplexos basados en O15 se obtuvieron mayores porcentajes relativos de transfección en comparación a los

poliplexos basados en O25, lo cual no se correlaciona con los resultados de captación. Esta ausencia de correlación entre captación celular y eficiencia de transfección ya ha sido reportada por otros autores [24], y nos indica la necesidad de entender el proceso de disposición intracelular de nuestros vectores no-virales.

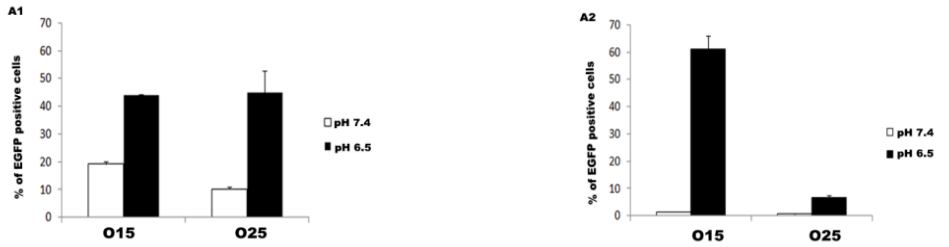


Figure 4: Eficiencia de transfección de los poliplexos UOC O15 y O25 /pCMS-EGFP (N/P=20) a diferentes pHs en células HEK293 (A1) y ARPE19 (A2). Los datos de transfección fueron normalizados frente a los datos con Lipofectamina 2000^{TM}.

A parte de ser eficientes, otro requisito que deben cumplir los vectores no-virales es la ausencia de toxicidad. Por ello, evaluamos la citotoxicidad de nuestros vectores y el efecto de la disminución del pH en la viabilidad celular. Los resultados mostraron que los poliplexos basados en UOC no son tóxicos para las líneas celulares utilizadas (HEK293 y ARPE19) a ninguno de los pH utilizados.

En este primer capítulo de la tesis doctoral, hemos descrito, con la mayor exactitud posible, los efectos de algunos factores importantes sobre las características fisicoquímicas que más influyen en la eficiencia de transfección, como son el tamaño y potencial zeta. Además, hemos determinado que es posible mejorar la eficiencia de transfección de nuestros poliplexos mediante un ligero descenso del pH del medio de transfección. A simple vista, la aplicación clínica de este primer estudio puede no estar del todo clara, pero nosotros proponemos las siguientes: Nuestros resultados sugieren que los poliplexos basados en UOC podrían ser utilizados para la transfección de

células que se encuentran en un ambiente ligeramente ácido, como pueden ser las células tumorales [25]. Por ejemplo, una de las aplicaciones *in vivo* más directas sería la administración local de los poliplexos en el tejido tumoral [26]. Otra posible aplicación consistiría en el uso de los poliplexos basados en UOC para una terapia génica *ex vivo*. En este tipo de terapias, las condiciones de transfección pueden ser moduladas con el objetivo de conseguir altas eficiencias de transfección *in vitro*, para, posteriormente, implantar las células transfectadas al paciente.

2. La importancia del plásmido: ¿es posible transportar un plásmido de 40 Kbp que codifica un adenovirus mediante oligoquitosanos ultrapuros?

Considerando que una de las posibles aplicaciones de los poliplexos basados en UOC es la vehiculización de genes a tumores, decidimos enfocar la segunda parte de esta tesis doctoral en la posibilidad de utilizar los poliplexos de UOC en terapia génica dirigida contra el cáncer. Una estrategia emergente en este campo es el uso de adenovirus (Ad) oncolíticos capaces de reproducirse y multiplicarse selectivamente en las células tumorales; además, la progenie viral tiene la capacidad de extenderse por el tejido tumoral infectando otras células cancerosas [27, 28]. Sin embargo, existen ciertos problemas a la hora de inyectar Ads directamente al torrente sanguíneo, como las interacciones con células Kupffer, plaquetas, eritrocitos, complemento y anticuerpos neutralizantes [29]. Por ello, el trabajo de investigación se ha enfocado en vehiculizar un plásmido que codifica el adenovirus oncolítico mediante vectores no-virales. Esta estrategia combina la alta eficacia terapéutica de los Ads oncolíticos y su administración sistémica gracias al vector no-viral [30, 31]. El mayor problema de esta novedosa estrategia es que el tamaño de estos plásmidos que codifican el adenovirus es muy grande, alrededor de 40 kbp. Como la capacidad de carga de los vectores no-virales es teóricamente ilimitada [32], en la segunda parte de esta tesis doctoral, quisimos dar a conocer la habilidad de los poliplexos basados en UOC para transportar un plásmido de 40 kbp que codifica un adenovirus de replicación incompetente

(pAdTLRGD), el cual expresa la proteína reportera EGFP, y transfectar las células HEK293 y A549.

En la primera parte del trabajo, determinamos que factores como el ratio N/P, pH y concentración de sales afectan al tamaño y potencial zeta de los poliplexos UOC/pCMS-EGFP. Nos preguntamos si esto también ocurriría al elaborar los poliplexos con el plásmido pAdTLRGD. Llevamos a cabo un segundo diseño experimental, y observamos que el tamaño y potencial zeta de los poliplexos claramente variaban en función de las condiciones de la solución (Tabla 2).

Tabla 2: Valor de los efectos e interacciones sobre el tamaño y potencial zeta de los poliplexos UOC/pAdTLRGD. X_1e: efecto del N/P, X_2e: efecto de [NaCl], X_3e: efecto del pH, X_1X_2i: interacción N/P-[NaCl], X_1X_3i: interacción N/P-pH, X_2X_3i: interacción [NaCl]-pH.

	Tamaño (nm)		Zeta (mV)	
Efectos e Interacciones	**O15**	**O25**	**O15**	**O25**
X_1 e	-17.1	-10.7275	3.6575	2.8875
X_2e	32.3	4.0325	20.8475	-2.2375
X_3e	-31.46	5.8725	-33.8325	-33.5875
X_1 X_2i	22.15	5.7225	-7.3725	-0.0125
X_1 X_3i	-9.19	-14.0175	10.2025	-1.5625
X_2 X_3i	10.29	-5.6775	-18.2025	-0.0875

En cuanto al tamaño, todos los complejos estaban en el rango nanométrico (alrededor de 200 nm), y este variaba dependiendo del ratio N/P, la concentración de sales y el pH de la solución. En cuanto al tamaño de partícula, al aumentar la N/P de 10 a 60 el tamaño de los poliplexos O15 y O25 disminuyó 17,1 nm y 10,73 nm, respectivamente. El cambio de la concentración de sales únicamente afectó a los poliplexos basados en el Ch O25 (un incremento de 32,2 nm al pasar de una concentración de 5 mM a 150 mM). La alcalinización de la solución provocó un incremento del tamaño de partícula de los poliplexos basados en O15 (31,46 nm). En lo referente al potencial zeta, la carga de las partículas disminuyó, alrededor de 33 mV, al pasar de un valor de pH ácido a uno valor neutro. Las interacciones entre los distintos

factores, nos indican que existen claras dependencias entre ellos, tanto para el tamaño como el potencial zeta de las partículas. Curiosamente, no observamos diferencias significativas en las propiedades fisicoquímicas de los poliplexos elaborados con el plásmido pAdTLRGD (40 Kbp) y los formados con el pCMS-EGFP, el cual es 10 veces más pequeño. Otros autores también reportaron la ausencia de diferencias en la estructura y tamaño de lipoplexos al comparar un plásmido de 52,5 Kbp con otro de menor tamaño [33].

Aparte del tamaño y potencial zeta, la afinidad de unión entre el vector no-viral y el pDNA es otro factor importante a tener en cuenta. Para lograr un transfección eficiente, el vector no-viral debe ser capaz de condensar, liberar y proteger el ADN frente a la degradación enzimática [24]. Además, debe existir un delicado equilibrio entre la capacidad de unión del ADN y su liberación [34]. Debido al gran tamaño del plásmido pAdTLRGD, nos planteamos la siguiente pregunta: ¿son capaces los UOC de condensar, liberar y proteger un plásmido de un tamaño 10 veces mayor a los que se han utilizado hasta el momento? La electroforesis en geles de agarosa reveló que la habilidad de los UOC de condensar, liberar y proteger el pAdTLRGD depende del ratio N/P (Fig. 5). Con los poliplexos elaborados con O15, el UOC de menor tamaño (5,7 KDa), se necesitaron ratios N/P más altos para condensar y proteger completamente el ADN (Fig. 5 A), mientras que con los elaborados con O25 (7,3 KDa) menores ratios N/P eran suficientes para el mismo propósito (Fig. 5 B). Además, debido a las diferencias en el peso molecular, los poliplexos basados en O15 fueron capaces de liberar el plásmido a los ratios N/P 5:1 y 10:1, lo cual no ocurrió con los poliplexos de O25. Por todo esto, concluimos que los poliplexos elaborados con Ch de alto peso molecular son muy estables y excelentes protectores del ADN. Sin embargo, la liberación del ADN puede verse comprometida debido a la alta estabilidad de la formulación [9]. En conjunto, la electroforesis reveló que los UOC son capaces de condensar, liberar y proteger el plásmido pAdTLRGD, a pesar de su gran tamaño.

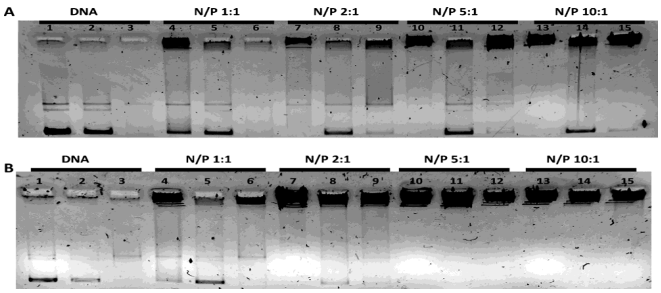


Figura 5: Electroforesis en gel de agarosa donde se muestra la capacidad de condensación, liberación y protección de los poliplexos basados en UOC O15 (A) y O25 (B) a diferente ratios N/P. Los poliplexos fueron tratados con SDS (líneas 2, 5, 8, 11 y 14) y con SDS + DNase I (líneas 3, 6, 9 y 15).

Una vez que verificamos que los poliplexos UOC/pAdTLRGD eran fisicoquímicamente adecuados para el transporte de plásmidos, pasamos a evaluar su eficiencia de transfección en células HEK293, por citometría de flujo. Teniendo en cuenta que el microambiente de los tumores es, en general, más ácido que en los tejidos normales [25], y basándonos en nuestros resultados previos en los cuales la eficiencia de transfección de los UOC aumentaba a valores de pH ácidos, los estudios de transfección se llevaron a cabo a un pH de 7,1. Los mayores porcentajes de células EGFP positivas (alrededor de un 6 %) e intensidad media de fluorescencia (MFI) (Fig. 6) se obtuvieron con el ratio N/P 5:1. La menor eficiencia de transfección a los ratios N/P más altos puede deberse a la fuerte interacción entre el polímero y el plásmido a estos ratios, lo que impediría la liberación del ADN del vector [24]. Si comparamos ambos UOC, la expresión de EGFP fue superior con el O15, lo cual puede deberse al menor peso molecular de este Ch [35]. Así, los resultados de MFI nos indicaron que a pesar de que ambos UOC son de bajo peso molecular, pequeños cambios en su peso molecular pueden tener un importante efecto en la eficiencia de transfección.

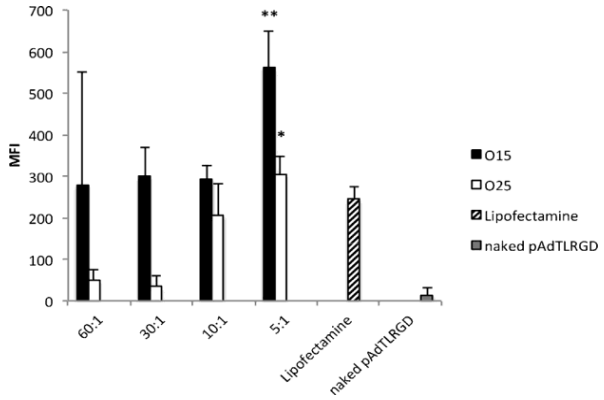


Figura 6: Intensidad media de la fluorescencia (MFI) de células HEK293 transfectadas con poliplexos UOC/pAdTLRGD elaborados con O15 (barras negras) y O25 (barras blancas) a diferentes ratios N/P. Las barras de error representa la desviación estándar (n=3). *P ‹ 0.05 comparado con el plásmido pAdTLRGD desnudo. **P ‹ 0.01comparado con el plásmido pAdTLRGD desnudo.

Posteriormente, los estudios de transfección se llevaron a cabo en un línea celular humana de carcinoma pulmonar, la línea A549 concretamente. El porcentaje de células EGFP positivas fue menor en comparación a las células HEK293. A pesar de esto, en la figura 7 se puede observar que los poliplexos a un ratio N/P 5:1 fueron capaces de transportar el plásmido que dio lugar a la expresión de la proteína reportera EGFP (Fig. 7). A pesar de la baja eficiencia de transfección obtenida, está fue la primera vez que se transportó un plásmido de 40 Kbp con UOC. Además, no se observó expresión EGFP cuando se administró el plásmido pAdTLRGD desnudo, ni en las células A549 ni en las HEK293, lo que nos indica que el vector es esencial para que el proceso de transfección sea exitoso.

En esta segunda parte de la tesis doctoral, observamos que la eficiencia de transfección con el plásmido que codifica un adenovirus de replicación incompetente

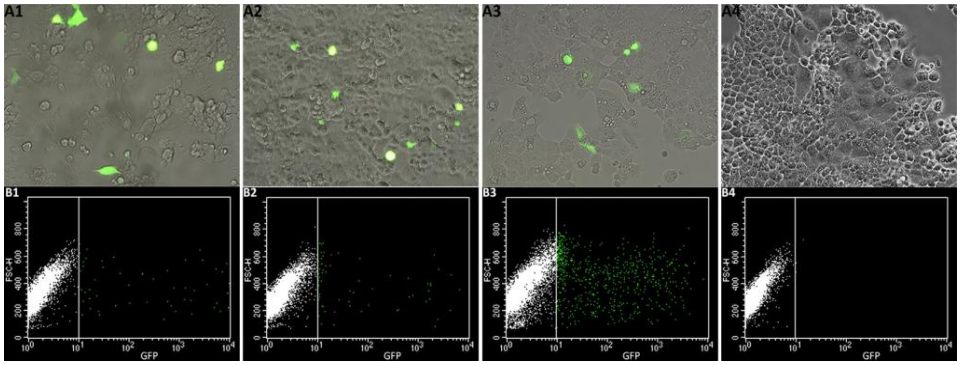


Figura 7: Eficiencia de transfección de los poliplexos UOC/pAdTLRGD en la línea celular A549. (A) Superposición de la imagen de contraste de fases con la imagen de fluorescencia (canal GFP) de células transfectadas con: (A1) O15 UOC/pAdTLRGD al ratio N/P 5:1; (A2) O25 UOC/pAdTLRGD al ratio N/P 5:1; (A3) Lipofectamine 2000; (A4) pAdTLRGD desnudo. (B) Diagrama de puntos de la citometría de flujo (GFP *vs* FSC) de células transfectadas con (B1) O15 UOC/pAdTLRGD al ratio N/P 5:1; (B2) O25 UOC/pAdTLRGD al ratio N/P 5:1; (B3) Lipofectamine 2000, (B4) pAdTLRGD desnudo.

(40 Kbp) fue menor en comparación a la eficiencia obtenida, en la primera parte de la tesis, con el plásmido reportero pCMS-EGFP (5,5 Kbp). No obstante, a pesar del gran tamaño del pAdTLRGD, los poliplexos fueron capaces de cruzar la membrana plasmática, translocar el plásmido al núcleo y producir la proteína EGFP, siendo nuestro grupo de investigación el primero en lograr este objetivo con UOC. En este trabajo hemos destacado una de las ventajas que presentan los vectores no-virales frente a los virales, ya que es improbable poder transportar ácidos nucléicos de 40 Kbp con un vector viral. Para hacer posible una aplicación real de esta terapia combinada, los siguientes experimentos deben realizarse con el plásmido de replicación competente. La capacidad de la progenie viral de propagarse por el tumor ofrecería la oportunidad de utilizar estos vectores no-virales *in vivo* como terapia oncolítica, a pesar de la baja eficiencia de transfección. Mirando al futuro, la administración

sistémica de los vectores no-virales, como los UOC, combinados con el plásmido que codifica un adenovirus de replicación competente sería el principal objetivo para el tratamiento oncolítico de tumores primarios y metastásicos. Gracias al vector no-viral, el genoma del virus oncolítico alcanzaría el foco tumoral mediante la circulación sistémica donde se traslocaría a los núcleos celulares para comenzar su replicación. Este proceso daría lugar a una progenie de adenovirus oncolítocos infecciosos que lisarían las células malignas a la vez que infectarían otras células cancerosas vecinas. Todavía hay mucho trabajo por delante para poder superar las limitaciones que presentan nuestros poliplexos para ser administrados sistémicamente, como su baja estabilidad y el rápido aclaramiento. Por ello, mejorar la tecnología de transporte actual sería necesario para poder alcanzar este ambicioso objetivo.

3. El proceso de transfección paso a paso: un estudio comparativo entre lipoplexos, nioplexos y poliplexos en células precursoras neuronales humanas NT2.

El uso de la línea celular humana NTera2/D1 derivada de teratocarcinoma humano (o células NT2) está muy extendido para obtener plataformas de liberación de proteínas exógenas en el sistema nervioso central (SNC). Estas células pueden diferenciarse a poblaciones de células neuronales bien establecidas (células NT2-N) que se injertan y maduran cuando son trasplantadas en el SNC adulto [36, 37], sin ningún signo de tumorigenicidad [38]. Además, estas células poseen un tropismo preferente por la glía, lo cual las convierte en una herramienta interesante para el tratamiento del glioblastoma. Hasta el momento, todos los trabajos relacionados con la ingeniería genética de las células NT2 se basan en el uso de vectores virales. No obstante, el desarrollo de estrategias no-virales tiene claras ventajas [39, 40]. Por lo tanto, considerando las favorables características de estas células, nos preguntamos si sería posible transfectar las células precursoras neuronales humanas NT2 con los poliplexos basados en UOC, como un primer paso en el desarrollo de una posible

plataforma para terapia génica *ex vivo* en el SNC.

Como se ha mencionado anteriormente, la eficiencia de transfección de los vectores no-virales, especialmente de los vectores basados en Ch, es relativamente baja, por lo que es necesario un conocimiento profundo del propio proceso de transfección para poder diseñar unos vectores no-virales con éxito. La expresión génica final estará influenciada por la captación de los vectores, la vía por la cual estos son captados, su habilidad para salir del endosoma y el paso del ácido nucleico al núcleo celular [41]. Con el objetivo de determinar el cuello de botella en el proceso de transfección en las células NT2, en este tercer y último capítulo de la tesis doctoral, llevamos a cabo un estudio comparativo entre poliplexos (basados en UOC, Novafect O15), nioplexos (basados en una formulación lipídica de niosomas) y lipoplexos (basados en liposomas de Lipofectamine 2000). En la tabla 3 se resumen los principales componentes, ratios peso/peso (w/w) y cantidades de ADN utilizados (Tabla 3).

Tabla 3: Resumen de los principales componentes, ratios w/w y cantidades de ADN utilizados para la elaboración de las tres formulaciones.

Formulación	Componente principal	Ratio w/w	Cantidad de ADN (μg)
Lipoplexos	Lipofectamine 2000	2:1	1.25
Nioplexos	2,3-di(tetradecyloxy)propan-1-amine cationic lipid	20:1	1.25
Poliplexos	Novafect O15 oligoquitosano ultrapuro	13:1	1.25

Las propiedades fisicoquímicas y la composición del vector pueden afectar a la interacción con las membranas y a su comportamiento intracelular [42]. Por lo tanto, las tres formulaciones fueron caracterizadas en base a su tamaño de partícula, potencial zeta e índice de polidispersión (PDI) (Fig. 8). Los lipoplexos y nioplexes presentaron un tamaño de partícula alrededor de los 140 nm con un PDI bajo (0,21),

mientras que los poliplexos tuvieron un tamaño (294 nm) y un PDI (0,44) mayor, como se puede observar en la curva de distribución de tamaños (Fig. 8 B). La carga varió de valores positivos a negativos en función del tipo de vector: los lipoplexos estaban cargados negativamente (-29,13 mV) y los nioplexos y poliplexos mostraron un potencial zeta positivo, +35,4 mV y +15,1 mV, respectivamente (Fig. 8 A).

A

	Size (nm)	pdi	zeta ptential (mV)
Lipoplexes	144.1±6.3	0.21±0.04	-29.13±1.43
Nioplexes	142.5±0.64	0.21±0.04	35.4±0.7
Polyplexes	294.8±23.36	0.44±0.04	15.1±3.05

B

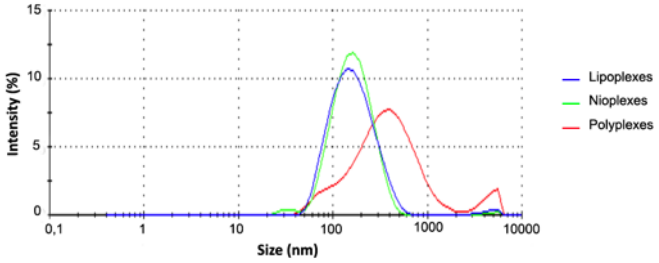


Figura 8: Caracterización de los complejos. (A) Valores de tamaño, PDI y potencial zeta. Cada valor representa la media ±desviación estándar de tres medidas. (B) Curva de distribución de tamaño de los complejos.

Una vez que caracterizamos las formulaciones, se analizaron la eficiencia de transfección y producción de EGFP a lo largo del tiempo, por citometría de flujo y Western blot (Fig. 9). Tal como se esperaba, los resultados revelaron que los lipoplexos exhibían los mayores porcentajes de transfección (Fig. 9 A) y niveles de expresión de proteína (Fig. 9 B). De hecho, la Lipofectamina es un conocido agente para transfección que presente altas eficiencias de transfección. A pesar de que en nuestros estudios de viabilidad está formulación no mostró signos de toxicidad *in vitro,* su uso *in vivo* es limitado [44, 45]. Sorprendentemente, la eficiencia de los nioplexos fue muy baja (3 %

de células EGFP positivas) (Fig. 9 A) y no se observó expresión de EGFP en los estudios de Western blot (Fig. 9 B). Pese a que está bien descrito que la eficiencia de transfección de las formulaciones lipídicas es superior a las poliméricas, contra todo pronóstico, los poliplexos basados en UOC fueron más eficientes que los nioplexos a la hora de transfectar células NT2. Aunque los porcentajes de transfección obtenidos con los poliplexos fueron menores que los obtenidos con los lipoplexos (15 % vs 35 % a las 24 h post-transfección), la biocompatibilidad, biodegradabilidad y la baja toxicidad del Ch favorecen su posible aplicación clínica. Teniendo en cuenta las diferencias que observamos en la expresión final de EGFP mediada por las tres formulaciones, nos preguntamos qué paso en el proceso de transfección sería el determinante.

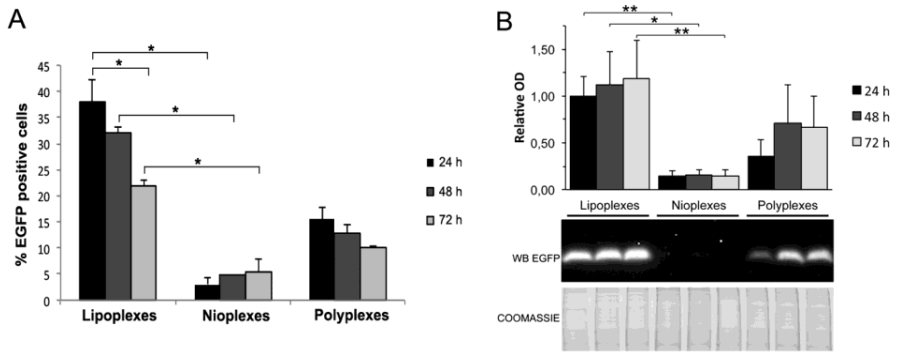


Figura 9: Eficiencia de transfección y expresión de EGFP a las 24, 48 y 72 horas post-transfección. (A) Gráfica de barras donde se muestran los porcentajes de células EGFP positivas 24, 48 y 72 horas post-transfección. Las barras de error representan la desviación estándar (n=3). *P ‹ 0.05. (B) Gráfica de barras en la que se ilustra los resultados semicuantitativos del análisis de EGFP por Western blot. Un Western blot representativo se muestra debajo de la gráfica. Las barras de erros representan la desviación estándar (n=4). *P ‹ 0.05, **P ‹ 0.01.

La primera barrera que deben superar los vectores no-virales es la captación celular [47]. Analizamos la captación de los tres vectores no-virales tras 1 y 4 horas de

incubación con las células, utilizando para ello el plásmido pCMS-EGFP marcado con FITC. Los resultados de citometría de flujo mostraron que la captación celular era tiempo-dependiente (Fig. 10 A), lo cual ya había sido descrito para otras formulaciones [48]. Comparando los tres vectores, pudimos comprobar que la captación de los lipoplexos (70 % a 4 horas) era superior a la de los poliplexos (30 % a 4 horas) y nioplexos (10 % a4 horas), como se observa en la gráfica de barras (Fig. 10 A) e histograma de citometría de flujo (Fig. 10 B). Estos resultados fueron respaldados por las imágenes de fluorescencia (Fig. 10 C). En líneas generales, los resultados de captación mostraron una clara correlación con respecto a las correspondientes eficiencias de transfección.

Una vez que confirmamos que los vectores eran capaces de entrar en las células NT2, el siguiente paso fue determinar qué mecanismo de internalización estaba involucrado en la captación de cada formulación. Considerando las diferentes propiedades fisicoquímicas y composición de los vectores, nos preguntamos si esto podría tener algún tipo de influencia en su mecanismo de internalización y tráfico intracelular [49]. Los nioplexos se descartaron para este estudio debido a su baja captación celular lo cual no permitía el análisis. En cuanto a los mecanismos de internalización, la endocitosis mediada por clatrinas (CME) y la endocitosis mediada por caveolas (CvME) son las vías más comunes [50]. Mediante microscopía confocal y análisis de colocalización (determinando el coeficiente de Manders, M1), encontramos que los lipoplexos (140 nm, -29 mV) entran en las células NT2 por la vía CME (M1=0,471), preferentemente; por el contrario, los poliplexos (290 nm, +158 mV) utilizan la vía CvME (M1=0,697), mayoritariamente (Fig. 11). Según Rejman *et al.* la vía CME está implicada en la internalización de microesferas de un diámetro ‹200 nm, mientras que al aumentar su tamaña se produce un cambio a la vía CvME [51]. A parte del tamaño de partícula, las características de la superficie también juegan un papel importante en el proceso de internalización. Al contrario de lo que ocurre con nuestras formulaciones, en la literatura está descrito que las partículas positivas entran en las

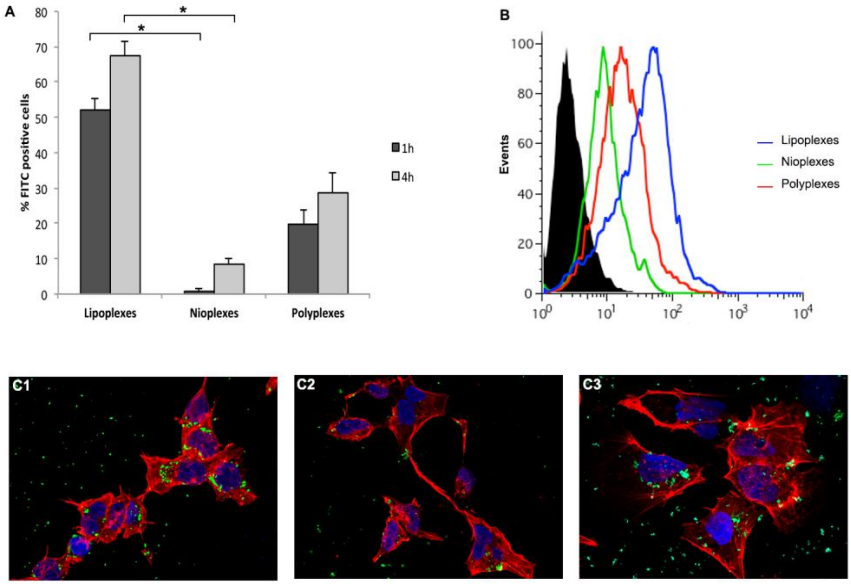


Figura 10: Captación celular de los complejos. (A) Gráfica de barras donde se muestra el porcentaje de células NT2 FITC positivas tras una incubación de 1 y 4 horas con los complejos. Las barras de error representan la desviación estándar (n=3). *P ‹ 0.05. (B) Histogramas de fluorescencia de la citometría de flujo que representan la captación celular de los complejos (formados con el ADN marcado con FITC) tras 4 horas de incubación. La curva negra representa las células sin tratar. (C) Imágenes combinadas de células NT2 4 horas después de la adición de (C1) lipoplexos, (C2) nioplexos y (C3) poliplexos. Las imágenes se tomaron con un aumento 63X. El color azul muestra los núcleos celulares teñidos con DAPI; el color rojo muestra la F-actina teñida con Faloidina; el color verde muestra el ADN marcado con Cy3 acomplejado con el correspondiente vector no-viral.

células, preferentemente, por la vía CME; las negativas, en cambio, utilizan la vía CvME, teniendo en cuenta que la vía de entrada y subsecuente tráfico puede estar influenciado por la línea celular [52]. Sin embargo, no podemos olvidar que otros mecanismos de internalización, independientes a las clatrinas y caveolas, también podrían estar implicados en la captación de nuestros vectores no-virales.

Tal y como ha sido extensamente descrito en la literatura, tras el proceso de internalización, las partículas que entran a través de la vía CME quedan confinadas en

endosomas que sufrirán un proceso de maduración que implica una acidificación del compartimento, para dar lugar a endosomas tardíos y finalmente lisosomas [53].

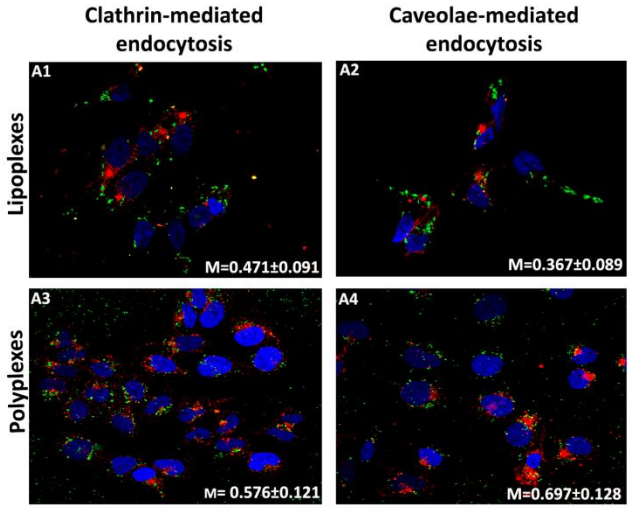


Figura 11: Colocalización entre los complejos (formados con el ADN marcado con FITC) (verde) y los marcadores específicos de vías endocíticas (rojo): endocitosis mediada por clatrinas (Alexa Fluor 546-Transferrina a 50 μg/ml) y endocitosis mediada por caveolas (Alexa Fluor 555-colera toxina a 10 μg/ml). Las imágenes se sacaron tras 4 horas de incubación con los respectivos complejos. Las imágenes se tomaron con un aumento 63X. Los núcleos están teñidos con DAPI (azul). La presencia de color amarillo representa la superposición del marcador de la vía de entrada y el vector. Los valores de colocalización se muestran como la fracción de complejos que colocalizan con los marcadores fluorescentes de la estructura endocítica, representada por el coeficiente de Manders (M).

Consecuentemente, tras 4 horas de incubación, los lipoplexos que entran a través de CME se localizaron en los endosomas tardíos (Fig. 12 A). Hasta hace poco, la comunidad científica aceptaba, en líneas generales, que la partículas que entraban mediante la vía CvME estaban libres de sufrir degradación lisosomal. Sin embargo, recientemente se ha postulado que las caveolas también fusionan con los endosomas ácidos haciendo posible la transferencia del material de su interior a los lisosomas [54].

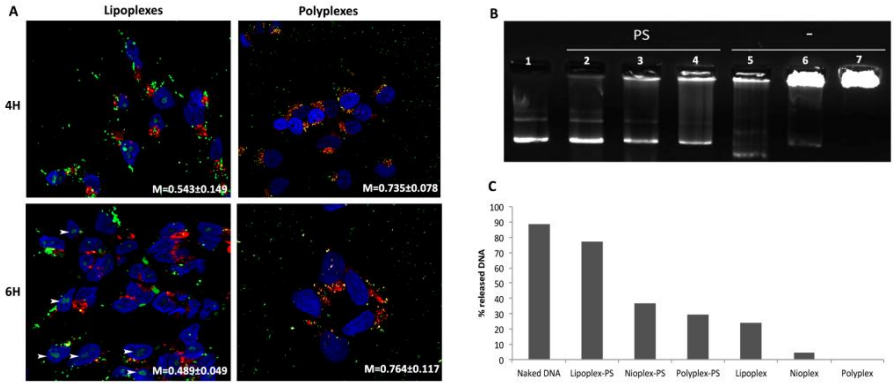


Figura 12: (A) Colocalización de los complejos (formados con el ADN marcado con FITC) (verde) con Lysotracker Red-DND-99, marcador específico del endosoma tardío (rojo). Las imágenes se sacaron después de 4 y 6 horas de incubación. Los marcadores se añadieron 1 hora antes del final del tiempo de incubación. Las imágenes se tomaron con un aumento 63X. Los núcleos están teñidos con DAPI (azul). La presencia de color amarillo representa la superposición del marcador del endosoma y el vector. Los valores de colocalización se muestran como la fracción de complejos que colocalizan con los marcadores fluorescentes de la estructura endocítica, representada por el coeficiente de Manders (M1). (B) Perfiles de liberación de ADN obtenidos mediante electroforesis en gel de agarosa. Carril 1: ADN desnudo; carril 2: lipoplexos incubados con PS; carril 3: nioplexos incubados con PS; carril 4: poliplexos incubados con PS; carril 5: lipoplexos; carril 6: nioplexos; carril 7: poliplexos. PS corresponde a las micelas de fo sfatidilserina. (C) Cuantificación del ADN liberado para lo cual se utilizó el software Image Lab ^{TM}.

Este último trabajo apoya nuestros resultados, ya que los poliplexos que entran preferentemente por la vía CvME fueron localizados en los endosomas tardíos tras 4 horas de incubación (Fig. 12 A).

La siguiente barrera en el proceso de transfección es la liberación del ADN del endosoma, necesario para evitar la degradación lisosomal. Este paso puede variar dependiendo del tipo de partícula [55]. Simulando del compartimento endosomal, elaboramos unas micelas aniónicas basadas en fosfatidilserina (PS) para determinar la habilidad de las formulaciones de interaccionar con el endosoma y liberara la carga genética [56]. Como se observa en la figura 12 B y 12 C, al incubar los vectores con las

micelas de PS se produjo una liberación del ADN de los vectores, lo cual no ocurrió cuando los vectores no se incubaron con las micelas.

Finalmente, los resultados de este último trabajo revelaron que existen diferencias en la velocidad de internalización de los lipoplexos y poliplexos. Los valores de colocalización que obtuvimos con los lipoplexos y los marcadores específicos a las 4 y 6 horas de incubación fueron menores que los que obtuvimos con los poliplexos (Fig. 12 A), lo que significa que a los tiempos testados los lipoplexos no se encontraban localizados en los compartimentos endosomales. Además, 6 horas después de añadir los complejos a las células NT2, el ADN marcado con FITC (transportado por lipoplexos) se encontraba localizado en el núcleo. En cambio, el ADN transportado por los poliplexos se encontraba aún en los endosomas tardíos (Fig. 12 A). Estos resultados sugieren que el tráfico intracelular de los lipoplexos es más rápido en comparación al de los poliplexos, lo cual concuerda con otros trabajos en los que se describe que la entrada de partículas pequeñas (alrededor de 100 nm, similar a nuestros lipoplexos) es más rápida que la de partículas de mayor tamaño, como en el caso de nuestros poliplexos. [51].

En resumen, este trabajo de investigación aporta conocimiento sobre el proceso de transfección de diferentes vectores no-virales en las células NT2, lo cual podría ser muy valioso para mejorar el diseño y desarrollo de nuevos vectores más eficientes. Concretamente, hemos podido concluir que los poliplexos basados en UOC son capaces de transfectar células precursoras neuronales NT2 humanas. Además, sugerimos que el porcentaje de captación celular es un paso limitante en el proceso de transfección mediado por UOC, el cual puede aumentarse mediante diferentes estrategias para mejorar la eficiencia de transfección. En los siguientes experimentos deberíamos dar un paso adelante y enfocar nuestro trabajo en una enfermedad específica utilizando los genes terapéuticos adecuados. Sin embargo, este trabajo podría ser el comienzo del desarrollo de una terapia génica *ex vivo* para el tratamiento

de enfermedades del SNC.

En conjunto, esta tesis doctoral describe los primeros pasos en el desarrollo de un vector no-viral basado en UOC para terapia génica *in vivo* o *ex vivo*. Somos conscientes de que hay mucho trabajo por delante y que existen limitaciones que deben ser superadas. Creemos que este polímero ofrece un amplio abanico de posibilidades que pueden ser explotadas para progresar en el desarrollo de vectores basados en Ch de bajo peso molecular.

El listado de referencias se encuentra en las páginas 244-248.

Gene therapy is the therapeutic strategy that consists in the delivery of genetic material into the cells, which results in a therapeutic effect by either correcting a genetic defect, by over-expressing therapeutically required biological agents or by suppressing the expression of an unwanted gene [1]. Gene therapy approaches, currently under development can be classified as *in vivo* and *ex vivo*. The *in vivo* strategy consists of the direct injection to the patient, while the ex vivo, or cell-mediated gene delivery, involves the *in vitro* genetic manipulation of cells followed by the introduction of this cells into the injury site of the patient. The success of gene therapy is largely dependent on the development of a vector that can selectively and efficiently deliver the corresponding gene to the target cells with minimal toxicity [2]. Gene delivery vectors can be classified into two main groups: viral and non-viral. Viral vectors have been widely used due to their high transfection efficiencies. Advances in genetic engineering have lead to the development of Glybera, the first gene therapy medicine approved by the European Medicines Agency (EMA) in 2012 [3], which is based on AAV for the treatment of lipoprotein lipase deficiency (LPLD). However, safety concerns related to viral vectors, such as insertional mutagenesis and immunogenicity could not be forgotten [4,5]. Also, the restricted insert size (4Kb) [6] and the high production costs of viral vectors limit their clinical applications. These complications have made the scientific community focus their attention on the development of non-viral vectors [7], which do not contain viral contaminants, do not stimulate specific immune response, do not have size limit on the amount of DNA they can deliver and have an easier large scale production compared to their counterparts [6].

Nevertheless, the use of non-viral vectors for *in vivo* gene transfer is relatively sparse due to their low transfection efficiencies, as it is hindered by numerous extracellular and intracellular obstacles [1]. Therefore, the appropriate vectors need to be carefully developed, characterized, evaluated and optimized.

This doctoral thesis work is based on the use of ultrapure oligochitosan (UOC) as non-viral vectors for gene delivery purposes. Chitosan (Ch) is a natural cationic polymer composed of β-D-glucosamine and β-N-acetyl-D-glucosamine subunits connected by a (1-4) glycosidic bond. Thanks to the positively charged amine groups of Ch, it is possible to form polyplexes by electrostatic interactions with negatively charged molecules, such as DNA (Ch/DNA polyplexes) [8]. It has been reported that the molecular weight (Mw) and the degree of deacetylation (DDA) of Ch affect the physicochemical stability and transfection efficiency of Ch based vectors [9]. Along this doctoral thesis, we have worked with low Mw and highly deacetylated Ch, Novafect O15, and O25 specifically, as we have considered that this type of Ch presents interesting characteristics for gene delivery purposes. Although numerous works have described the promising characteristics of this polymer for gene transfer, the problem related to the low transfection efficiency of Ch based polyplexes remains. Thus, the knowledge of the main factors that may influence the transfection efficiency and, the understanding of the whole transfection process itself, would be very valuable to progress in the design and development of Ch based non-viral vectors.

1. The influence of physicochemical factors on the transfection efficiency of ultrapure oligochitosan based polyplexes

The most important properties that affect the transfection efficiency of Ch/DNA polyplexes are the size and zeta potential. Generally, it is believed that smaller particles are more efficient at transfecting cells [10,11]. In addition, the particle size will determine the ability of the vectors to reach various organs [12]. Concerning zeta potential, the high

surface charge of polyplexes determines their colloidal stability and, frequently, in the case of positively charged particles, it correlates with higher cellular uptake and transfection efficiencies *in vitro* [13]. However, the exact influence of physicochemical factors on these properties has not been clearly described. Taking this into account, in the first part of this doctoral thesis, we wanted to answer the following question: how do factors like pH, salt concentration and N/P ratio (nitrogen to phosphate ratio) affect the size and zeta potential of UOC based polyplexes?

An orthogonal experimental design was carried out to evaluate the effects of the pH, salt concentration, and N/P ratio and, the interactions between the factors on size and zeta potential of O15 and O25 UOC/pCMS-EGFP polyplexes. Regarding the independent influence of the factors on the particles size (Table 1), the increase of the pH from 6.2 to 7.2 increases the size of the polyplexes the increase of the N/P ratio from 10 to 60 reduces the size; and the rise of the salt concentration hardly affects the particle size. The interactions between the factors were quite high; therefore, the effect of each factor on the particle size depends on the rest of the analyzed factors (Table 1).

Table 1: The value of the effects and interactions on size and zeta potential of the UOC/pCMS-EGFP polyplexes. X_1e: pH effect, X_2e: N/P effect, X_3e: [NaCl] effect, X_1X_2i: pH-N/P interaction, X_1X_3i: pH-[NaCl] interaction, X_2X_3i: N/P-[NaCl] interaction.

	Size (nm)		**Zeta (mV)**	
Effects and Interactions	**O15**	**O25**	**O15**	**O25**
X_1 e	180.37	28.73	-12.86	-14.91
X_2e	-139.98	-85.31	3.86	-0.65
X_3e	-21.93	13.31	-4.80	-6.16
X_1 X_2i	-128.12	-44.98	2.26	-0.25
X_1 X_3i	-57.57	66.58	0.50	0.73
X_2 X_3i	42.88	55.43	-0.60	2.60

Concerning the influence of the size of the complexes in the transfection efficiency, conflicting results have been published [14,15]. Nevertheless, most of the research groups agree with the idea that the zeta potential of the complexes is an important factor to

consider. Our results showed that the zeta potential of the polyplexes was highly influenced by the pH value of the solution. An increase in the pH value from 6.2 to 7.2 resulted in a decrease of the zeta potential of 12.86 and 14.91 mV for UOC O15 and O25, respectively (Table 1). Furthermore, it should be stressed out the absence of interactions between the analyzed factors for the zeta potential.

Another essential property that should be considered when working with polymers for gene delivery is their buffering capacity, as it will determine the ability of the polyplex to escape from the endosomes once inside the cells [16]. Despite the reduced buffering capacity of Ch [17], we demonstrated that UOC (O15 and O25) had a significantly higher buffer capacity compared to G214 high Mw Ch (340 kDA and > 90 DDA) (Fig. 1). These results reinforced the idea that UOC could be excellent candidates for gene delivery.

Taking into account that the pH of the solution alters the charge of our polyplexes and considering that their zeta potential may affect the stability of the suspension, the adhesion of the particles to biological membranes and thus, the final transfection efficiency [18], we wondered if the acidification of the transfection solution could alter, somehow, the transfection efficiency of UOC based polyplexes.

In order to elucidate this hypothesis, firstly, we evaluated the influence of the pH decrease in the cellular uptake of UOC/pCMS-EGFP-FITC polyplexes in HEK293 and ARPE19 cells. In previous experiments, we established that the optimal N/P ratio was 20:1, and thus, the following assays were carried out at this ratio. G214 was employed as a high Mw Ch control. According to our results, the percentage of FITC positive cells was higher at pH 6.5 compared to pH 7.4 in both cell lines, as it can be observed on in figure 2 A1 and A2 (Fig. 2A). Fluorescent images corroborated flow cytometry data, as it can be seen in figure 2 B (Fig. 2B).

These differences could be explained by the increase in the zeta potential of the polyplexes after the acidification of the medium solution (Fig. 3, lines). We concluded that

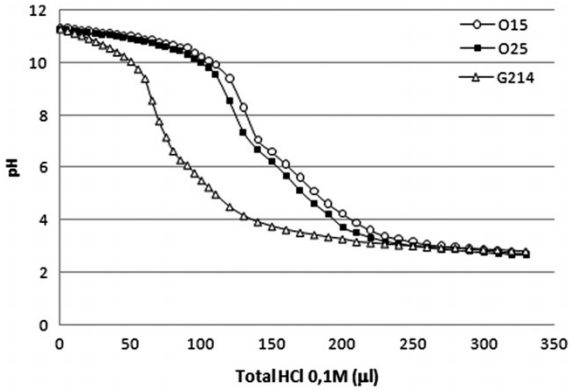


Figure 1: Acid–base titration profiles of O15 UOC, O25 UOC and G214 chitosan in 150 mM NaCl solution.

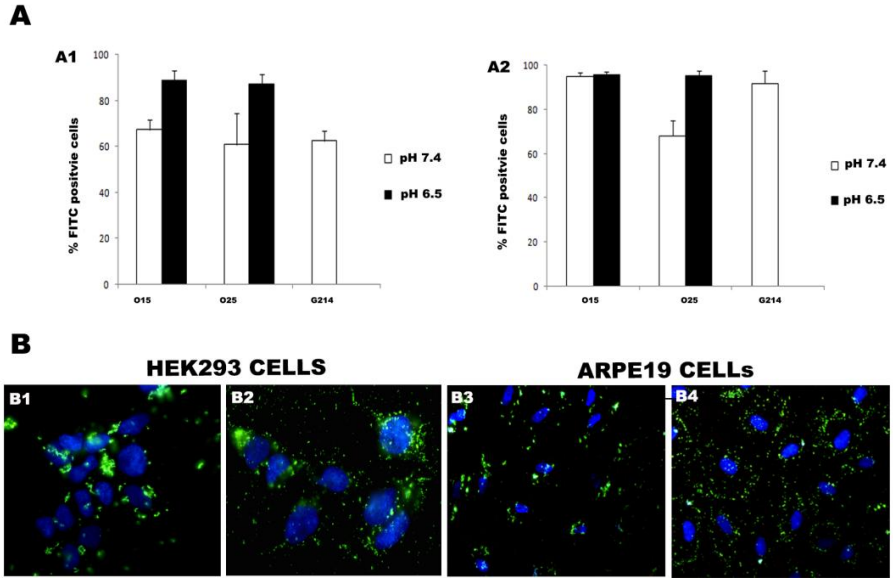


Figure 2: Cellular uptake of FITC-labeled pCMS-EGFP delivered by UOC polyplexes (N/P = 20). Percentage of FITC-positive cells treated with O15 and O25 UOC and G214 chitosan at pH 6.5 and 7.4 in HEK293 (A1) and ARPE19 (A2) cell lines. Fluorescent images of FITC-pCMS-EGFP uptake: O15 UOC/FITC-pCMS-EGFP at pH 7.4 in HEK293 (B1); O15 UOC/FITC-pCMS-EGFP at pH 6.5 in HEK293 (B2); O15 UOC/FITC-pCMS-EGFP at pH 7.4 in ARPE19 (B3); O15 UOC/FITC-pCMS-EGFP at pH 6.5 in ARPE19 (B4).

the positive charge of the complexes allowed the electrostatic interaction with the negatively charged cell membrane, increasing the percentage of cellular uptake [19,20]. Additionally, UOC based polyplexes elaborated at pH 6.5 presented a smaller particle size compared to those elaborated at pH 7.4 (Fig. 3, bars). This reduction of the particle size could also explain the higher cellular uptake achieved at the lower pH value. However, in ARPE19 cells, the cellular uptake of higher polyplexes such as, UOC O15 at pH 7.4 (250 nm) and polyplexes based on G214 (400 nm), was similar to those with lower size, indicating that the particle size may not be a critical factor for the cellular uptake process in ARPE19 cells. Based on these results, a large cell type dependent internalization of the polyplexes should be considered [21].

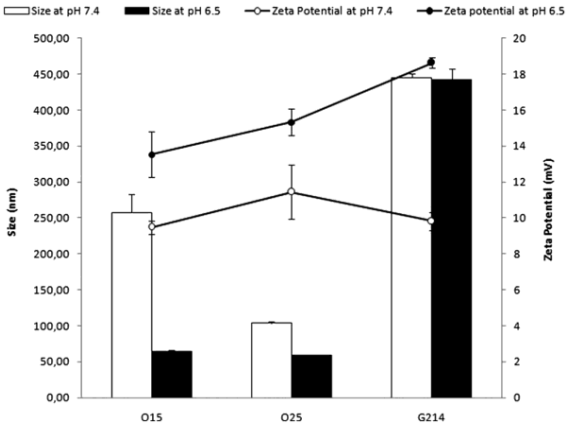


Figure 3: Size and zeta potential of O15 and O25 UOC/pCMS-EGFP and G214/pCMS-EGFP polyplexes (N/P ratio of 20) at pH 7.4 and 6.5.

Once we observed that the pH value had an apparent effect on the cellular uptake, the next experiments were designed to investigate if the observed increase in the cellular uptake correlated with transfection efficiency results. In simple terms, we wanted to answer the following question: could the transfection efficiency of UOC bases polyplexes be improved by acidifying the transfection solution? The percentage of EGFP positive cells 72 hours

post-transfection was evaluated by flow cytometry. A significant increment of the percentage of EGFP positive cells occurred when the pH was decreased from 7.4 to 6.5, with both UOC in HEK293 (Fig. 4 A1) and ARPE19 (Fig. 4 A2), in accordance with previously reported works [13, 22]. Differences between the three employed Chs were also observed. No transfection was obtained with the high Mw G214 Ch (data not shown), which could be explained by the low capacity of this high MW Ch to release the DNA once inside the cell [23] and, its low buffering capacity which could hinder the endosomal escape [17]. Regarding UOC, O15 based polyplexes showed the higher relative percentage of EGFP positive cells compared their O25 UOC based counterparts, which did not correspond to cellular uptake levels. The absence of correlation between cellular uptake and transfection efficiency has been reported by other authors [24], and it indicates the need for understanding the intracellular process of our non-viral vectors.

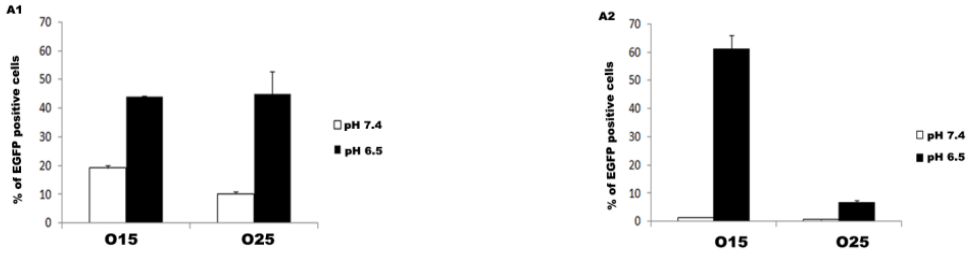


Figure 4: Transfection efficiency of O15 and O25 UOC/pCMS-EGFP polyplexes (N/P = 20) at different pH values in HEK293 (A1) and ARPE19 cells (A2). Transfection data were normalized to Lipofectamine 2000™.

Apart from being efficient, another prerequisite of non-viral vectors is their lack of toxicity. Therefore, we assessed the potential cytotoxicity of our polyplexes and the influence of the pH decrease in cell viability. We concluded that UOC based polyplexes were nontoxic for the employed cells (HEK293 and ARPE19) regardless the pH value of the transfection solution.

In this first part of the doctoral thesis, we describe the effects of some important factors that affect the main physicochemical characteristics involved in the transfection process, such as the size and zeta potential. We conclude that it was possible to improve the transfection efficiency of our polyplexes by acidifying the transfection solution slightly. At first sight, the clinical application of this approximation could not be clear at all. However, we propose the following: our result suggested the potential use of UOC based polyplexes for delivering genetic materials into cells that are in acidic environment, which is the case of tumor cells [25]. For instance, one of the most direct *in vivo* applications could be the local administration of the polyplexes in tumor tissues [26]. Another possible application consists in the use of UOC based polyplexes for an *ex vivo* gene therapy. In this kind of therapy, the transfection conditions could be modulated to obtain the higher transfection efficiencies in vitro, for the posterior implantation of the transfected cells into the patient.

2. The importance of the plasmid: is it possible to deliver an adenoviral vector plasmid of 40 Kbp with ultrapure oligochitosan?

Considering that one of the potential applications of UOC based polyplexes is related to gene delivery to tumor, we decided to focus the second part of this doctoral work on the possibility of using UOC based polyplexes for cancer gene therapy. An emerging approach in this field is the use of oncolytic adenoviruses (Ad) that can replicate selectively in tumor cells; moreover, the progeny adenovirus has the capability to spread through the tumor tissue infecting other cancer cells [27,28]. Nevertheless, there are several problems when injecting Ads directly into the blood stream, such as interactions with Kupffer liver cells, platelets, erythrocytes, complement and neutralizing antibodies [29]. Thus, research is being focused on the delivery of the oncolytic Ad plasmid by non-viral vectors. This approach could synergize oncolytic Ads mediated high therapeutic efficacy and non-viral vector mediated systemic delivery [30,31]. The main concern related to this novel approach is the fact that the size of oncolytic adenovirus plasmids is very high, around 40 kbp. As the packing capacity of non-viral vectors is theoretically unlimited [32], in the second part of

this doctoral thesis, we wanted to throw light upon the ability of UOC based polyplexes to deliver 40 kbp replication-incompetent adenoviral plasmid pAdTLRGD, which expresses the reporter protein EGFP, and transfect HEK293 and A549 cell lines.

In the first part of the work, we found that factors like N/P ratio, pH and salt concentration influence the size and zeta potential of UOC/pCMS-EGFP polyplexes. We wonder whether this influence was also present in polyplexes elaborated with the pAdTLRGD plasmid. We carried out a second orthogonal experimental design, and we observed that both the size and zeta potential were clearly modified by changes in the solution of the polyplexes (Table 2).

Table 2: The value of the effects and interactions on size and zeta potential of the UOC/pAdTLRGD polyplexes. X_1e: N/P effect, X_2e: [NaCl] effect, X_3e: pH effect, X_1X_2i: N/P-[NaCl] interaction, X_1X_3i: N/P-pH interaction, X_2X_3i: [NaCl]-pH interaction.

	Size (nm)		**Zeta (mV)**	
Effects and Interactions	**O15**	**O25**	**O15**	**O25**
X_1e	-17.1	-10.7275	3.6575	2.8875
X_2e	32.3	4.0325	20.8475	-2.2375
X_3e	-31.46	5.8725	-33.8325	-33.5875
X_1X_2i	22.15	5.7225	-7.3725	-0.0125
X_1X_3i	-9.19	-14.0175	10.2025	-1.5625
X_2X_3i	10.29	-5.6775	-18.2025	-0.0875

Concerning the size, all complexes were in the nanorange (around 200 nm), and this was influenced by the N/P ratio, the salt concentration and the pH of the formulation. According to the N/P ratio, when it was raised from 10 to 60, the particle size of O15 and O25 UOC based polyplexes decreased 17.1 nm and 10.73 nm, respectively. Changes in the salt concentration only affected the size of polyplexes based on O25 (an increment of 32.2 nm when the salt concentration was changed from 5 to 150 mM). The basification of the solution, lead to an increase in the particle size of O15 based polyplexes (31.46 nm). According to the zeta potential data, the charge of the particles decreased around 33 mV when the pH was raised from acid to neutral values. The observed interactions either for the

size or the zeta potential indicated that an explicit dependency between the factors exists. Interestingly, we did not found significant differences in the physicochemical properties of the polyplexes elaborated with pAdTLRGD plasmid (40 Kbp) and polyplexes elaborated with pCMS-EGFP, which is ten times smaller. Other authors also reported no differences in the structure and particle size of lipoplexes when using 52.5 Kbp plasmids compared to smaller pDNA [33].

Apart from the size and zeta potential, the binding affinity between the non-viral vector and the pDNA is another important property that should be considered. To achieve an efficient transfection, the non-viral vector must be able to condense, release and protect the DNA against enzymatic digestion [24]. Also, a delicate balance between DNA binding and release is required [34]. Due to the big size of the pAdTLRGD, we posed the following question: are UOC able to condense, release and protect a plasmid that is ten times larger than the ones that have been used so far? The agarose gel electrophoresis assays revealed that the ability of UOC based polyplexes to bind, release and protect the pAdTLRGD depended on the N/P ratio (Fig. 5). Polyplexes based on O15 UOC, which has a slightly lower Mw (5.7 KDa) than O25 UOC (7.3), required higher N/P ratios to completely bind and protect the pDNA (Fig. 5 A) (Fig. 5B). In addition, due to the difference in the Mw, polyplexes based on O15 UOC were able to release the DNA at N/P ratios of 5:1 and 10:1, but that did not occur with O25 UOC based polyplexes. Thus, we concluded that polyplexes formulations elaborated with higher Mw Ch are very stable and excellent DNA protectors. However, the release of the DNA cargo could be restricted due to a high stability of the formulation [9]. Overall, the agarose gel electrophoresis revealed that despite the high size of the pAdTLRGD, UOC based polyplexes are able to bind, release and protect it from enzymatic digestion.

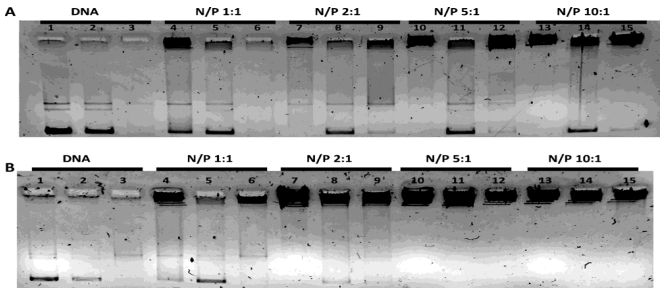


Figure 5: Binding, release and protection capacity of (A) O15 and (B) O25 UOC based polyplexes at different N/P ratios visualized by agarose gel electrophoresis. Polyplexes were treated with SDS (lanes 2, 5, 8, 11 and 14) and SDS + DNase I (lanes 3, 6, 9 and 15).

After we verified that UOC/pAdTLRGD polyplexes were physicochemically suitable for plasmid delivery, we move onto the evaluation of the transfection efficiency in HEK293 cell line, by flow cytometry. Taking into consideration that the microenvironment of tumors is generally more acidic than normal tissues [25], and based on our previous results where UOC mediated transfection was improved in acidic pH values, transfection experiments were carried out at pH 7.1. The highest percentage of EGFP positive cells (around 6 %) and mean fluorescent intensity MFI were obtained at N/P ratio 5 (Fig. 6). The low transfection efficiency and EGFP expression at high N/P ratios could be explained by the strong interactions between the polymer and the pDNA at high N/P ratios, which could hamper the release of the DNA from the vector [24]. Comparing both UOC, the expression of EGFP was higher with O15, which could be due to the lower Mw of this Ch [35]. Thus, MFI results indicated that although both UOC had low Mw, slight differences in their Mw could have a significant effect in the transfection efficiency.

Transfection experiments were then carried out in a lung adenocarcinoma cell line, A549. The percentage of EGFP positive cells was lower compared to the percentage obtained in HEK293. However, as it is shown in figure 7, at N/P 5:1 UOC based polyplexes were able to deliver the, 40 Kbp, plasmid for the subsequent production of the reporter

protein, in A549 cells (Fig. 7). Despite the low transfection efficiency, the delivery of a 40 Kbp plasmid with UOC was achieved for the first time. No EGFP expression was observed with the naked pAdTLRGD plasmid, neither in A549 nor in HEK293, which indicates that the non-viral vector is essential for the transfection to be successful.

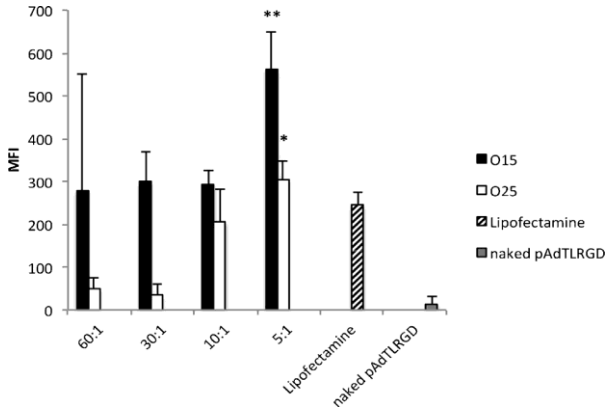


Figure 6: Mean fluorescent intensity (MFI) of HEK293 cells transfected with O15 (dark bars) and O25 (white bars) UOC/pAdTLRGD polyplexes at different N/P ratios. Error bars represent ±SD (n=3). *P < 0.05 compared to naked pAdTLRGD.**P < 0.01 compared to naked pAdTLRGD.

In this second part, we observed that the transfection efficiency with the replication-incompetent adenoviral plasmid (40 Kbp) was lower compared to the percentages that we obtained with the pCMS-EGFP plasmid (5.5 Kbp), in the first part of this work. However, despite the big size of the pAdTLRGD plasmid, the formed polyplexes were able to cross the cell membrane, translocate the plasmid into the nucleus and produce the EGFP reporter protein. Our research group was the first to achieve this objective with UOCs. With this work, we have highlighted one of the advantages of the non-viral carriers over viral counterparts, as the delivery of 40 Kbp nucleic acids with viral vectors is improbable. In order to make possible a real application of this combined therapy, the next experiments should be carried out with the replication competent adenoviral plasmid. The capacity of

the adenovirus progeny to spread through the tumor would offer the opportunity to use the non-viral vectors *in vivo* for oncolytic therapy, despite their low transfection efficiencies. Looking at the future, the systemic administration of non-viral vectors, such as UOC, combined with the replication competent adenoviral plasmid would be a major goal for the oncolytic treatment of primary and metastatic tumors. Thanks to the non-viral carrier, the oncolytic viral genome would arrive through the systemic circulation to the tumor foci and then translocate to the nucleus where they would replicate. This process would generate infectious oncolytic Ad progeny that lyses tumor cells while infecting neighboring cancer cells. There is still a lot of work ahead to overcome the limitations of the systemic administration of our polyplexes such as the poor stability *in vivo* and rapid blood clearance. Therefore, an improvement in the existing delivery technology would be required to achieve this ambitious objective.

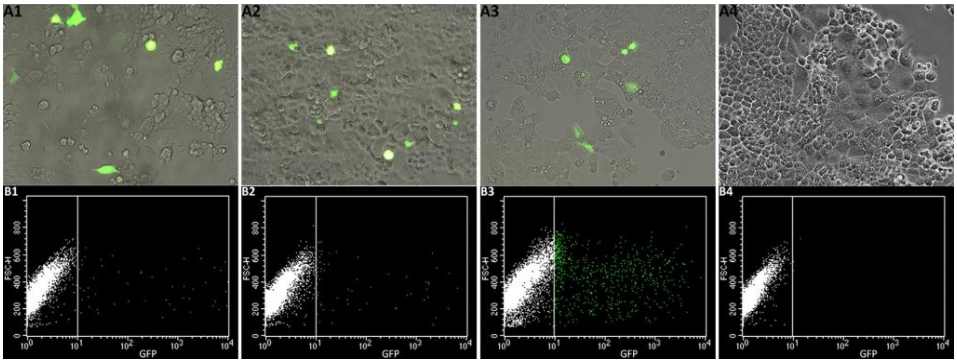


Figure 7: Transfection efficiency of UOC/pAdTLRGD polyplexes in A549 cell line. (A) Overlay of phase contrast image with fluorescent illumination (GFP channel) of cells transfected with (A1) O15 UOC/pAdTLRGD at N/P ratio 5; (A2) O25 UOC/pAdTLRGD at N/P ratio 5; (A3) Lipofectamine 2000; (A4) naked pAdTLRGD plasmid. (B) Flow citometry dot plots (GFP vs FSC) of cells transfected with (B1) O15 UOC/pAdTLRGD at N/P ratio 5; (B2) O25 UOC/pAdTLRGD at N/P ratio 5; (B3) Lipofectamine 2000; (B4) naked pAdTLRGD plasmid.

3. The transfection process step by step: a comparative study between lipoplexes, nioplexes, and polyplexes in human neuronal precursor NT2 cells

The human NTera2/D1 teratocarcinoma-derived cell line (or NT2 cells) is widely used to obtain suitable platforms for the delivery of exogenous proteins into the central nervous system (CNS). These cells can be differentiated into well-established populations of neuron-like cells (or NT2-N cells) that engraft and mature when transplanted into the adult CNS [36,37] with no signs of tumorigenicity [38]. Also, these cells possess a glioma tropism that makes them an interesting tool to derive new cellular vehicles for clinical glioblastoma therapy. Until now, most of the studies based on genetically engineered NT2 cells rely on the use of viral vectors. Nevertheless, the development of non-viral strategies to produce NT2 cells expressing exogenous gene products has distinct advances [39,40]. Hence, considering the flattering properties of this cell line, we wondered if it was possible to transfect human neuronal precursor NT2 cell line with UOC based polyplexes, as a first step in the development of a suitable platform for an *ex vivo* gene therapy in the CNS.

As we have mentioned before, the transfection efficiency mediated by non-viral vectors, and especially by Ch based vectors, is relatively low and thus, the successful design of the non-viral carrier requires a profound understanding of the whole transfection process. The final gene expression will be highly influenced by the uptake of the non-viral vector, the pathway by which these are internalized, their ability to escape from endosomes and the import of the genetic material to the cell nucleus [41]. In order to determine the bottleneck in the transfection process in NT2 cell line, we carried out a comparative study between polyplexes (based on UOC, Novafect O15), nioplexes (based on a lipidic niosome formulation) and lipoplexes (based on Lipofectamine 2000 liposomes). In table 3, the main components, w/w ratios, and employed DNA quantities are summarized (Table 3).

The physicochemical characteristics and composition of the non-viral vector could influence their interaction with the cell membrane and their intracellular behavior [42].

Table 3: Summary of the main components of the formulation, w/w ratios and DNA quantities employed for the elaboration of the three formulations.

Formulation	Main component	w/w ratio	DNA quatity (μg)
Lipoplexes	Lipofectamine 2000 reagent	2:1	1.25
Nioplexes	2,3-di(tetradecyloxy)propan-1-amine cationic lipid	20:1	1.25
Polyplexes	Novafect O15 ultrapure oligochitosan	13:1	1.25

Therefore, the three formulations were characterized in terms of size, zeta potential and polydispersity index (PDI) (Fig. 8). Lipoplexes and nioplexes presented a particle size around 140 nm with a low PDI (0.21), while polyplexes had a bigger size (294 nm) and PDI (0.44) as it can be observed in the size distribution curve (Fig. 8 B). The surface charge varied from negative to positive values depending on the vector: lipoplexes were negatively charged (-29.13 mV) and nioplexes and polyplexes had a positive zeta potential, + 35.4 mV and + 15.1 mV, respectively (Fig. 8 A).

Once the formulations were characterized, the transfection efficiency and the EGFP production were studied along the time, by flow cytometry and western blot analysis (Fig 9). As expected, the results revealed that lipoplexes exhibited the highest transfection efficiencies (Fig. 9 A) and protein expression levels (Fig. 9 B). In fact, Lipofectamine is a well-known transfection agent with high transfection efficiencies. Although we did not show significant toxicity signs with this formulation *in vitro*, its use *in vivo* has been limited [43]. As an alternative to liposomes, niosomes have shown promising results *in vitro* and *in vivo* [44,45]. Surprisingly, transfection efficiency of nioplexes was very low (3 % of EGFP positive cells) (Fig. 9 A), and no EGFP expression was observed in the Western blot studies (Fig. 9 B). Although the superior transfection efficiency mediated by lipid

Discussion

A

	Size (nm)	pdi	zeta ptential (mV)
Lipoplexes	144.1±6.3	0.21±0.04	-29.13±1.43
Nioplexes	142.5±0.64	0.21±0.04	35.4±0.7
Polyplexes	294.8±23.36	0.44±0.04	15.1±3.05

B

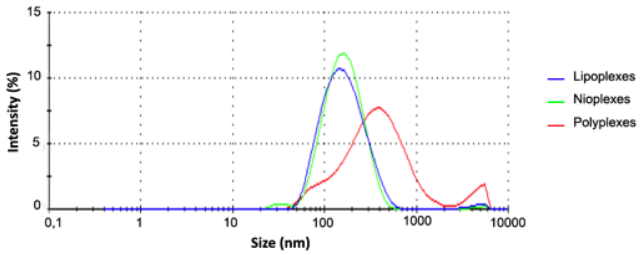


Figure 8. Characterization of the complexes. (A) Size, PDI and zeta potential values. Each value represents the mean ± standard deviation of three measurements. (B) Size distribution curve of the complexes.

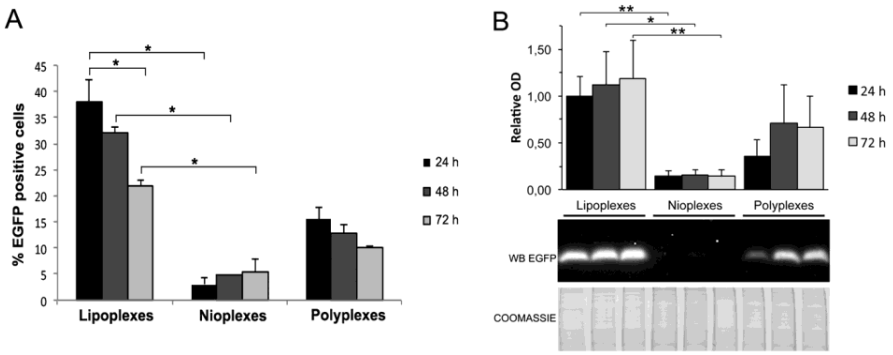


Figure 9. Transfection efficiency and EGFP expression at 24, 48 and 72 hours post-transfection. (A) Bar graphs showing percentage of EGFP positive live cells at 24, 48 and 72 hours post-transfection. Error bars represent ± SD (n=3) **P* < 0.05. (B) Bar graph depicts results of semiquantitative analysis of EGFP protein expression by Western Blot. A representative Western Blot is shown below the bar graph. Error bars represent mean ± SD (n=4). **P* < 0.05, ***P* < 0.01.

formulations compared to polymeric has been widely described [46], against all predictions, polyplexes based on UOC were more efficient than nioplexes at transfecting NT2 cells. Even though the transfection efficiency mediated by polyplexes was lower than the one obtained with lipoplexes (15 % vs. 35 % at 24 h post-transfection), the biocompatibility, biodegradability and low cytotoxicity of Ch enhances its possible clinical application. Considering the differences that we observed in the final EGFP expression between the three vectors, we wondered which step in the transfection process was the bottleneck.

The first barrier in the transfection process mediated by non-viral vectors is the cellular uptake [47]. We analyzed the uptake of our non-viral vectors after 1 h and 4 h of incubation with the cells, using the pCMS-EGFP plasmid labeled with FITC. Flow cytometry results showed a time-dependent cellular uptake of the three formulations (Fig. 10 A), which was previously reported by other authors [48]. Comparing the three formulations, the cellular uptake of lipoplexes (70 % at 4 h) was higher than the uptake of polyplexes (30 % at 4 h) and nioplexes (10 % at 4 h), as it can be observed in the bar graphs (Fig. 10 A) and flow cytometry histograms (Fig. 10 B). These results were supported by the confocal microscope images (Fig. 10 C). Overall, the cellular uptake results showed a clear correlation with respect to the corresponding transfection efficiencies.

Once we confirmed that our formulations were able to enter NT2 cells, the next step was to determine which internalization mechanism was involved in the cellular uptake. Taking into account the different physicochemical characteristics and composition of the vectors, we wondered if this could influence the internalization mechanisms and intracellular traffic [49]. Nioplexes were discarded for these experiments due to their low cellular uptake that did not allow the measurement. Regarding nanoparticle uptake mechanisms, clathrin-mediated endocytosis (CME) and caveolae-mediated endocytosis

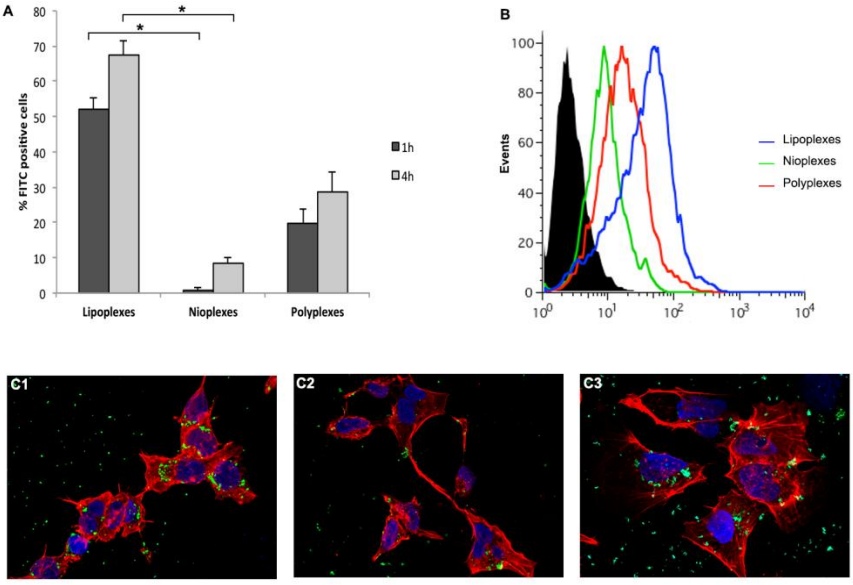


Figure 10. Cellular uptake of the complexes. (A) Bar graphs showing the percentage of FITC positive NT2 cells after the incubation for 1 hour or 4 hours with the complexes. Error bars represent ± SD (n=3). *P < 0.05. (B) Flow cytometry histograms representing the cellular uptake of the complexes (formed with FITC labelled plasmid DNA) after 4 h of incubation. Black filled curve represents untreated cells. (C) Merged images of NT2 cells 4 hours after the addition of (C1) lipoplexes, (C2) nioplexes and (C3) polyplexes. Images are at 63X magnification. Blue colouring shows cell nuclei stained with DAPI; red coloring shows F-actin stained with Phalloidin; green coloring shows Cy3 labelled plasmid DNA complexed with the corresponding non-viral vector.

(CvME) are the most common endocytosis routes [50]. By confocal imaging and colocalization assays (determining the Manders coefficient, M1), we found out that lipoplexes (140 nm, -29 mV) enter NT2 cells mainly through CME (M=0,471), while polyplexes (290 nm, +15 mV) by CvME (M=0,697) (Fig. 11). According to Rejman et al. internalization of microspheres with a diameter < 200 nm involved CME, while increasing the size shift to CvME became apparent [51]. Apart from the size, the surface characteristics of nanoparticles can also influence their internalization mechanisms. Unlike

our results, according to the literature, positively charged vectors predominantly internalize through CME while negatively charged formulations utilize CvME, taking into account that the entry pathway and subsequent traffic could also depend on the cell type [52]. However, we cannot forget that other internalization pathways, clathrin- and caveolae- independent endocytosis routes, could also take part in the internalization of our non-viral vectors.

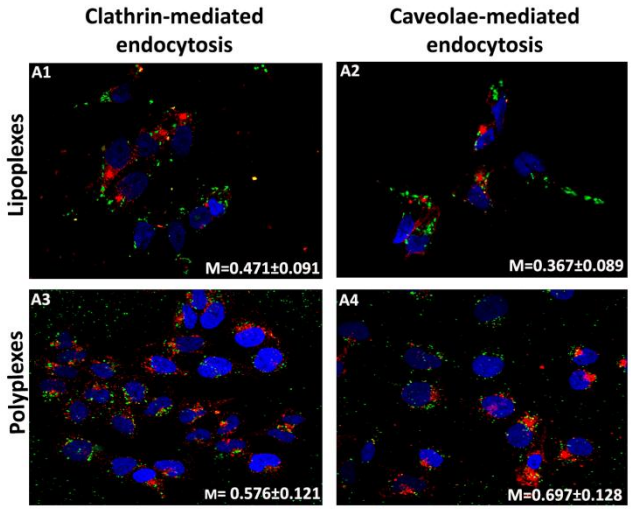


Figure 11. Colocalization of test complexes (formed with FITC labelled plasmid DNA) (green) with the specific markers of endocytic pathways (red): clathrin-mediated endocytosis (Alexa Fluor 546-Transferrin at 50 μg/ml) and caveolae-mediated endocytosis (Alexa Fluor 555-cholera toxin at 10 μg/ml). The images were taken after 4 hours of incubation with the test complexes. Images are at 63x magnification. Nuclei are stained with DAPI (blue). Presence of yellow color represents the overlay of the endocytic pathway marker and the vectors. The colocalization values are given as the fraction of complexes colocalizing with fluorescently labelled endocytic structure, represented by Mander´s (M1) coefficient.

It has been broadly reported in the literature that after the internalization process, particles that enter the cell via CME are confined within endosomes that will suffer a maturation process involving the compartment acidification resulting in late endosomes and finally, lysosomes [53]. Accordingly, after 4 hours of incubation, lipoplexes, which enter the cell via CME, were located at the late endosome (Fig. 12 A). Until recently, it was generally accepted by the research community that nanoparticles that enter the cell via CvME were away from lysosomal degradation. However, it has been recently postulated that caveolae also fuse with normal acidified endosomes being able to transfer material to lysosomes [54]. This work supports our results since the polyplexes that enter the cell via CvME were located at the late endosome after 4 h of incubation (Fig. 12 A).

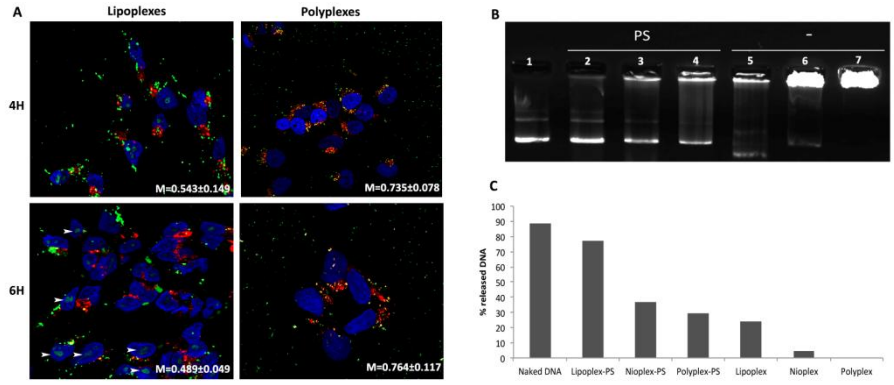


Figure 12. (A) Colocalization of test complexes (formed with FITC labelled plasmid DNA) (green) with Lysotracker Red-DND-99 that is a specific marker of the late endosome (red). The images were taken after 4 and 6 hours of incubation with the test complexes. Markers were added 1 hour prior to the end of the incubation time. Images are at 63 x magnification. Nuclei are stained with DAPI (blue). Presence of yellow colour represents the overlay of the endocytic pathway marker and the vectors. The colocalization values are given as the fraction of complexes colocalizing with fluorescently labelled endocytic structure, represented by Mander´s (M1) coefficient. (B) DNA release profiles measured with gel electrophoresis. Lane 1: naked DNA; lane 2: lipoplexes incubated with PS; lane 3: nioplexes incubated with PS; lane 4: polyplexes incubated with PS; lane 5: lipoplexes; lane 6: nioplexes; lane 7: polyplexes. PS refers to phosphatidyl serine micelles. (C) The percentage of released DNA quantified from the agarose gel using the Image Lab ^{TM} software.

The next barrier in the transfection process is the endosomal escape of the DNA in order to avoid lysosomal degradation, which could vary depending on the nanoparticle nature [55]. Thus, as an analogue of the endosomal compartment, we elaborated anionic micelles based on phosphatidylserine (PS) to simulate the ability of our formulations to interact with the endosome and release the DNA cargo [56]. As it can be observed in figure 12B and 12C, the incubation of the vectors with the PS micelles led to the release of the DNA from the carriers, which did not occur when the vectors were not incubated with the micelles.

Finally, our results revealed differences in the internalization velocity of lipoplexes and polyplexes. The colocalization values that we obtained with lipoplexes and the specific markers after 4 h and 6 h of incubation were lower compared to the values obtained with polyplexes(Fig. 12 A), which means that at the tested times most of the lipoplexes were not located within the endosomal compartments. Moreover, 6 h after the addition of the complexes to NT2 cells, the FITC-labelled DNA carried by lipoplexes was situated in the nucleus, while the DNA carried by polyplexes was still located in the late endosome (Fig. 12 A). These results suggested that the internalization process of lipoplexes is a relatively rapid process compared to polyplexes, which is in agreement with other authors who reported that smaller particles, (around 100 nm, as our lipoplexes) enter the cell more rapidly than bigger particles, like our polyplexes [51].

Overall, this research work gives some knowledge about the transfection process of different non-viral vectors in NT2 cells, which could be valuable to improve the design and development of more efficient vectors. Concretely, we have concluded that UOC based polyplexes can transfect human neuronal precursor NT2 cells. Moreover, we suggested that the percentage of cellular uptake is a limiting step in the transfection process of UOC based polyplexes that could be enhanced by different strategies in order to improve their transfection efficiency. In the following experiments, we should take a step forward and focus our research on a specific disorder utilizing the appropriate therapeutic genes.

Nevertheless, this work could be the beginning of the development of an *ex vivo* gene therapy for the treatment of CNS disorders.

As a whole, this doctoral thesis describes the first steps in the development of a non-viral vector based on UOC for either *in vivo* or *ex vivo* gene therapy. We are aware that there is still much work to be done and limitations that must be overcome. We believe that this polymer offers a broad range of possibilities that could be exploited to progress in the development of low Mw Ch based non-viral vectors.

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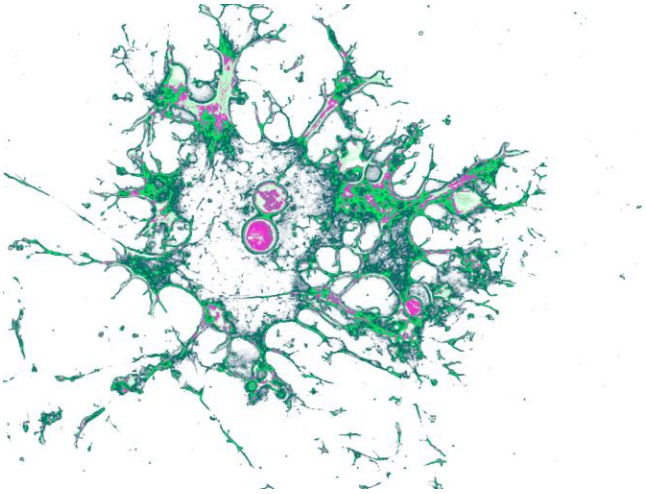

Ondorioak
Conclusiones
Conclusions

1. N/P ratioa, soluzioaren gatz kontzentrazioa eta pH-a bezalako faktoreek, UOC-etan oinarritutako poliplexoen tamainan eta zeta pontenzialean aldaketak eragiten dituzte, plasmidoa edozein izanda ere. Bereziki, pH-aren baloreak eragin sendoa dauka poliplexoen zeta potentzialean. pH-aren balioak pixka bat azidotuz, 6.5 aldera, zeta potentzial balore altuagoak lortzen dira.

2. HEK293 eta ARPE19 zeluletan egindako *in vitro* entseguek, transfekzio soluzioaren pH-aren jeitsiera txiki batek UOC/pCMS-EGFP poliplexoen barneratze zelularra handiagotzen duela ezagutzera eman zuten. Hau, UOC-etan oinarritutako polipexoen transfekzio efizientziaren handipena ekarri zuen berekin.

3. UOC-etan oinarritutako poliplexoak 40 Kbp-ko adenobirus baten bektore den plasmidoa lotzeko eta babesteko gai dira. Poliplexoek 40 Kbp-dun plasmidoa garraiatzeko eta HEK293 eta A549 zelulak transfektatzeko gaitasuna dute.

4. Lipoplexo, nioplexo eta poliplexoekin egindako entsegu konparatioak ezagutzera eman zuen UOC-etan oinarritutako komplexuak NT2 giza neuronen zelula prekurtsoreak transfektatzeko gai direla. Transfekzio prozesuaren ikerketak adierazi zuen, UOC-etan oinarritutako poliplexoek eragindako transfekzioaren pausu mugatzailea zelula-barneratzea zela. Behin zelula barnean daudela, poliplexoek zelula barneko garraio eraginkor bat dute, intereseko proteinaren expresioa ahalbidetzen duena.

1. El tamaño y potencial zeta de los poliplexos basados en UOC se ven afectadas por varios parámetros como el ratio N/P, la concentración de sales de la solución y el valor del pH, independientemente del plásmido utilizado para elaborar los complejos. En particular, el potencial zeta de los poliplexos está fuertemente influenciado por el valor del pH. Mediante un ligero descenso del pH a valores alrededor de 6.5 se obtienen potenciales zeta más altos.

2. Los estudios *in vitro* en células HEK293 y ARPE19 revelaron que un pequeño descenso en el pH de la solución de transfección conlleva un aumento en la captación celular de los poliplexos UOC/pCMS-EGFP. Esto se traduce en una mejora en la eficiencia de transfección de los poliplexos basados en UOC cuando el pH del medio de transfección es ligeramente acidificado.

3. Los poliplexos basados en UOC son capaces de condensar y proteger eficientemente un plásmido de 40 Kbp que codifica un adenovirus. Estos poliplexos pueden transportar el plásmido de 40 Kbp y transfectar las células HEK293 y A549.

4. El estudio comparativo entre los lipoplexos, nioplexos y poliplexos mostró que los UOC son capaces de transfectar de forma eficiente las células humanas precursoras neuronales NT2. El estudio del proceso de transfección reveló que el paso limitante en el proceso de transfección mediado por poliplexos basados en UOC en células NT2 es la captación celular. Una vez dentro de la célula, los poliplexos presentan un tráfico intracelular eficiente que dar lugar a la expresión de la proteína de interés.

1. The size and zeta potential of UOC based polyplexes are affected by several parameters like the N/P ratio, the salt concentration of the solution and the pH value, regardless the plasmid used to elaborate the complexes. In particular, the zeta potential of the polyplexes is strongly influenced by the pH value. Higher zeta potential values are obtained when the pH is slightly acidified to values around 6.5.

2. The *in vitro* studies in HEK293 and ARPE19 cells revealed that a small decline in the pH value of the transfection solution leads to an increased cellular uptake of UOC/pCMS-EGFP polyplexes. This translates into an improved transfection efficiency mediated by UOC based polyplexes when the transfection medium is slightly acidified.

3. UOC based polyplexes are able to efficiently bind and protect a large adenovirus vector plasmid of 40 Kbp. The polyplexes are capable of delivering the 40 Kbp plasmid and transfecting HEK293 and A549 cells.

4. The comparative study between lipoplexes, nioplexes and polyplexes showed that UOC based complexes are able to efficiently transfect the human neuronal precursor NT2 cells. The study of the transfection process revealed that the limiting step in the transfection mediated by UOC based polyplexes in NT2 cell line is the cellular uptake. Once the polyplexes are inside the cell they present an efficient intracellular trafficking which leads to the expression of the desired protein.