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Recent Advances and Prospects in NanoDrug Delivery Systems using Lipopolyoxazolines

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Abstract

Facing the growing demand in nano drug delivery systems (nDDS), hybrid excipients based on natural molecules and well-defined synthetic polymers are intensively investigated. Lipopolyoxazolines (LipoPOx) composed of a polyoxazoline block (POx) and a lipid or lipid-like derivative are detailed in this review. The nature of lipids used, the route to synthesize LipoPOx and their advantages for the formulation of drugs are reported. The place of POx family in nanomedicine is discussed compared to PEG, considered as the gold standard of hydrophilic polymers. LipoPOx nanoformulations including liposomes, mixed micelles, lipid nanocapsules are provided alongside discussion of the nDDS for intravenous or topical administration.

Keywords

drug delivery system, hybrid material, lipid, lipid nanocapsules (LNC), liposomes, micelles, polyoxazoline (POx), LipoPOx.

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Abbreviations

AUC	area under curve
BCS	biopharmaceutics classification system
CHEMS	cholesterol hemisuccinate
Chi-aDDnS	chimeric advanced drug delivery nanosystem
CHMC	cholesteryl methyl carbonate
CL	conventional liposome
CMC	critical micellar concentration
CPE	chemical penetration enhancer
CROP	cationic ring-opening polymerization
DAG	diacylglycerol
DecenOx	2-(dec-9-enyl)-2-oxazoline
DodOx	2-dodecyl-2-oxazoline
DOX	doxorubicin
DSPE	1,2-distearoyl-sn-glycero-3-phosphoethanolamine
EPC	egg phosphatidylcholine
FBS	fetal bovine serum
HLB	hydrophilic lipophilic balance
HSPC	hydrogenated soy phosphatidylcholine
IND	incorporated drug
IV	intravenous
LCST	lower critical solution temperature
LipoPOx	lipopolyoxazoline
LNC	lipid nanocapsules
MAG	monoacylglycerol
MM	mixed-micelles
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MPS	mononuclear phagocyte system
MW	molecular weight
nDDS	nano drug delivery system
NonOx	2-nonyl-2-oxazoline

PcPrOx	poly(2-cyclopropyl-2-oxazoline)
PBS	phosphate buffered saline
PEG	poly(ethylene glycol)
PEI	poly(ethyleneimine)
PEtOx	poly(2-ethyl-2-oxazoline)
PPhOx	poly(2-phenyl-2-oxazoline)
PiPrOx	poly(2-isopropyl-2-oxazoline)
PLA	poly(glutaric acid)
PLL	poly(*L*-lysine)
PLGA	poly(glutaric acid)
PMOx	poly(2-methyl-2-oxazoline)
PnPrOx	poly(2-n-propyl-2-oxazoline)
POx	poly(2-oxazoline)
PPhOx	poly(2-phenyl-2-oxazoline)
PSLs	pH-sensitive liposomes
PVP	poly(vinyl pyrolidone)
RBC	red blood cells
Rc	hydrophobic core
RES	reticuloendothelial system
Rh	hydrodynamic radius
ROS	reactive oxygen species
RNS	reactive nitrogen species
SC	*stratum corneum*
SOPC	1-Stearoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine
SoyOx	soy-based oxazoline
SUV	small unilamellar liposome
S75, S100	Soybean phosphatidylcholine
TAG	triacylglycerol
TEC	thiol-ene coupling
TPOS	D-tocopheryl-Polyoxazoline
T_{cp}	cloud point temperature
UndOx	2-undecyl-2-oxazoline

1.Introduction

Among drug formulations, nano drug delivery system (nDDS) offer many advantages such as (i) high drug loading, (ii) long circulation time in the body, (iii) targeting the pathological site, (iv) release of encapsulated drugs in the pathological site (pH sensitivity, thermal response) and (v) reduction of side effects (Torchilin, 2009). The most common polymers used in nDDS are poly(-*L*-lysine) (PLL), poly(ethyleneimine) (PEI) (Kunath et al., 2003), poly(lactic acid) (PLA), poly(glutaric acid) (PLGA), poly(vinyl pyrrolidone) (PVP) and poly(ethylene glycol) (PEG). PEG is considered as the "gold standard" of hydrophilic polymers in medical applications. Nevertheless, many side effects of PEG have been identified such as the presence of PEG antibodies in blood due to immune response in the case of patients treated with PEG asparginase (Armstrong et al., 2007) and PEG uricase (Sherman et al., 2008). Another drawback called the PEG dilemma has been identified leading to a poor endosomal escape of the nanoformulations with PEG via membrane fusion and the degradation in lysosomes. These phenomena induce a reduction of the treatment efficiency because of a degradation of the drug encapsulated in the formulation and an accelerated blood clearance; furthermore, the non-biodegradability of PEG induces an accumulation of high-molecular mass PEG in the body (Armstrong et al., 2007).

Hence, researches were made to find alternative stealth polymers to PEG. Aware of this clinical awareness, researchers proposed poly(amino acid)s, poly(glycerol) or poly(oxazoline)s as alternatives (Knop et al., 2010). The family of poly(2-oxazoline)s also named poly(2-R-2-oxazoline)s, or poly(*N*-acyl or *N*-aroyl ethyleneimine)s is abbreviated as POx (or PAOx). The chemical structure is regarded as synthetic polyamide or polypeptide see more pseudopeptide or bioinspired polymers (Schlaad et al., 2010). They aroused considerable enthusiasm since the beginning of the 21th century with the occurrence of the polymerization of oxazolines by microwave process (Hoogenboom et al., 2005). The

production of PEO was an old and well-controlled industry. Nevertheless, the use of volatile and toxic precursors such as the ethylene oxide monomer stays a potential industrial risk as still illustrated in January 2020 in Iqoxe manufactury (Tarragona, Spain) with the explosion of a 20-ton tank of this precursor. The maturity of POx technology is not enough succeed than that of PEO but some advantages can be mentioned with the use of low toxic monomer and reactants with a potential gainful business by using micro-wave activation shortening the time of production until only a few minutes. One drawback of the polymerization of oxazolines was the use of toxic and harmful for the environment solvents. The recent successful polymerization in presence of ethyl acetate, viable green solvent, being non-harmful and non-toxic opens up new ways for POx.(Ethyl acetate as solvent for the synthesis of poly(2-ethyl-2-oxazoline) (Vergaelen et al., 2020). Moreover, for LipoPOx, it is not the addition of a lipid on the POx which increases the environmental impact of these materials by using either as previously described CROP process (initiator route) or by a simple esterification reaction (termination route).

An excellent recent review details the fundamental properties that POx possess for biomedical fields, such as hemocompatibility, low cytotoxicity and non immunogen character (Lorson et al., 2018). Nowadays, one goal is to evaluate this family of polymers related to PEG when hydrophilic polymer was required in biomedical application. The underlying ambition is to avoid the massive use of PEG (Barz et al., 2011) at the origin of the appearance of PEG antibody and the resulting loss of furtivity towards immune system (Adams and Schubert, 2007), (Luxenhofer et al., 2012).

Formulation of drug necessitates to combine excipients such as polymers, lipids, surfactants etc. Among them, promising excipients are lipopolymers, which possess several advantages: (i) increase of the entrapment of drugs with low aqueous solubility and low permeability (BCS IV), (ii) enhancement of the protection of the drug against degradation during diffusion

across membrane (intestinal, skin etc.) and (iii) allowing grafting of ligands to target cells (Xu et al., 2015), (Lorson et al., 2018), (Han et al., 2019). Lipopolymers can be considered as a homogeneous mixture of natural glycolipids found in cell membranes and can be used in the building of artificial membranes such as vesicles or liposomes. They stabilize surfaces of complex biological matrices or increase the free circulation of nanoobjects in blood or across skin by stealth behavior towards immune system (Gupta and Roy, 2020). This review will focus on lipopolyoxazolines (LipoPOx)s defined as amphiphilic macromolecules composed of polyoxazoline (POx) block and lipid derivatives (fatty esters, acids, alcohols…) or lipid-like moieties such as (un)saturated long alkyl chains. From the lipids point of view, polyoxazoline-tethered lipids require the functionalization of lipids by oxazolines or polyoxazolines. The relevance of the wedding between POx (case of 2-methyl- and 2-ethyl-2-oxazoline) and lipids results from the antagonism of behavior at the interface - hydrophilicity and lipophilicity, respectively - offering amphiphilic polymers able to self-organize in water. In 2011, Hoogenboom et al. (Hoogenboom, 2011) reviewed on "Poly(2-oxazoline)s based on fatty acids" in which the properties of POx and LipoPOx were summarized. Numerous teams in the world study LipoPOx as schematized in Figure 1, where each point represents the number of publications in this topic. We noted the gathering of LipoPOx research in the north of America and even more in Europe with numerous teams in France, Germany and Belgium.

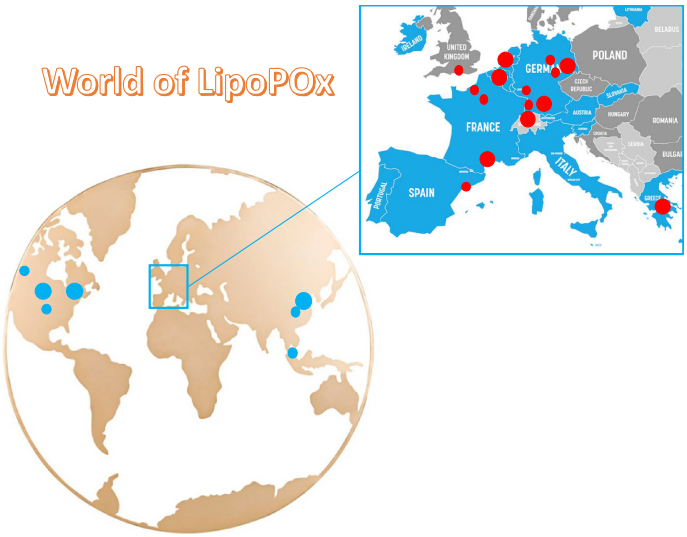


Figure 1. Teams studying LipoPOx in the world. The size of the point corresponds to the number of papers of each team working on LipoPOx.

This review aims to give a comprehensive and critical update of LipoPOx for applications in the formulation field. To do so, we detailed the nature of the lipids and the POx that can be used, then we described the LipoPOx macromolecular engineering, including linear, ramified and brush polymers architectures. All along this review, the position of LipoPOx related to PEG homologues is discussed. Advantages of POx in terms of stability for new nDDS development were given and additional benefits regarding tunability, pH-sensitivity without PEG dilemma were highlighted. The goal of the review is to demonstrate how LipoPOx can be appropriated for nanoformulations like liposomes, mixed micelles (MM) and lipid nanocapsules (LNC) to act as nDDS for drugs being intravenously (IV) and topically delivered.

2.POx and lipid precursors features

2.1. Generalities on polyoxazolines

POx success arises from the versatility of its synthesis based on a cationic ring-opening polymerization (CROP) of the corresponding 2-R-2-oxazoline monomers. Different synthesis strategies were designed from the initiation step, terminating agent or monomer itself. More especially, oxazoline monomers polymerize by CROP process using an initiator R_1-X where X corresponds to a good leaving group such as tosylate, mesylate, triflate or halide reactive unit (mainly iodide) (Guillerm et al., 2012). The polymerization process enables to control the length of the POx chains by fixing the initial monomer/initiator ratio and thus the preprogrammed HLB (Hydrophilic Lipophilic Balance) of LipoPOx. To stop the propagation of the polymer chain an end terminating agent (amine, carboxylic acid, thiol…) is required. The resulting POx are non-ionic and stable polymers, whose tunable physicochemical properties vary with the chemical structure. For instance, fine tuning the HLB of POx by modifying the aliphatic side chains from the 2-oxazoline monomer allows to easily control the polymer properties such as its solubility in water. Indeed, short methyl group leads to highly hydrophilic POx whereas alkyl side chains longer than C4 result in water-insoluble polymers (Luxenhofer et al., 2012), (Viegas et al., 2011). For intermediate side chain lengths, the water solubility of POx decreases as: PEtOx > PiPrOx > PcPrOx > PnPrOx (Foreman et al., 2003), (Viegas et al., 2011). More interestingly, the solubility can be impacted by temperature of the medium conferring stimuli responsive properties to the polymers with a lower critical solution temperature (LCST). As the LCST phase transition is entropy driven by the water release from polymer chains dehydration upon heating, the cloud point temperature decreases as the hydrophobic character of the alkyl side chains is increased. Thus, the HLB of POx can be easily tuned to produce thermoresponsive polymers presenting interesting properties for drug delivery applications, as recently reviewed by Hoogenboom and Schlaad (Hoogenboom and

Schlaad, 2017). POx are also soluble in a broad range of organic solvent from low polarity (chloroform) to polar (acetonitrile, dimethylacetamide). This specificity is useful regarding formulation of water insoluble-drug (BCS class IV). Interestingly, it has been reported that linear and branched PEtOx (MW of 10, 20, 30 and 40 kDa) at a concentration as high as 40% w/v exhibit a significantly lower viscosity than PEG (Viegas et al., 2011). This physical property of POx plays a crucial role in formulating drugs in low viscosity formulation as it determines the filterability and syringeability characteristics.

Concerning cyto- and hemocompatibility, many studies involving PEG have been published but only few publications report on such experiments using POx (Gaertner et al., 2007), (Woodle et al., 1994). The effect on the integrity of erythrocyte membrane was investigated with an hemolysis study conducted under standardized conditions with PEtOx and PEG with pharmaceutically relevant molar mass (0.4 to 40 kDa). No clear correlation between molar mass and cytotoxicity was evidenced, but medium molar masses presented higher cytotoxicity for unknown reasons. None of the polymer concentration modified the behaviour of erythrocytes or led to significant hemolysis: PEtOx up to 40 kDa and 80 g/L did not cause erythrocyte aggregation and PEtOx of kDa only led to minor erythrocyte aggregation at 40 g/L (Bauer et al., 2012). Similar cytotoxicity and hemocompatibility studies were assessed using the same conditions with PMOx varying from 2 to 20 kDa even for a concentration of 80 g/L (Bauer et al., 2013). Polymers were found to slightly reduce the cells viability only for higher concentrations and in long term treatments (MTT assay) (Bauer et al., 2013). None of the PMOx and PEtOx polymers induced a haemoglobin release. Cytotoxicity studies confirmed the high cytocompatibility of PMOx and PEtOx.

For topical application, evaluations on L929 mouse fibroblasts have been conducted. Only 2 kDa PMOx (80 g/L) reduced the cell viability of L929 mouse fibroblasts to 60 and 44% after 3 and 12 h incubation, respectively. Globally, a similar behaviour was observed for PEtOx

(Bauer et al., 2012). Even if studies are difficult to compare since they used different test conditions, cell types, and polymers of different origin and purity, POx rather exhibit excellent cyto and hemocompatibility developed in the past 5 years as reported above.

In vivo behaviour of POx was also evaluated. For example, in acute dose studies, PEtOx (10 and 20 kDa) solution was administered intravenously (Sprague Dawley rats) in single and multiple injections at doses of 500, 1 000 and 2 000 mg/kg. Neither toxicity nor adverse effects were observed (Viegas et al., 2011). Furthermore, the body weights, food intake levels of each animal, blood and serum compositions were normal when compared to the control group that received 0.9% (w/v) sodium chloride. The organ sizes, weights, morphology, and texture of liver, kidney, lung, spleen, heart, and gastrointestinal tract were normal. In the multiple dose essay, rats received seven intravenous doses of 50 mg/kg of PEtOx 20 kDa (14 days). The blood counts and serum chemistry at days 15 and 21 were normal when compared to the control group. The key organ sizes, weights, morphology, and texture were also normal, and the histopathology of the kidney and spleen showed no microscopic differences when compared with the organs of control animals.

Finally, many studies have evaluated the biodegradability of POx towards hydrolysis or oxidation to predict POx behaviour when administered *in vivo*. POx are amenable to acidic and basic hydrolysis but in simulated stomach and intestine fluids no significant hydrolysis was observed. It has been suggested that the enzymatic degradation may be hindered by the tertiary amide of the pseudo-polypeptides structure, inducing non-biodegradability of the polymers (Adams and Schubert, 2007), (Hoogenboom, 2009). However, some oxidative pathways may induce mechanisms towards polymer biodegradation. Experiments investigate the effects of ROS/RNS on pseudo-polypeptides and compare their degradation behaviour with PEG (PEtOx) and PEG with a degree of polymerization of approx. 50 and 120 (corresponding to 211 kDa) under physiologically relevant conditions. It was concluded that

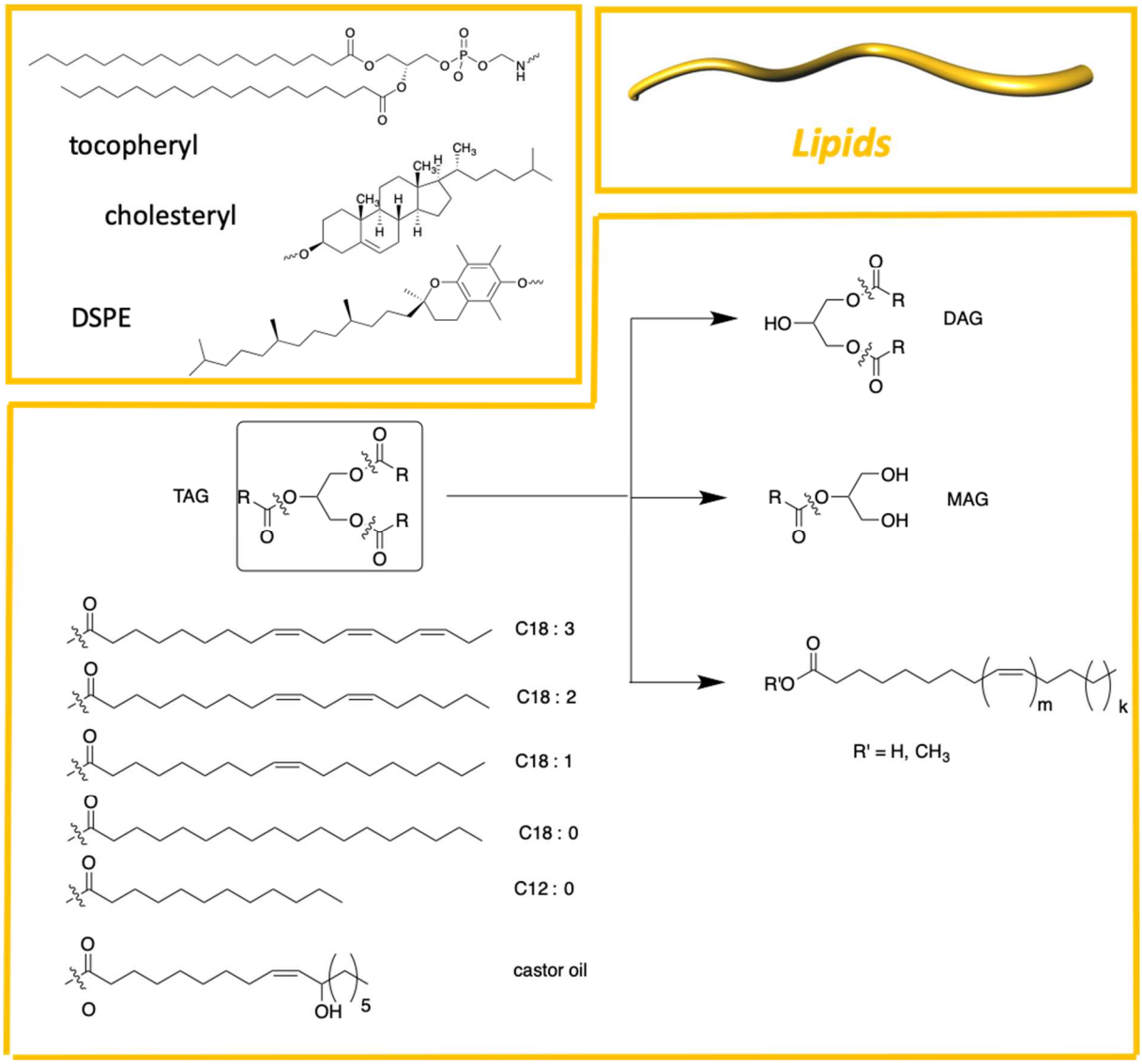
PEG and POx are degradable by oxidative degradation (Ulbricht et al., 2014). Another oxidative degradation route of POx was established by reactive oxygen species (ROS) in macrophages (Van Kuringen et al., 2012), (Shah et al., 2015). One interesting aspect of POx related to PEG is the by-products resulting from the hydrolytic process. For POx, degradation resulted in PEI and carboxylic acid derivatives (*e.g.* acetic acid for PMOx, propanoic acid for PEtOx) whereas PEG produce the toxic 1,4-dioxane, one of the seven common ingredients to avoid in cosmetics. As POx are biodegradable, they do not accumulate in tissues and are rapidly cleared from the blood by glomerular filtration in the kidneys. Only minimal amounts of POx are taken up by the reticuloendothelial system (RES) (Gaertner et al., 2007).

However, it should never be assumed that these results can be simply extended to POx with different chemical structures, architectures or end groups and must always be assessed on a case-by-case basis. Nonetheless, POx are promising polymers for biomedical applications as they are easy to synthesize and they present not only interesting physico-chemical properties as high stability and low viscosity but also a good cyto- and hemocompatibility, biodegradability and an excellent *in vitro* toxicological feature.

2.2. Nature of lipids

Lipids originate from biochemical subunits: ketoacyl and isoprene groups. Among them only the fatty acids such as glycerolipids (TAG: triacylglycerol and ramified derivatives), glycerophospholipids (DSPE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine) and sterol lipids (cholesterol, tocopherol (named TPOS when associated to POx)) (Scheme 2) are used to design LipoPOx. TAG produce ramified structures such as diacylglycerol (DAG) and linear ones like monoacylglycerol (MAG), fatty alcohol, amine, ester and fatty oxazoline as detailed in § 2.2. Some other original lipids are not considered in this review even if LipoPOx

synthesis with them has been described. For example, it is the case for cardanol for which its aromatic ring connected to a fatty unsaturated chain (Delage et al., 2019). The review mainly focuses on glycerolipids because a lot of lipid derivatives are available for oxazoline chemistry. This broad variety comes from the extended length of the fatty chain from n = 10 to 22 carbon atoms as well as the number of unsaturations ranging from m = 0 (saturated lipids) to 1 (e.g. C18:1, oleic), 2 (e.g. C18:2, linoleic) and 3 (e.g. C18:3, linolenic) (unsaturated lipids). It is known that all vegetable oils (grapeseed oil, sunflower oil, soy oil…) only differ from one to another by lipid composition of saturated and unsaturated fatty chains. LipoPOx produced from fatty chains bearing n carbon atoms and m unsaturations are written as Cn:mPOx. Furthermore, LipoPOx can also be synthesized from lipids bearing specific functional groups such as castor oil possessing an alcohol group (Giardi et al., 2009). In this review, the petrosourced and biosourced saturated chains (C12:0, C16:0, C18:0…) are indifferently considered.



Scheme 2. Nature of lipids used for LipoPOx.

3. Overview of LipoPOx

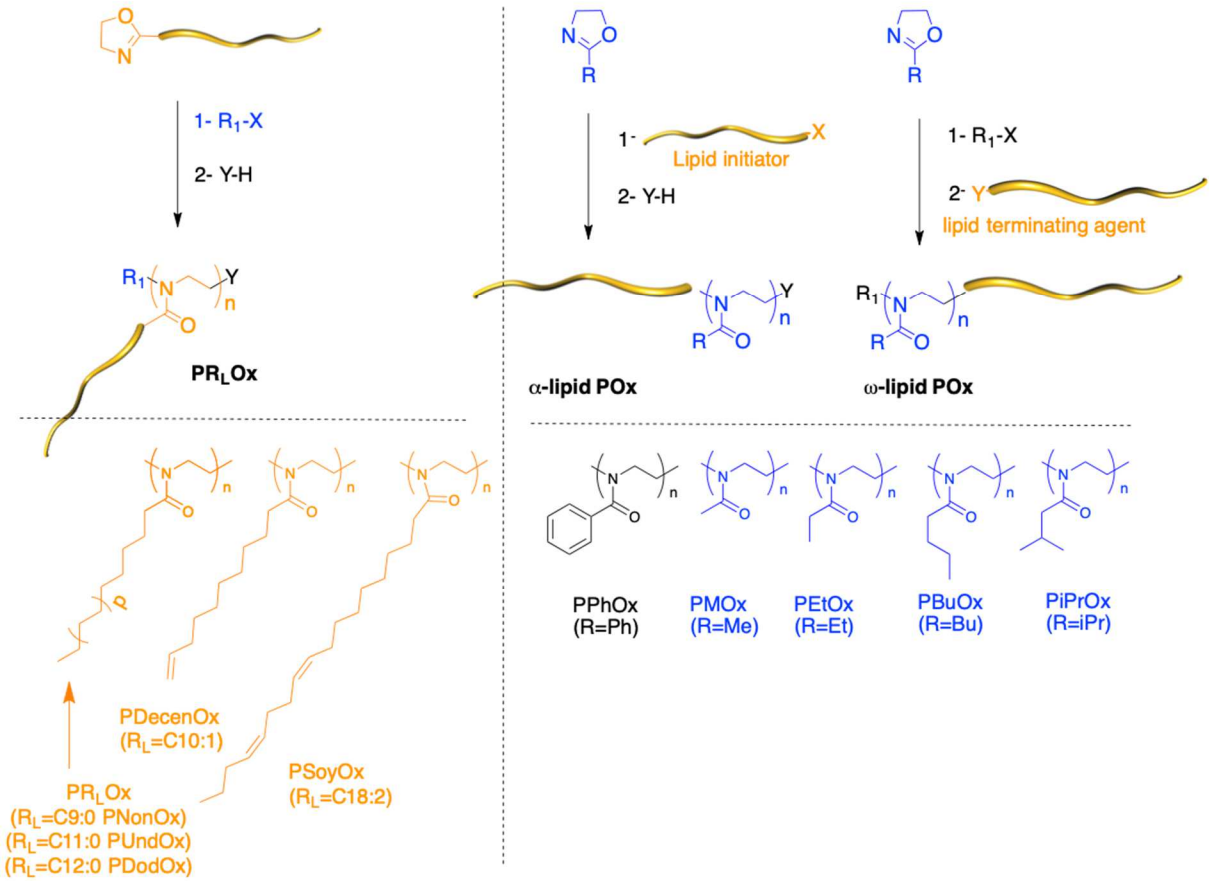
Hybrid materials are extensively studied to build well-shaped liposomes or polymersomes especially using lipids, glycopolymers. This review focuses on the hybrid polymers resulting from a lipid derivative coupled to a POx block excluding polymersomes fully based on POx chemistry (Le Meins et al., 2013).

3.1. Synthesis of LipoPOx

LipoPOx can be produced by three different routes of synthesis: using functional initiator (α-lipid POx), terminating agent (ω-lipid POx) and functional oxazoline (PR_LOx) (Scheme 3). It is important to highlight one advantage of POx chemistry over PEG's one: the additional functionalization from the R group of 2-R-2-oxazoline monomer in PR_LOx route. As a result, a new architecture of brush polymers with lipid pending chains has emerged, which are written as 2-R_L-2-oxazolines. The 2-R_L-2-oxazolines are synthesized by condensation between fatty acids and ethanolamine leading to pending chains in monomers that are either saturated (R_L=C9:0, 2-nonyl-2-oxazoline (NonOx), C11:0, 2-undecyl-2-oxazoline (UndOx), C12:0, 2-dodecyl-2-oxazoline (DodOx)), and unsaturated (R_L=C10:1, 2-(dec-9-enyl)-2-oxazoline (DecenOx), C18:2, soy-based oxazoline (SoyOx)) (Huang et al., 2006), (Hoogenboom et al., 2007). Additional modifications of C10:1 pending chain have been undertaken by thiol-ene coupling (TEC) on terminal unsaturation (Del Rio et al., 2011), (Kempe et al., 2011). The two other routes are complementary with either a lipid initiator and non functional terminating agent for α-lipid POx route or a non functional initiator and a lipid terminating agent for ω-lipid POx route. The last two routes occurred with non functional 2-R-2-oxazoline where R corresponds to methyl (MOx), ethyl (EtOx) and isopropyl (iPrOx) groups. In this review, we consider that poly(2-phenyl-2-oxazoline) (PPhOx) can constitute the hydrophobic part of LipoPOx, resulting in PPhOx-b-PMOx copolymers. Indeed, they are able to self-assemble and are used in lipid based nanoformulations such as liposomes (§ 3).

The advantages and drawbacks of initiator and termination routes also apply for LipoPOx. The initiation route allows to have a lipid block (lipid initiator) on all the POx chains. Unfortunately, lipid initiators have a lower reactivity than classical initiators such as MeOTs potentially resulting in ill-defined polymers. One explanation for the low reactivity could be the lipophilic nature of the initiator by contrast with the hydrophilicity of oxazoline monomer.

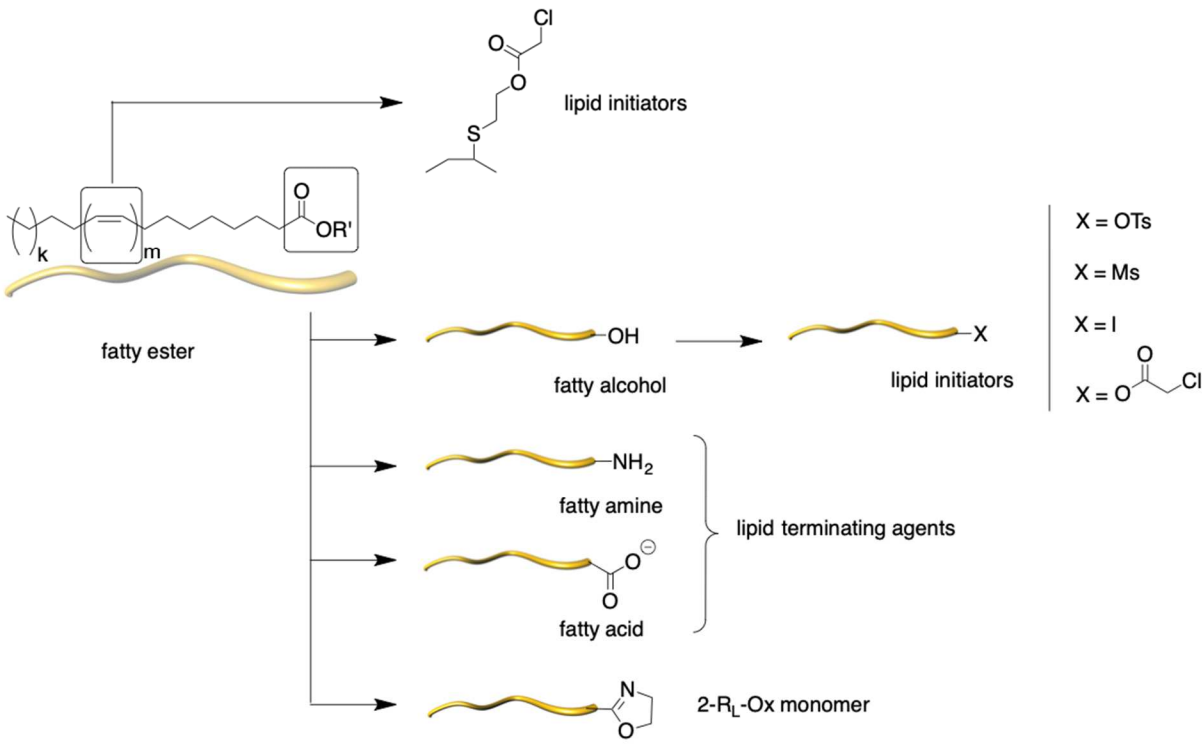
Regarding the termination route, a good molecular weight control of POx chain was reached with an efficient initiator such as MeOTs. Nonetheless, it is necessary to highlight that a low reactivity of the lipid terminating agent on the hydrophilic POx chain can generate an uncompleted functionalization of the end group. Moreover, during the purification of the resulting polymer, the separation of non terminated POx and LipoPOx is difficult.



Scheme 3. The three chemical routes for LipoPOx. (The nature of lipid initiators and terminating agents were described in Scheme 4).

Lipids can be functionalized through many ways depending on the synthetic route followed. For the initiation route, the ester groups of TAG can be converted into alcohol and then into tosylate, mesylate (El Asmar et al., 2016), iodate (Volet et al., 2005) or chlorate initiators after *trans*-esterification (Stemmelen et al., 2013), (Travelet et al., 2013). The unsaturations (the

other reactive sites of TAG) can be modified into good leaving groups by thiol-ene coupling (Stemmelen et al., 2013) (Scheme 4). For the termination route, the ester groups of TAG can be transformed into carboxylic acids and fatty amines acting as terminating agents (Waschinski and Tiller, 2005). A specific case has been reported where native alcohol groups of castor oil were directly converted into tosylates (Giardi et al., 2009).

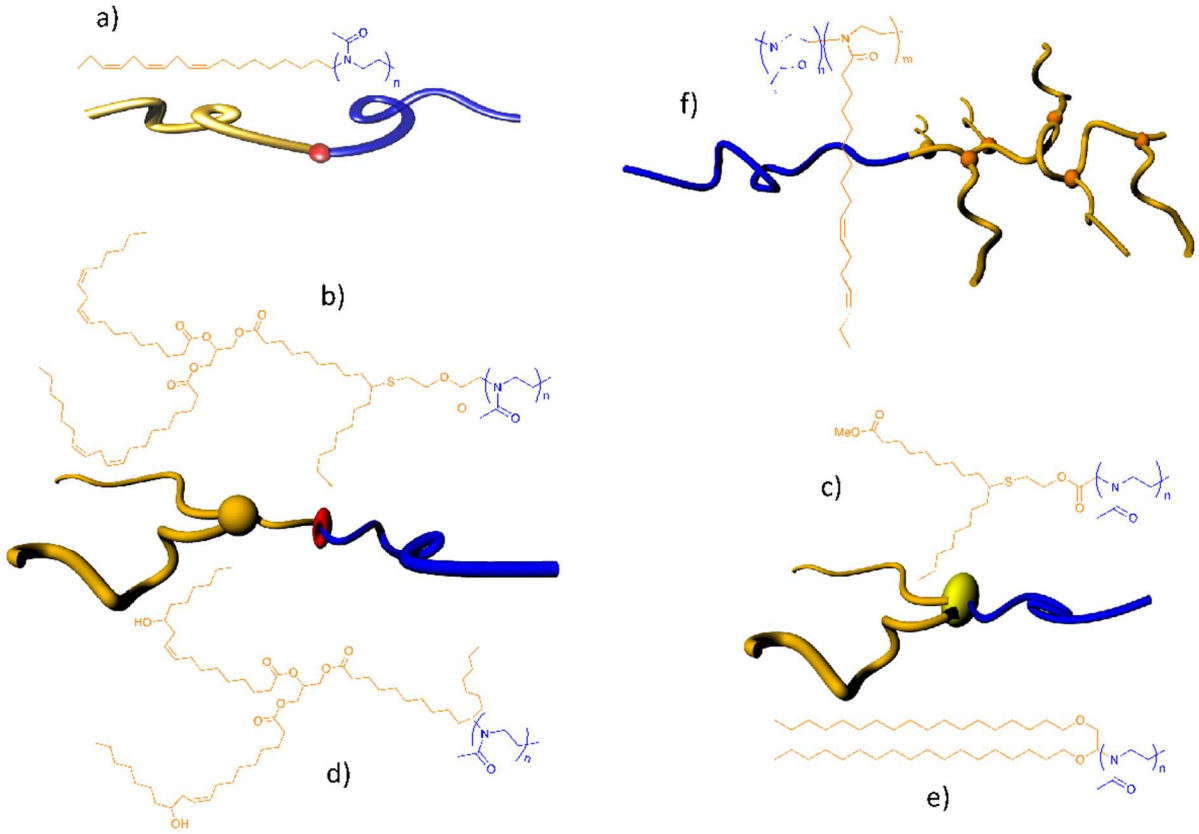


Scheme 4. Lipids modifications for the synthesis of LipoPOx.

3.2. Macromolecular engineering of LipoPOx

Linear or ramified LipoPOx can be synthesized with various macromolecular architectures (Scheme 5). The complexity of the structure originates from the lipid. Using fatty esters as lipid initiators or terminating agents linear diblock LipoPOx are produced (Scheme 5a). One example of ramified LipoPOx from fatty ester or native triacylglycerol, TAG, was represented on Scheme 5b and 5c. The unsaturation was converted by thiol-ene coupling into chloride initiator, and further modified *in situ* into iodide. In the specific case of castor oil the synthesis

of tosylate initiator from the native alcohol group produces a similar structure of ramified LipoPOx as represented in Scheme 5d (Giardi et al., 2009). In other cases, ramified LipoPOx results from diacylglycerol, DAG, converted into tosylate initiator (Einzmann and Binder, 2001), (Lüdtke et al., 2005) (Scheme 5e). The last class of ramified LipoPOx concerns the brush polymers obtained by the copolymerization of hydrophilic oxazoline and 2-R_L-2-oxazolines (Baekmark et al., 1998) (Scheme 5f). An alternative route to obtain them is based on the coupling of hydrophilic POx on the terminal reactive function of lipophilic POx.



Scheme 5. Linear and ramified macromolecular architectures of LipoPOx.

This part took stock of the chemical strategies to yield original and well-defined LipoPOx based on various lipid structures from fatty ester group to unsaturated fatty chains and the versatility of oxazoline chemistry with initiation, termination and monomer routes. All these ways produce linear and ramified LipoPOx able to self-assemble in water in nano-objects acting as nDDS thanks to their specific properties described in the following part.

3.3. Surfactant properties of LipoPOx

Since the initial report by Kobayashi et al. in 1986, amphiphilic poly(2-oxazoline) block copolymers have been recognized as non-ionic surfactants (Kobayashi et al., 1986). Some of them have been evaluated concerning their physico-chemical properties, such as critical micelle concentration (CMC) and surface tension (Aoi and Okada, 1996). Naka et al. reported the CMC values and micellization behavior of block copolymers having PMOx backbone and hydrophobic block constituted of short monomer chains like 2-butyl-, 2-octyl- or 2-phenyl-2-oxazoline (Naka et al., 1997). Spherical micelles with a diameter of 25 nm were obtained for 2-phenyl-2-oxazoline, while 2-butyl-, 2-octyl-2-oxazoline formed a mixture of spherical and rodlike micelles. Most of the studied block copolymers led to the formation of large aggregates as the hydrophobic content and molecular weight increases (Binder and Gruber, 2000). Introducing lipophilic moieties such as C12 or C18 alkyl chains at the end of highly soluble PMOx result in micelle aggregates at a CMC that depends on their HLB. It decreases with a decrease of the hydrophilicity of the polymer backbone or by an increase of the alkyl chain length. For example, replacing a C12 chain by a C18 in LipoPOx of *ca.* 5 kDa the CMC decreases from 4×10^{-2} to 7×10^{-4} wt % (Volet et al., 2005). Alternatively, hydrophilicity of the polymer backbone can be tuned by the length and the chemical nature of the polymer. Indeed, substituting the aliphatic side chains of C18-POx 10 kDa by iPr instead of Et decreases the CMC from 4×10^{-3} to 1.6×10^{-3} wt %, whereas reducing the C18-PiPrOx length from 10 to 7 kDa marginally reduces the CMC at 1.2×10^{-3} wt % (Obeid et al., 2009) %. The group of Winnik reported another critical parameter concerning the temperature-dependence aggregation phenomenon (defined by the cloud point temperature, T_{cp}) in the case of C18-based LipoPOx (Obeid et al., 2009). Below T_{cp}, the micellar aggregates form star-like micelles of hydrodynamic radius (Rh) ~ 7 - 11 nm depending on alkyl groups and polymer size, which are constituted by a hydrophobic core (Rc) of ~1.2 – 1.4 nm surrounded by a

corona of dangling POx chains. Heating through the T_{cp} induces aggregation of the micelles forming rigid objects of constant size of about 260 nm for C18-PiPrOx. It has been suggested that micelles aggregation form through bridging of water molecules, inducing rearrangement of the rigid micellar core to a more fluid one. This mechanism is supported by the extended conformation of the POx chains in micelles observed from SANS data in semi-dilute regime (Volet et al., 2009) and microcondensation of the alkyl chains from surface rheological properties measured at the air/water interface (Lüdtke et al., 2008). The well-defined behavior and self-organisation in water of POx and LiPOx demonstrated their reassuring uses in nanoformulations.

3.4. *In vitro* biological properties of LipoPOx

The use of LipoPOx in biomedical fields arose for 10 years. They found applications for the formulation of different DDS but only a few basic biological and stability studies were reported such as (i) hemocompatibility, (ii) cytotoxicity and (iii) buffering capacity.

(i) hemocompatibility: In a recent study (Han et al., 2019), a LipoPOx associating PMOx to tocopherol named D-tocopheryl-POx (TPOS) was performed to evaluate its safety compared to DSPE-PEG towards red blood cells (RBC) for *in vivo* administration. Human RBC were used as they simulate more accurately *in vivo* environment than animal RBC. From these results, TPOS and DSPE-PEG (FDA approved) presented similar behavior towards human RBC within 5% concentration, indicating TPOS safety to meet the requirements of intravenous injection.

(ii) cytotoxicity: A systematic investigation of the toxicity of a panel of LipoPOx based on homo- and block copolymers has been investigated *in vitro* in three immortal mammalian cell

lines (Barz et al., 2011). It was found that LipoPOx are very well tolerated even at rather high concentrations ≥ 10 g/L.

(iii) buffering capacity: nDDS such as liposomes entered the cells mainly through energy-dependent endocytosis phenomenon followed by fusion with lysosomes. Liposomes are rapidly degraded by the acidic endolysosomes content. The buffering capacity of the formulation is essential to improve the endosomal escape and the rapid release of the drugs into the cytoplasm without acid degradation. Replacing PEG by TPOS-based LipoPOx in DSPE-PEG formulations resulted in new nDDS formulations that will be described in § 3. TPOS displayed a slower downtrend and gentler slope in the titration curve than DSPE-PEG over the pH range 5.0–7.4, indicating that TPOS exhibits a good buffering capacity (Han et al., 2019). Moreover, it indicates that TPOS would also be pH-sensitive in the acidic environment of lysosomes (pH 5) of cancer cells (see § 3.1.).

4. Nanoformulations of LipoPOx

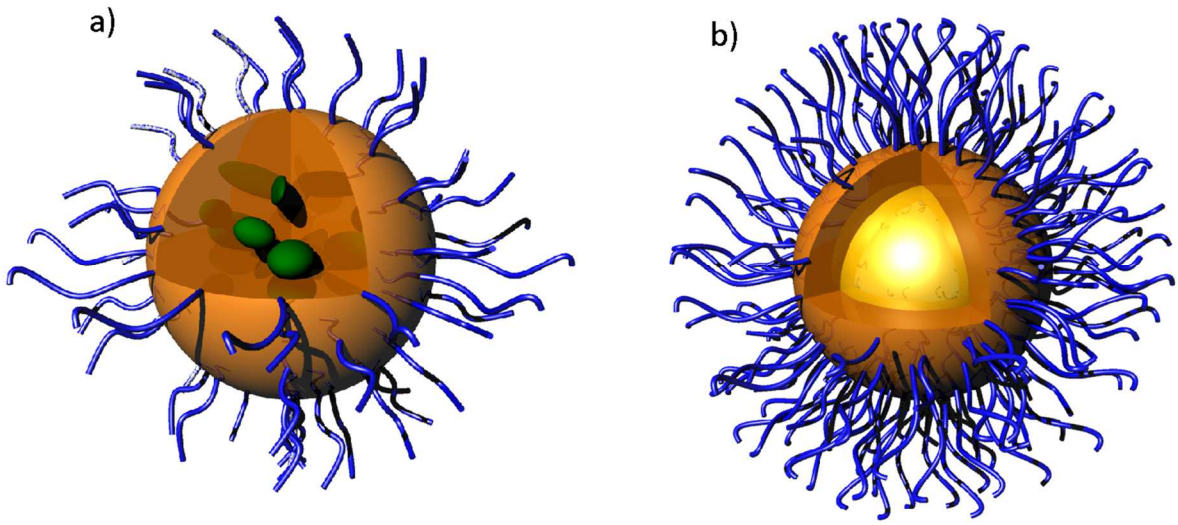
As LipoPOx hemocompatibility and cytotoxicity have been evaluated and thanks to their physico-chemical properties and synthesis possibilities, LipoPOx are very interesting for formulation work and help designing stable nDDS.

LipoPOx self-assembly into well-defined nano-objects such as micelles which help to solubilize active drugs with hydrophobic properties. Furthermore, the flexible POx backbone (Luxenhofer et al., 2010) with a $-CH_2-CH_2-N-$ repetitive unit ensures the stability of the formulation even at a high curvature as it is the case for small spherical nano-objects. The dense corona of POx creates steric repulsion enhancing the stability. The modulation of the LipoPOx physical properties is also a very attractive characteristic as it can introduce thermal stimuli response for formulation. As introduced in part 1.1, depending on the monomer, the hydrophobic and hydrophilic properties can be fine tuned. This directly impacts the

crystallinity but also the LCST of the LipoPOx. The formulation behavior with LipoPOx is modified from water soluble to water insoluble upon temperature change. The LCST feature is important to design formulation with a control release of drug at the body temperature (Tokuyama and Kato, 2008). Moreover, the versatility of the LipoPOx synthesis leads to formulations with various organizations such as spherical or lamellar shapes (Huang et al., 2006). The addition of excipients (phospholipids, oil, surfactants) to micelles permit further innovant formulations. It is also possible to conjugate molecules such as proteins, peptides or drugs onto the LipoPOx by covalent coupling during its synthesis (Viegas et al., 2011). This increases the drug solubility and helps to control the drug delivery. Finally, as most of the amphiphilic polymers, LipoPOx also adopts a "frozen behavior" (Nicolai et al., 2010). For the development of formulations, it means that the LipoPOx sometimes require energy supply to interact with the other constituents of the formulation. This can be easily balanced with an increase of agitation, temperature or ultrasound, to disperse the mixture that further reorganizes.

Up-to-date, different types of nanoformulations were designed with LipoPOx such as liposome, mixed-micelle (MM) and lipid nanocapsule (LNC) as represented on Scheme 6. Liposomes are currently made of a lipid bilayer of phospholipids (S75, S100, HSPC) and cholesterol (molar ratio around 3:2) enclosing an aqueous core (Zhai and Zhai, 2014) with LipoPOx inserted into small unilamellar liposomes (SUV). This formulation of about 100 nm in size enables encapsulation of hydrophilic, amphiphilic and lipophilic drugs with a high drug loading (Zalipsky et al., 1996), (Xu et al., 2014), (Han et al., 2019), (Xu et al., 2015). Mixed-micelles are a mixture of lipids, more especially phospholipids and amphiphilic LipoPOx, forming a single vesicle of about 30 nm (Simon et al., 2019) and 100nm (Pippa et al., 2013b) (Scheme 6a). This matrix system can be seen as a hybrid system combining features of liposome and polymersome. Finally, LNC are composed of a liquid lipid core

surrounded by a solid membrane formed by lecithin and LipoPOx (Scheme 6b). The phase inversion process was readapted to LipoPOx. This core/shell system has a size of approximately 20 nm and its oily core considerably improved the solubility of hydrophobic drugs (Simon et al., 2020). In all of the three formulations LipoPOx coexist with phospholipids but in various ways: insertion in liposomes, entanglement in MM and interaction in LNC. These formulations are applied in most of the cases as nDDS for intravenous administration and more recently for topic applications as respectively described in § 3.1 and § 3.2.



Scheme 6. a) Mixed micelle loaded with drugs, b) Lipid nanocapsule enclosing drugs in its core.

4.1. LipoPOx nanoformulations designed for intravenous route

Reduction or suppression of biological recognition mechanisms is of first importance for the efficiency of nDDS. As conventional DDS preferentially accumulate in organs of the reticuloendothelial system (RES), mainly liver and spleen, and to avoid the short blood lifetimes, nDDS are often pegylated to reduce protein adsorption and recognition by the immune system (Cheng et al., 2012), (Knop et al., 2010). As a result, such nDDS present

enhanced circulation times inducing drug release over a longer time period. Furthermore, different types of nDDS, which belong to the class of soft colloidal nanomaterials such as liposomes, micelles and lipid nanocapsules (Bonacucina et al., 2009), (Mishra et al., 2010) were tested to enhance drug solubility of hydrophobic drugs and targeting (Veronese, 2001), (Torchilin, 2004). These formulations have been recently studied using LipoPOx as a new excipient to improve their properties and also reduce side effects (PEG dilemma). Indeed, the lipoPOx major advantage over PEG is its ability to overcome the PEG dilemma which will present a barrier to the development of PEGylated nanoformulations. As represent in Scheme 7, PEGylated nanoformulations with their PEG hydrophilic corona induced first a weak cellular uptake by the target cells compared to LipoPOx nanoformulations. After the cellular uptake a poor and late endosomal escape is observed in presence of PEGylated nanoformulations while LipoPOx nanoformulations by interacting directly with the endosome membrane, lead to a rapid escape of the drug in the cytoplasm before the lysosomal degradation (pH 5.5). LipoPOx nanoformulations improve the cellular uptake and the integrity of the drug loaded and therefore the efficiency of the treatment. In a first study published in 1994 (Woodle et al., 1994), PMOx and PEtOx amphipathic polymers were investigated. Both POx bore a carboxylic group end group on which DSPE was covalently linked. LipoPOx was incorporated readily (5 mol %) into liposomes bilayer containing HSPC and cholesterol. PMOx-DSPE and PEOx-DSPE exhibited long plasma lifetimes and low hepatosplenic uptake. PMOx-DSPE was more effective at decreasing blood clearance rates than PEtOx-DSPE and best results were reached with PMOx-DSPE 3.26 kDa. LipoPOx liposomes were demonstrated to have similar and even better advantages compared to PEGylated liposomes. These results stimulated the keen interest of nDDS to overcome the PEG dilemma and improve drug delivery. Indeed, for many years, liposomes were coated with PEG on surfaces to improve pharmacokinetic and pharmacodynamic properties of

liposomes (Mo et al., 2013) but many side effects were observed. The intersurface aqueous layer and the dense PEG corona partly inhibited the ability of cellular uptake (Mishra et al., 2004) leading to a poor endosomal escape via membrane fusion and favor the degradation of drugs in lysosomes referred as the "PEG dilemma" (Chen et al., 2017). To overcome this phenomenon, pH-sensitive liposomes based on TPOS have been investigated improving endosomal escape. As previously described (section 2.4), TPOS had satisfactory biocompatibility and pH sensitivity. The cellular uptake of TPOS-liposomes increased with pH compared to conventional liposomes and PEG-liposomes. The intracellular distribution of liposomes was evaluated. Contrary to PEG-liposomes (and conventional liposomes), TPOS-liposomes were localized into the cytoplasm. Thus, the modification of liposomes with TPOS improves the cellular uptake capabilities. Furthermore, after cell internalization, TPOS promoted liposome destabilization leading to endosomal escape and cytoplasmic release. In this study, the cellular uptake and endosomal escape were weaker for the PEG-liposomes and conventional liposomes (Han et al., 2019).

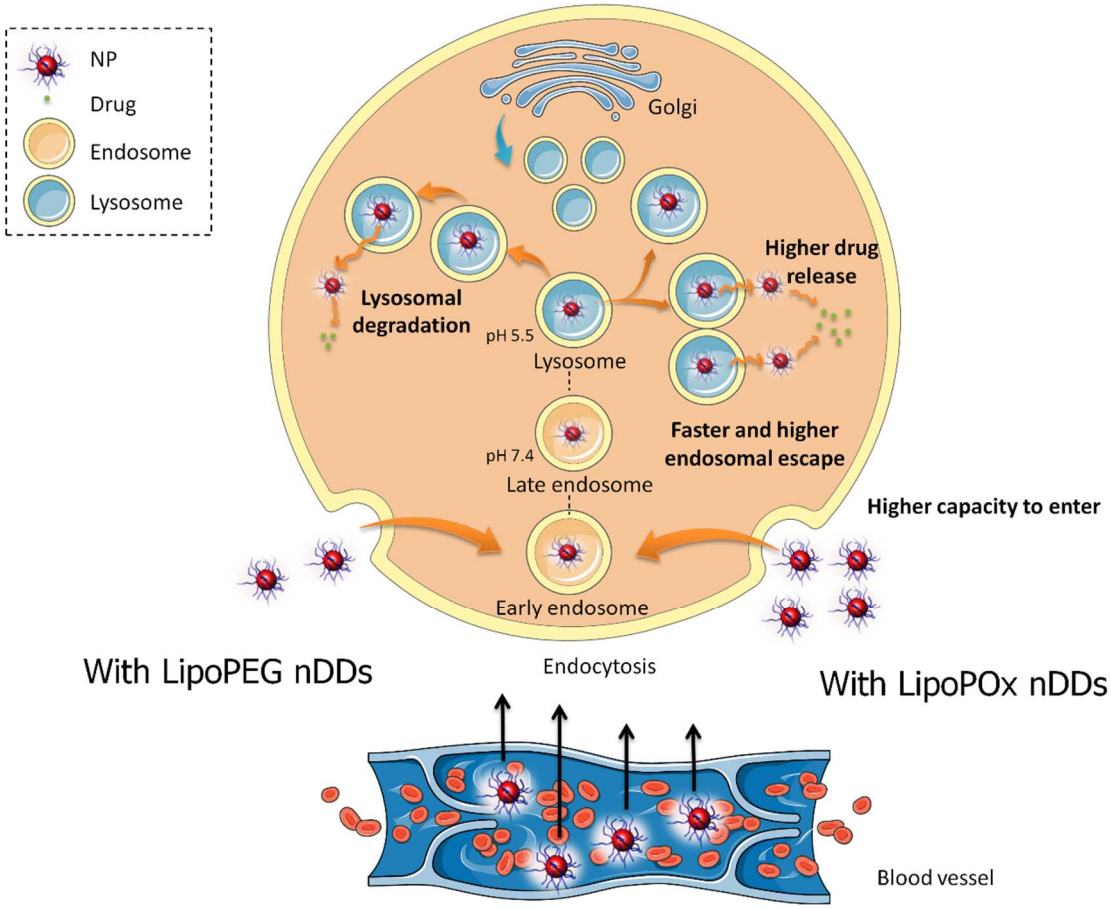
Based on this property, increasing research interest on pH-sensitive liposomes has emerged to improve cancer treatment efficiency. Thus, the use of liposomes loaded with new effective biological anti-cancer drugs, such as genes or proteins, has received increasing attention for two main reasons. Firstly, when pH-sensitive liposomes are ingested by cancer cells, and because the pH in endosomes can reach values below 5.5, the encapsulated genes or proteins must be released rapidly into the cytosol after having entered the endosome to avoid their digestion and metabolization by lysosomes and their subsequent loss of anticancer activity. Secondly, the extracellular pH values of most solid tumors range from 5.7 to 7.8, whereas the pH of normal tissues is at pH 7.4 (Wang et al., 2012). Therefore, LipoPOx based on PEtOx is suitable for pH-sensitive drug delivery systems as recently reported for liposomes and mixed micelles.

Concerning liposomes, a first study reports on a bifunctional liposome using PEtOx-CHMC with long-circulating and pH-sensitive properties. PEtOx-CHMC modified liposomes (PEtOx-CHMC-lipo) were loaded with calcein and an increase of calcein release at low pH was observed. Flow cytometric analysis results showed that the fusion and cellular uptake of PEtOx-CHMC-lipo could be promoted significantly at pH 6.4 compared with those at pH 7.4. Confocal laser scanning microscopy first revealed that PEtOx-CHMC-lipo could be sensitive to low endosomal pH and directly released the fluorescent probe into the cytoplasm of HeLa cells. Then, MTT assays with HeLa cells demonstrated that doxorubicin hydrochloride (DOX) loaded in PEtOx-CHMC-lipo exhibited stronger anti-tumor activity at pH 6.4 than 7.4 (culture cell medium). PEtOx-CHMC-lipo also remained stable when incubated in calcium chloride solution. The pharmacokinetic experiments of liposomes in rats showed that $t_{1/2}$ and AUC of PEtOx-CHMC-lipo were 4.13 and 4.71 times higher than those of classic liposomes.

In a second study, PEtOx was applied in pH-sensitive liposomes named PEtOx-cholesterol hemisuccinate (PEtOx-CHEMS) (Xu et al., 2015) loaded in DOX. Compared to conventional and PEGylated liposomes, PEtOx-CHEMS can fuse with the endosomal membrane under acidic conditions inducing DOX release in the cytoplasm. In addition, *in vivo* experiments using rats demonstrated that pH-sensitive PEtOx-CHEMS-Lipo were promising formulations for intracellular targeted delivery system to overcome the “PEG dilemma”.

In a last liposomes study, Han et al. (Han et al., 2019) investigated a liposomal formulation with TPOS (TPOS-L) to yield a sustained and controlled *in vitro* drug delivery. The characteristics of TPOS-L were compared to PEG liposomes (PEG-L). TPOS-L presents a strong hydrophilic shell and conformation cloud increasing its stability. The pH-sensitivity of TPOS-L was attributed to the carbonyl group of TPOS which had a non shared electron pair. When pH decreases, the carbonyl group binds to the hydrogen and undergoes protonation, and the charge transfer changes “N” to “N+”, which induces the molecular chain to possess

positive charges. The transfer of charge density and the greater steric hindrance around the carbonyl group make the polymer molecules change from hydrophilic to hydrophobic in an acidic environment. As a result, TPOS will induce the destruction of the liposomes bilayer and the release of the anticancer drug loaded in liposomes (Docetaxel) into the cytoplasm. PEtOx modified liposomes exhibit excellent long circulating and pH-sensitive properties as described in Table 1 summarizing all the studies related to this topic.



Scheme 7. Illustration of PEG dilemma.

Table 1. LipoPOx used in nanoformulations for intravenous route.

Reference	Formulation	Composition	Entraped drug	Nature of LipoPOx	Results
(Woodle et al., 1994)	Liposomes	EPC/chol (3:1)	None	DSPE-PEtOx DSPE-PMOx	POx for protection from rapid recognition and clearance
(Zalipsky et al., 1996)	Liposomes	HSPC/Chol (3:1)	None	DSPE-PEtOx DSPE-PMOx	POx for long plasma lifetimes low hepatosplenic uptake. Decrease blood clearance.
(Kierstead et al., 2015)	Liposomes	Water soluble polymers	None	DSPE-PEtOx DSPE-PMOx	Comparison of soluble polymers -POxylated liposome -furtivity
(Adams and Schubert, 2007)	Micelles	POX-PEI	None	POX-PEI	Review on membranes, nanoparticles, DDS, stimuli responsive systems
(Pippa et al., 2013a)	Chi-aDDns	DPPC:MPOx	Indomethacin	MPOx = PPhOx-g-PMOx	Thermoresponsive behavior of POx -Understanding POx-lipid interactions in bilayer liposome

(Pippa et al., 2013b)	Chi-aDDns	DPPC:MPOx	Indomethacin	MPOx = PPhOx-g-PMOx	Preparation of liposomes nanovectors
(Pippa et al., 2018)	Chi-aDDns Liposomes	DPPC:MPOx	Indomethacin	DPPC:MPOx	-Comparison diblock and gradient polymers -Understanding POx-lipid interactions in bilayer liposome
(Xu et al., 2014)	Liposomes	S75/chol (3:2)	Calcein	PEtOx-CHMC	Liposomes exhibit stronger anti tumor activity
(Xu et al., 2015)	Liposomes	Phospholipid/chol (3:1)	Doxorubicin	PEtOx-CHEMS	
(Han et al., 2019)	Liposomes	S100/chol (3:1)	Docetaxel	TPOS	New pH sensitive DDS for anti tumor activity

A series of papers reported the work of Pippa et al. on advanced DDS named “chimeric advanced drug delivery nanosystems” (chi-aDDnSs) entrapping indomethacin (IND) drug. Two first publications focus on chimeric nano-assemblies composed of DPPC and gradient copolymer: PMOx-*g*-PPhOx named MPOx (be careful of the confusion with the homopolymer PMOx) (Pippa et al., 2013a), (Pippa et al., 2013b). The impact of the molar ratio between the components, the temperature, the concentration and the medium (PBS and fetal bovine serum (FBS)) on the size and the stability of chimeric nanocarriers was evaluated. The penetration of PPhOx into lipid domains increases the stability of liposomes of similar nano-objects size except at the higher molar DPPC:MPOx ratio. This increased stability was interpreted by morphological perturbations of the biomolecular sculpture originating from several entry and exit points in the lipid membrane. The thermoresponsive behavior of the chimeric nanocarriers was also highlighted with a change of structural characteristics under increased temperature. Drug release was also detailed with a variation of the MPOx content. chi-aDDnSs were found to be efficient DDS as MPOx allows the release control of the incorporated drug.

In a more recent paper, these chimeric vesicles were compared to DPPC liposomes modified by an amphiphilic block polymer guest (PEO-*b*-PCL) (Pippa et al., 2018). The study of various macromolecular architectures was motivated by the understanding of the polymer-lipid interactions because the phospholipid bilayer can be modified with the number of entry points of polymer chains into bilayer - low for block polymer and multiple for gradient polymers. The impact can be measured with the size of the liposomes as well as its thermotropic behavior. A reduction in size was observed with a copolymer architecture dependence (compared to DPPC). Gradient copolymers have intuitively low cooperative interaction with phospholipid bilayer regarding the appearance of a pre-transition enthalpy. However, the main transition of lipid membrane increased from 41 (DPPC) to 52 °C

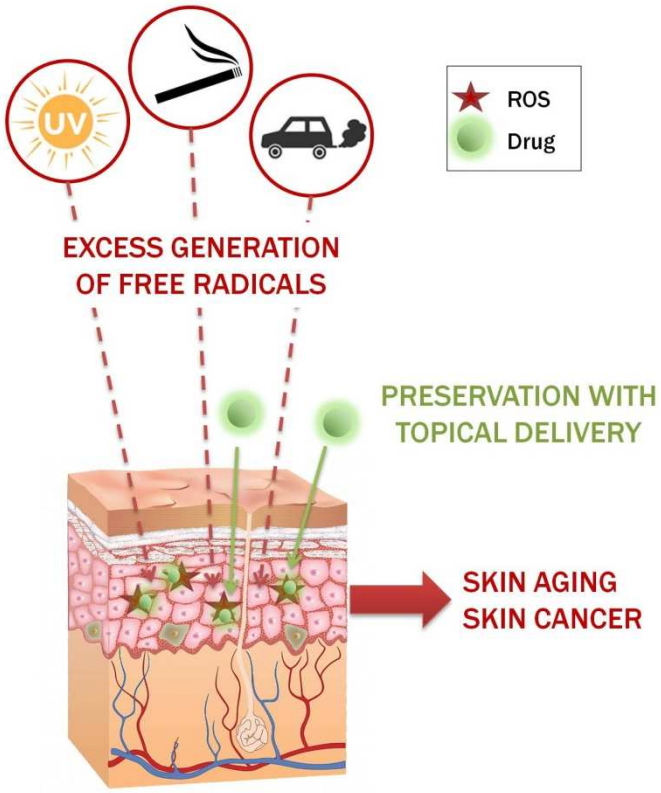
(DPPC/MPOx) and to 54°C (DPPC/PEO-*b*-PCL) and the enthalpy associated with this transition decreased. The conclusions of this detailed work are the key role of the composition and the fractal sculpture of these chimeric systems for designing future nanovectors. Even if this investigation concerns phospholipid with amphiphilic polyoxazoline and not LipoPOx it was very instructive to better understand the behavior of POx in lipid bilayer.

Currently, there is no commercialized nanoformulations with LipoPOx even if Ultroxa company designs and produces high-quality, well-defined, ultra-pure poly(2-oxazoline)s. Moreover, the Serina Therapeutics company investigated a technology for drug delivery based on POx, drug conjugates delivered by injection (Harris et al., 2019). They reach the FDA approval and are currently in clinical trials phase 2 for Parkinson disease treatment (Moreadith et al., 2017). It holds great promises for further nDDs developments based on LipoPOx. LipoPOx were mainly used in formulations for IV administration, no other routes of administration were explored. Consequently, our group was interested in evaluating the potential of LipoPOx for topical delivery of active compounds.

4.2. Opening towards nanoformulations for topical route

To topically deliver a molecule to the epidermis or the dermis, the main barrier to overcome is the *stratum corneum* (SC). It acts as a shield to protect the skin from chemical and mechanical aggressions and prevent from water loss. This role is ensured by its unique structure and composition of a series of corneocytes layers embedded in a lipid matrix that limits drug permeability. In addition, most of the therapeutic molecules such as the BCS IV are poorly soluble and have a low penetration capacity, which further enhances the penetration issue (Roberts et al., 2017) (Scheme 8). Therefore, the main challenge is to design a formulation able to cross the SC without altering its integrity and deliver the drug to the targeted layer (epidermis or dermis). Taking up this challenge to create new skin treatment becomes a

growing concern especially with the increase of the environmental pollutants impacting skin health (Krutmann et al., 2017). Air pollution, UV irradiation, tobacco are over generating reactive oxygen species (ROS) in the skin potentially leading to skin aging and cancer (Baudouin et al., 2002) as represented in Scheme 8.



Scheme 8. Enhanced skin damages requiring effective topical formulation treatment.

Therefore, nDDS of LipoPOx were evaluated as a potential solution to reach a topical delivery in the case of C16:0PMOx15 (Simon et al., 2019). First, the cell viability of the LipoPOx was tested by our group on skin cells, more especially on NiH3T3 mice fibroblasts. After 24 hours, cell viability was impacted (according to the limit of 70% of cells alive, ISO 10993-5) with a concentration of C16:0PMOx15 over 0.2 g/L. Looking at the cellular study on PMOx23 conducted by Bauer et al (Bauer et al., 2013) on L929 mice fibroblasts for which PMOx23 had no impact on cell viability until 20 g/L, it evidenced the impact of the alkyl chain of LipoPOx on cell viability. With the same cell source and close polymerization

degree, the alkyl chain decreased by a factor of 100 the cell viability. Therefore, as highlighted in part 1.1, each LipoPOx has its own properties and behaves differently, and no general conclusion can be drawn before conducting cell viability experiments. However, when our group compared LipoPOx to LipoPEG with the same alkyl chain, LipoPOx had significantly less impact on cell viability than LipoPEG (Simon et al., 2019).

To reach the skin targeted layer, a formulation interacts, enhances permeability and penetrates the SC. Looking at the literature, LipoPOx were proven to have a good interaction with lipid membranes of various compositions (DSPE, SOPC). Used at lipopolymer tethers, the lipid head group of the LipoPOx incorporates into the lipid bilayer by hydrophobic interactions to support phospholipid bilayers (Förtig et al., 2004). The morphology, stability and physical properties (lipid diffusivity, mobile lipid fraction) of the supported lipid membrane can be modulated with the LipoPOx HLB (Lüdtke et al., 2005). Indeed, it is possible to fine-tune the LipoPOx HLB depending on the choice of the monomer, the degree of polymerisation and the lipid head group (Förtig et al., 2004). This first evidences the potential of LipoPOx to incorporate and destabilize a lipid membrane. As the SC is often modeled as a simple lipid bilayer, LipoPOx promise to definitely interact with the skin as well. Moreover, the literature on chemical penetration enhancers (CPE) supports this assumption since it was demonstrated that the alkyl chain of fatty acid improves skin penetration. For example, it has been proven in several studies that fatty acid such as oleic acid is an effective skin penetration enhancer (Kanikkannan et al., 2000). Based on those facts, a LipoPOx with oleic acid as lipid chain should raise hope for skin penetration. In addition, non ionic surfactants composed of lipophilic alkyl chain and hydrophilic head group are known to solubilise lipophilic active ingredients and so they should be able to solubilise lipids with the lipids of SC and enhanced penetration (Williams and Barry, 2012).

Since LipoPOx have never been tested on skin, there is no evidence of enhanced penetration. However as described above, the different works conducted on supported lipid membrane and evaluation of CPE suggest a potential penetration for LipoPOx regarding topical delivery of drugs. Oftenly, lipid based formulations are used for their capacity to enhance penetration thanks to their composition close to that of the SC (Roberts et al., 2017). Therefore lipid based nanoformulations stabilized by LipoPOx were recently developed for topical delivery: mixed-micelles and lipid nanocapsules as represented in Scheme 8. We aimed to conjugate the penetration enhancement of lipid based formulation and the potential effect of LipoPOx for example with the design of LipoPOx mixed-micelles and LipoPOx lipid nanocapsules.

5. Conclusion

Lipopolyoxazolines are readily obtained thanks to an adjustable chemistry of lipids and preprogrammed POx CROP. The broad macromolecular engineering allows to synthesize LipoPOx with fine tuned chemical structure. This high degree of modulation combined with the suitable resulting physico-chemical and biological properties place LipoPOx as a valuable asset for formulation work. Indeed, lipid based nanoformulations such as liposomes, mixed micelles and lipid nanocapsules were designed with LipoPOx and ensured stability, encapsulation of drugs and efficacy. Thus, liposomes were successfully designed to match the requirements for IV delivery with appropriate pH sensitivity and even exceed expectations by overcoming the current main issue known as PEG dilemma. LipoPOx also hold great promise for further skin application as a potential chemical penetration enhancer to improve topical drug delivery.

As a new challenging perspectives, LipoPOx also start helping the design of nDDS either for pulmonary route such as for potential lung cancer treatment (Silva et al., 2017) or for oral administration to improve pharmaceutical applications (Moustafine et al., 2019).

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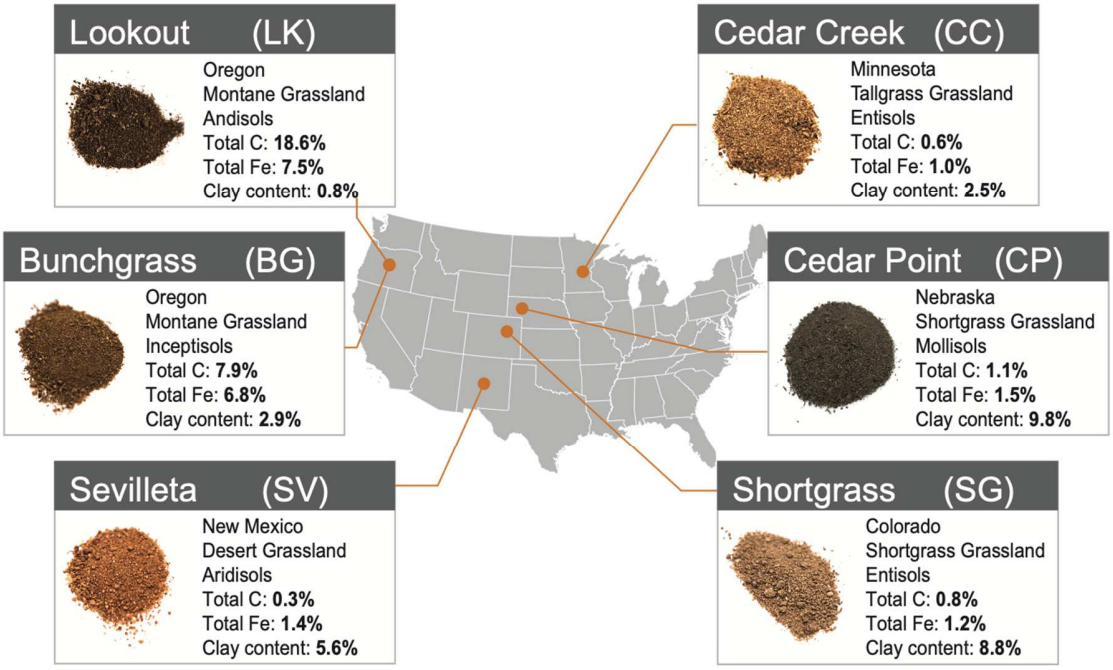

Figure 2: Locations of six study sites from the Nutrient Network project with site information and soil properties.