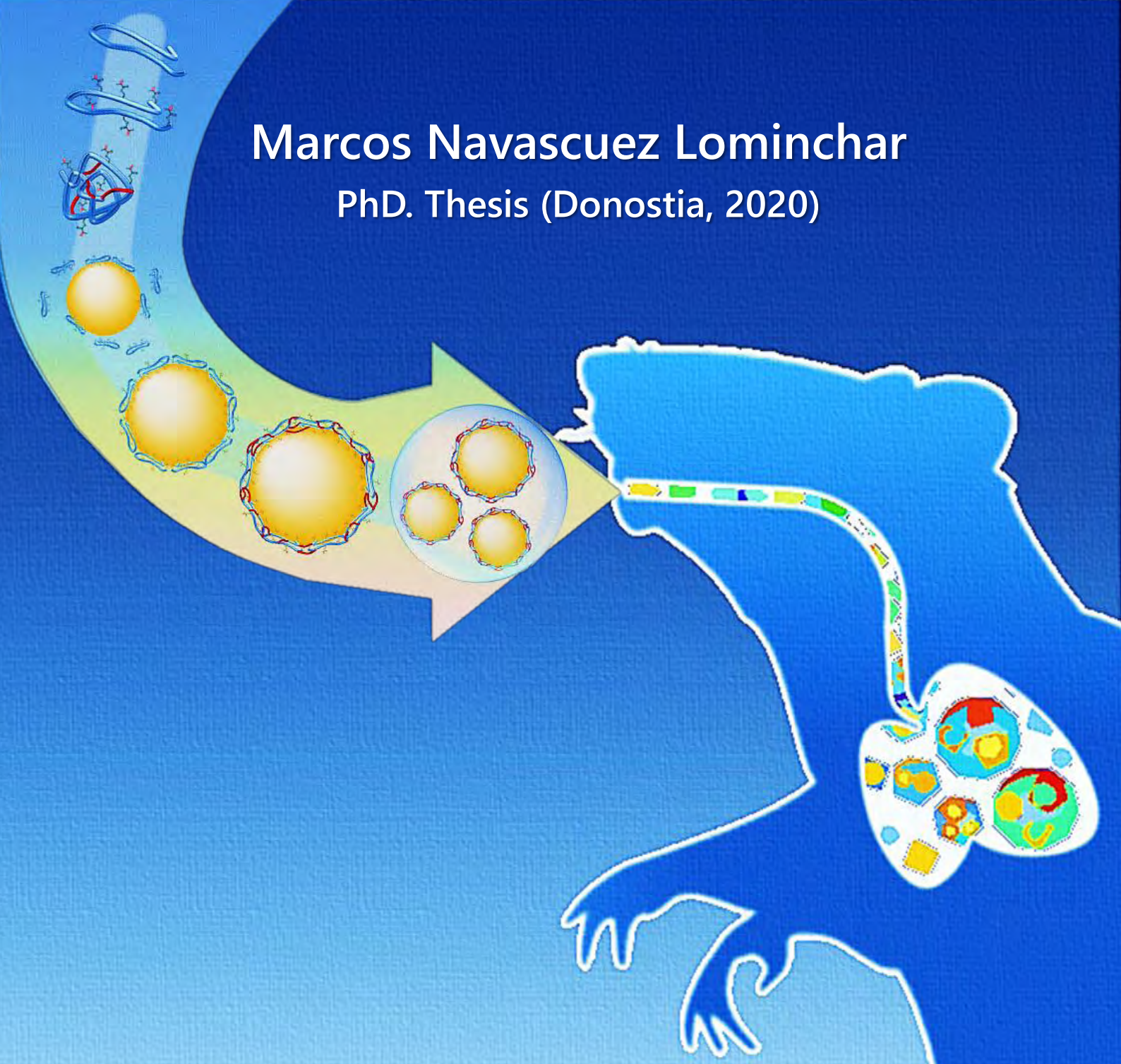
Marcos Navascuez Lominchar

PhD. Thesis (Donostia, 2020)



Nanoemulsions as drug carriers: preclinical evaluation of pulmonary delivered drug-loaded nanocapsules

eman ta zabal zazu

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Nanoemulsions as drug carriers: Preclinical evaluation of pulmonary delivered drug-loaded nanocapsules

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by

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List of Abbreviations and Acronyms

ALI	Acute lung injury
AMF	Ammonium formate
ARDS	Acute respiratory distress syndrome
Asp	Aspartate
BCS	Biopharmaceutical Classification System
BFCs	Bifunctional chelators
CMC	Critical Micelle Concentration
CALB	Candida Antarctica Lipase B
CL	Cross-linked
CT	Computational Tomography
COPD	Chronic obstructive pulmonary disease
COSAN	Cobalt *bis*(dicarbollide) anion ($[3,3'\text{-}Co(C_2B_9H_{11})_2]^-$)
Dh	Hydrodynamic diameter
Dh[4;3]	Volume diameter
DHA	Docosahexaenoic acid
DLS	Dynamic light Scattering
DMAP	4-(*N*,*N*-dimethylamino)pyridine
Dv50	Median diameter by volume
DODT	3,6-dioxa-1,8-octane-dithiol
DOTA	1,4,7,10-tetraazacyclododecane-tetraacetic acid
DS	Degree of substitution
DXT-MA	Dextran random coil polymer (MA functionalized)
DXT-F	Dextran random coil polymer (COOH functionalized)
DXT-SCPN-MA	Dextran single chain polymer nanoparticle (MA functionalized)
DXT-SCPN-F	Dextran single chain polymer nanoparticle (COOH functionalized)
DXT-R	Dextran derivatives
D (number)	Dextran derivatives labelling
E (number)	Emulsions labelling
FDG	2-deoxy-2-[^{18}F]fluoro-D-glucose
FES	16α-[^{18}F]Fluoroestradiol
FFA	Free fatty acid
FFA_{max}	Maximum percentage of free fatty acids
Glc	Glucose
GMA	Glycidyl methacrylate
Glu	Glutamate
His	Histidine

LD	Laser diffraction
MA	Methacrylate
MAG	Monoglyceride
MeCN	Acetonitrile
MPA	3-mercaptopropionic acid
MMSE	3-methoxymethyl-16β,17β-epiestriol-O-cyclic sulfone
NPs	Nanoparticles
NCs	Nanocarriers
NEs	Nanoemulsions
O/W or W/O	oil-in-water or water-in-oil
PBS	Phosphate buffer saline
PDI	Polydispersity index
PET	Positron Emission Tomography
PLA	Polylactide acid
PL	Pancreatic Lipase
PTA	Phosphotungstenic acid
RML	Rhizomucor miehei Lipase
ROL	Rhizopus oryzae Lipase
SCPNs	Single chain polymer nanoparticles
SEC	Size exclusion chromatography
SEM	Scanning Electron Microscopy
Ser	Serine
SEAr	Aromatic electrophilic substitution
SMEs	Small and medium enterprises
SPECT	Single Photon Emission Computerised Tomography
ST	Surface tension
TAG	Triacylglyceride
TEM	Transmission Electron Microscopy
TLC	Thin layer chromatography
TLL	Thermomyces lanuginosus Lipase
VOI	Volume of interest

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Summary

The use of nanomaterials for biomedical applications has been widely studied during the last decades. In medicine, nanomaterials constitute a very promising research field from an academic point of view in areas such as the development of medical devices, molecular imaging or drug delivery, among others. Advanced drug delivery systems can improve the pharmacological properties of conventional "free" drugs by modifying their biodistribution and pharmacokinetics. Ideally, nanocarriers should increase the bioavailability of the drug in the target organ while decreasing toxic and side effects. To this purpose; a proper size, shape and surface functionality of the carrier are required to prolong the biological half-life of the drug and to achieve the optimal dose in the diseased tissue while minimizing off-target side effects.

The administration route also plays a pivotal role in nanomedicine, and pulmonary delivery is attractive both for local and systemic therapies. The low thickness of the epithelium (0.1–0.5 µm in the alveolar region) and its huge surface area (75–150 m^2) lead to a faster onset of therapeutic action while avoiding the first-pass metabolic barriers. One of the main challenges in inhalation when the lungs are the target organ is to avoid or reduce the extremely fast clearance in the alveoli region. This effect is especially relevant for poorly water-soluble drugs which are highly permeable to biological membranes. However, the therapeutic efficacy of poorly-water soluble drugs can be significantly improved by optimizing the formulation design. In this context, oil-in-water (o/w)-nanoemulsions (NEs) have gained attention as hydrophobic drug carriers because of their high loading capacity. Emulsion stabilization usually requires the use of surfactants (surface-active substances) which are amphiphilic molecules able to reduce interfacial tension between the immiscible phases. Regarding to this, natural polymer grafting has appeared as novel strategy to produce biodegradable surfactants relevant to biomedical applications.

In this PhD thesis, pulmonary administration and *in vivo* molecular imaging have been pushed forward, with the ultimate aim of exploring the suitability of polymers and polymeric NPs to stabilize (o/w)-nanoemulsions as potential drug carriers for enhanced transport and delivery of poor water soluble drugs.

First, the capacity of dextran polymers and dextran-based single-chain polymer nanoparticles (SCPNs) to stabilize NEs was investigated (chapter 3). The main goal of this chapter was to produce stable and monodisperse dextran-based (o/w)-nanoemulsions below 500 nm suitable for pulmonary delivery. As pristine dextran (DXT, hydrophilic polysaccharide) is not able to sufficiently decrease the oil/water interfacial tension to produce (o/w)-nanoemulsions, chemical modification of dextran with hydrophobic or hydrophilic moieties (DXT-R) were carried out. Four dextran derivatives with the same degree of functionalization were synthesized while changing the functional group (methacrylic or carboxylic acid) and the three-dimensional structure (polymer coil or single chain polymer nanoparticle). Besides surfactant optimization (surfactant selection, determination of surfactant weight percentage and functionalization degree), other experimental conditions such as the parameters of the oil phase (selection of the oil and the oil/water proportions) were evaluated in order to fulfill droplet size and stability requirements for intratracheal instillation. Big efforts were conducted to achieve long-term stable emulsions. However, emulsion stability was compromised in highly ionic strength media, and NEs were rapidly destabilized under physiological conditions.

Aiming at improving the media stability of the NEs, cross-linking strategies were explored (chapter 4). More concretely, cross-linking was performed on the polymers after adsorption at the oil/water interface under mild reaction conditions using a surface active dithiol as the linker. Two different strategies for crosslinking (via the aqueous phase or the organic phase) were evaluated in order to avoid inter-droplet crosslinking. Successful intra-droplet crosslinking via the organic phase could be achieved, with consequent extensive improvement in emulsion long-term stability. The resulting cross-linked emulsions proved to be stable for months (up to 1 year) in any physiologically relevant medium. Moreover, the effect of the reaction on emulsion stability was assessed, both *in vitro* and *in vivo*, the latter using Positron Emission Tomography (PET). Fluoroestradiol was selected as model drug due to its hydrophobicity (log Kow=4.01) and easy labelling with the positron emitter ^{18}F (half-life of 109.8 minutes). Copper-64, a positron emitter with physical half-life of 12.7 hours,

was anchored to the polymeric surfactant to investigate the biodistribution of the nanocarrier.

Moving forward towards potential controlled drug delivery, the enzyme-responsive properties of the NEs were studied (chapter 5). Due to the presence of ester bonds generated during dextran functionalization and also present in the oil phase, our emulsions can be destabilized in the presence of esterase enzymes. Hence, we designed an experimental set up to unravel the mechanism of enzymatic destabilization by evaluating the response of the individual components (oil and surfactant) towards Candida Antarctica Lipase B (CALB) and the effect of the enzyme on droplet size. Finally, the studies were extended to Pancreatic Lipase.

Finally, with the aim of exploring less conventional amphiphiles as emulsion stabilizers, and taking advantage of the expertise of the research team in boron chemistry, the suitability of the boron-rich anionic complex cobalt bis(dicarbollide) (COSAN) to stabilize NEs was also assayed (chapter 6). Nanoemulsion stability was evaluated towards a wide range of stimuli: overtime, in relevant physiological media, in presence or absence of light, at different temperatures and in presence of lipases. Moreover, the capability of the resulting NEs to modulate the biodistribution of entrapped hydrophobic drugs after pulmonary administration was investigated in rodents using *in vivo* nuclear imaging. Previous results obtained with FES as model drug encouraged us to use the same model compound using COSAN-based NEs as pulmonary carriers. To get further information of the nanocarrier, we also explored the biodistribution of the stabiliser, i.e. the COSAN. In this case, COSAN was firstly functionalized with iodine and later on radiolabelled with the positron emitter ^{124}I (half-life of 4.2 days) via isotopic exchange. Our promising results suggest that the newly developed NEs can be applied for drug delivery of poor water soluble drugs when the lung is the target organ.

Resumen

La utilización de nanomateriales en aplicaciones biomédicas ha suscitado un gran interés durante las últimas décadas. En medicina, los nanomateriales constituyen un campo de investigación muy prometedor desde un punto de vista académico en áreas

como el desarrollo de dispositivos médicos, la imagen molecular o la liberación controlada de fármacos. Los nanomateriales avanzados para la administración controlada de medicamentos (*nanocarriers*) pueden mejorar las propiedades farmacológicas de los mismos en comparación con la administración del compuesto activo libre, debido a que consiguen modificar su biodistribución y farmacocinética. Idealmente, los *nanocarriers* deberían aumentar la biodisponibilidad del fármaco en el órgano a tratar, al mismo tiempo que disminuyen los efectos secundarios en el resto de los órganos. Para este fin se requieren una forma y tamaño adecuados, además de una funcionalización apropiada de la superficie del *nanocarrier* con el objetivo de prolongar la vida media biológica del fármaco. Esto ayudaría a alcanzar la dosis adecuada en el tejido/órgano afectado, minimizando a su vez los efectos secundarios fuera de la diana terapéutica.

La ruta de administración juega un papel fundamental en el uso de nanomateriales como *nanocarriers,* y en este contexto la inhalación de aerosoles a través del sistema respiratorio es considerada una ruta de administración muy atractiva tanto para terapias locales como sistémicas. El bajo grosor del epitelio (0.1–0.5 µm en la región alveolar) y su enorme área superficial (75–150 m^2) proporcionan un inicio más rápido de la acción terapéutica evitando las principales barreras metabólicas del cuerpo. Uno de los principales desafíos a superar cuando los pulmones son el órgano a tratar consiste en evitar o reducir la rápida eliminación del fármaco en la región de los alvéolos. Este efecto, que es especialmente relevante en fármacos poco solubles en agua que son altamente permeables a membranas biológicas, puede mitigarse optimizando el diseño de su formulación. Una opción para conseguir una formulación adecuada consiste en utilizar nanoemulsiones de aceite-en-agua (NEs) como portadores de fármacos hidrofóbicos, cuyo uso ha suscitado un gran interés científico debido, principalmente, a su gran capacidad de carga. La estabilización de la emulsión generalmente requiere del uso de tensoactivos, que son moléculas anfifílicas capaces de reducir la tensión interfacial entre fases inmiscibles. En este aspecto, el grafting o funcionalización de polímeros naturales ha emergido como una novedosa estrategia

para la producción de tensoactivos o estabilizadores biodegradables que puedan ser útiles en aplicaciones biomédicas.

En esta tesis doctoral, se ha utilizado la administración pulmonar y las técnicas de imagen molecular *in vivo* con el objetivo final de explorar la idoneidad de polímeros y NPs poliméricas basadas en dextrano para estabilizar nanoemulsiones de aceite en agua cuya principal función es la encapsulación, transporte y liberación controlada de fármacos poco solubles en agua.

Los polisacáridos son las biomacromoléculas más comunes en todo el mundo. La gran variedad de grupos reactivos y composición química, junto con su biodegradabilidad, han propiciado su uso en diferentes aplicaciones biomédicas. El dextrano (DXT) es un polisacárido hidrofílico incapaz de disminuir suficientemente la tensión interfacial entre agua y aceite como para producir emulsiones estables; en consecuencia, requiere de modificaciones químicas que le confieran propiedades como tensoactivo. El primer objetivo de la tesis, abordado en el capítulo 3, ha sido el de producir nanoemulsiones estabilizadas con derivados de dextrano (DXT-R), estables, monodispersas y con un diámetro inferior a 500 nm, con lo que serían adecuadas para la administración pulmonar. Con este propósito, se sintetizaron cuatro derivados de dextrano con el mismo grado de funcionalización pero cambiando de grupo funcional (metacrilato o ácido carboxílico) y la estructura tridimensional (polímero *random coil* o nanopartícula polimérica de cadena única). Además de la optimización del tensoactivo (selección del tensoactivo, determinación del porcentaje en peso del tensoactivo y su grado de funcionalización), se evaluaron otras condiciones experimentales como los parámetros relacionados con la fase oleosa (selección del aceite y las proporciones de aceite/agua) con el objetivo de cumplir con los criterios de tamaño, polidispersidad y estabilidad necesarios para la nebulización del aerosol. Todos los DXT-R han demostrado su capacidad para actuar como emulsificantes, incluidos los modificados hidrofílicamente. Sin embargo, DXT-MA (modificado con grupos metacrilatos, MA) ha sido seleccionado como el mejor estabilizante debido a la simplicidad en su síntesis química (sólo una etapa de funcionalización) y a su capacidad para generar las emulsiones con menor polidispersidad y menor diámetro de gota (**Figura 1**).

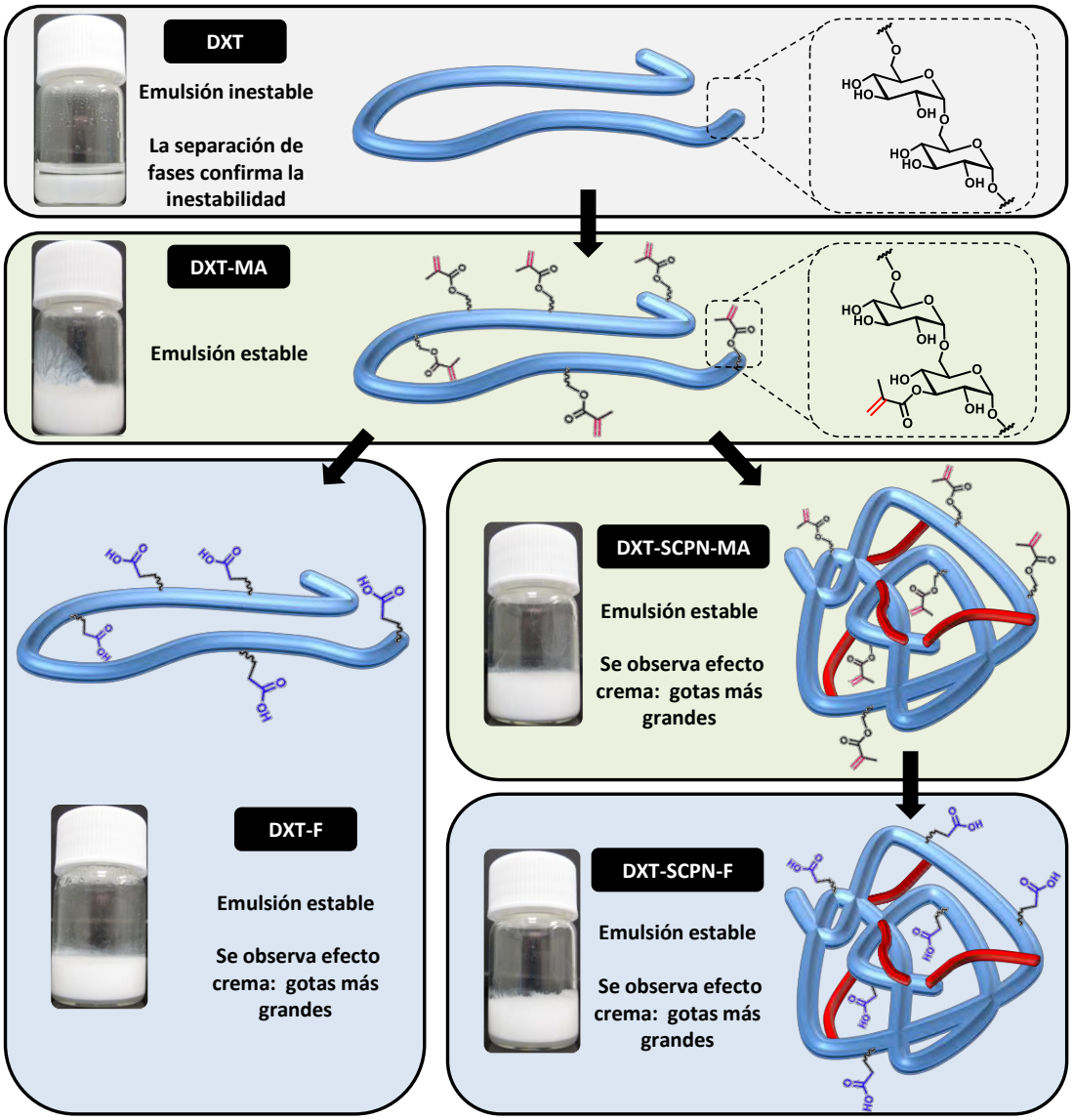


Figura 1. *Rutas sintéticas seguidas para la funcionalización del dextrano (DXT) y el uso de los diferentes derivados como estabilizadores de emulsiones de aceite en agua. Representación esquemática de la estructura fisicoquímica y los pasos sintéticos necesarios para obtener los diferentes derivados de dextrano (DXT, DXT-MA, DXT-F, DXT-SCPN-MA y DXT-SCPN-F) combinados con fotografías digitales de las emulsiones resultantes después de 24 horas en reposo. Las emulsiones se prepararon al 10% en peso de dodecano (fase aceite) y estabilizadas con 0,5% en peso de dextrano funcionalizado (emulsificante).*

Además, se han probado diferentes cantidades de estabilizante para encontrar el porcentaje en peso óptimo del mismo, que finalmente ha sido fijado en 0.5 wt. %. Por otro lado, el aceite de pescado (cuyo componente principal es el ácido docosahexenoico, DHA) dio como resultado las gotas más pequeñas y más estables con el porcentaje de fase oleosa del 10 wt. %, previamente seleccionado. Además, se

ha sintetizado DXT-MA con diferentes grados de sustitución (DS) para evaluar el efecto de los grupos MA en las propiedades de la emulsión. Los resultados sugieren que se necesita un DS mínimo para formar emulsiones estables. El DS mínimo se ha establecido como la sustitución mínima necesaria para obtener valores de tensión superficial (ST) inferiores a 60 mN/m a una concentración de 5 g/L. Nuestros resultados confirman que hay un número mínimo de grupos MA, en el rango de 10-15%, necesarios para producir emulsiones estables. Además, el diámetro de la gota aumenta al aumentar el DS lo que significa que existe un rango de sustitución óptimo (DS = 15-25%) que permite la producción de emulsiones estables, más pequeñas y menos polidispersas. Por último, se realizaron grandes esfuerzos para lograr emulsiones estables a largo plazo. Sin embargo, la estabilidad de la emulsión se vio comprometida en medios con elevada fuerza iónica, comprobando así que las nanoemulsiones se desestabilizaban rápidamente en condiciones fisiológicas.

Con el objetivo de mejorar la estabilidad en medios fisiológicos de las nanoemulsiones, en el capítulo 4 se exploraron dos estrategias diferentes para la reticulación (a través de la fase acuosa o la fase oleosa), procurando evitar la reticulación entre polímeros pertenecientes a distintas gotas. Más concretamente, dicha reticulación se llevó a cabo directamente sobre los polímeros adsorbidos en la interfase aceite/agua bajo condiciones suaves de reacción y utilizando el ditiol 2,2'-(etilendioxi)dietanetiol (DODT), con cierta actividad interfacial, como agente reticulante. Los resultados mostraron que se puede conseguir estabilizar la emulsión siguiendo la estrategia de adición del reticulante en la fase oleosa, permitiendo la reticulación polimérica intra-gota (**Figura 2**). Para demostrar la reticulación de las nanogotas se ha utilizado el método de DMSO/dioxano (70:30) que permite eliminar agua y aceite dejando aislado el polímero reticulado. Gracias a esto, se ha podido llevar a cabo la cuantificación por ^{1}H-RMN de la agente reticulador unido al polímero. Además, este método nos permitió visualizar la reticulación del material a través de las microgafías SEM del coloidosoma colapsado (**Figura 2c**). La estabilidad de las emulsiones reticuladas aumentó en medios de elevada fuerza iónica como en el tampón fosfato salino (PBS, 10 mM en fosfato), mientras que las emulsiones no reticuladas mostraron una rápida desestabilización

(efectos de coalescencia). Tal y como se planteó en las hipótesis de partida, las emulsiones reticuladas también demostraron ser estables en el tiempo al permanecer inalterado su tamaño durante meses (hasta 1 año) en cualquier medio fisiológicamente relevante (Figura 2).

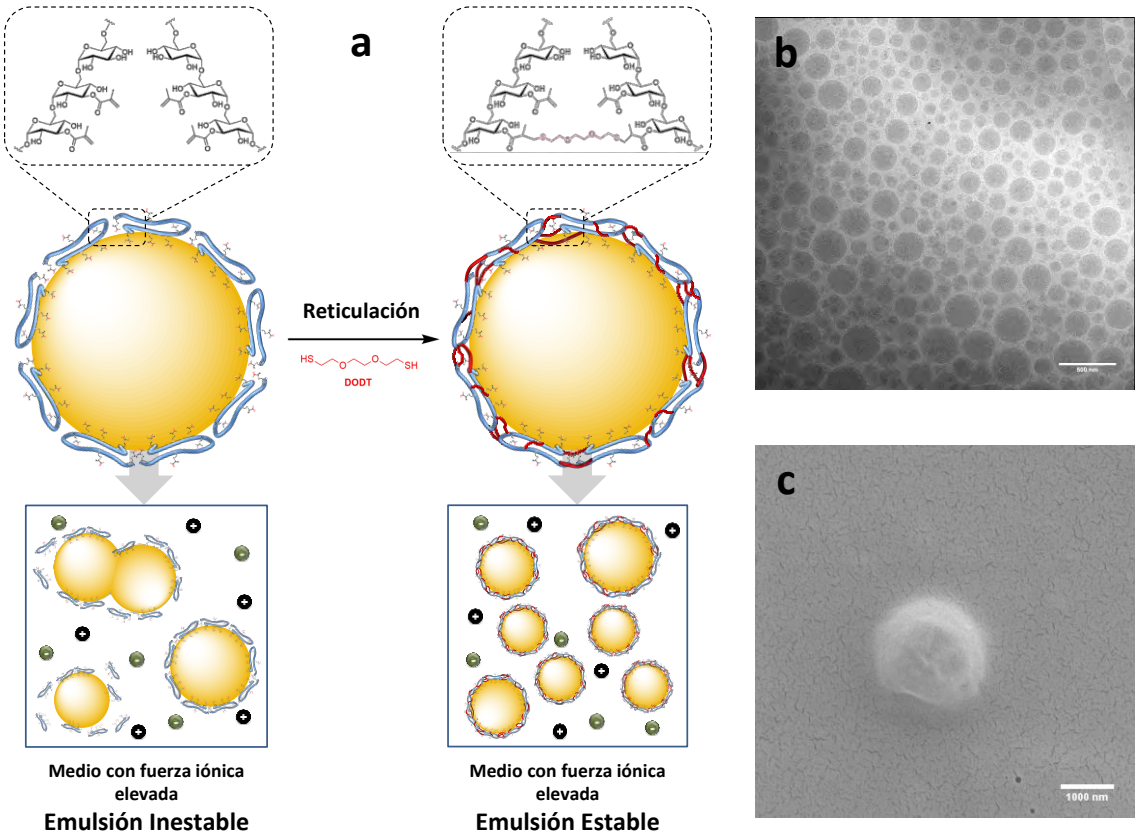


Figura 2. *a) Representación esquemática de la mejora de la estabilidad tras la reticulación en la interfase a través de la fase de oleosa. b) Micrografía Crio-TEM de una nanoemulsión reticulada. c) Imagen SEM del coloidosoma colapsado obtenido después de la extracción del aceite mediante el método del DMSO/dioxano.*

Además, este efecto estabilizador se evaluó *in vivo* utilizando tomografía por emisión de positrones (PET). El fluoroestradiol (FES) fue seleccionado como fármaco modelo debido a su hidrofobicidad (log Kow = 4.01) y a la facilidad para ser funcionalizado con el isótopo emisor de positrones ^{18}F (vida media de 109.8 minutos). La nanoemulsión prolongó 10 veces el tiempo de residencia del FES en los pulmones. Por otro lado, el cobre-64 que es un emisor de positrones con una vida media de 12.7 horas, se unió al tensioactivo polimérico mediante un agente quelante con el objetivo de investigar la biodistribución del *nanocarrier*. Para ello, se utilizó un derivado tiolado de DOTA

(DOTA-SH) que permitía por un lado la formación del complejo con el radiometal, y por otro su unión al polímero a través de los grupos metacrilato presentes en el DXT-MA. La biodistribución del DXT-MA confirmó que el estabilizador también es eliminado de los pulmones a una velocidad similar a la del FES encapsulado, lo que parece indicar que los componentes individuales de la emulsión permanecen juntos tras la administración *in vivo*.

Idealmente, los materiales utilizados para liberación controlada de fármacos deberían acumularse selectivamente en el órgano o tejido a tratar liberando su carga bajo estímulos concretos. La sobreexpresión de ciertas enzimas generalmente está relacionada con trastornos fisiológicos. Cuando dicha sobreexpresión se produce en el órgano diana, se puede utilizar como estímulo para iniciar la liberación del fármaco. Con esta idea, se utilizaron diferentes enzimas para evaluar si eran capaces de desestabilizar las nanoemulsiones. Debido a la presencia de enlaces éster generados durante la funcionalización del dextrano y los presentes en los triglicéridos de la fase oleosa, nuestras emulsiones podrían desestabilizarse en presencia de enzimas de tipo esterasa. Dentro de la familia de las esterasas se encuentran las de tipo lipasa, que son especialmente eficientes en la hidrólisis de enlaces éster procedentes de triglicéridos generando ácidos grasos y glicerol. En general, las lipasas necesitan ser activadas interfacialmente. Sin embargo, la presencia de nuestro tensoactivo basado en dextrano en la interfase nos llevó a estudiar el principal mecanismo de desestabilización enzimática (capítulo 5). Para ello, se evaluó la respuesta de los componentes individuales de la emulsión, aceite y surfactante, frente a la enzima *Candida Antártica Lipasa B (CALB)* y el efecto que ésta generaba en el tamaño de gota. La investigación del tamaño mediante difracción láser confirmó una disminución de la población principal con el tiempo, lo que sugiere que la estabilidad de la emulsión se ve comprometida como consecuencia de la actividad enzimática. Las principales diferencias en la velocidad de la demulsificación están relacionadas con las propiedades fisicoquímicas en la interfase, la naturaleza del enlace éster y el tamaño de gota inicial. El tamaño de la gota determina la superficie total disponible para el reconocimiento enzimático. En consecuencia, las gotas más pequeñas se degradan

más rápido debido al aumento del ratio superficie/volumen. El impedimento estérico del enlace éster presente en el emulsificante también juega un papel fundamental en la velocidad de degradación. Estos estudios se extendieron hacia el uso de una lipasa presente en humanos como es la lipasa pancreática y los resultados obtenidos demostraron que la velocidad de demulsificación puede ajustarse mediante un diseño apropiado de la nanoemulsión.

Por último y con el objetivo de explorar la utilización de compuestos anfifílicos poco convencionales, se evaluó la idoneidad del anión anfifílico conocido como COSAN, cobalto *bis*(dicarballuro), para la estabilización de nanoemulsiones (capítulo 6). La estabilidad de la nanoemulsión fue testeada frente a una amplia gama de estímulos: tiempo, medios fisiológicos relevantes, en presencia o ausencia de luz, a diferentes temperaturas y en presencia de lipasas. Además, se estudió la capacidad de las nanoemulsiones resultantes para modular la biodistribución de fármacos hidrófobos. Las técnicas de imagen nuclear sirvieron de nuevo para comprobar las diferencias obtenidas en las biodistribuciones del fármaco libre o encapsulado tras la administración pulmonar. Los resultados obtenidos anteriormente con FES, fármaco hidrofóbico modelo, nos animaron a repetir los estudios utilizando nanoemulsiones basadas en COSAN. Los estudios de imagen PET *in vivo* realizados con *[^{18}F]FES* y *NE-[^{18}F]FES* demostraron que las nanoemulsiones son capaces de prolongar el tiempo de residencia en los pulmones del FES después de la aerosolización intratraqueal (**Figura 3**). Para obtener más información sobre el *nanocarrier*, también se estudió la biodistribución del estabilizante, es decir, el COSAN. En un primer paso, el COSAN fue funcionalizado con yodo y posteriormente se realizó el marcaje radiactivo con el emisor de positrones ^{124}I (vida media de 4.2 días) mediante intercambio isotópico. Posteriormente se generó la nanoemulsión ([^{124}I]NE-FES). Los resultados de imagen demostraron que el emulsificante se elimina rápidamente de los pulmones después de la liberación del fármaco (**Figura 3c**). El conjunto de los resultados posiciona a las nanoemulsiones estabilizadas con COSAN como potenciales agentes de liberación controlada de fármacos poco solubles en agua cuando el pulmón es el órgano a tratar.

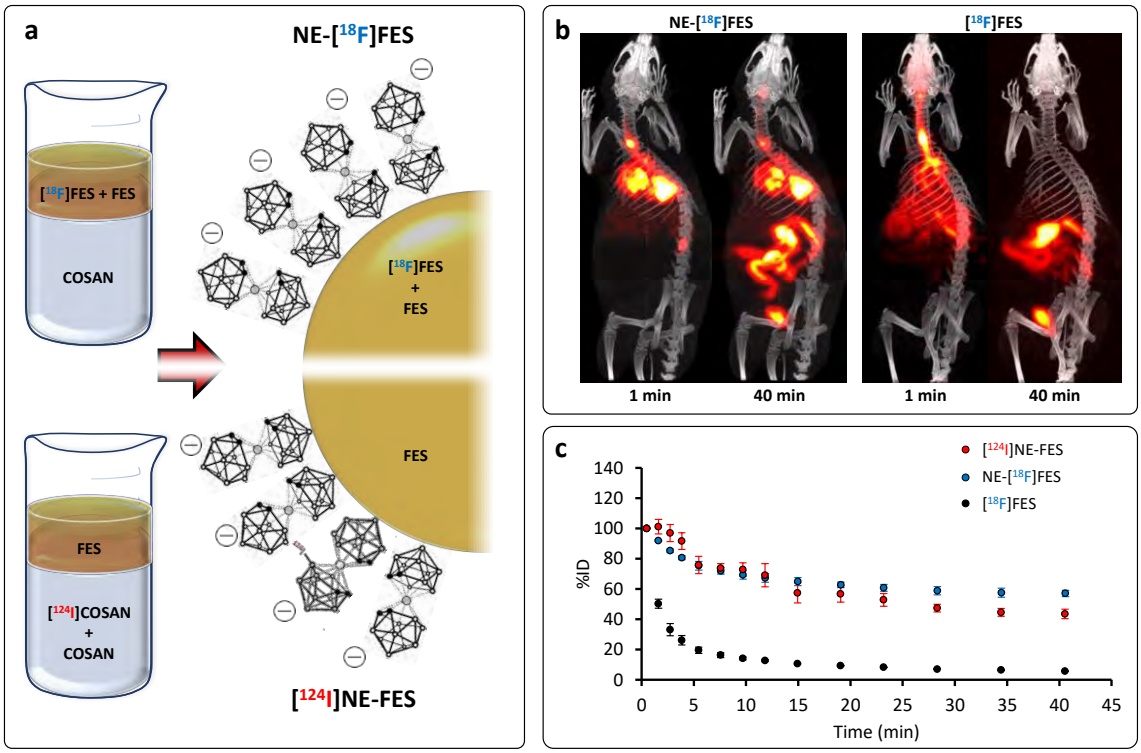


Figura 3. *a) Representación esquemática de la preparación de NE-[^{18}F]FES y [^{124}I]NE-FES; (b) imágenes de PET-CT representativas de la biodistribución de la radiactividad en diferentes puntos temporales tras la administración intratraqueal de NE-[^{18}F]FES (izquierda) y [^{18}F]FES (derecha). Las proyecciones de máxima intensidad de las imágenes PET se han corrregistrado con las imágenes CT; c) Curvas de la radioactividad en pulmón frente al tiempo después de la administración intratraqueal de [^{124}I]NE-FES, NE-[^{18}F]FES y [^{18}F]FES. Los resultados se expresan en porcentaje de la dosis inyectada (% DI). Los valores mostrados corresponden a la media ± desviación estándar, n = 2 por compuesto.*

Chapter 1. General introduction

1.1. Nanoparticles and nanomedicine

1.1.1. Nanoparticle: definition and applications

Nanoparticles (NPs) are generally defined as particles between 1 and 100 nanometres (nm) in size, in at least one dimension.[1] Due to their high surface-to-volume ratio, nanoparticles (NPs) have physico-chemical properties that differ from their respective bulk materials, turning them into very interesting materials in different market sectors such as energy, electronics, food, agriculture or health.[1,2] In addition, the properties of NPs can be tuned by functionalization of their surface, making them stable in different media and enabling the attachment of bioactive molecules which can develop a specific role for the final application. For these reasons, NPs have emerged as excellent candidates for their use in medicine.

1.1.2. Nanoparticles in medicine

The use of NPs for biomedical applications has been widely studied during the last decades. In medicine, NPs constitute a very promising research field from an academic point of view in areas such as the development of medical devices, molecular imaging or drug delivery, among others.[2] The unique properties of NPs, together with their size, turn NPs into genuine materials having a three-dimensional structure that confers certain advantages over small-molecule therapeutic or diagnostic agents.[3] These facts have resulted in the emergence of a new research field, nanomedicine, in which nanomaterials are designed to solve medical problems. Nanomedicine, as defined by the European Commission, includes different application areas: Drug delivery, drugs and therapies, *in vivo* imaging, *in vitro* diagnostics, biomaterials, and active implants.[4] The field has evolved creating nanomaterials to help medical doctors with early detection and prevention, improved diagnosis, personalized medicine and the follow-up of diseases.[5,6]

Nanomedicine research is not only focused on the academic area and has been also translated into industry. A recent study carried out by the European Commission

identified around 200 companies with nanomedicine activities, including 92 start-ups (44%), 67 small and medium enterprises (SMEs; 32%) and 41 large pharmaceutical or medical device companies (21%) (**Figure 1**). A detailed data analysis revealed that the relative proportion of corporations, SMEs and start-ups are similar in the US and the EU.[4] Drug delivery is the field of nanomedicine with higher development; 76% of the publications and 59% of the patents are related to drug development. In second position, and contributing only with 11% of the publications and 14% of the patents, the field of *in vitro* diagnosis has significantly lower economic impact than drug delivery.[4,7]

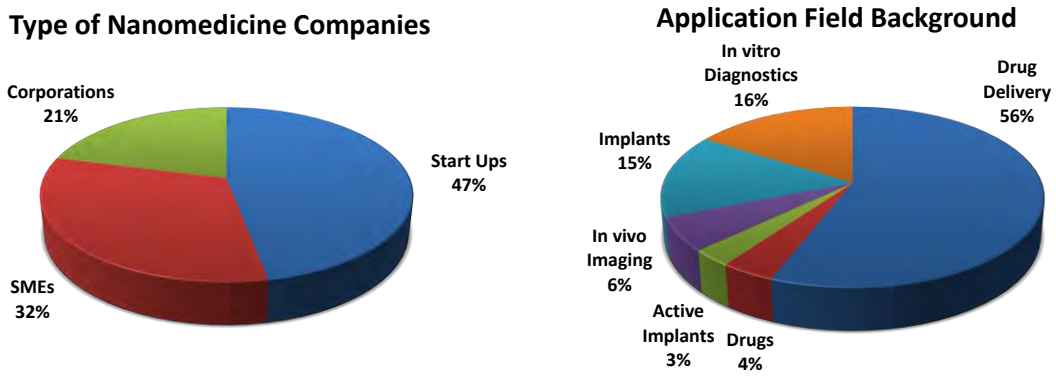


Figure 1. *Nanomedicine Sector: Types of companies and application field breakdown. Figure adapted from a European Commission report.*[4]

Advanced drug delivery systems can improve the pharmacological properties of conventional "free" drugs. This is because they are designed to modify the biodistribution and pharmacokinetics of their associated drugs. An ideal carrier should increase the bioavailability of the drug in the target organ while decreasing toxic and undesired side effects in the rest of the organs. Proper size, shape and surface functionality of the carrier contribute to prolong the biological half-live of the drug and may help in achieving the optimal dose in the targeted diseased tissue while minimizing off-target side effects.[8]

A wide variety of drug delivery systems with sizes below 1 micrometer have been reported as nanocarriers.[9–11] These systems can be classified depending on the final application, type of material or the type of drug that they carry. The active ingredients

can be either attached or adsorbed onto inorganic particle surfaces or dissolved, encapsulated and/or entrapped inside soft matter systems.[12] Drug-encapsulated liposomes and polymer–drug conjugates such as polyethyleneglycol-functionalized (PEGylated) drugs are predominant in clinical trials. Besides liposomes and polymeric conjugates, other nanocarriers such as polymeric nanoparticles, micelles, nanoemulsions, nanoshells, dendrimers, engineered viral nanoparticles, albumin-based nanoparticles, polysaccharide-based nanoparticles, metallic nanoparticles, and ceramic nanoparticles have been investigated.[13–15] A few examples of these drug delivery systems are represented in **Figure 2**.

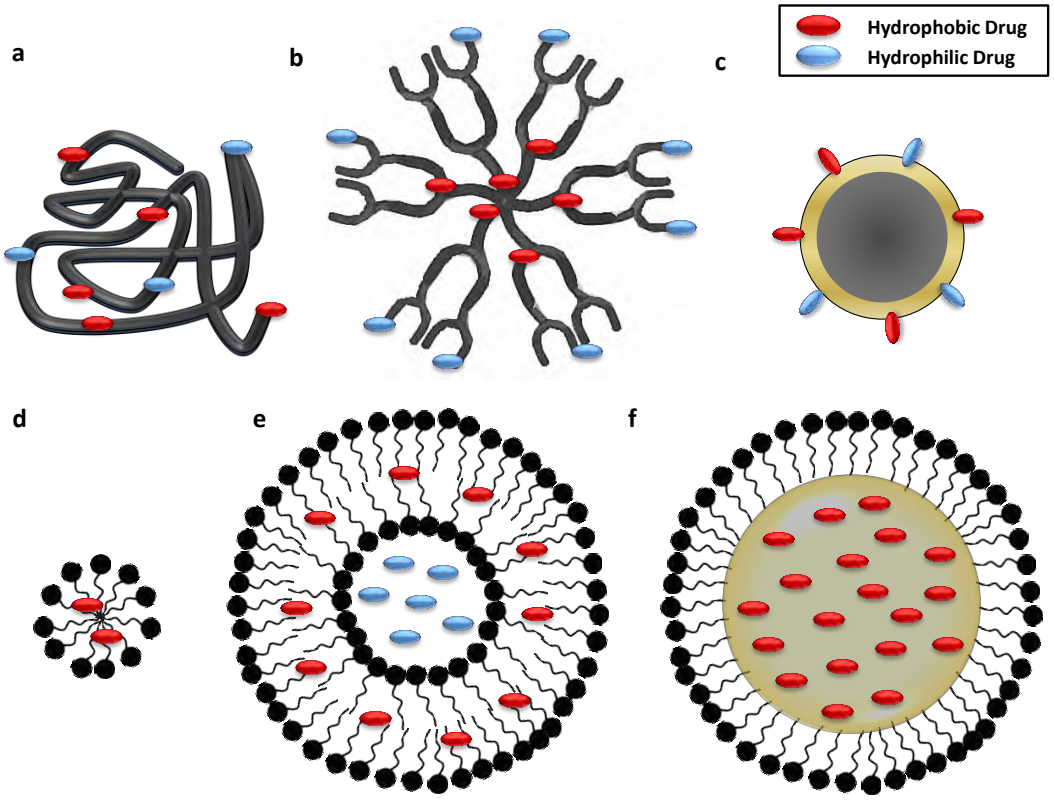


Figure 2. *Schematic illustration of therapeutic nanoparticle platforms in preclinical development: (a) polymer-drug conjugate, (b) dendrimer, (c) inorganic nanoparticles, (d) Micelle, (e) liposome and (f) nanoemulsion. Red dots represent hydrophobic drugs and blue dots represent hydrophoilic drugs.*

1.1.3. Nanocarriers for poorly water-soluble drugs

As mentioned above, NP formulations are being deeply investigated as drug delivery systems. This is especially relevant for poorly water-soluble drugs, for which direct administration has difficulties. The use of nanoformulations is advantageous due to three main reasons: (1) their size, which facilitates the accumulation in the target organ or tissue, e.g. preferential accumulation in the tumor tissue due to the well-known enhanced permeability and retention (EPR) effect;[16] (2) their high surface-to-volume ratio, which can confer genuine physical properties and enables multi-functionalization;[3] and (3) the possibility of loading a high amount of cargo while increasing its bioavailability, which turns NPs into ideal carriers for hydrophobic drugs.[17] Indeed, the presence of poorly water-soluble drugs has increased the pipeline of the drug discovery process.[18] According to US pharmacopeia, more than 40% of the drugs in the market are poorly water-soluble, and it is estimated that 60% of the drugs currently under development in pharmaceutical chemical laboratories are insoluble or poorly soluble in water.[18–20] Many of these, classified by the Biopharmaceutical Classification System (BCS) as BCS Class II-IV drugs (**Figure 3** [21]) have been successfully tested *in vitro*; however, they finally fail *in vivo* due to their poor solubility in blood (92% wt. content of water). To overcome this problem, different solubilization strategies have been used in pharmaceutical industry including prodrug formation, complexation and use of co-solvents and/or surfactants which usually generate undesirable side effects. For this reason, the use of nanodelivery systems (nanocarriers) is currently drawing attention.

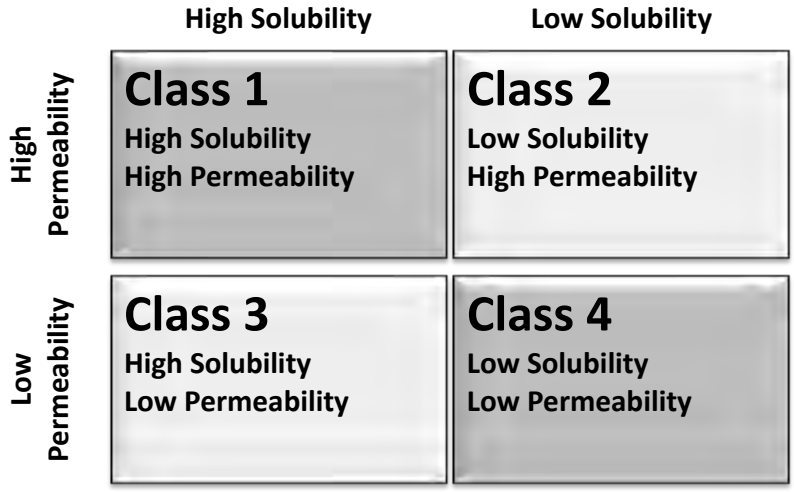


Figure 3. *Biopharmaceutics classification system of drugs.*[21]

In this PhD thesis, we have focused in the use of nanocarriers to improve the bioavailability of drugs classified as BCS Class II, which are highly permeable, and hence its bioavailability can be significantly enhanced by optimizing the formulation design to mitigate low water solubility. It is important to mention that BCS classification is mainly related to orally administered drugs but, in the absence of alternatives, it would be a good approach for inhalation. One strategy to achieve this is by using NPs. Historically; lipid-based nanocarriers have been mostly used for this purpose. Most commonly used lipid components are phospholipids, cholesterol and triglycerides.[22] These materials often derive from natural sources and hence are biodegradable and biocompatible *in vivo*. Thus, the use of physiological lipids as part of the nanocarriers prevents certain negative side effects. Lipid-based delivery systems include liposomes, micelles, solid lipid nanoparticles, nanosuspensions and nanoemulsions.[23,24]

Liposomes are vesicles that contain an aqueous core surrounded by phospholipid bilayers.[23] Vesicles can be mono- or multi-lamellar in a range of sizes between 50–2000 nm. Lipid and phospholipid micelles are nanomaterials (5–50 nm) generally composed by single units containing hydrophobic and hydrophilic regions that self-assemble spontaneously in aqueous media above their critical micelle concentrations (CMCs). In this case lipid monomers are able to form the micelle core while polar groups face outwards to the aqueous media constituting the outer layer of the particle (micelle).

On the other hand, when polar groups are self-oriented inside and outside of the lipidic membrane the final structure relies in the formation of a liposome.[25,26] Solid lipid nanoparticles are colloids that consist of a matrix based on lipids at solid state (50–1000 nm) dispersed in aqueous media. Some of these complexes demand the use of emulsifiers for increasing physical stability of the system.[23] Lipid-based nanosuspensions are submicron colloids (100–500 nm) of hydrophobic drug particles coated by lipid-derived surfactants which prevent self-aggregation.[25] Finally, a nanoemulsion consists in 2 immiscible liquids (typically oil and water) where the lipidic phase is dispersed in the aqueous phase (in the case of oil-in water nanoemulsion) in the form of nanosized droplets around 100-500 nm. Surfactants and/or co-surfactants are adsorbed at the oil/water interface in order to stabilize the system. Nanoemulsions are thermodynamically unstable but kinetically stable, and hence these nanosystems are suitable for drug delivery purposes.[25] This PhD thesis is based on the use of nanoemulsions as nanocarriers for poorly-water soluble drugs.

1.1.4. Nanoemulsions in nanomedicine

As mentioned above, lipid-based formulations have been widely used to improve the bioavailability of poorly water-soluble drugs,[24] and a wide range of lipid-based materials to generate such lipid-based formulations have been developed. Also, one other nanosystem widely investigated is emulsion based, which according to the IUPAC, are ***"fluid systems in which liquid droplets are dispersed in a liquid"***.[27] Liquids tend to minimize their surface area generating the geometrical form with the smallest surface/volume ratio. Therefore, a typical emulsion consists in two immiscible liquids (usually oil and water) where one phase is dispersed as small spherical droplets within the other.[12,24,28–30] In addition to the oil and water phases, stabilizers are needed in order to maintain the properties of the emulsion. Most common stabilizers are surfactants, which are surface-active substances that reduce the interfacial tension between the two immiscible liquids. Other stabilizers alter the properties of the continuous phase thus retarding emulsion breakdown processes. One example is emulsifiers, which are absorbed at the interface of the emulsion droplet forming a protective coating that prevents droplet aggregation and facilitates the disruption of

the initial emulsion droplets during homogenization, generating smaller droplets.[29] However, an energy input is required for the adsorption of the emulsifier at the oil-water interface. This is because the interfacial area between the two phases is enlarged during the homogenization of the bulk materials, resulting in an increment of the interfacial free energy of the system.[31]

Generally speaking, **surfactants comprise the simultaneous presence of hydrophilic and hydrophobic** parts in the same molecule. These molecules are defined as amphiphiles but these characteristics are not the only requirement to be surface active. Surfactants also need an adequate three-dimensional structure. In aqueous phase, surfactants tend to self-assembly as micelles, after overtaking the critical micelle concentration (CMC), where the hydrophobic parts form the core of the aggregate and the hydrophilic regions are in contact with the surrounding liquid. Other types of aggregates can also be formed, such as spherical or cylindrical micelles or lipid bilayers.[32] Thus, the final shape of the self-assembled structure also depends on the physicochemical properties of the surfactants. In droplet generation, the formation of spheres is resisted by the interfacial tension as determined by the Young-Laplace[33] equation:

Equation 1 $$p_\alpha - p_\beta = \frac{2\gamma}{r}$$

where γ is the surface (air-liquid) or interfacial (liquid-liquid) tension, p_α and p_β are the internal and external pressures of the spherical surface and r is its radius. This equation describes the pressure difference sustained across the interface between two static fluids. The surface tension acts to reduce the surface area and hence the volume of the drop, while the pressure difference (p_α-p_β) acts to increase the volume of the drop. The equilibrium condition is achieved when these two tendencies counterbalance each other. The Young–Laplace equation shows that the pressure difference increases if the radius becomes smaller and tends to infinity when r tends to zero. Following this, an extra energy is required to form smaller (highly curved) droplets for overtaking the large pressure that results from their small radius. For this reason, droplet formation is highly affected by the presence of surfactants because they lower the interfacial

tension and therefore the internal pressure of the droplets. Thus, surfactants act diminishing the stress required to break up a droplet and preventing new droplets from coalescing.[33,34]

Macromolecules containing both hydrophilic and hydrophobic parts are generally referred as polymeric surfactants. Compared to their low-molecular weight analogous, polymeric surfactants are structurally more complex (e.g. number and distribution of hydrophilic and hydrophobic moieties along the chain), which can result in different properties.[5] Usually, homopolymers, either hydrophilic or hydrophobic, have not enough interfacial activity to be adsorbed at the oil/water (o/w)-interface. However, copolymers or grafted polymers which contain both hydrophilic and hydrophobic segments are usually more surface active molecules. The adsorption of the polymer at the air-liquid or liquid-liquid interfaces strongly depends on the chemical segments but also on the structural conformation in the liquid media (see **Figure 4**).[5,31]

Other molecules or even particles without the abovementioned amphiphilic character have enough interfacial activity to stabilize emulsions. Particle stabilized emulsions, also known as Pickering emulsions, have been described for more than a century.[35,36] These solid particles tend to self-assembly at the oil/water interface depending on their wettability, contact angle and the total energy delivered to the system.[37–39] In this work we have tested polymers, polymeric particles and organometallic clusters as emulsion stabilizers for drug delivery purposes.

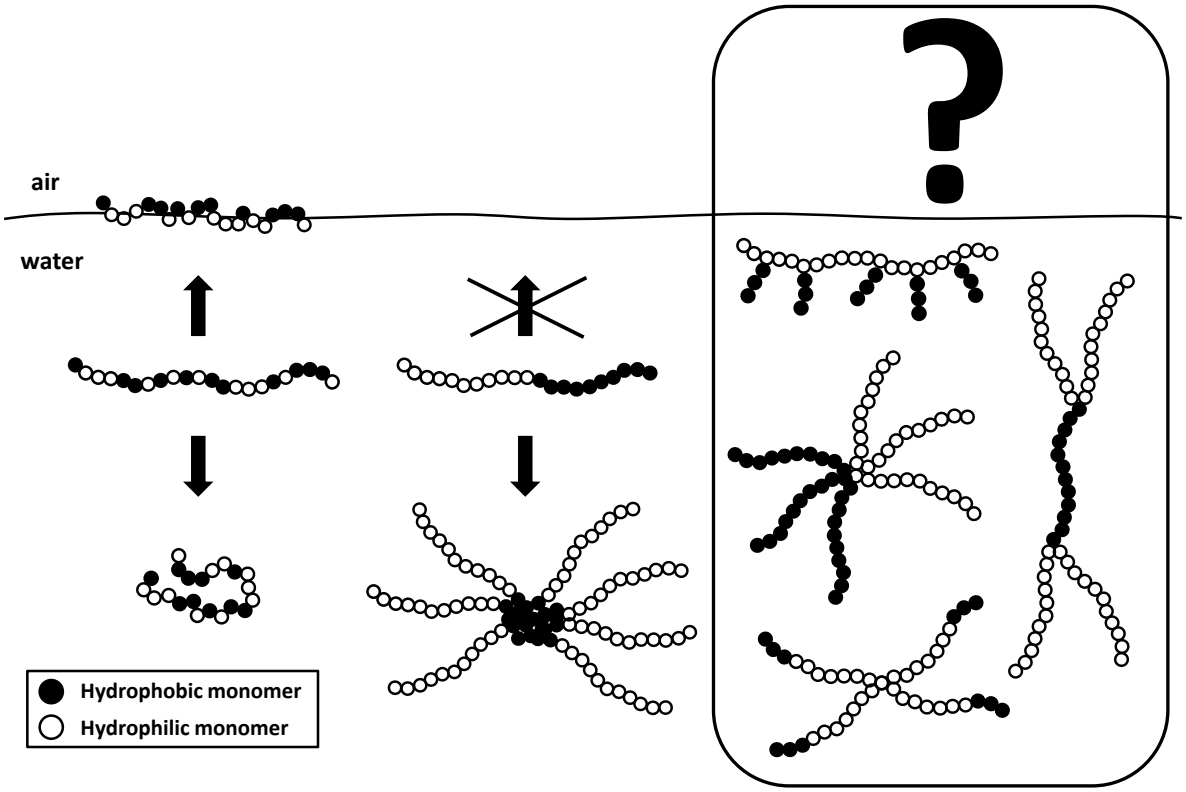


Figure 4. *Schematic representation of the different behavior displayed in solution and at the air/water interface by randomly amphiphilic polymers (left) and macrosurfactants (center). Very few data about the behavior of complex architectures is available (right). Figure adapted from Raffa et al.* [5]

Emulsions are mainly categorized depending on the characteristics of the dispersed phase or their droplet size. Regarding the dispersed phase, there are two principal emulsion types; oil-in-water (O/W) emulsions, where oil droplets are dispersed in an aqueous phase, and water-in-oil (W/O) emulsions if the water droplets are dispersed in an oil phase. On the other hand, emulsions can be classified by their hydrodynamic diameter as macro-emulsions (1-100 µm), mini-emulsions (50-1000 nm) and/or micro-emulsions (1-100 nm). In addition, the term nano-emulsions has been used for emulsions in the range of 100-500 nm despite of its discrepancy with recent definitions of a nanomaterial (one dimension below 100 nm). This terminology has been accepted in literature because previous categorization for micro-emulsions (1-100 nm) was appointed before scientists discover their nanoscale size.[30–32] Macroemulsions are integrated by large droplets and thus they are “unstable”; as a consequence, the dispersed and continuous phases tend to coalescence within time periods from a few

seconds to a few hours leading finally in phase separation. Miniemulsions are meta-stable systems which are thermodynamically unstable but kinetically dependent (stabilized against diffusion degradation) and they are usually stable for at least several days. Microemulsions are isotropic and thermodynamically stable systems where the domains of the dispersed phase are either globular or interconnected.[27] Recent trends suggest that NEs in the range 100-500 nm should be described as miniemulsions.[25,32,40,41]

The term "emulsion stability" is usually referred to the capability of an emulsion to resist changes in its physicochemical properties over time.[31] For this reason, a complete emulsion characterization requires the investigation of its long-term stability under storage conditions. Emulsions for drug delivery use to be thermodynamically unstable but kinetically stable systems and size is the parameter that mainly drives emulsion breakdown processes. Other parameters also related with destabilization are the volume concentration of the dispersed phase, properties and composition of both phases, and interactions among dispersed phase particles or between particles and continuous phase constituents.[29,42] As dispersion quality criteria, the state and properties of the dispersion must be the same throughout the whole material.[42]

Knowledge about the destabilization mechanisms is paramount to achieve a proper characterization of the emulsion and to improve such stability. By identifying the dominant physical and/or chemical destabilization mechanism, effective strategies to improve stability can be implemented. Most typical mechanisms involved are flocculation, coalescence, partial coalescence, gravitational separation (creaming or sedimentation), phase inversion and Ostwald ripening [29] which have been summarized in **Figure 5**.

Flocculation is the process whereby two or more droplets come closer to each other because of attractive interactions and finally form an aggregate, in which the integrity of the individuals is maintained but they move as single entity. In contrast, during coalescence two or more droplets merge to form a single larger droplet. Partial coalescence is usually produced by partly crystalline droplets which form a single

irregularly shaped aggregate due to the penetration of solid crystals from one droplet into a fluid region of another.

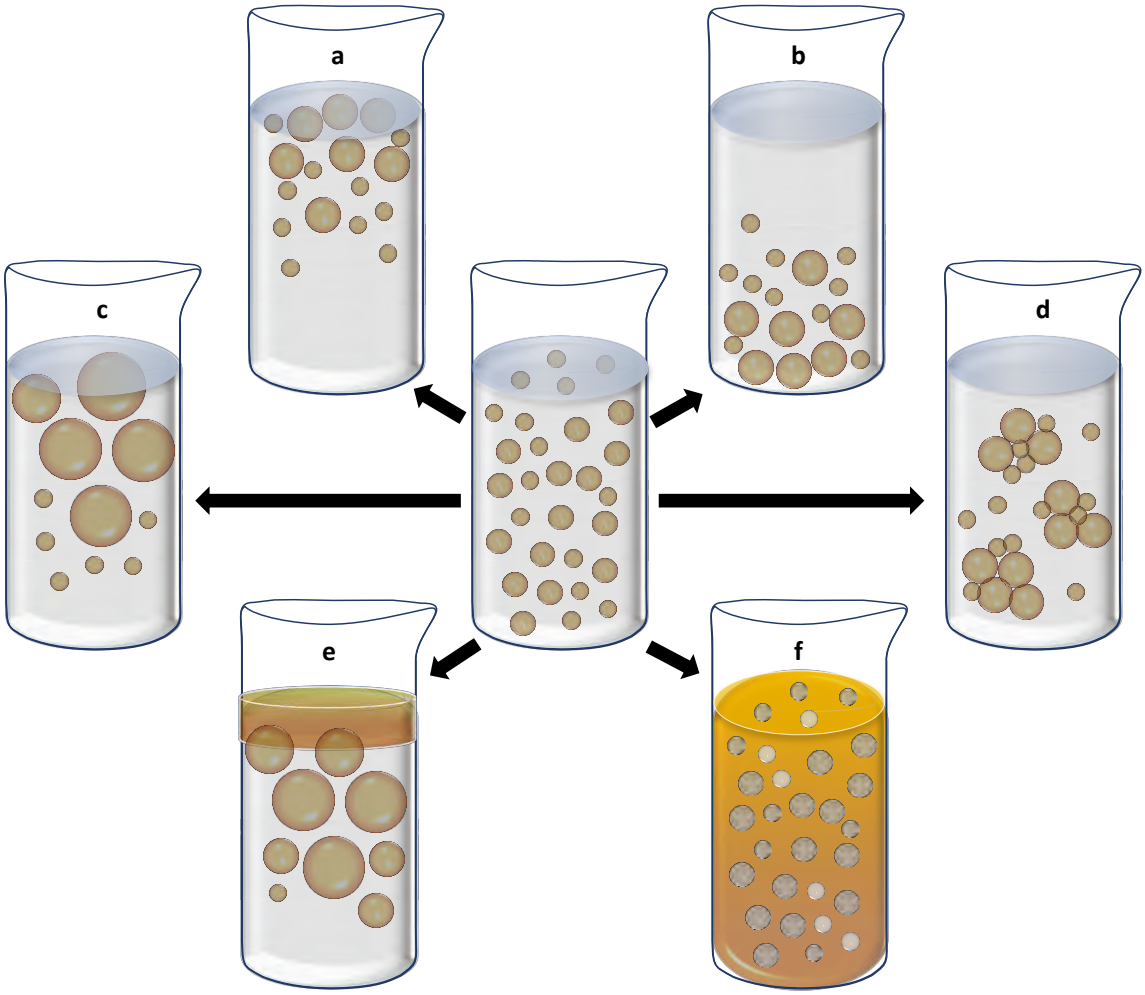


Figure 5. *Different unstabilization pathways due to physical and/or physicochemical phenomena: (a) Creaming, (b) Sedimentation, (c) Ostwald ripening, (d) Flocculation/agglomeration, (e) Coalescence + phase separation and (f) phase inversion.*

Often, the presence of an emulsifier layer at the interface induces repulsive forces due to steric interactions between the droplets which prevent flocculation and coalescence. This phenomenon is stronger in (nano/micro)-emulsions as the thickness of the emulsifier layer (≈ 10 nm) is similar to droplet size.[29,32] Gravitational separation processes in dispersions mainly depend on density differences between dispersed and dispersant phases. If droplets have lower density than the dispersant phase, they move upwards by buoyancy and the process is called “creaming”. On the contrary, if droplets

have higher density than the dispersant phase, they move downwards and the process is called “sedimentation”. Creaming predominates when buoyant force dominates over the Brownian motion, and it is insignificant in miniemulsions with droplet size below a few microns.[32] Phase inversion is produced when an oil-in-water emulsion changes to a water-in-oil emulsion, or vice versa. Ostwald ripening is the process whereby larger droplets grow at the expense of smaller droplets due to mass transfer of dispersed phase material through the continuous phase. There are many factors which can contribute to Ostwald ripening as for example: dispersed phase solubility, polydispersity and ionic strength. Solubility of the dispersed phase in the continuous phase is critical for Ostwald ripening rate, because dispersed phase molecules with high solubility in the continuous phase will lead to faster destabilization. Ostwald ripening is also faster at higher temperatures because both solubility and diffusivity are temperature dependent. Polydispersity affects the Ostwald ripening rate significantly because higher polydispersity represents higher chemical potential differences between droplets. Finally, ionic strength of the continuous phase also promotes Ostwald ripening by reducing the repulsive barrier between droplets. It is important to mention that these instability mechanisms are often interrelated. For example, an increment of the main droplet population size due to flocculation, coalescence or Ostwald ripening usually leads to an increment of the instability of the droplets by gravitational separation. However, if droplets due to gravitational separation or flocculation come closer and stick together for long enough, they become more susceptible to coalescence. Consequently, the instability mechanism responsible for the visible manifestation of emulsion breakdown is not unique and depends of each system.[29,32,42]

At the industrial level, the traditional method employed for emulsion production consists of the homogenization of the bulk materials (oil and water) by applying energy to the system. Usually, this energy is implemented by mechanical agitation or ultrasonication of the liquid mixture.[29,40] Food, pharmaceutical and cosmetic industries have been developing and using emulsions for drug delivery since the last century. This is because the production is simple and robust; furthermore, emulsions have higher

loading capacity than other drug delivery systems such as liposomes and vesicles (only surfactant shell conforms the hydrophobic domain while the inner core is hydrophilic) or micelles (small volume of the hydrophobic inner core).[32,40] Regarding to this, *Rosenblatt et al.* described that the concentration of encapsulated ibuprofen was almost double for emulsions (14 mg/mL) than for other colloidal suspensions (2-8 mg/mL).[43] Generally, mini-emulsions (50-1000 nm) are extensively used because their size makes them kinetically stable over a time period (e.g., a few days, weeks, months or years) so they can suit the shelf-life requirements for pharma industry. Specifically, (o/w)-miniemulsions, which can carry poorly water-soluble drugs, have been employed to increase solubility and bioavailability.[25,32] Furthermore, emulsions are highly tunable systems, and their charge and rheology can be adapted to the requirements dictated by their final application. For medical purposes, (o/w)-emulsions below 1 µm are the most suitable drug delivery systems due to their long-term stability and adequate size. Nanoemulsions have been tested in a wide number of drug delivery applications using different administration routes including topical, dermal, intravenous, intranasal and oral.[44,45] In this work, we describe the use of (o/w)-nanoemulsions (150-400 nm) as hydrophobic drug carriers for *in vivo* applications.

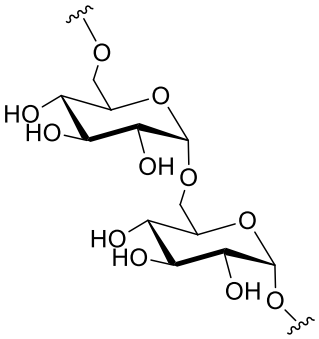
1.1.5. Polysaccharides

Polysaccharides are the most abundant biomacromolecules on the planet.[46,47] They are **biopolymers made of monosaccharides (sugars)** linked together through glycosidic bonds. Polysaccharides have a general formula of $C_x(H_2O)_y$ where x is usually a large number between 200 and 2.500. Considering that the monomer units in the polymer backbone are often six-carbon monosaccharides, the general formula commonly used to represent polysaccharides is $(C_6H_{10}O_5)_n$ where $40 \leq n \leq 3.000$ [48]. Depending on the bond type between sugar monomers, the polymeric structure can give random coil shapes (e.g. dextran), semiflexible chains (e.g. cellulose derivatives), or interrupted helical structures (e.g. amylose) (

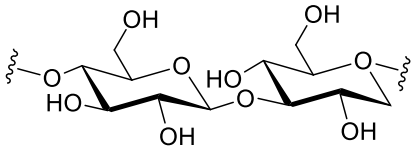
Figure 6).[47,49] Polysaccharides usually have a large number of reactive groups per molecule and they can cover a wide range of molecular weights. Both conditions determine their physicochemical structure and properties making them highly versatile

materials.[50] Due to this diversity, polysaccharides play diverse and important roles in a huge number of biological processes. Most common functions are storing energy (e.g. starch and glycogen) or their use as structural components (e.g. cellulose in plants and chitin in arthropods). However, they are also involved in signal recognition, communication between cells and different functionalities related with the immune system. As they come from biological sources they are highly biocompatible systems. Biocompatibility, together with availability and tuneability turn them into interesting materials for different *in vivo* applications.[46] Polysaccharides can also generate polymeric networks for improving drug bioavailability and targeting, stimuli-responsiveness, enhanced permeability and modulated drug release, among others.[51] Chemical or biochemical modifications have been widely studied in order to produce highly stable, safe, non-toxic, amphiphilic, ionic or non-ionic molecules and also to transform these polymers into more complex structures (e.g. hydrogels). This tuneability has to be aligned with their inherent material properties, biodegradability and/or bioactivity, especially for their use in drug delivery.[46,48,50]

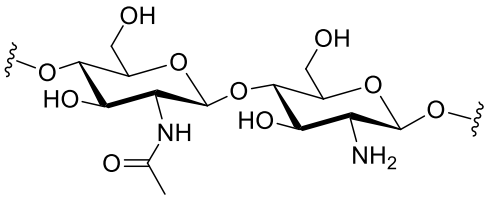
Proteins and polysaccharides are usually applied as emulsifiers/stabilizers in food and pharmaceutical industries. Many proteins can act as emulsifiers as far as they have amphiphilic properties that allow their adsorbance at the (o/w)-interface. However, polysaccharides act as stabilizers by increasing the emulsion viscosity and forming an extended network in the continuous phase, and not acting as surfactants.[52] Pioneering work of Landoll demonstrates that cellulose can also act as surfactant by the appropriate functionalization with alkyl chains.[53] Since this pioneering work, different studies have confirmed that hydrophobic modification of polysaccharides contributes not only to reduce surface and interfacial tension, but also to (o/w)-emulsion stabilization by thickening aqueous phase.



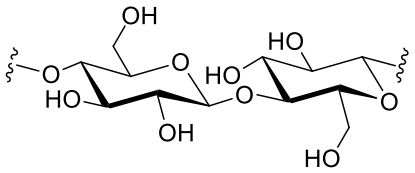
Dextran



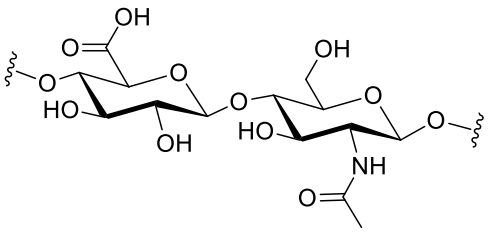
Amylopectin



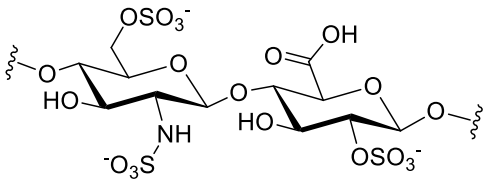
Chitin / Chitosan



Cellulose



Hyaluronic acid



Heparin

Figure 6. *Structures of polysaccharides used in the development of drug-delivery systems and the possible modified site.*

Recently, the **hydrophobic modification of polysaccharides** has gained increasing attention because functionalized polysaccharides show amphiphilic behavior, and hence can be applied as rheology modifiers, emulsion stabilizers, surface modifiers for liposomes and self-assembling units.[31] Intrinsic self-assembly at the aqueous phase (micelle formation) or adsorption at the interface of (o/w)-emulsions induces the

generation of hydrophobic cores surrounded by hydrophilic outer shells. Both conformations allow the incorporation of hydrophobic drugs in the inner core, because of the hydrophobic interactions between both, making them suitable for drug delivery. In this work, we have functionalized polysaccharides, especially dextran, generating sufficient interfacial activity for their adsorption at the oil/water interface stabilizing (o/w)-emulsions and preventing their coalescence.

Dextran (DEX) is a natural polysaccharide formed by the condensation of glucose monomers in a complex branched structure of varying lengths (from 3 to 2.000 kDa). According to the IUPAC dextran is defined as "Branched poly-α-D-glucosides of microbial origin having glycosidic bonds predominantly (1→6)".[54] This composition makes dextran hydrophilic and electrically neutral. These properties, together with its biodegradability and the possibility of functionalization promote its uses in drug delivery. Hydrophobically modified dextran has been previously used in the synthesis of hydrogels or as polymeric surfactant.[55,56] Dextran grafting with phenoxy groups,[57] n-butyl cyanoacrylate,[58] polylactic acid[59,60] and alkyl, dialkyl or bile acids[61] have generated amphiphilic derivatives with adequate properties to stabilize (o/w)-emulsions. For the use of modified dextran as a surfactant, the degree of functionalization, the hydrophobic character of the molecule attached and the chain length affect both, size and polidispersity of (o/w)-emulsions. Generally, long hydrophobic chains and high grafting grades result in more stable and less polydisperse. In this context, few authors have demonstrated that polylactide(PLA)-grafted dextran can stabilize (o/w)-nanoemulsions even with short-length PLA chains[59,60] while Rouzes and coworkers have proved the efficiency of low substituted phenoxy-grafted dextran to stabilize emulsions.[57,62] In our approach, a facile modification of natural dextran with relatively short hydrophobic but reactive functional groups (methacrylate moieties, MA) appeared sufficient to confer interfacial activity to the biocompatible polymer producing stable methacrylate-functionalized dextran (DXT-MA) stabilized emulsions.

1.1.6. Enzyme-responsive materials to trigger drug release

Stimuli-responsive or smart materials are versatile systems with dynamic properties which can be switched on or off depending on the external signals.[63] Main strategies to achieve smart materials are the combination of different material properties as forming composites or generating chemical modifications that change the physico-chemical properties of the original material. Nowadays, they have gained attention in different research areas being their use as drug delivery systems one of the most interested fields of application. This is because drug delivery systems are expected to selectively accumulate in the target organ or tissue and release their cargo in a controlled manner. To that aim, drug delivery systems have been developed to change its properties as response to their local environment (pH, temperature, shear forces, enzymes …) or remotely applied stimuli (magnetic or electric fields, light …).[63–65] Most common stimuli-responsive materials used in drug delivery are based on biodegradable polymers as i.e. polysaccharides. As described above, polysaccharides are natural polymers which have been extensively used in medicine for their biocompatibility and biodegradability. This is because, in most of the cases, natural origin confers a specific biodegradation route which can be used as stimulus to selectively trigger drug release.[66–68] Following this, enzymes have emerged as potential stimulus to trigger drug release. **Enzymes** are biological catalyzers which have been evolved for years to fulfil the selectivity and specificity requirements of living organisms. Thus, enzymes are highly specialized in biochemical reactions performed under mild conditions and in aqueous media.[69] Furthermore, enzymes can be classified depending on their specific substrate (e.g. lipase hydrolyzes lipids generating fatty acids and glycerol) or englobing a family of reactions (e.g. esterases hydrolyze ester bonds).[70] Overexpression of certain enzymes is generally related to physiological disorders.[71] When such overexpression occurs in the target organ, this stimulus can be used to trigger drug release by using enzyme-responsive materials. In this process, the interaction between the enzyme and the substrate often results in degradation of the carrier (triggering drug release), and this interaction can be some extent controlled by the modification of either involved in the catalytic cycle.

Lipases are carboxyl-esterases naturally designed for lipid digestion. Generally speaking, lipid digestion consists in the cleavage of long-chain acylglycerides (mono-, di-, and triglycerides) into polar lipids (fatty acids). Triglycerides can be cleaved at all three ester bonds or specifically at only one or two positions. Moreover, lipases show very different specificities depending on the lengths of the fatty acids. Opposite polarity between the enzyme (hydrophilic) and substrates (hydrophobic) demands reaction occurs at the oil-water interface.[72–75] Lipases, on the contrary to other esterases which primarily hydrolyze water-soluble esters, demand the presence of (o/w)-emulsions to be activated in a phenomenon called "interfacial activation". A close contact between the enzyme and the hydrophobic interface lead in the movement of the lid domain that is covering the active site. This enzymatic recognition is paramount to leave the active site available for substrate binding and processing.[74] In the first step the water-soluble enzyme is adsorbed at the interface leading to a more favorable state of energy (E*) (**Figure 7**). Secondly, active site of the adsorbed enzyme binds the substrate molecule (S) achieving the complex enzyme-substrate (E*S).

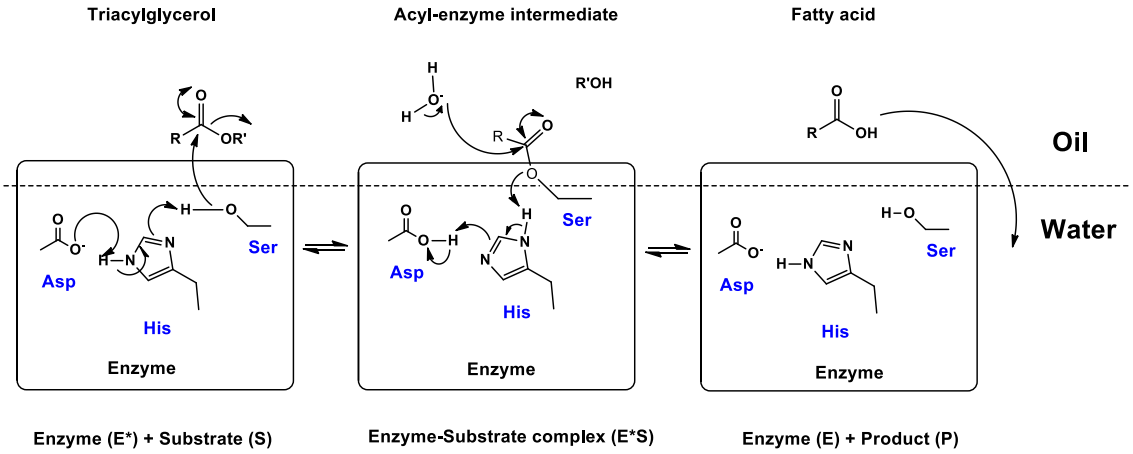


Figure 7. *Mechanism of lipase catalysis adapted from De Simone.[76] The active site of lipase is formed by catalytic triad of aspartic acid, histidine and serine. First, His deprotonates the OH group of Ser. Then, Ser attacks the carboxyl group of the triacylglycerol and an acyl-enzyme intermediate is formed (center). Diacylglycerol (R'OH) is released during this step. Subsequently, a deprotonated water molecule acts as nucleophile, adding to the acyl group. Finally, a free fatty is released and the catalytic residues are regenerated.*

Then, lipase catalytic triad (composed of serine, histidine and aspartate or glutamate) facilitates ester hydrolysis following the mechanism described in **Figure 7**. Finally, ester hydrolysis generates the product (P*) that is solubilized and released into the aqueous phase (P).[76]

In this PhD thesis two different lipases have been employed, Candida Antarctica Lipase B (CALB) and Pancreatic Lipase (PL), as both enabled ester hydrolysis of (o/w)-emulsions. Thus, a brief description of the characteristics of each lipase is included below. **Candida Antarctica Lipase B** is a monomeric protein composed by 317 amino acids with a molecular weight of 33 kDa. CALB structure appears to be in an "open" conformation with a rather restricted entrance to the active site. This is because, unlike most lipases, CALB has no lid to cover the entrance to the active site and thus do not show interfacial activation. The relatively low activity of the enzyme on large triglyceride substrates together with the absence of lid suggests that CALB may be an intermediate between an esterase and a true, interfacially activated lipase.[77,78] **Pancreatic Lipase** is a single-chain glycoprotein composed by 449 amino acids with a molecular weight of 51 kDa. As most of lipases, PL has the hydrolytic site covered by a lid which loop (opening/closing motion) in water is closed being inaccessible to the solvent. This loop is opened upon interfacial adsorption leading in enzyme activation. Furthermore, this enzyme is located in the duodenum of mammals where various amphiphiles (bile salts, soaps, phospholipids, etc.) are hindering interfacial activation and thus demanding the use of cofactors (colipases) to promote *in vivo* digestion.[75,79,80]

1.1.7. Nanoparticles in pulmonary delivery

Pulmonary delivery is an attractive administration route for either local or systemic therapies.[81,82] Lung administration for systemic delivery has many advantages in comparison with other noninvasive routes as oral. The low thickness of the epithelium (0.1–0.5 µm in alveolar region) and its huge surface area (75–150 m^2) lead to a faster onset of therapeutic action while avoiding the first-pass metabolic barriers.[82,83] Furthermore, **pulmonary delivery is the main administration route when the lung is**

the target organ, as local administration of therapeutics reduce the overall dose and negative side effects by decreasing systemic drug exposure.[84]

After direct administration, lung clearance can take place following different mechanisms such as mucociliar clearance, phagocytosis by macrophages, intracellular catabolism or permeation through the alveolar epithelium, among others.[82] One of the main challenges of topic treatments in lungs by inhalation is to avoid or reduce the extremely fast clearance in the alveoli region. In this context, NPs have emerged as attractive tools because they can be retained within the lungs, thus prolonging the residence time of the therapeutic agent and providing controlled drug release. In parallel, they can aid in administering poorly water-soluble drugs. Thus, there are many studies involving NPs and focused on pulmonary delivery for the treatment of respiratory diseases such as asthma, interstitial lung diseases (e.g. cystic fibrosis), acute lung injury (ALI), acute respiratory distress syndrome (ARDS) or chronic obstructive pulmonary disease (COPD).[83–85] Therefore, the nebulization of submicron (o/w)-emulsions is becoming an upcoming research area. However, successful formulations of inhalable NEs have to be studied deeply in order to understand how particles are deposit along the respiratory tract and overcoming possible adverse effects of surfactants and oils on lung alveoli function (adverse interactions with lung surfactant).[83,84,86,87]

1.2. Tracing NPs *in vivo*: Nuclear Imaging Techniques

1.2.1. Why we need to track NPs?

Nanoformulations for *in vivo* applications have to fulfill strict requirements to reach the market, as any other drug. NPs for drug delivery need to be biocompatible and accumulate a sufficient amount of drug in the target organ or tissue while minimizing accumulation in off-target organs to minimize adverse effects. Furthermore, they should be cleared from the body to enable subsequent treatments to the patient. Because of this, a complete understanding of the pharmacokinetics and biological fate of the nanocarrier and the drug is extremely important in order to ensure the safety

and efficacy of the treatment and to select the therapeutic doses. However, nanoformulations are difficult to track once distributed *in vivo*. One strategy to overcome this limitation consists of labeling the different components of the nanoformultaion with radionuclides, which enable the detection and quantification of the different components with high sensitivity and in a non-invasive way by using molecular imaging techniques such as single-photon emission computed tomography (SPECT) and positron emission tomography (PET). The use of these techniques is gaining relevance in the pharmacokinetic evaluation of novel nanosystems.[10,88,89]

1.2.2. Nuclear imaging

Nuclear imaging techniques rely on the administration of a trace amount of the molecule/material to be investigated previously labeled with a radionuclide, i.e. a positron or gamma emitter. Radionuclides spontaneously decay, resulting in the emission of positrons or gamma rays. The annihilation of a positron with an electron occurs rapidly and results in the formation of gamma rays. Hence, both positron and gamma emitters ultimately lead to emission of gamma rays, which have high penetration power and can "escape" from the body and reach the detectors. By detecting such gamma rays, three dimensional, time-resolved images accounting for the concentration of the radioactivity in different regions can be generated. Positron Emission Tomography (PET) and Single Photon Emission Computerised Tomography (SPECT) are the most commonly used nuclear imaging techniques. In the context of this PhD thesis, only PET has been used, and hence a brief introduction on the principles is included in this chapter. For a detailed description of SPECT the reader is referred to *Israel et al.* [90]

1.2.3. Positron Emission Tomography

Positron emitters are radioactive nuclides that spontaneously decay by emitting a positron, the electron antimatter with equivalent mass but positive electrical charge. Positrons interact with the media and progressively lose their kinetic energy; when it is almost at rest, a positron annihilates with an electron, a phenomenon that results in

the emission of two gamma rays traveling in opposite directions with an energy of 511 keV each.[91]

When a positron emitter-labeled molecule is injected into a living subject, millions of annihilations occur within the organism, thus generating millions of gamma-ray pairs. Images are acquired with PET cameras, which consist of an array of detectors arranged in a ring surrounding the organism. Because gamma ray pairs are emitted in opposite directions, the simultaneous detection (within i.e. 1 nanosecond) of two gamma rays by two detectors defines a "line of response", in which the annihilation took place[92] (**Figure 8**). This is known as "electronic" collimation. The detection of hundreds of thousands of gamma ray pairs can be translated into a three-dimensional, time-resolved image that accounts for the regional concentration of radioactivity.

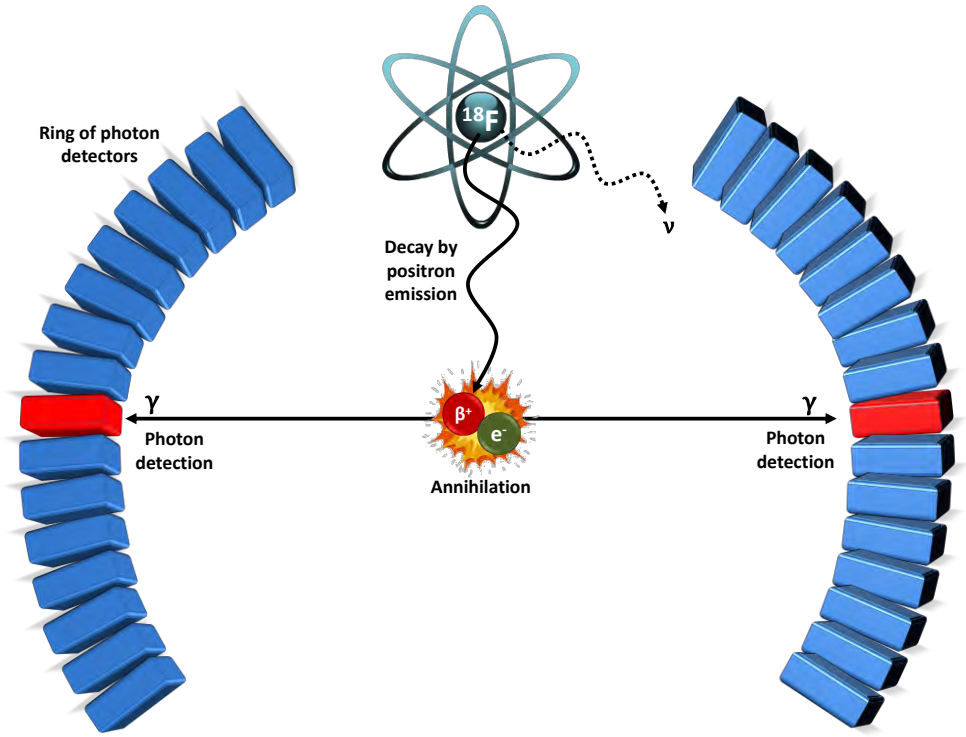


Figure 8. *Schematic representation of positron/electron annihilation and detection in Positron Emission Tomography (PET).*

PET images offer molecular information. In order to unambiguously localize the radioactive signal, PET images are usually corregistered with anatomical images, typically obtained by computerized tomography (CT). Hence, typical practice both in the pre-clinical and clinical fields consists of acquiring consecutive PET and CT images

and analyze them together. The anatomical image can be used to define volumes of interest (VOIs) in different regions. Such VOIs are then translated to the PET images, in order to obtain quantitative information regarding the concentration of radioactivity as a function of time.

One key parameter in PET radionuclides is their **physical half-life**, defined as the time period required for the amount of radioactivity to decrease to one half of the initial value. Half-life values for PET radionuclides typically used in biomedical applications range from few minutes to few days (

Table 1).

Table 1. Typical positron emitters used in nuclear imaging (with half-life and positron energy).

Isotope	Half-Life	β^+ Energy$_{max}$ (β^+ Fraction)*
^{18}F	109.8 min	0.63 MeV (0.97)
^{64}Cu	12.7 h	0.66 MeV (0.18)
^{11}C	20.4 min	0.96 MeV (1.00)
^{13}N	9.97 min	1.20 MeV (1.00)
^{15}O	122 s	1.73 MeV (1.00)
^{68}Ga	67.6 min	1.89 MeV (0.89)
^{124}I	4.18 days	2.14 MeV (0.23)

* The positrons are not emitted with a single energy. The energies of the positrons emitted by a radionuclide follow a Poisson distribution. The maximum value of the distribution is presented in the table.

The selection of the right isotope with adequate half-life is paramount to guarantee successful pharmacokinetic evaluation of new entities. Ideally, the radionuclide should have a physical half-life similar to the biological half-life of the molecule or nanosystem under investigation. If the physical half-life is too short, the radionuclide will completely decay before the compound is eliminated from the organism, and part of the information will be lost. If the half-life is too long, the subject under investigation will be exposed to an unnecessary dose of radiation.

In this PhD thesis ^{18}F, ^{64}Cu and ^{124}I have been employed, as they enabled the radiolabelling and *in vivo* investigations of the nanocarriers and drugs assayed. A brief description of the production and radiochemical applications of these radionuclides is included below.

1.2.4. Properties, production and radiochemistry of the positron emitters ^{18}F, ^{64}Cu and ^{124}I.

Fluorine-18 (^{18}F) is considered as an ideal PET radioisotope. It decays almost quantitatively by positron emission (97%) and it has a low β^+ energy ($E_{\beta max}$ = 0.635 MeV), enabling the acquisition of images with higher resolution than other positron emitters (see

Table 1). Moreover, its half-live of 109.8 min is very convenient for different uses such as drug development or *in vivo* diagnosis. Indeed, ^{18}F is the most commonly used positron emitter world-wide due to its application in the preparation of the glucose analogue 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG, an indirect proliferation marker which is used in the early diagnose and the evaluation of the response to treatment of different types of cancer, inflammatory processes and other diseases.[93]

^{18}F can be generated in biomedical cyclotrons in two chemical forms, i.e. [^{18}F]F^- and [^{18}F]F_2, by using different nuclear reactions. Usually, production of [^{18}F]F^- is achieved by irradiation of ^{18}O-enriched water (95-98%) with protons in an energy range of 8-18 MeV following the nuclear reaction of $^{18}O(p,n)^{18}F$.[94] Once the nuclear reaction is produced, the enriched water containing $^{18}F^-$ is transferred to a shielded hot cell and processed. $^{18}F^-$ is then used to prepare ^{18}F-radiotracers using nucleophilic substitution reactions on a precursor bearing a good leaving group.[95,96] Production of [^{18}F]F_2 is based on a double irradiation approach. In a first step, [^{18}O]O_2 is irradiated with protons to generate ^{18}F, which remains absorbed on the walls of the target chamber. The oxygen gas is then removed from the target by cryogenic trapping, and the target is filled with a Ne/F_2 mixture. A second irradiation induces isotopic exchange reaction with the consequent formation of [^{18}F]F_2,[97] which is then transferred to the hot cell and used for labeling.

Radiolabeling with ^{18}F is generally approached by using two different strategies: "direct" or "indirect" fluorination. Direct fluorination consists of the introduction of ^{18}F, either as $[^{18}F]F^-$ or $[^{18}F]F_2$, in the target molecule in a single step;[95] indirect fluorination consists of radiolabelling a reactive prosthetic group, which is subsequently attached to the target molecule (multistep synthesis).[98,99] The latest is commonly applied to the radiolabelling of biomolecules that can be unstable under the harsh reaction conditions required for direct fluorination. ^{18}F-fluoroalkylation, ^{18}F-fluoroacylation, or ^{18}F-fluoroamidation of primary amino groups or thiol residues or cycloaddition reactions are usually exploited for indirect labeling.

For direct fluorination, the most commonly used reactions are based on the nucleophilic substitution of $[^{18}F]F^-$ on a molecule bearing a good leaving group;[95,96] the most representative example is the production of [^{18}F]FDG, in which $^{18}F^-$ is reacted with 1,3,4,6-tetra-*O*-acetyl-2-*O*-trifluoromethanesulfonyl-beta-D-mannopyranose (mannose triflate) in acetonitrile.[93] In some occasions, when nucleophilic substitution reactions are not feasible, ^{18}F is introduced to the target molecule via electrophilic aromatic substitution reactions using $[^{18}F]F_2$,[94] although this strategy is technically more challenging due to difficulties associated with the generation and manipulation of $[^{18}F]F_2$.

In the current PhD thesis, **direct fluorination of 17β-estradiol was achieved via nucleophilic substitution**. The ^{18}F atom was incorporated after the reaction of $[^{18}F]F^-$ with the precursor 3-methoxymethyl-16β,17β-epiestriol-O-cyclic sulfone (MMSE) followed by acid hydrolysis.[100]

Copper-64 (^{64}Cu) is a radionuclide which exhibits three different decay routes and a relatively long half-life (12.7 hours). It can undergo by electron capture (ε, 43.8%), β^+ emission to ^{64}Ni (17.8%), and β^- emission to ^{64}Zn (38.4%). The half-life and positron-branch turn ^{64}Cu into a suitable candidate for diagnostic imaging, while the β^- and electron capture decay modes are useful for therapy. Copper-64 production can be done by reactor-based or accelerator-based methods. Reactor-based methods consist in the irradiation of the target material with thermal (relatively low energy) or fast

(highly energetic) neutrons. In the first case, the irradiation of stable ^{63}Cu (69.1% natural abundance) with thermal neutrons produces ^{64}Cu with low specific activity *via* the ^{63}Cu(n,γ)^{64}Cu nuclear reaction. Irradiation of ^{64}Zn with high energy neutrons generates ^{64}Cu with high-specific activity *via* the ^{64}Zn(n,p)^{64}Cu nuclear reaction. As an alternative to reactor-based production methods, biomedical cyclotrons can be used to produce ^{64}Cu *via* the ^{64}Ni(p,n)^{64}Cu nuclear reaction. This methodology firstly proposed by Szelecsenyi *et al.*[101] is based on the irradiation of enriched ^{64}Ni, previously electroplated onto a gold disk. Because of the low natural abundance of ^{64}Ni, it has a high cost (a few tens € per mg). Thus, after irradiation, the target material is dissolved in concentrated HCl, and the resulting solution is eluted through an anion exchange column with different acid concentrations, allowing the separation of ^{64}Cu from the nickel fraction which can be reused.[102]

^{64}Cu has a suitable half-life for tracking both small and large molecules. This advantage, together with its well-known coordination chemistry and redox behaviour, dominated by oxidation states I and II, make ^{64}Cu very attractive in comparison with other metal radioisotopes, especially for the radiolabelling of NPs, which can be achieved via one of the following routes: (i) incorporation of a chelator in the nanoparticle and subsequent radiolabeling by formation of a radiometal-chelator complex;[103–105] (ii) formation of a pre-labeled chelator bearing a reactive residue and subsequent attachment to the NP;[106] and (iii) direct incorporation of the radionuclide within the NP,[107] typically within the crystal lattice of inorganic NPs.

The use of chelators finds its main application in the radiolabelling of soft-matter NPs, and to date a wide variety of chelating agents bearing a reactive moiety (so called bifunctional chelators, BFCs) have been developed and are currently commercially available.[108] The choice of the chelator is critical for *in vivo* applications, as dissociation of the chelator-radiometal complex could lead to misinterpretation of the imaging results. Thus, stability studies to guarantee that the labelled NP remains intact over the duration of the study need to be performed. Due to its relatively large cavity and chelating properties, 1,4,7,10-tetraazacyclododecane-tetraacetic acid (DOTA) and its

derivatives (**Figure 9**) are widely used for medical diagnostics and in the preclinical setting.[109]

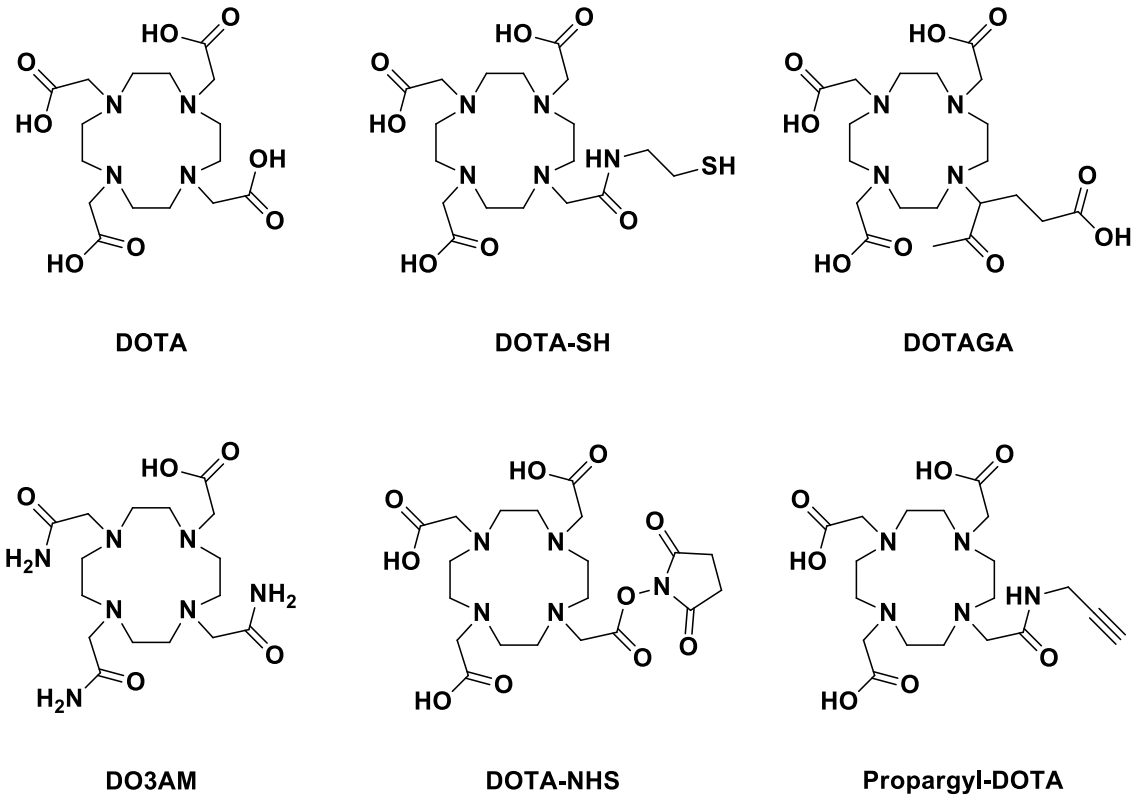


Figure 9. *DOTA and commercially available derivatives of DOTA functionalized for use as bifunctional chelating agents.*

In this PhD thesis, ^{64}Cu has been used to radiolabel the polymers employed to stabilize the emulsions, and this has been achieved by using bifunctional chelators. Due to the presence of double bonds in DXT-MA, which are susceptible to react with thiol groups via thio-Michael addition, we used a pre-labeled thiolated DOTA (DOTA-SH) as bifunctional chelator (**Figure 9**).

Iodine has mainly four radioisotopes with interest in the field of *in vivo* imaging, namely: ^{123}I, ^{124}I, ^{125}I and ^{131}I. For PET imaging **^{124}I**, which is a positron emitter, has become a very useful labelling tool due to its long half-life (4.17 days). Its decay route entails the emission of high energy γ-rays (0.603 MeV, 63.0% abundance) and high energy positrons ($E_{\beta max}$ = 2.14 MeV, 23% abundance). Due to its relatively long half-life, Iodine-124 can be obtained from commercial suppliers. The production is carried out

in solid targets *via* the ^{124}Te(d,2n)^{124}I or the ^{124}Te(p,n)^{124}I nuclear reactions.[110] The target material consist of tellurium or tellurium oxide which is irradiated and thereupon recovered by heating at 750^{o}C (dry distillation) or by dissolution in an oxidizing alkaline medium with the subsequent chemical reduction to I^- state and the final purification by solid phase extraction.[111] Despite the methodology for the production of this isotope looks simple, different parameters must be optimized and, as a consequence, the cost remains relatively high.

Labeling with radioiodine has been widely described in the literature.[111–113] The main radiolabelling routes (see **Figure 10** for scheme) are: (i) in situ oxidation of the anionic species (I^-) and subsequent aromatic electrophilic substitution (SEAr) in an activated aromatic ring;[113] (ii) indirect methods based on pre-labeling using a prosthetic group and further functionalization via covalent bond with the target molecule;[112] and (iii) catalyst-assisted isotopic exchange, only applicable when an iodine atom is already present in the target molecule.[114] Methods (i) and (ii) usually result in high yields, and have been widely applied to the radiolabelling of proteins and peptides, among others. Isotopic exchange often leads to low specific activity values of the final radiotracer and is less often employed.

In this PhD thesis, ^{124}I has been used for the radiolabeling of the cobalt *bis*(dicarbollide) anion ([3,3'-Co($C_2B_9H_{11}$)$_2$]$^-$ (COSAN), a metallocarborane compound consisting of a central cobalt (Co^{3+}) atom sandwiched between two η^5-bonding $[C_2B_9H_{11}]^{2-}$ moieties which has been used as stabilizer of (o/w)-emulsions. The labeling was carried out by isotopic exchange.

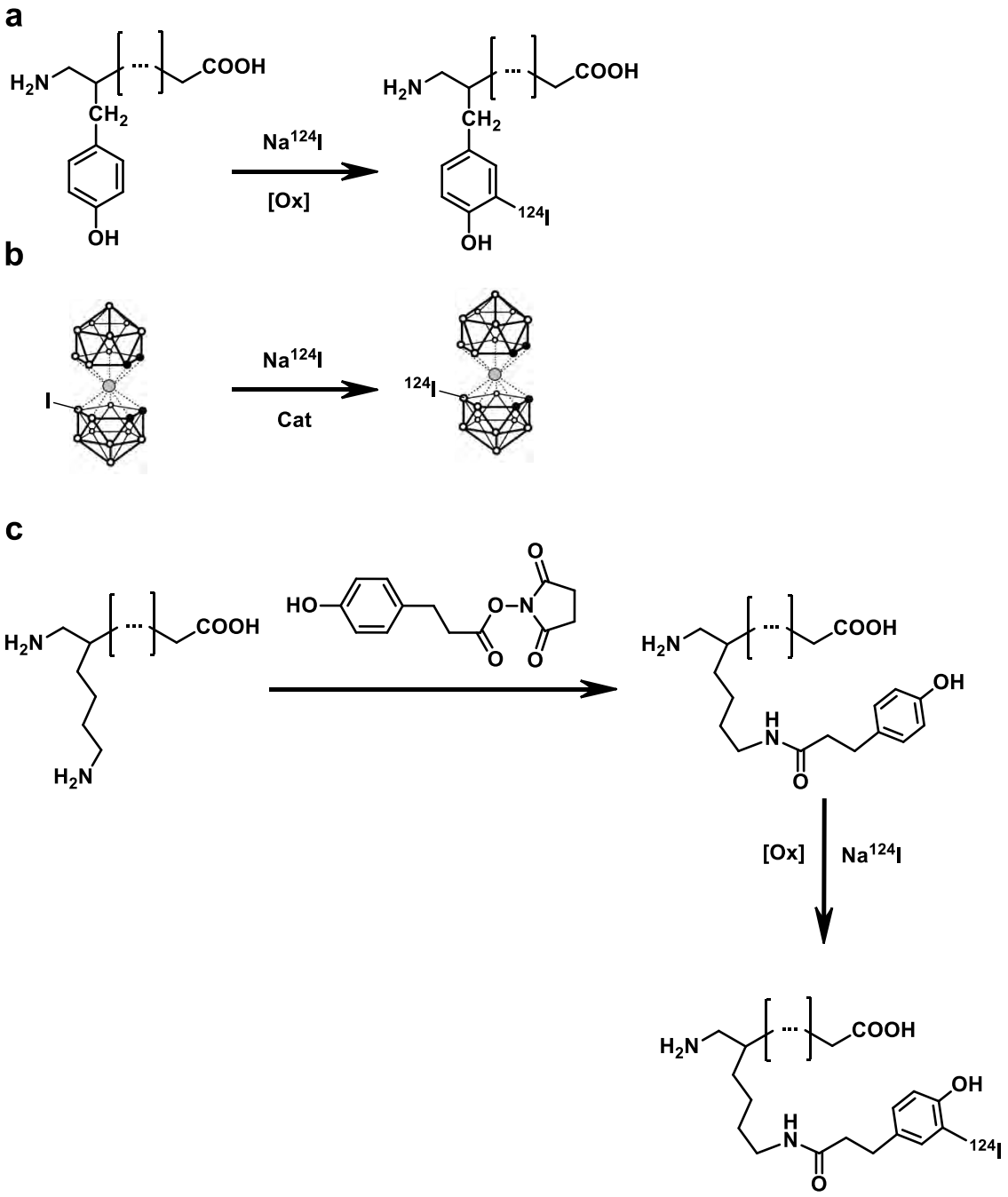


Figure 10. *Schematic representation of the main strategies used for the radioiodination of different molecules; (a) electrophilic substitution, (b) isotopic exchange and (c) pre-labeling using a prosthetic group. Examples are shown with ^{124}I; these strategies can be extended to other iodine radioisotopes.*

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Chapter 2. Motivation, Hypothesis and Objectives of the Thesis

2.1. Motivation and Hypothesis

The work carried out in this PhD thesis has been co-supervised by Dr. Iraida Loinaz (Director of the Nanomedicine Institute, CIDETEC) and Dr. Jordi Llop (Principal Investigator of the Radiochemistry and Nuclear Imaging group, CIC biomaGUNE). In a previous collaborative effort in the context of an EU funded project, both groups worked on the development of polymeric nanoparticle-based formulations for direct pulmonary administration of new antibiotics to treat antibiotic-resistant bacterial infections. This previous work, which was conducted in a cross-sectorial and international effort and in tight collaboration with European experts in areas such as lung infection, pulmonary delivery and nanotechnology, brought to the groups a strong expertise and tracked record in the use of nanovehicles for direct pulmonary administration of drugs and the use of imaging modalities for their investigation *in vivo*.[1,2] Both groups explored the use of single-chain polymer nanoparticles based on dextran for lung delivery. This strategy was proven ideal for lung-delivery of hydrophilic peptides.[3,4] However, it was not adequate for the delivery of poorly water-soluble drugs where the loading was reduced and hardly reproducible. In order to force the loading and control drug release, other groups employed hydrophobic backbone to force drug loading through non covalent hydrophobic interactions with a maximum loading of 16%.[5]

The use of dextran modified with hydrophobic motives was previously described to reduce interfacial tension and to generate nanoemulsions.[6–8] Furthermore, hydrophilic particles were reported as Pickering stabilizers for (o/w)-emulsions being those hydrophobic useful for (w/o)-emulsions.[9]

With the aim of solving this limitation of dextran based single chain polymer nanoparticles, in this PhD thesis, we proposed to explore the suitability of polymers and polymeric NPs to stabilize oil-in-water (o/w)-nanoemulsions as potential drug carriers for enhanced transport and delivery of poor water soluble drugs.

We hypothesized that nanoemulsions could be stabilized by dextran polymers and dextran-based NPs in order to obtain monodisperse droplets below 500 nm, suitable for nebulization. We envisaged that introduction of hydrophobic modifications on dextran (hydrophilic polymer) could decrease the interfacial tension between oil and water[8] when compared to the hydrophilically-modified homologous polymers. Altogether, encourage us to study the use of functionalized dextran-based polymers and NPs for (o/w)-emulsion stabilization.[10] Furthermore, we also hypothesized that long-term stability in different media could be improved by using cross-linking strategies at the oil/water interface,[11] as this could avoid adsorption/desorption equilibrium and slow down phase separation.[12] Moving forward towards potential controlled drug delivery, we hypothesized that esterase enzymes may trigger nanoemulsion destabilization due to the presence of ester bonds in both surfactant and oil phase.[13] Finally, and with the aim of exploring less conventional amphyphiles as emulsion stabilizers, we envisioned the formation of nanoemulsions using the anionic complex cobalt bis(dicarbollide) as the stabiliser.[14,15] Our final hypothesis was that the newly generated (o/w)-nanoemulsions should be able to modulate the biodistribution of entrapped hydrophobic drugs after pulmonary administration in rodents, and that this hypothesis could be confirmed with the aid of *in vivo* nuclear imaging.

2.2. Objectives

The main aim of the PhD thesis was the development, characterization and evaluation of nanoemulsions as drug carriers for pulmonary administration of poor water-soluble drugs. To achieve this ambitious goal and prove our hypotheses, the following objectives were defined:

1. To evaluate the capacity of different dextran derivatives to stabilize (o/w)-emulsions, and optimize experimental conditions to achieve small-sized, monodisperse nanodroplets.
2. To optimize the methodology for polymer cross-linking at the O/W interface to improve nanoemulsion stability in strong ionic media and over time.

3. To evaluate the *in vivo* stability of cross-linked nanoemulsions and their capacity to modulate the biodistribution of entrapped drugs by using *in vivo* imaging techniques
4. To investigate the demulsification mechanism of polymeric nanoparticle-stabilized oil-in-water emulsions in the presence of selected Lipases and to evaluate the effect of composition and crosslinking on the stability.
5. To prepare and characterize COSAN-stabilized (o/w)-emulsions and evaluate the *in vivo* stability and the capacity of the resulting nanoemulsions to modulate the biodistribution of entrapped drugs.

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