European Journal of Pharmaceutics and Biopharmaceutics 113 (2017) 211–228

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Review article

Polymeric mixed micelles as nanomedicines: Achievements and perspectives

Maximiliano Cagel [a,c], Fiorella C. Tesan [b], Ezequiel Bernabeu [a,c], Maria J. Salgueiro [b], Marcela B. Zubillaga [b,c], Marcela A. Moretton [a,c], Diego A. Chiappetta [a,c,*]

[a] Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Tecnología Farmacéutica I, Buenos Aires, Argentina
[b] Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Física, Buenos Aires, Argentina
[c] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 18 October 2016
Revised 1 December 2016
Accepted in revised form 4 December 2016
Available online 11 January 2017

Keywords:
Polymeric mixed micelles
Nanomedicines
Pharmaceutical applications
Cancer
HIV/AIDS
Central nervous system diseases
Imaging techniques
Preclinical studies
Clinical trials

ABSTRACT

During the past few decades, polymeric micelles have raised special attention as novel nano-sized drug delivery systems for optimizing the treatment and diagnosis of numerous diseases. These nanocarriers exhibit several *in vitro* and *in vivo* advantages as well as increased stability and solubility to hydrophobic drugs. An interesting approach for optimizing these properties and overcoming some of their disadvantages is the combination of two or more polymers in order to assemble polymeric mixed micelles. This review article gives an overview on the current state of the art of several mixed micellar formulations as nanocarriers for drugs and imaging probes, evaluating their ongoing status (preclinical or clinical stage), with special emphasis on type of copolymers, physicochemical properties, *in vivo* progress achieved so far and toxicity profiles. Besides, the present article presents relevant research outcomes about polymeric mixed micelles as better drug delivery systems, when compared to polymeric pristine micelles. The reported data clearly illustrates the promise of these nanovehicles reaching clinical stages in the near future.

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* Corresponding author at: Departamento de Tecnología Farmacéutica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, 956 Junín St., 6th Floor, Buenos Aires CP1113, Argentina.
E-mail address: diegochiappetta@yahoo.com.ar (D.A. Chiappetta).

http://dx.doi.org/10.1016/j.ejpb.2016.12.019
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1. Introduction

In recent years, one of the most studied nanocarriers in diagnosis and pharmacotherapy of numerous diseases are polymeric micelles (PMs). These interesting vehicles are composed of amphiphilic polymers that self-assemble into nanostructures with sizes ranging between 20 and 200 nm [1–4]. This thermodynamically driven process occurs above a copolymer determined concentration, commonly known as critical micellar concentration (CMC) [5,6]. PMs are formed by an inner hydrophobic core, in which poorly-water soluble-drugs can be entrapped and by an outer hydrophilic shell which insolates the encapsulated drug from the external medium [1–3]. This outer hydrophilic corona can be functionalized with different moieties, such as folate (FOL), monoclonal antibodies (mAb) and monosaccharides (mannose, glucose, fructose), among others, as an attempt to achieve active targeting and/or pH/temperature responsive nanocarriers [7–9].

Over the past few years, PMs have raised special interest as nano-sized drug delivery systems, not only because they provide increased solubility and stability of hydrophobic drugs [10–13], but also due to their *in vivo* exhibited advantages *versus* the free drug [14]. As a consequence of their size, they are large enough to prevent premature elimination via glomerular filtration and sufficiently small to pass through certain blood vessels [4]. Furthermore, they are capable of both (i) improving cellular uptake of drug-loaded micelles and (ii) granting an alternative way of internalization (endosomes). This is of vital importance in several pathologies, where the pharmacotherapy is affected by drug reflux mechanisms related to multi-drug resistance (MDR) [15]. All these advantages are translated in altered pharmacokinetics: longer mean residence time (MRT) of the drug in the bloodstream [14–17]; increased bioavailability [18]; reduced administered dose and possible diminished of non-specific organ toxicity as a result of more precise drug delivery to target tissues [14].

The appropriate application of PMs as nanocarriers for drug delivery requires taking into account several parameters, such as micelle stability, micellar size distribution, drug loading capacity and presence of functionalities [19–22]. Micellar stability mainly depends on the copolymer self-aggregation tendency (CMC value). The CMC of the amphiphilic polymers is influenced by the hydrophilic-lipophilic balance (HLB) of the polymer [21]. In general, maintaining the hydrophilic portion, the longer the hydrophobic chain, the lower the CMC. Micelles-based in copolymers that present low CMC value may resist in a greater extent the dilution suffered when administered intravenously since PMs as dynamic systems. If the micelles disassemble, the drug is rapidly released and toxic effects might appear. On the other hand, micelle stability is also governed by the physical state of its hydrophobic core, the interactions between the lipophilic fractions and their molecular weight [19,22]. This point has been thoroughly detailed by several reviews that provide vast information on the stability of the micelles [10,23,24].

Furthermore, the affinity between the loaded drug and the polymer is one of the most relevant factors that determines the drug loading capacity [6,19], whereas the size and its distribution are affected by the molecular weight of the polymer, the proportion and length of both the lipophilic and hydrophilic segments, the drug loading capacity and the micelle aggregation number [6].

An interesting approach for optimizing these properties and overcoming some of the disadvantages of single micelles, as the dissociation suffered upon dilution [25], is the combination of two or more distinct amphiphilic polymers in order to assemble mixed micelles (Fig. 1) [14]. Ideally, their CMC may be calculated from the CMC values and molar fractions of their components [8]. In comparison to single PMs, the mixed micellar systems exhibit the following advantages: improvements in the thermodynamic (lower CMC) [26] and kinetic stabilities [27] (25), enhanced drug loading capacity [28], more accurate size control [29] and easier ways to incorporate different modifications [30]. In this review, several mixed micellar formulations will be fully analysed, with emphasis on type of copolymers, physicochemical properties, *in vivo* progress achieved so far, as better pharmacokinetic parameters and improved biodistribution and toxicity profiles.

2. Commonly used amphiphilic macromolecules

Among mixed micellar systems, there exist various block copolymers employed as drug delivery vehicles, with distinctive characteristics, which can be classified in different families. Fig. 2 shows the chemical structure of several copolymers and some surfactants that are used for the preparation of mixed micelles.

One of the most studied amphiphilic materials to prepare polymeric mixed micelles are derivatives of poly(ethylene oxide)–poly (propylene oxide)-poly(ethylene oxide) (PEO–PPO–PEO) block copolymers [31]. Among them, there are two commercially available families: (i) the linear and bifunctional PEO–PPO–PEO triblocks or poloxamers (Pluronic®) (Fig. 2A) and (ii) their branched 4-arm counterparts named poloxamine (Tetronic®) (Fig. 2B). The latter presents two tertiary amine groups in the center of the molecule, which contributes to the thermal stability and, more importantly, confers both temperature and pH sensitiveness to the copolymer [32]. Both families are available in a wide range of molecular weights and ethylene oxide/propylene oxide EO/PO ratios. Also, their advantages include low toxicity, suitable biocompatibility and appropriate availability [10]. In contrast, these block copolymers usually exhibit high CMC, making the formulation less stable, as the micelles tend to dissociate easier when diluted upon the bloodstream [33].

Other relevant amphiphilic macromolecules used to self-assemble into polymeric micelles are the ones formed by biodegradable hydrophobic blocks of polyesters covalently bonded to hydrophilic blocks, mainly of polyethylene glycol (PEG). Moreover, these hydrophobic polyesters such as poly(lactic acid)

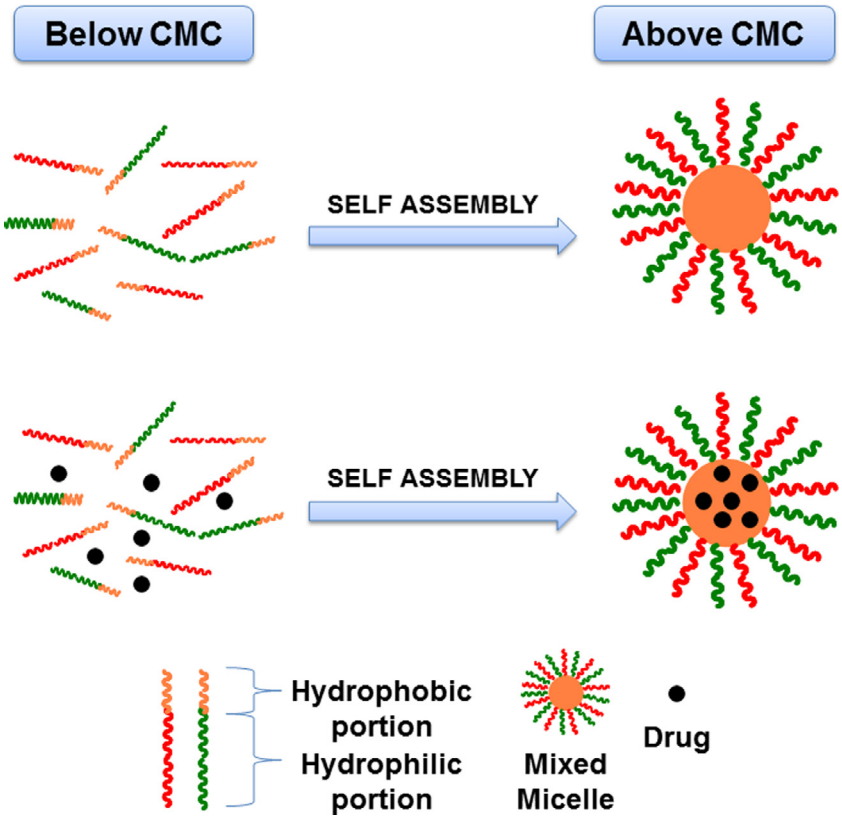

Fig. 1. Representative scheme of the components of polymeric mixed micelles and their self-assembly above the critical micellar concentration (CMC).

(PLA), poly(lactic-*co*-glycolic acid) (PLGA), poly(ε-caprolactone) (PCL) have been approved by the Food and Drug Administration (FDA) for biomedical applications in humans [2] (Fig. 2C–E).

Another interesting family of biodegradable hydrophobic block copolymers that are also covalently bonded to hydrophilic blocks of PEG and used to build polymeric micelles are the ones composed of polyanhydrides (Fig. 2F), such as poly sebacic anhydride (PSA). The hydrophobic polyanhydride segments exhibit the advantages of being biocompatible and degrading *in vivo* into non-toxic diacid compounds that are excreted from the organism as metabolites [34].

On the other hand, other block copolymers that have been employed as nanosized vehicles for numerous drugs, not only because they are biodegradable and biocompatible [35], but also because of their pH-sensitivity and potential as pH-dependent release at tumour sites [36], are the poly(L-amino acid) (PAA) family, which are also covalently bonded to hydrophilic blocks of PEG (Fig. 2G). They include poly(L-histidine) (polyHis), poly(L-aspartic acid) (polyAsp), poly(L-glutamic acid) (polyGlut) and poly(L-lisine) (polyLis). However, it should be taken into account that the PAA portion of the micelle must be either neutrally charged or conjugated to lipophilic fractions [35].

Apart from these, other macromolecules that self-assemble into nanostructured micelles are the PEGylated phospholipid based block copolymer family, such as PEG-distearoylphosphatidyletha nolamine (PEG-PE) (Fig. 2H). In comparison to liposomes, they exhibit the advantages of simpler and more reproducible preparation, avoidance of macrophage phagocytosis system (MPS) uptake, longer circulation times, biocompatibility and almost no toxicity [31].

In the past few years, different biomaterials have been employed to prepare mixed micellar systems, despite the fact that they are not strictly block copolymers. For example, D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) is a PEGylated-vitamin E (Fig. 2I) capable of loading hydrophobic drugs and inhibiting P-glycoprotein (P-gp) [37]. Furthermore, the *in vitro* and *in vivo* cytotoxic activity of TPGS has been reported on different cancer cell lines, as it has been affirmed that it promotes apoptosis [38]. Recently, a newly studied polymer with amphiphilic properties that has successfully solubilized some poorly water-soluble drugs is the polyvinyl caprolactam–polyvinylacetate–polye thylene glycol graft copolymer (Soluplus®) (Fig. 2J) [39,40]. Due to its low CMC value, the micelle forming aqueous dispersions of Soluplus® show high stability upon dilution [39]. Another example of a recently explored polymer used for the preparation of mixed micelles is the case of Solutol® HS 15, a mixture of PEG_{15} mono- and di-esters of 12-hydroxystearic acid and free PEG in the 70/30 wt.% ratio (Fig. 2K) [41]. This polymer has been widely employed in oral and intravenous drug delivery formulations, satisfactorily solubilizing hydrophobic drugs and vitamins [42]. In the past few decades, a class of surfactants referred as surfactant oligomers have been extensively applied in the pharmaceutical industry [43]. They are characterized by lower CMC values than their monomeric conventional counterparts. One of them known as Tyloxapol (4-isooctylpolyoxyethylene phenol formaldehyde polymer) (Fig. 2L) presents numerous biomedical applications and it has been satisfactorily used in the preparation of micellar systems [44]. Finally, biocompatible non-ionic polysorbate and polyoxyethylene ether surfactants, commonly known as Tween® (Fig. 2M) and Brij® (Fig. 2N) respectively, have been utilized in the preparation of micellar formulations [45,46]. The former can effectively solubilize lipophilic drugs and enhance the permeability of phospholipid membranes, improving drug permeation [47], while also the latter present minimum toxicity [48].

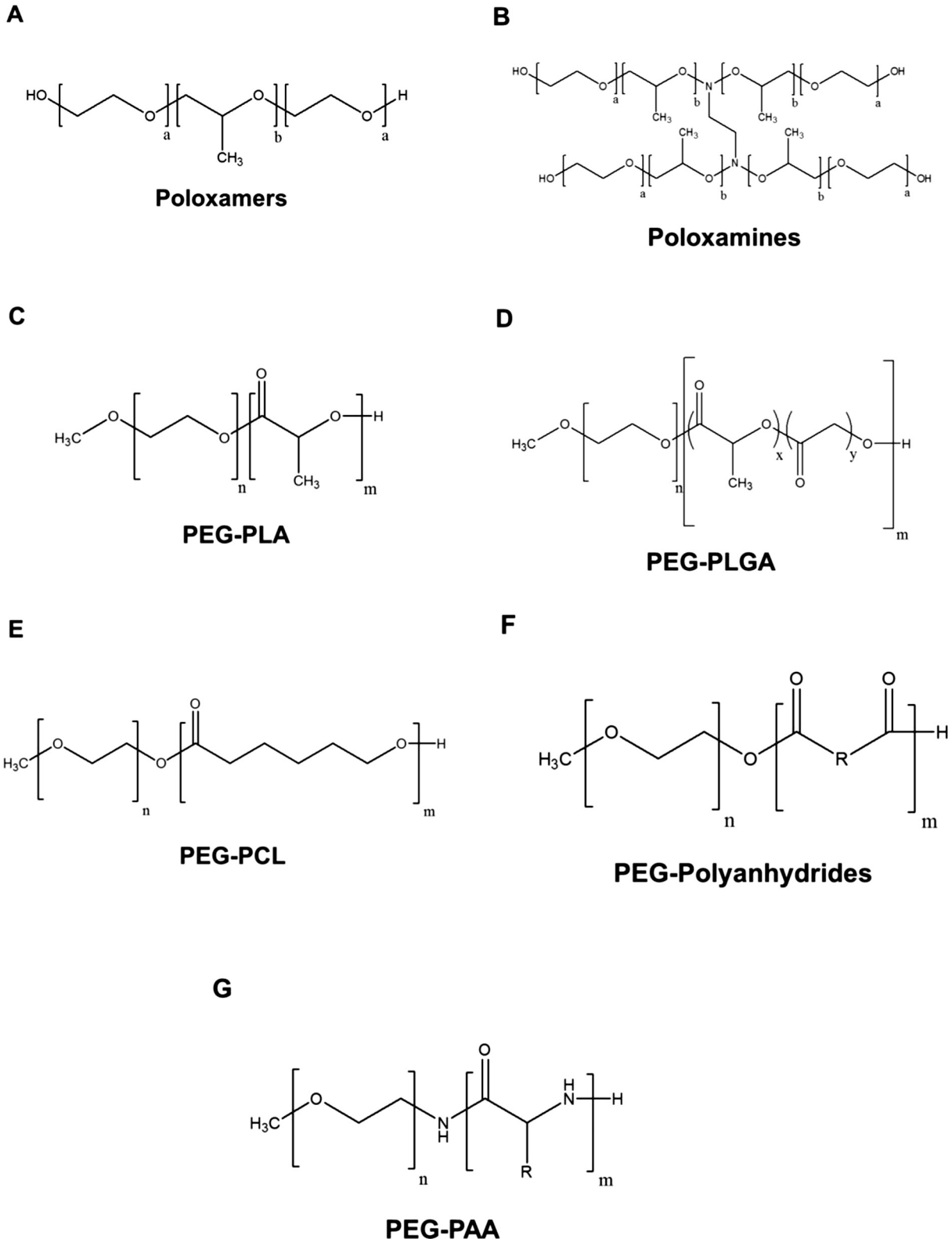

Fig. 2. Commonly used amphiphile macromolecules. (A) Poloxamers. (B) Poloxamines. (C–E) PEG-Polyesters. (F) PEG-Polyanhydrides. (G) PEG-Polyaminoacids. (H) PEG-Phospholipid based. (I) TPGS. (J) Soluplus®. (K) Solutol® HS 15. (L) Tyloxapol. (M) Polysorbates. (N) Polyoxyethylene Alkyl Ethers.

3. Techniques used for mixed micelles preparation

In accordance to the physicochemical characteristics of the polymers, there are two main classes of methods that can be applied to assemble micelles and, therefore, encapsulate drugs [19]. The first one involves the polymer dispersion in an aqueous solvent (Fig. 3A–C), while the drug may be also dispersed with the polymer or it might be dissolved in an organic solvent. In this case, the organic solution can be added dropwise to the copolymer dispersion and left under stirring or a rotary evaporator may be

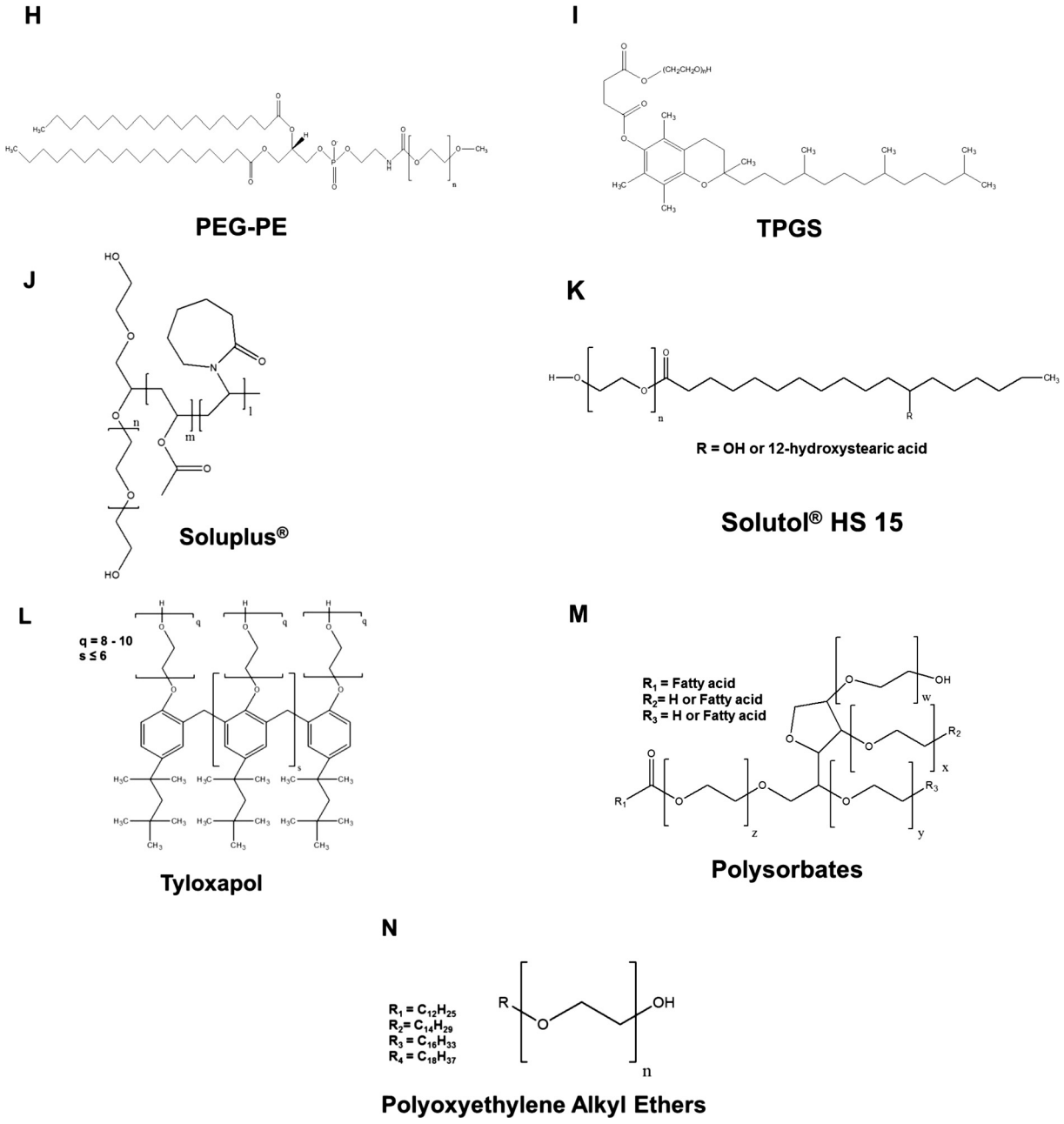

Fig. 2 (*continued*)

used to remove the organic solvent. This technique is usually employed for intermediate hydrophobic polymers, some of which may require heating of the aqueous dispersion and others low temperature (refrigeration) to form the micelles (e.g. poloxamers). For example, our group successfully prepared poloxamer:poloxamine mixed micelles to solubilize the antiretroviral drug efavirenz (EFV), attaining a sharp increment (8400-fold) in the apparent solubility, when adding the drug directly to the dispersed copolymers in aqueous medium [49]. Some of the advantages of this method are its simplicity and the relative short times during the process; the avoidance of organic solvent use, when the polymer is dispersed together with the drug in an aqueous solvent; the ease of scaling-up and the results that show, in some cases, satisfactory percentages of encapsulation efficiency [49]. These advantages are key points for the development of a pharmaceutical formulation. In contrast, one of the disadvantages of this technique is the fact that just a few drugs can be entrapped, obtaining high loading levels.

The second class of micelle formation techniques (Fig. 3D and E) involves the use of organic solvents (e.g. tetrahydrofuran, dimethyl sulfoxide, acetonitrile, methanol, acetone, N,N-dimethylformamide) to dissolve not readily water soluble amphiphilic polymers together with the drug. The mechanism of micelle formation depends primarily on the way in which the solvent is removed. In the case of water-miscible organic solvents, the solution can be dialyzed against deionized water. As the organic phase is removed, drug loaded micelles are assembled. This is known as "dialysis method". For instance, Lee et al. managed to successfully load doxorubicin (DOX) in pH-sensitive polyHis-PEG/poly(L-lactic acid)(PLLA)-PEG mixed micelles applying this technique [50]. Alternatively, the organic phase can be evaporated and a thin film, in which polymer-drug interactions are favoured, is produced.

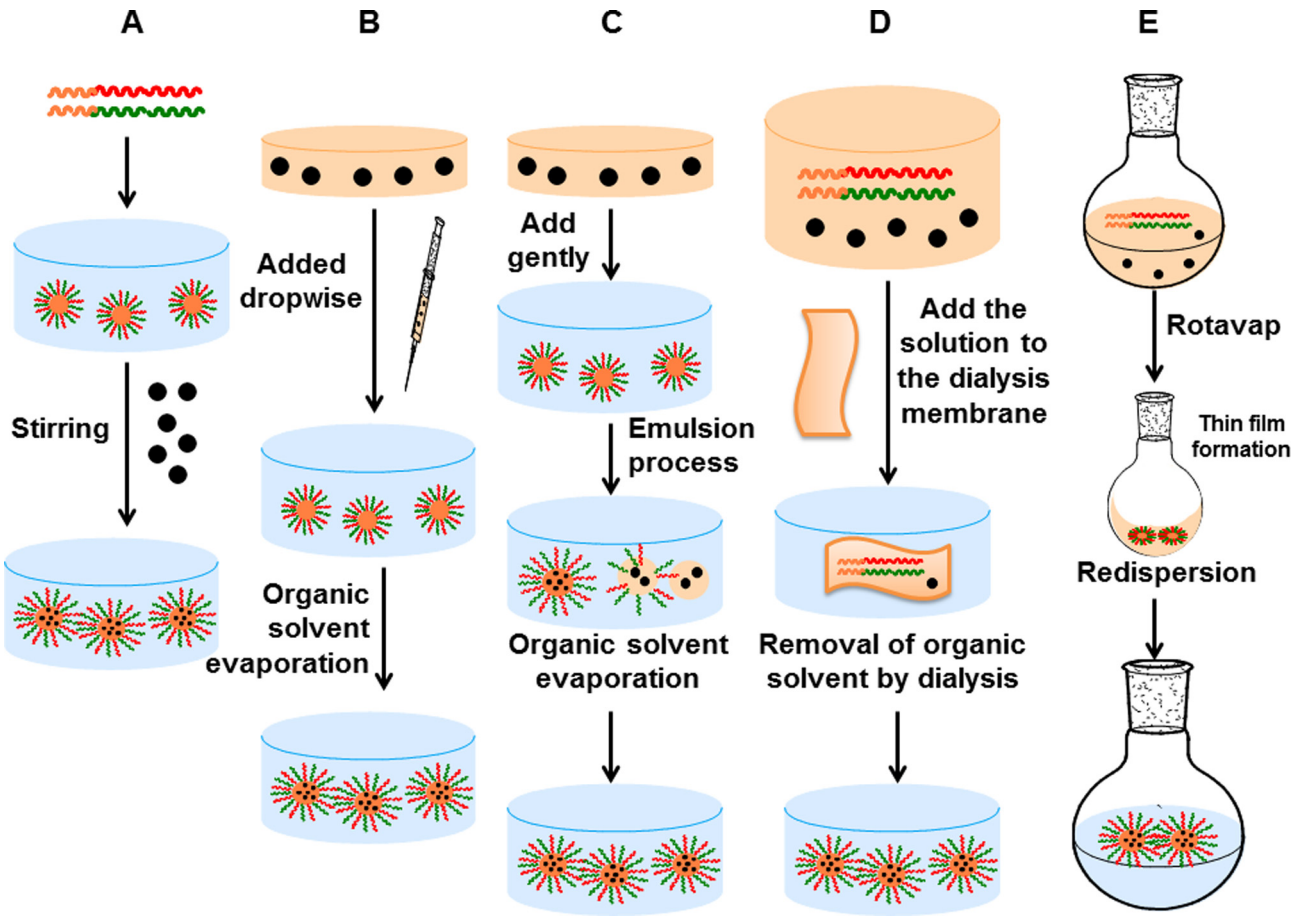

Fig. 3. An illustration of the different techniques to prepare polymeric micelles. (A) Self-assembly method. (B) Solvent evaporation method. (C) Oil in water (O/W) emulsion method. (D) Dialysis method. (E) Film hydration method.

When this film is rehydrated with an aqueous phase, the micelles are formed. This is known as the "thin film method". For example, employing this method, Zhang et al. achieved an important increase (~190.6-fold) in the apparent solubility of baicalin (BAI) using sodium taurocholate/Pluronic® P123 mixed micelles [51]. For water immiscible organic solvents (e.g. dichloromethane, chloroform, ethyl acetate), hydrophobic drugs may be physically entrapped utilizing an oil in water (O/W) emulsion process, usually known as "emulsifying method". Employing this technique, Hu et al. achieved an increase in the solubility of paclitaxel (PTX) by 6.17 times, when loading the drug in PLA-PEO-arginylglycylaspartic acid (RGD)/Pluronic® F68 mixed micelles [52]. It is worth stressing that all these techniques require both sterilization and lyophilization processes to obtain injectable formulations with a suitable stability [35]. Some of the most relevant advantages of these methods are the high drug loading levels achieved and the possibility of an intimate and favoured polymer-drug interaction, as both constituents of the micelles are solubilized in the same phase. On the other hand, the disadvantages that present these techniques are the use of organic solvents, which should be fully removed or, at least, below certain allowed levels; poor feasibility of scaling-up and more steps than with the "direct dissolution method".

Interestingly, a third one-step approach to form micelles was described a few years ago, in which both the drug and the polymer were dissolved in a water/*tert*-butanol (TBA) mixture, followed by the removal of the solvents using a freeze-drying process. The drug loaded micelles assemble spontaneously upon reconstitution of the obtained freeze-dried powder in an acceptable injectable vehicle [53]. To the best of our knowledge, there are no precedents of mixed micellar formulations prepared employing this technique.

4. Physico-chemical characterization of mixed micelles

4.1. CMC

One of the most relevant features to evaluate the stability of different micellar systems is the CMC. This is a fundamental parameter, because micelles usually suffer several environmental variations, such as changes in the pH, ionic strength, exposure to different media with numerous proteins and cells, important dilution upon oral administration or intravenous injection. For these reasons, micelles should remain unharmed as long as possible, to prevent a rapid drug release and to assure its appropriate delivery to the site of action.

The CMC of amphiphilic macromolecules that are generally used in the preparation of micellar formulations can be determined by measuring sharp changes in physical parameters that occur at the CMC, such as surface tension measurements, micellar size and optical clarity of a solution. The most common methods include fluorescence spectroscopy, surface tension measurement, UV–vis spectroscopy and dynamic light scattering (DLS) [49,54–56]. A preferred technique to determine the CMC, due to its high sensitivity, involves the use of fluorogenic dyes, such as pyrene [57]. This is a popular molecule that presents high hydrophobicity and acute sensitivity to the polarity of the surrounding medium [58]. When micelles are formed, pyrene is preferentially parti-

tioned on the hydrophobic core and thus, a CMC value can be determined [59]. Another approach based on dye molecules is the determination of CMC using UV–vis spectroscopy. In this case, hydrophobic dye molecules solubilized in organic solvent are added at low amphiphilie concentrations (below CMC); consequently, no dye partition takes place, due to the absence of micelles. However, as the polymer concentration increases, an augment in the absorbance is observed above the CMC. This sharp change in absorbance values is indicative of CMC [56].

DLS technique provides a sensitive, high-throughput mean to determine the CMC of samples [60]. In DLS measurements, the changes in light intensity are recorded and an abrupt increase in the scattering intensity indicates the formation of micelles [61], which corresponds to the concentration of amphiphiles, where the CMC is reached. This lowest observed concentration is taken as the CMC. Some of the advantages that exhibits the light scattering procedure are the small amount of utilized solution (~1 mL) and the fact that there is no contamination of the probe by dye molecules [62].

Besides, the CMC can also be determined by measuring surface tension, employing a Wilhelmy plate or a Du Noüy ring. In this case, various concentrations of amphiphile are used for measuring their surface tension. An increase in the concentration of amphiphile molecules in the solution produces a decrease in the surface tension, achieving a less pronounced change. This indicates the formation of micelles, being this point indicative of CMC [63]. Despite being one of the most used methodologies, depending on the copolymer, in some cases CMC values may differ when compared, for example, with spectroscopic techniques [64].

In the case of mixed micelles, both experimental and theoretical CMC values can be determined. The theoretical CMC value (CMC^*) for a mixed surfactant system can be calculated using the following equation [65]:

$$1/CMC^* = X_1/CMC_1 + X_2/CMC_2$$

being X_1 and X_2 the molar fractions of the components 1 and 2, and CMC_1 and CMC_2 the experimental CMC values of components 1 and 2, respectively.

The deviation from ideal behavior over the entire range of molar fractions *versus* the experimental behavior is indicative of favorable (negative deviation) and unfavorable (positive deviation) mixing in the system [66]. For example, our group observed a negative deviation from ideal behavior of mixed micelles composed of Pluronic® F127 and Tetronic® T304. Conversely, systems containing the same poloxamer and Tetronic T904 showed a positive deviation [49].

4.2. Cloud point

The cloud point (CP) is the temperature at which a non-ionic surfactant solution presents a cloudy appearance. This phenomenon is the result of the separation into two phases when the system is heated and it is caused by a decrease of the surfactant solubility in water, as the surfactant polar head and water hydrogen bond weaken, when the temperature rises (dehydration) [67]. The explanation given by some authors [68] is that the aggregation number of the micelles increases with the temperature (micelles growing), pointing out the instability of the micellar solution. At high temperature, large aggregates may be formed, exhibiting a cloudy appearance. Taking into account that the CP of a micellar dispersion depends on the interactions between the micelles, it is expected that the clouding behavior of a binary system will be completely different from that of a single micellar formulation [69]. This characteristic behavior was observed for poloxamer:poloxamine mixed micelles in different copolymer ratios [49].

Besides, the CP of a mixed micellar system intended to be intravenously administrated is a very important parameter, as it may give an idea of the growth of the micelles according to temperature and predict whether they will or will be not be able to be properly administered by this route.

Finally, it is important to mention that visual observation is employed for CP measurements [70]. From a practical standpoint, the formulations are placed in glass vials and submerged in an oil bath at room temperature. After that, the system is gradually heated (1 °C/min, approximately) from room temperature until the appearance of the preparation changes from clear to turbid [49].

4.3. Size and size distribution

The size of the micelles is one of their most important features, as an adequate size, depending on the route of administration, indicates a successful performance as drug delivery systems. For example, micelles with small size (10–100 nm) have the capacity to avoid clearance at the kidney and evade their capture at the reticuloendothelial system in the liver and spleen, making them suitable as drug delivery systems and, more particularly, appropriate for intravenous administration [71]. On the other hand, when speaking about an oral administration, it has been observed that nanocarriers with a size lower than 300 nm exhibit the ability to rapidly overcome gastrointestinal mucilliary clearance [72].

Furthermore, there are certain factors that can be utilized to control the size of the micelles, like the type of copolymer, molecular weight and aggregation number, varying from a few nanometers to hundreds [11,73]. In the case of mixed micelles, the size will depend on the type and ratio of their components [49]. In some cases, the presence of a unique population could be indicative of an appropriate mixed micelles formation. This behavior was evidenced by Soluplus®:TPGS mixed micelles (5%w/v) in 4:1 copolymer ratio, presenting a D_h of ~120 nm and a single population [74].

Size and size distribution can be obtained directly by DLS, being this technique the most preferred one to determine the hydrodynamic diameter of the micelles due to its reproducibility, simplicity and speed. This measurement includes the associated solvent molecules in the micellar corona [75]. Atomic force microscopy (AFM) and transmission electron microscopy (TEM) are two alternative techniques to estimate micellar size [76]. Micellar shape and size dispersity can all be characterized by these methods. Size and size distribution are usually affected during sample preparation in the case of TEM and with certain samples [11], as solvent evaporation and shrinkage of the structures occur [77]. Therefore, micelles are perceived to be smaller than they actually are. By contrast, taking into account that DLS includes the solvent molecules in the measurement, the particle size is overestimated.

5. Pharmaceutical applications

5.1. Drug-loaded polymeric mixed micelles in oncology

Nowadays, the use of chemotherapy is increasingly important in cancer therapy. While radiation and surgery are focused over a specific area, antineoplastic agents travel the body and so, can kill cancer cells that have spread from the primary site of origin. In current chemotherapeutic treatments, a large number of the employed drugs present low water solubility [78]. Consequently, many of them should be formulated using high concentrations of solubilizing agents for their intravenous administration. Moreover, some of them are toxic and tend to produce serious side effects. For these reasons, PMs have attracted considerable interest as an efficient means of improving these inconvenients. The present section

details, first, the different mixed micellar formulations pursued to overcome some specific limitations shown by the most common clinical drugs used in cancer therapy and finally, give an overview of the recently studied natural based compounds loaded in polymeric mixed micelles.

5.1.1. Doxorubicin

Among chemotherapy, DOX is one of the most effective antineoplastic drugs against different types of cancer, such as breast, lung, ovarian, hepatic, bladder, stomach, Hodgkin's and non-Hodgkin's lymphoma, certain types of leukaemias and other malignancies. However, it presents several adverse effects, including nausea, vomiting, stomatitis, alopecia, myelosuppression and dose-dependent cardiotoxicity (550 mg/m^2 is the maximum allowed cumulative dose) [79]. Considering the facts that dose limiting toxicities are myelosuppression and cardiotoxicity and that DOX is part of the first-line pharmacotherapy of the previously mentioned types of cancer, several studies have been carried out in order to develop novel micellar nanocarriers with improved physicochemical and pharmacological parameters.

In vitro studies in human colon cancer (Colo 205), human cervix cancer (ME180) and human oral cancer cell lines (Gurav) revealed that DOX loaded micelles composed of polysorbate 80 and sodium deoxycholate were 2- to 10-fold more cytotoxic than free DOX [80]. Similar results were observed using Pluronic® F127/TPGS mixed micelles in breast cancer (MCF-7) and leukaemia cell lines (THP-1), as they were 3.9-fold and 12.2-fold more cytotoxic, respectively [81]. Depending on the applied nanomaterials, these differences in the cytotoxicity are even more significant when the utilized cell lines exhibit certain resistance. DOX-loaded hyaluronic acid (HA)-polyHis/TPGS2K mixed micelles showed a 39.5-fold in the cytotoxicity, in comparison to free DOX, using a resistant breast cancer cell line (MCF-7/ADR) [82].

Alternately, several studies have focused their work on exploring the release profiles of their formulations in search of pH-dependent drug delivery systems for cancer therapy. Lin et al. investigated the influence of pH in the drug releasing behavior of polyGlut-*b*-PPO-*b*-polyGlut and PEG-*b*-PPO mixed micelles. They observed that the drug releasing rates were considerably accelerated when the pH of the solution decreased from 7.0 to 4.0, with an appropriate polyGlut-*b*-PPO-*b*-polyGlut/PEG-*b*-PPO proportion [83]. Coincident results were obtained using DOX-loaded mixed micelles constituted of polyHis-PEG and PLA-PEG. Consequently, this last micellar formulation showed minimal cytotoxicity in an *in vitro* cell viability study at pH 7.4 and a higher cell killing effect at more acidic extracellular pH of tumours (pH 6.6–7.2) [50].

In regards of active targeting, some studies revealed that conjugating FOL groups to the outer corona of mixed micelles using DOX-PLGA-PEG and PLGA-PEG; PLGA-PEG and TPGS; Pluronic® P407 and TPGS, resulted in an increased cellular uptake and/or cytotoxicity, as compared to the unconjugated counterparts [30,84,85]. When combining this strategy with a pH-sensitive copolymer, such as polyHis, the uptake of resistant tumour cells (MCF-7/ADR) was significantly improved, when compared to the micelles lacking polyHis [86].

5.1.2. Paclitaxel

Another first-line antineoplastic agent that has proved excellent therapeutic outcomes against a wide range of tumours, such as breast, ovary, lung, head and neck, is PTX. Nonetheless, this drug presents some physicochemical, pharmacokinetic and pharmacodynamic inconvenients: low aqueous solubility, variable half-life (1.3–8.6 h), high plasma protein binding (higher than 90%) and several side effects, such as neutropenia (dose-limiting toxicity), peripheral neuropathies at doses greater than 200 mg/m^2, vomiting, diarrhea, mucositis, alopecia, paralytic ileus and myalgia [87]. In order to overcome the solubility issue, the first commercial formulation was developed: Taxol®. In this preparation, the drug was dissolved in a 50:50 mixture of Cremophor® and ethanol. Together with the adverse effects of PTX, the volume of this non-ionic surfactant (~26 mL) at the needed doses of the drug may produce serious negative consequences on the patient, such as type I hypersensitivity reactions, neurotoxicity and nephrotoxicity [88]. The use of Cremophor® has also been associated with neutropenia, hyperlipidemia, altered levels of plasmatic high density lipoproteins (HDL), respiratory difficulties and vasodilatation [89]. Taking into account all these issues, several studies have been carried out with the objective of improving some of the physicochemical and pharmacokinetic parameters of PTX.

Our group, for example, has achieved a sharp increase on the solubility of PTX (38,000 times), when it was formulated in mixed micelles prepared with Soluplus® and TPGS (5%w/v) [74]. On the other hand, Hou et al. have reached a higher increase on the solubility of PTX (82,947 times) with a mixture of Soluplus®:Solutol® HS 15, but employing a much higher copolymer concentration (13.3%) [90]. Other works have also increased in a lesser extent (6.17- and 1000-fold) the solubility of the drug, using Pluronic® F68/PLA-PEO-RGD and PLLA/PEG and PDLA/PEG mixed micelles [29,52].

Several studies have focused their work on improving the cell killing effect against different cell lines by comparing their preparations with both commercial formulations and free PTX. Wei and co-workers used Pluronics® F127 and P123, achieving increases of 4 and 17 times in the cytotoxicity of their PTX loaded mixed micelles against human lung carcinoma cells A-549, when compared to Taxol® and free PTX, respectively [91]. Similar results were obtained in human oral epithelial cancer cell line KBv with PTX loaded PEG-PLA/TPGS mixed micelles, as the cytotoxicity resulted 8.6-fold and 30.5-fold lower than that of Genexol® and free PTX, respectively [92]. Besides, when employing pH-sensitive copolymers, as polyHis-PEG/PEG-PE, it was observed that at pH 5.8 (early endosomal compartment) these mixed micelles showed higher cell killing effect (24–56% cell viability) than the same micellar formulation at pH 7.4 (normal tissue; 60–80% cell viability) and at pH 6.8 (tumour extracellular medium; 60–70% cell viability) against murine breast cancer cells 4T1 [93].

Regarding active targeting, most of the studies employed Pluronics® as copolymers (F127, P123, P105 or L121) and FOL as ligand conjugated to the outer corona of the micelles. Cellular uptake was evaluated in MCF-7/ADR cell line and the results showed that PTX loaded FOL-P105/L101 mixed micelles exhibited higher uptake than non-conjugated mixed micelles [28]. *In vitro* cytotoxicity was assayed against MCF-7, KBv and human epitheloid cervix carcinoma HeLa cells, using FOL-P105 and L101; P123 and F127; F127-polyethylenimine (PEI)-FOL and P123 mixed micelles, respectively. In all cases, the micellar formulations presented significantly higher cytotoxic effect than non-conjugated micelles, Taxol® and free PTX, respectively [28,94,95]. *In vivo* studies were also carried out in Sprague Dawley (SD) and Wistar rats and it was observed that the oral bioavailability of the FOL conjugated mixed micelles was about 3 times greater than Taxol® and free PTX, respectively [94,95]. Moreover, in BALB/c mice bearing KBv MDR tumour xenografts, the researchers observed that the volume of tumour treated with the functionalized P123/F127 mixed micelles was 2.1-fold smaller than that of Taxol® [94]. Similar results were obtained using PEG-PE and egg phosphatidylcholine (PC) mixed micelles, covalently modified with the nucleosome specific monoclonal antibody 2C5 and deoxycholic acid-modified chitooligosaccharide (COS-DOCA)/mPEG-poly(D,L-lactic acid) (PDLLA) mixed micelles [96,97].

5.1.3. Docetaxel

Docetaxel (DTX) belongs to the taxane family of drugs and is a semi-synthetic analogue of PTX [98]. It is considered as one of the most important cytostatic agents, as its clinical efficacy was proved against numerous types of cancer, including metastatic breast cancer, advanced gastric cancer, non-small cell lung cancer (NSCLC), among others [99,100]. However, due to its poor aqueous solubility, the commercial formulation (Taxotere®) is prepared with the non-ionic surfactant polysorbate 80 (Tween® 80) and 13% ethanol. Consequently, together with the adverse effects of DTX (neutropenia, neurotoxicity, fluid retention and musculoskeletal toxicity), Taxotere® also presents the negative effects of its excipients (hypersensitivity reactions and fluid retention) [101]. Moreover, as many other chemoterapeutic drugs, it distributes nonspecifically all through the organism and presents the inconvenient of being affected by MDR, limiting its effectiveness [102,103]. Taking into account all these factors, novel nanosystems have been developed in order to improve its drug delivery to the tumour site. *In vivo* anti-tumour efficacy was tested in KBv cells bearing BALB/C nude mice using DTX loaded mPEG-PLA/P85 mixed micelles and a 3.4-fold reduction of the volume of the tumour was observed with the micellar formulation, as compared with Taxotere® [104]. Similar results were obtained in A-549/Taxol cell-bearing BALB/c nude mice with DTX-loaded P105/F127 mixed micelles, as the tumour inhibition rate of the micellar preparation was significantly superior (69%) to that of Taxotere® (34%) [105].

5.1.4. Natural based compounds

In the last years, several investigations have been conducted taking into account the properties of certain bioactive compounds present in different herbal species. These are the cases of curcumin (CUR), the active ingredient extracted from the rhizomes of *Curcuma longa* [106]; BAI, one of the main flavone gucuronides derived from the radix of *Scutellaria baicalensis* [107] and gambogic acid (GA), a natural compound obtained from gamboge resin [108]. Despite not having the approval of the FDA, these compounds have raised particular attention not only for their well-known properties, but especially for their recently demonstrated antitumour effects.

5.1.4.1. Curcumin. CUR is a poorly water-soluble drug with antibacterial, anti-inflammatory and antioxidant properties that can also inhibit proliferation of cancer cells, due to its ability of inducing apoptosis via inhibition of Bcl-2 and Bcl-XL proteins overexpression [109]. Nevertheless, low aqueous solubility of CUR, coupled with its rapid degradation, restricts the clinical use of this drug [110]. Therefore, various micellar formulations have been developed, with the objective of improving the solubility of the drug [111]. For example, Lai et al. have prepared mPEG-PLLA and mPEG-PSA mixed micelles, reaching an increased in the solubility of CUR by 4650-fold (50 μg/mL) [112]. However, the highest increase was achieved employing FOL-PEG_{3000}-PLA_{2000} and $mPEG_{2000}$-PLA_{2000} mixed micelles, as it was about 1,800,000 times the solubility of free CUR (11 ng/mL) [113]. Other recent studies have focused their work on improving cytotoxicity and bioavailability of CUR. For instance, Patil et al. achieved a 3-fold improvement in the cytotoxicity of CUR against a human lung cancer cell line (A549) and a 55-fold enhancement in the oral bioavailability of the drug in male Wistar rats, in comparison to free CUR using F127/Gelucire® 44/14 mixed micelles [114].

5.1.4.2. Baicalin. In the past few years, BAI has been attributed numerous beneficial properties, like antioxidant, anxiolytic, anti-inflammatory and neuroprotective activities [115]. Furthermore, it has been recently demonstrated in numerous studies that it also presents anti-proliferative and anti-apoptotic effects [116,117]. However, the low water-solubility of this drug may hinder its clinical usage. Bearing this in mind, Zhang et al. achieved a sharp increase in the solubility of BAI (from 53.5 μg/mL to 10.2 mg/mL) with their sodium taurocholate/P123 mixed micelles [51]. Later, the same research group confirmed higher absorption rates of this mixed micellar system in an intestinal perfusion experiment carried out in Wistar rats. Moreover, they achieved a 1.5-fold increase in the $AUC_{0\text{-}48}$ and a 1.6-fold augment in the T_{Max} with their mixed micelles, when orally administered and compared to a BAI suspension in male Wistar rats [118].

5.1.4.3. Gambogic acid. In the last years, GA has been described as a potential anticancer candidate, due to its high antineoplastic activity in different types of cancer [119]. With the objective of overcoming some of the mechanisms of multidrug resistance (MDR) in breast cancer, like overexpression of P-glycoprotein (P-gp) and of anti-apoptotic proteins, such as survivin, Wang et al. developed GA loaded PEG-polyHis-PLGA/$TPGS_{1000}$ mixed micelles. This system showed a pH-dependent behavior, as it remained stable at pH 7.4 and rapidly dissociated at a more acidic environment (pH 5.5). Moreover, the mixed micellar system exhibited increased cytotoxicity against DOX-resistant MCF-7/ADR breast cancer cell line, when compared to free GA (12.92% vs 59.85% cell viability). Besides, the mixed micelles down-regulated the expression of two anti-apoptotic proteins (survivin and Bcl-2) and inhibited the expression and the activity of P-gp in MCF-7/ADR cells [120].

5.1.5. Co-encapsulation

Considering the heterogeneity of cancer cells and the appearance of acquired drug resistance, in some types of cancers, single agent chemotherapy results inadequate [121]. In order to achieve a better long-term prognosis, improve the therapeutic outcomes and effectiveness and overcome mechanisms of resistance, a combined therapy is becoming progressively imperative, due to the molecular complexity of this disease [122].

Regarding co-encapsulation of two drugs, PTX and parthenolide (PAL), another antineoplastic agent that induces apoptosis, were co-loaded into PEG_{2000}-DSPE/TPGS mixed micelles and explored their cytotoxicity against sensitive (A549) and resistant (A549-T24) NSCLC cell lines. These *in vitro* studies indicated that their formulation caused higher cellular death than the one caused by the free drugs solutions in the sensitive cell lines and similar results were gathered from the resistant cell lines [123]. PTX was also loaded with CUR in PEG-phosphatidylethanolamine (PE)/Vitamin E (VE) mixed micelles. It was demonstrated that the micellar co-delivery of both drugs achieved a 3-fold decrease in the IC_{50} against the multi-drug resistant version of human ovarian adenocarcinoma SKOV-3 cells, SKOV-3-paclitaxel-resistant (SKOV-3TR), as compared with free PTX. Furthermore, it was confirmed that the inhibition rate of tumour growth of the combined treatment in female nude mice bearing SKOV-3TR tumours was almost 3-fold higher than that of pristine PTX micelles [124]. When PTX was loaded with DOX in P105-DOX conjugate/F127 mixed micelles, the micellar system showed the highest cytotoxicity against MCF-7, MCF-7/ADR, KB and KBv, as compared to single PTX mixed micelles, single DOX mixed micelles and a solution of DOX and PTX. Using MCF-7/ADR tumour-bearing BALB/c nude mice, an *in vivo* tissue distribution assay revealed that DOX was accumulated in the heart in a lesser extent using the PTX and DOX mixed micellar system, in comparison to free DOX and PTX. Additionally, using the same mice, an *in vivo* anti-tumour efficacy assay showed that the inhibition rate of tumour of the mixed micelles was even higher than that of single DOX and single PTX mixed micelles [125].

Some of these systems in which two drugs are transported in the same nanocarrier were also functionalized with different moi-

eties. Actively targeted (anticancer mAb 2C5-modified) PEG_{2000}-PE/VE mixed micelles loaded with PTX and Cyclosporin A (CyA), one of the most effective P-gp inhibitors, were studied exploring their cytotoxicity against Madin-Darby canine kidney (MDCKII) cells and concluding that the 2C5-conjugated mixed micelles showed the highest cytotoxicity, in comparison to other formulations [126]. When PTX was loaded with CUR in transferrin (TF) modified PEG-PE mixed micelles, the results obtained in *in vitro* cytotoxicity assays using monolayers of resistant ovarian cancer cell line (NCI-ADR-RES) were compared to *in vitro* multicellular 3D cancer cell culture (spheroids) and to *in vivo* tumour inhibition studies on nude mice SKOV-3TR bearing tumours. After several observations, they concluded that the cytotoxicity of the TF-modified micelles was enhanced only in the monolayers, while these mixed micellar system had no significant effect neither on the viability against 3D resistant cancer cell spheroid nor on the volumes of the tumours, confirming that there was only a correlation between the spheroid model and the *in vivo* model [127]. Finally, DOX and thioridazine (THZ), which was reported to kill cancer stem cells, were co-loaded in acid-functionalized poly(carbonate)/PEG and urea-functionalized poly(carbonate)/PEG mixed micelles. Cell killing effect assays showed that these mixed micelles were more cytotoxic than free DOX and free THZ against cancer cells and cancer stem cells from BT-474 and MCF-7 human breast cancer cell lines. Moreover, DOX and THZ mixed micelles exhibited a significantly higher anti-tumour activity against nude mice bearing BT-474 xenografts, in comparison to free DOX, free THZ, a solution of DOX and THZ, THZ micelles and DOX micelles, after 16 days [128].

5.2. Antiglaucoma drugs

Antiglaucoma drugs can be orally or topically administered. The former usually exhibit systemic adverse effects, such as depression, gastrointestinal irritation, metabolic acidosis, metallic taste, fatigue, etc., depending on the drug, while the latter frequently present local adverse effects, such as dry eye, burning, stinging sensations, tearing and allergic reactions. An interesting approach used for lowering the occurrence of these local side effects is to reduce the number of instillations, thus improving the ocular tolerance. In order to achieve the greatest therapeutic response with the lowest dose of a particular drug and the least number of adverse effects, nanotechnological strategies, particularly PMs, can be applied to enhance the aqueous solubility of the antiglaucoma agent and therefore, reduce the number of drops.

In this sense, the antiglaucoma agent ethoxzolamide (ETX) was encapsulated in single and mixed Tetronic® micelles for topical ocular application. It was found that Tetronic® T904 micelles exhibited a 50-fold increase in the apparent solubility of the drug and that combining T904 with a more hydrophilic polymer, for example T1107 or T1307, led to mixed micelles with an improved solubility, as compared with T1107 or T1307 single micelles. Besides, it was observed that the mixed micelles presented greater stability, retaining almost 100% of drug solubilized after 28 days, than single micelles [129].

5.3. Immunomodulatory drugs

As in the case of cancer therapy, many immunomodulatory drugs exhibit low aqueous solubility and, hence, their commercially available formulations utilize the same non-aqueous cosolvents, entailing identical adverse effects. With the aim of increasing their aqueous solubility and avoiding the usage of these cosolvents, PMs are applied as a potential and novel alternative as drug delivery systems.

On this subject, Lee et al. replaced the toxic solubilizing agent of a commercially available intravenous dosage form of CyA, by formulating dipalmitoyl phosphatidylcholine/phosphatidic acid (DPPC/PA) liposomes and a mixed micellar preparation composed of dimyristoyl phosphatidylcholine (DMPC)/PEG-PE. These mixed micelles showed a 2-fold increase in the drug's solubility, as compared to the liposomal formulation. Moreover, the mixed micelles presented similar pharmacokinetic parameters in male Sprague Dawley rats, as compared to the commercial formulation. Therefore, they concluded that these mixed micelles could be useful as an acceptable intravenous dosage form, replacing the commercial formulation, with the toxic solubilizing agent [130].

5.4. Antibiotics

Together with side effects, one of the most concerning issues about antibiotics is the resistance developed by microorganisms. Among others, the misuse of antibacterial agents (used when they are not needed, wrong prescribed or dispensed drug, insufficient doses) threatens our ability to treat common infectious diseases, resulting in an increase in the number of disabilities and deaths all over the world. What is more, a great number of antibiotics belong to classes III and IV of the biopharmaceutical classification system (BCS), negatively affecting their therapeutic efficacy. To overcome this, PMs have emerged as a promising alternative as drug delivery systems.

In this regard, amphotericin B was loaded into (AmB) PEG_{5000}-*b*-PCL/PEG_{5000}-PE mixed micelles and a solubility of almost 0.2 mg/mL was achieved [131]. For their part, Mehta et al. fully explored anti-tuberculosis drugs (ATDs) loaded lecithin/tyloxapol mixed micelles employing physicochemical and spectroscopic measurements. They obtained high encapsulation efficiency (EE) for the tested ATDs: 97.8, 93.4 and 96.6%EE for rifampicin (RIF), isoniazid (INH) and pyrazinamide (PZA), respectively. Additionally, *in vitro* drug release assays, revealed that INH and PZA were rapidly released from the micelles at the beginning, followed by a plateau during the second phase of the release profile (87.2% of INH and 88.5% of PZA were released within the first 5 h). However, RIF presented a slender initial burst in first phase and a faster release rate during the second phase of the kinetic profile (74.5% of RIF was released in the first 5 h) [132].

5.5. Antiviral drugs

Most of the antiviral drugs used against viral diseases present drawbacks on their solubility, permeability and stability. Unfortunately, these characteristics may vary certain pharmacokinetic parameters, such as drug absorption and biodistribution, resulting in viral resistance to these drugs and, hence, a poor clinical outcome [133].

Taking into account the above, we studied the synergistic performance of EFV loaded Pluronic® F127/Tetronic® T304 and Pluronic® F127/Tetronic® T904 mixed micelles. Our results showed that the solubility of this widely used anti-HIV drug was remarkably augmented from 4 μg/mL to more than 30 mg/mL (8430-fold increase). In addition, we confirmed that drug-loaded mixed micelles exhibited higher physical stability than pure poloxamine ones, after being monitored at 25 °C over 28 days [49]. Furthermore, we carried on analyzing the pharmacokinetic parameters, including drug-loaded T904 single micelles and F127/T904 mixed micelles, taking advantage of the capacity of T904 to load such amount of drug and the physical stability granted by F127 [134]. Continuing with the investigation on EFV loaded F127/T904 mixed micelles, we aimed for intranasal administration of the first line anti-HIV drug for anatomical targeting to the central nervous system (CNS). Using male Wistar rats, we reached a 1.4-fold and 2.1-

fold increase in the $AUC_{CNS\ 0\text{-}2}$ with the mixed micelles, as compared with drug-loaded F127 single micelles, after intranasal administration, with an EFV concentration of 20 mg/mL and 30 mg/mL, respectively [135].

5.6. Analgesic drugs

The main disadvantages of this family of drugs are the relative short plasma half-life and the fact that many of them exhibit very low water solubility. This, coupled with their respective adverse effects, make these kinds of drugs a perfect candidate to develop topical formulations or systems allowing a controlled release, useful in high dose-dependent treatments and chronic diseases, such as rheumatoid arthritis. Bearing this in mind, PMs arise as potential drug delivery systems for their application in this biomedical field.

On this matter, the solubilization of naproxen (NAP) was tested and compared by micellar dispersions using single and mixed surfactants preparations. Non-ionic (Brij 35, Brij 56 and Brij 58), cationic (CTAB, DTAB and TTAB) and anionic surfactants (SDS and SDBS) were utilized. After quantifying the solubilization capacity of each system with the molar solubilization ratio (MSR = moles of solute dissolved per mole of surfactant), the research group noticed that cationic surfactants showed greater solubilization capacity than non-ionic and anionic ones and that when the chain length increased, the MSR augmented as well. When mixing these surfactants, it was observed that equimolar cationic-non-ionic combinations exhibited the highest values of MSR (0.234 for Brij58-CTAB and 0.193 for Brij35-CTAB) [136]. Working with a different analgesic, Kulthe et al. studied aceclofenac (ACL) loaded Pluronic® L81/P123 mixed micelles. They achieved an ACL concentration of 4.70 mg/mL with the formulation containing 0.5% wt. of L81 and 0.3% wt. of P123 and found that this preparation remained clear for more than 4 weeks [137]. Aiming for something different, Chopra et al. investigated whether dexamethasone (DEX) loaded sodium taurocholate/egg lecithin mixed micelles were able to promote a sustained drug delivery in transscleral iontophoresis *in vitro*. Their results showed that less than 20% of the drug was released within around 2 h from the sclera after cathodal iontophoretic delivery from the mixed micelles, while more than 50% of indomethacin (IDM) was released from the control during the same period of time under the same conditions [138].

5.7. Anesthetic drugs

Certain drugs of this family tend to present high lipophilicity and, thus, a low water solubility or miscibility, depending on their physical state. This drawback was initially overcome by developing formulations based on the same harmful organic cosolvents as some antineoplasic agents. Later, anesthetics were emulsified in oil/water preparations consisting of soya bean oil, glycerol, and egg phosphatide. However, emulsions also exhibit various limitations, such as poor physical stability and the potential of embolism. Therefore, PMs appear as potential nanocarriers for the solubilization of poorly water-soluble drugs.

In this regard, propofol (PPF) was loaded into mPEG-PLA/Solutol® HS15 mixed micelles. After the optimization of the micelle composition and its characterization, this research group managed to reduce the concentration of free PPF in the aqueous phase of the system as much as 3-fold lower than that of the commercial lipid emulsion (less than 5 μg/mL vs more than 15 μg/mL, respectively) with 1% of PPF in each formulation. Furthermore, using male SD rats, they evaluated the mean time values of loss (around 0.5 min for both formulations) and recovery (7.17 min for the mixed micelles and 7.29 min for the commercial lipid emulsion) of righting reflex and concluded that there was no significant difference in these values [139].

5.8. Drugs used for CNS diseases

From the whole list of therapeutic agents used in CNS disorders and diseases, a considerable number of these drugs present numerous disadvantages, such as significant first-pass metabolism, low water solubility, irregular and delayed or reduced absorption and gastric instability, limiting oral bioavailability and, possibly, their therapeutic performance. Among all the explored nanotechnological approaches (nanoparticles, microemulsions, liposomes, etc.), PMs have become an interesting strategy for oral delivery of insoluble and/or instable drugs.

On this field, El-Dahmy et al. formulated mixed micelles with Pluronics® L121, F127 and P123 to encapsulate vinpocetine (VPC). After the optimization of the systems, they chose the preparation with L121 and F127 to fully characterize it and employ the mixed micelles to perform *in vivo* pharmacokinetic studies in New Zealand male rabbits. The research group observed in these studies that the drug-loaded micelles presented a 1.8-fold increase in the MRT, when compared to the market product [140]. Using a different drug, Huang and co-workers loaded nimodipine (NMP) in mPEG-PLA micelles and in mPEG-PLA/TPGS mixed micelles to explore their pharmacokinetic parameters and compare them with a nimodipine solution. After intraperitoneal administration to twenty SD rats, the authors affirmed that the $AUC_{0\text{-}t}$ and the MRT were higher and the clearance was lower in both micellar formulations (47.2 and 48.2 mg/mL/min; 49.7 and 49.1 min; 77.7 and 65.6 mg/min/kg, for the single and mixed micelles, respectively) than in the drug solution (42.8 mg/mL/min; 45.3 min 81.7 mg/min/kg) [141]. Similar results were obtained employing NMP loaded PC/F127/P123/Polysorbate 80 mixed micelles, as their $AUC_{0\text{-}\infty}$ in plasma and in brain resulted 3.2-fold and 2.0-fold higher than those of free NMP [142]. For their part, Zhang et al. demonstrated that stiripentol (STP) loaded mPEG-*b*-PCL/sodium oleate mixed micelles improved the drug's solubility by almost 203.4-fold and its stability in acidic medium, as compared to free STP (pH 1.0). Moreover, they carried out pharmacokinetic studies in SD rats and reported a 1.56-fold and 4.44-fold increase in the $AUC_{0\text{-}t}$, in comparison to the commercial formulation (Diacomit®) and a STP suspension, respectively [143]. Applying a different strategy, Abdelbary et al. prepared olanzapine (OZ) entrapped Pluronic® L81/P123 mixed micelles for intranasal delivery. An *ex vivo* estimation of nasal toxicity performed in sheep mucosa revealed that no apparent histopathological changes were observed in the pseudostratified epithelium or in the underlying tissues, when treated with the micellar formulation, in comparison to a negative control (PBS) and a positive one (isopropyl alcohol). Further, the research group found that the C_{max}, MRT and $AUC_{0\text{-}\infty}$ were significantly increased by 1.4-, 3.2- and 2-fold with the micellar system, as compared to an OZ solution, when following intranasal administration to male albino Wister rats [144].

Regarding co-encapsulation of two drugs, carbamazepine (CBZ) and nifedipine (NFD) were co-loaded in sodium cholate binary or ternary micellar systems with non-ionic polysorbate (Tween 20 and Tween 40) and polyoxyethylene surfactants (Brij 30, Brij 35, Brij 56 and Brij 58). The research group characterized all the formulations in terms of solubility, and drug-surfactant and drug-drug interactions. They concluded that, when co-entrapping CBZ and NFD, the solubility of the first decreased down to 40.6%, in the case of Brij 46/Tween 40/sodium cholate mixed micelles, as compared to the same micelles loaded only with CBZ. Nevertheless, the solubility of NFD increased up to 6.41-fold for the CBZ/NFD co-encapsulated Tween 20/sodium cholate mixed micelles, when compared to the same NFD loaded micelles [145].

5.9. Biopharmaceuticals

In the past few years, protein-based therapies emerged as a potential strategy for improving the treatment of numerous human diseases, as many of them remain incurable by small molecule drugs [146]. However, the *in vivo* pharmacokinetic parameters of therapeutic proteins are significantly affected by enzymatic proteolysis, denaturation and their inability to penetrate cell membranes, thus hindering their therapeutic efficacy [147]. Therefore, PMs appear as an interesting kind of drug delivery system for enhancing the physical and chemical stabilities of therapeutic proteins and improving their pharmacokinetic properties.

Several studies have focused their work on increasing the mean residence time of the therapeutic protein in the organism. For example, Lee et al. developed a pristine micellar system composed of exendin-4, a potent agonist of GLP-1 receptor, modified with palmitic acid, achieving an 11.1-fold increase in the t1/2 of the protein, in comparison to non-modified exendin-4 [148]. For their part, Harada and co-workers partially substituted PEG-polyglutamate block co-polymers with octyl or benzyl groups to encapsulate granulocyte colony-stimulating factor (G-CSF) in these micelles and achieved an almost 7-fold increase in the t1/2 of the protein [149]. Other investigations aimed to improve the targeting efficiency of the protein to a specific tissue. For instance, Weissig et al. found that their single micellar formulation comprised of PEG-DSPE and soybean trypsin inhibitor (STI) modified with N-glutaryl-phosphatidyl-ethanolamine (NGPE), delivered the model protein more efficiently to the targeted tissue than PEG-DSPE liposomes, in a Lewis lung carcinoma mouse model [150]. Finally, some other works intended to design a drug delivery system for an alternative and non-invasive route of administration, different from intravenous. In this regard, Andrade and co-workers developed and extensively characterized powders for pulmonary administration of insulin-based pristine micelles composed by Soluplus®, Pluronic® F68, F108 and F127 [151]. Moreover, the same research group continued their work studying the efficacy and safety of these systems *in vivo* and the aerosolization profile of the powders through *in vitro* deposition simulation [152]. Another example on this matter is reported by Xiang et al., as they investigated the feasibility of employing PLA-F127-PLA single micelles for oral delivery of insulin. The results indicated that their micellar formulation was able to maintain the blood glucose concentration at about 4.5 mmol/L for at least 18.5 h, while a subcutaneous injection of free insulin returned to the initial glucose level after about 10 h [153].

In spite of the numerous works on the applications of PMs to therapeutic proteins, as far as we are concerned, there are no studies on mixed micellar systems and biopharmaceuticals. In this framework, there exists an enormous field to be exploited.

6. Mucoadhesion (non-parenteral administration)

Non-parenteral mucosal administration of therapeutic agents is usually hindered by the mucus layer that acts as an efficient barrier, immobilizing and removing conventional drug delivery systems by mucus clearance mechanisms [154]. In the past few years, mucoadhesive PMs have gained special attention as potential nanocarriers for the topical delivery of drugs in mucosal surfaces (e.g. eyes, pulmonary, gastrointestinal and female reproductive tracts). This point has been thoroughly detailed by some reviews that provide vast information of mucoadhesive PMs [155,156]. In this regard, several co-polymers have been synthesized with natural or synthetic polymers in order to develop mucoadhesive single micellar formulations. Songsurang et al. designed and characterized a hydrophobic cationic aminocellulose micellar preparation loaded with the antineoplasic agent camptothecin (CMP) for oral delivery [157]. Using the same drug, Barreiro Iglesias et al. conjugated Pluronic® F127 and L92 to terminal blocks of poly (acrylic acid) (PACR) to increase the solubility and stability of CMP [158]. Employing similar biomaterials, Eshel-Green and Bianco-Peled fabricated single micelles of Pluronic® F127 with acrylate end groups loaded with IDM to mucus covered tissues [159]. For their part, Yang et al. prepared PEG-PLLA micelles modified with mucus cysteine binding dithiol terminal groups for DEX ocular delivery [160]. Aiming also for ocular administration, Prosperi-Porta et al. designed CyA loaded PLLA-*b*-poly (methacrylic acid-co-3-acrylamidophenylboronic acid) block copolymer micelles [161]. Dufresne et al. developed PEG-block-poly(2-(N,N-dimethylamino)ethyl methacrylate) copolymer conjugated to a thiol group at the end of the PEG chain to improve the mucoadhesion of the system and to form pH-sensitive micelles as a depot formulation [162]. Another biomaterial that has been extensively employed to synthesize mucoadhesive co-polymers is the polysaccharide chitosan (CS), due to its relatively low cost, adequate biocompatibility and biodegradability [163]. For instance, CS-stearic acid pristine micelles were reported to enhance pulmonary delivery of AmB [164], while acetylcysteine functionalized CS-TPGS single micelles were employed for enhancing PTX oral delivery [165]. However, to the best of our knowledge, there are no studies on mucoadhesive mixed micellar systems. In this context, mucoadhesive co-polymers appear as a promising, though underexploited resource to expand the applicability of mixed micelles in non-parenteral mucosal administration.

7. Applications in imaging techniques

Imaging techniques arise as complementary tools to asses new drug delivery systems [166]. Tests of biological or *in vivo* nature can be performed in a non invasive way, providing information that would not be available by other techniques. The main goals of this approach are usually to dilucidate pharmacokinetic parameters, biodistribution and uptake in specific organs [167].

Particularly polymeric mixed micelles can be evaluated using imaging techniques by building a micellar system that carries (i) a signal emitting compound and thus it can mimic biodistribution and uptake mechanism of drug loaded micelles; (ii) a contrast agent in order to reduce its toxicity; (iii) a signal emitting compound or labeled in a way that constitutes itself an imaging probe suitable for diagnostics; (iv) a therapeutic drug and a signal emitting compound in a way that constitutes itself an imaging probe and a therapeutic agent (theranostic). Considering this, near infrared (NIR) dyes can be encapsulated in polymeric micelles as a strategy to visualize micellar *in vivo* biodistribution or to elucidate uptake mechanisms. In this sense, Kim et al. demonstrated the ability of PEO-poly[(R)-3-hydroxybutyrate](PHB)-PEO and Pluronic® F127 mixed micelles to encapsulate hydrophobic drugs for tumour-delivery applications [168] by means of noninvasive optical imaging. Micelles were comprised of PEO-PHB-PEO and F127 encapsulating the hydrophobic NIR fluorophore indocyanine (IND) as the imaging probe. Thus, authors compared the efficiency of different formulations for tumour accumulation as well as the uptake of other organs and clearance from blood. With a similar approach, Qiu et al. developed a pH-sensitive mixed micelles composed of HA-polyHis and TPGS 2000 copolymers to co-deliver DOX and TPGS 2000 in order to overcome drug resistance mediated by P-glycoprotein (Pgp). In this way, the authors evaluated the tumour targeting efficiency by using a NIR probe for optical imaging to perform successive studies, at different time points, in a drug resistant tumour composed by MCF-7/ADR human cell line. This technique allowed them to confirm that their mixed micelles could

be considered a highly efficient carrier to overcome MDR [169]. Moreover optical imaging can be useful to investigate which variables affect micellar biodistribution that can be related with their applications as drug delivery carriers. For example, Zhang et al. studied the effect of chemically substituted polyHis with 2,4-dinitrophenol (DNP) on the physiochemical and biological properties of the polyHis based micelles by assessing the biodistribution of the triblock copolymer of mPEG-*b*-PLA-*b*-DNP-polyHis using NIR optical imaging. In this case, results indicated that the mPEG-*b*-PLA-*b*-DNP-polyHis micelles showed a reduced passive targeting to the tumour, due to their larger particle size, compared with mPEG-*b*-PLA-*b*-PHis [170]. Similarly Arranja et al. used another imaging strategy to evaluate the influence of PEO block length and aggregation state in the pharmacokinetics and biodistribution of Pluronic® nanocarries composed of PEO-PPO-PEO. In this case the imaging technique involved the use of single photon emission computed tomography (SPECT) to asses *in vivo* biodistribution and pharmacokinetics of ^{111}In-Pluronic® nanocarriers [171]. Similarly, the group of Hoang et al. demonstrated the high sensitivity and high resolution of microSPECT/computed tomography (CT) imaging for tracking the *in vivo* pathway and fate of ^{111}In-labeled amphiphilic diblock copolymer micelle formulation composed of diethylene-triaminepentaacetic acid (DTPA)-PEG-*b*-PCL. Moreover, authors remarked that the integration of advances in imaging and this delivery technology provided unique insight into the intratumoural distribution of the micelles *in vivo* [172].

For diagnostic purposes, mostly in magnetic resonance imaging (MRI) and CT modalities, contrast agents and metals can be chelated and delivered by micellar systems as well. Trubetskoy et al. aimed to obtain small polymer-stabilized particulate carriers for organic iodine, to serve as a contrast agent for CT. For this purpose, they designed a carrier based on polymeric micelles (two blocks of the copolymer of mPEG and poly[ε,N-(triiodobenzoyi)-L-lysine] (poly-3I-Lys) and discussed their results performed in rabbits and the possible use of those particulates as contrast medium for computed tomography [173]. With regard to MRI, Anelli et al. reported physicochemical and pharmacokinetic properties of different formulations of mixed micelles prepared with lipophilic gadolinium complexes and postulated their potential as MRI contrast agents. Authors performed biodistribution and metabolic studies with radioactive ^{153}Gd and evaluated haemolytic effect in rats and also magnetic resonance coronary angiography (MRCA) in pigs concluding that the composition of the gadolinium complex affected the MRI studies results and the micelles polymer composition affected biodistribution and ^{153}Gd metabolism [174].

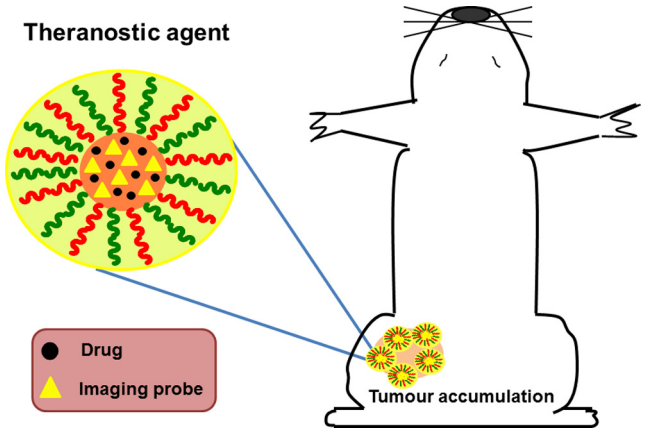

Fig. 4. Schematic representation of a drug-loaded mixed micellar system that carries a signal emitting compound, constituting itself an imaging probe and a therapeutic agent (theranostic), selectively accumulated in a solid tumour.

Multifunctional micelles have the potential to act as true theranostic agents given that they can carry a therapeutic drug and simultaneously a signal that can be detected from imaging devices, with the aim of performing a diagnosis or an evaluation of therapeutic performance (Fig. 4). A type of multifunctional micelle for DOX active delivery was constructed by Tsai et al. consisting of mPEG-*b*-PLA, Cy5.5–PEG–PLA, FOL–PEG–PLA and poly(2-hydroxyethyl methacrylate) (poly(HEMA)-co-His-g-PLA as a polymeric carrier for tumour targeting delivery. Authors used *in vivo* optical imaging to determine micelles biodistribution and tumour accumulation. For therapeutic efficacy tumour size was measured as well. Results revealed the active targeting of FOL carrying micelles by non invasive imaging and that a sufficient amount of DOX reached the tumour in order to achieve a therapeutic effect [175].

One of the most interesting examples in imaging applications is constituted by micelles carrying a photosensitizer that allows conducting photodynamic therapy combined with NIR dyes [176,177]. Briefly, Yang et al. studied copolymers of mPEG and alkylamine-grafted polyAsp assembled with carbocyanine dyes into theranostic micelles. According to the authors, these theranostic micelles presented small size (around 40 nm), high loading capacity (20% w/w), a sustained release profile and enhanced cellular uptake [178].

Table 1
Current clinical status of single and mixed micellar formulations.

Type of micelle	Drug	Biomaterials	Size (nm)	Name	Indications	Current phase	Ref.
Single	DOX	PEG-*b*-poly(α,β-aspartic acid)	40	NK911	Metastatic pancreatic cancer	II	[180]
	PTX	PEG-*b*-poly(aspartate-4-phenyl-1-butanolate)	85	NK105	Gastric carcinoma	II	[181]
					Metastatic or recurrent breast cancer	III	[182]
	SN-38 (metabolite of irinotecan)	PEG-*b*-poly(l-glutamic acid)	20	NK012	Metastatic colorectal cancer	I	[183]
					Metastatic triple negative breast cancer	II	[184]
					Relapsed small cell lung cancer	II	[185]
	Epirubicin (EPI)	PEG-*b*-poly(aspartate-hydrazone)	60–70	NC 6300	Hepatic tumour	I	[186]
	Cisplatin (CIS)	PEG-*b*-poly(l-glutamic acid)	28	NC 6004	Pancreatic cancer	III	[187]
					Advanced solid tumours or non-small cell lung cancer	I/II	[188]
	(*Trans*-l-1,2-diaminocyclohexane)platinum (II) (DACHPt)	PEG-*b*-poly(l-glutamic acid)	40	NC 4016	Advanced solid tumours or lymphoma	I	[189]
Mixed	DOX	Pluronic® L61 and F127	22	SP1049C	Esophagus or gastroesophageal junction (GEJ) adenocarcinoma	II (finished)/III (under FDA Special Protocol Assessment)	[190,191]

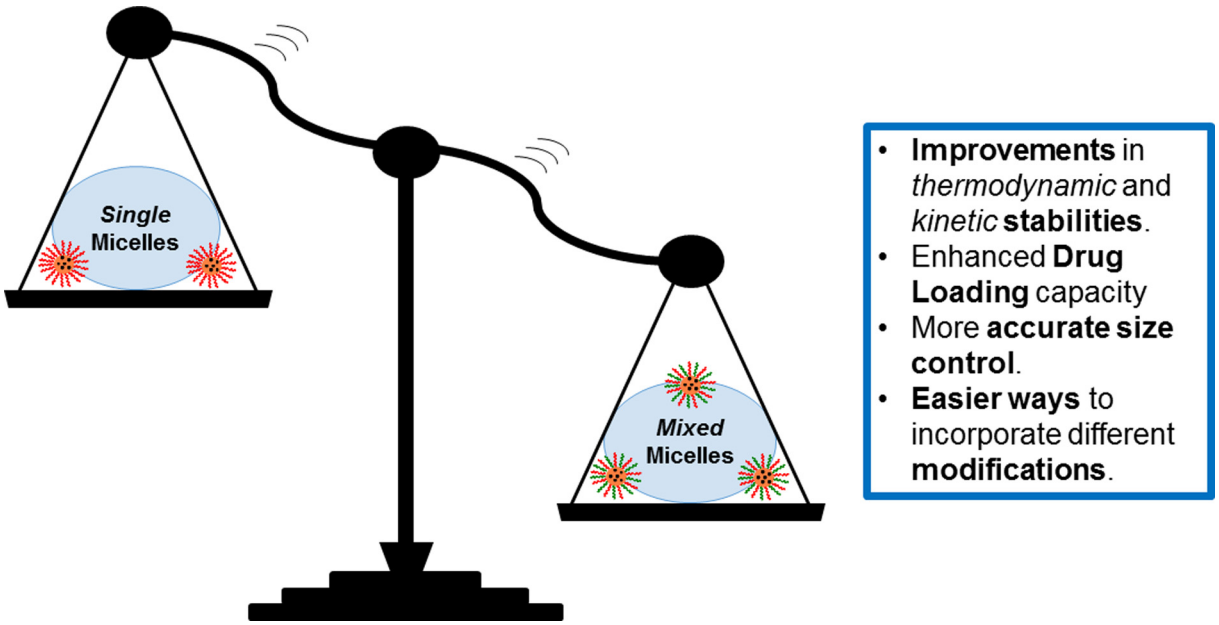

Fig. 5. Schematic representation of the advantages of polymeric mixed micelles in comparison to polymeric single micelles.

Imaging techniques include a wide variety of strategies that are useful in the pipeline of development of new drug delivery systems. Particularly mixed micelles research has started to make use of them since drug loaded micelles can constitute themselves a theranostic or diagnostic agent, which in addition is susceptible to be labeled, and therefore they can be tracked in living organisms to perform biological tests (preclinical phase of pharmaceutical research).

8. Mixed micelles in clinical trials

As shown in Table 1, there are numerous polymeric single micellar formulations in different phases of clinical studies. However, to the date, there is only one micellar system that has been approved in Bulgaria, Hungary and South Korea and are being evaluated in Phase II trials in the US, known as Genexol-PM®, which consists of 20–50 nm PEG-PLA micelles loaded with PTX [74]. In contrast, there is only one mixed micellar formulation that has passed through clinical phases I and II and is currently being studied for esophagus or gastroesophageal junction (GEJ) adenocarcinoma, in phase III. It is known as SP1049C. Alahkov et al. developed a novel formulation composed of Pluronic® L61 and F127 mixed micelles loaded with DOX (SP1049C). Pluronic® L61 copolymer was selected, because they observed that it induced a 7.2-fold higher drug uptake in Chinese hamster ovary $CH^{R}C5$ (resistant) cells, while F127 granted physicochemical stability to the formulation, as it prevented liquid phase separation and preserved the effective size of the micelles below 30 nm., without significantly affecting the cytotoxicity of the micellar system [179].

9. Concluding summary and perspectives

Nowadays, mixed micelles are used to improve the applications of PMs as smart drug delivery systems. This concept is based on the rational combination of two or more polymers in order to form mixed micelles capable of optimizing thermodynamic (lower CMC) [26] and kinetic stability [27], enhancing drug loading capacity [28], controlling size distribution [29] and enabling the incorporation of different ligands for active targeting (Fig. 5) [30]. In this review article, we have fully summarized the current pharmaceutical applications of mixed micelles for pharmacotherapeutic treatments of various diseases and their relevance in imaging techniques. Also, different preparation methods of mixed micelles have been described, evaluating their advantages and disadvantages.

A large number of biomaterials have been satisfactorily employed to prepare mixed micelles with the objective of transporting drugs used in numerous diseases, such as different types of cancer, HIV, tuberculosis and other bacterial infections, glaucoma, CNS pathologies, among others. However, not all these biomaterials exhibit the same chances to reach clinical phases. For instance, those who have been approved by regulatory agencies present the highest possibilities to reach these stages. Furthermore, the latter may overcome time demanding and expensive toxicity studies related to their biodegradability and biocompatibility, in order to get the approval. This aspect, together with efficacy and pharmacokinetic evaluations, should therefore be one of the main focuses of researchers working with these kind of "nano" vehicles.

Finally, when analyzing the above described investigations, it results clear that the vast majorities are related to parenteral cancer therapy. Thus, it is not a coincidence that, until now, the only mixed micellar formulation (SP1049C) that has reached clinical trials transports a chemoterapeutic agent (DOX). Considering that (i) these drug delivery systems represent a scalable, versatile and studied nanotechnology platform and (ii) there is a vast number of polymers approved by FDA or European Medicine Agency (EMA), the advent of mixed micelles to clinical stages of other pathologies different from cancer, appears to be encouragingly applicable in the near future.

Acknowledgments

Authors thank the Universidad de Buenos Aires (Grant UBACyT 20020130200038BA). Maximiliano Cagel and Fiorella Tesan are supported by doctoral scholarships of CONICET and Universidad de Buenos Aires, respectively. Ezequiel Bernabeu, Marcela A. Moretton and Diego A. Chiappetta are partially supported by CONICET, Argentina.

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