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Liposomes characterization for market approval as pharmaceutical products: Analytical methods, guidelines and standardized protocols

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ABSTRACT

Liposomes are nano-sized lipid-based vesicles widely studied for their drug delivery capabilities. Compared to standard carries they exhibit better properties such as improved site-targeting and drug release, protection of drugs from degradation and clearance, and lower toxic side effects. At present, scientific literature is rich of studies regarding liposomes-based systems, while 14 types of liposomal products have been authorized to the market by EMA and FDA and many others have been approved by national agencies. Although the interest in nanodevices and nanomedicine has steadily increased in the last two decades the development of documentation regulating and standardizing all the phases of their development and quality control still suffers from major inadequacy due to the intrinsic complexity of nano-systems characterization. Many generic documents (Type 1) discussing guidelines for the study of nano-systems (lipidic and not) have been proposed while there is a lack of robust and standardized methods (Type 2 documents). As a result, a widespread of different techniques, approaches and methodologies are being used, generating results of variable quality and hard to compare with each other. Additionally, such documents are often subject to updates and rewriting further complicating the topic. Within this context the aim of this work is focused on bridging the gap in liposome characterization: the most recent standardized methodologies suitable for liposomes characterization are here reported (with the corresponding Type 2 documents) and revised in a short and pragmatical way focused on providing the reader with a practical background of the state of the art. In particular, this paper will put the accent on the methodologies developed to evaluate the main critical quality attributes (CQAs) necessary for liposomes market approval.

1. Introduction

Liposomes are self-assembled drug vesicles characterized by an internal aqueous compartment enclosed in a bilayer (uni-lamellar) and/or a concentric series of multiple bilayers (multilamellar) of (phospho) lipids [1] (Fig. 1). The size of liposomes ranges from 30 nm to the micrometer scale, while the phospholipid bilayer is 4–5 nm thick [2] and is usually composed of glycerophospholipids (GP), sphingomyelin (SM) and cholesterol (Chol) [3]. Liposomes are biocompatible and exhibit outstanding properties as drug delivery systems [4,5]. They are able to protect an encapsulated drug from physiological degradation and at the same time they can provide selective delivery of the drug reducing its side effects, thus elevating the maximum-tolerated dose, and improving therapeutic benefits [6].

Starting from the 90 s the implementation of liposomes as carriers for anticancer drug led to the market approval of numerous lipid-based products. In recent years the interest in liposomes further increased (Fig. 2) in facing the delivery challenges provided by new therapeutic approaches based on oligonucleotides [7], DNA and mRNA antigens [8] and CRISPR [9].

Although the interest in nanomedicines has steadily grown in the last two decades the development of documents regulating and standardizing all the phases of their development and quality control, including those related to liposome drugs, still suffers from major lacks, [11]. In fact, the complexity of liposomal preparations often does not allow a straightforward characterization approach. Liposome formulations may vary in size, composition, surface coating, charge, drug load and many other properties all affecting their behavior causing the inability to develop robust analytical methods always applicable to such a wide and diverse array of systems [12]. As result a widespread of different

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techniques, approaches and methodologies are being used, generating results of variable quality and difficult to compare. Consequently, the development of standardized and regulatory accepted methodologies is extremely important to optimize the regulatory process and thus accelerate the clinical translation of nanomedicines while reducing the possible risks for the patients.

Within this context the first part of this review will be focused on the possible application of liposomes to nanomedicine, highlighting the products that have received market approval by international agencies (EMA, FDA) and the future challenges within this field. The second part instead will resume and discuss the main methodologies and regularity aspects involved in the physicochemical characterization of liposomes. The aim is to assist the reader with a compendium of the available techniques listed by CQA, their application and specific advantages, and reference of their application towards definition of the attribute of interest.

2. Liposomes as vector in pharmaceutical products

As previously mentioned, liposomes are biodegradable, biocompatible, non-toxic and composed of amphiphilic non-immunogenic compounds (such as cholesterol and phospholipids) improving solubility and tissue penetration of both lipophilic and hydrophilic drugs [13]. These features allowed their successful exploitation in numerous areas of nanomedicine and at present twenty liposomes-based formulations have been approved by FDA and/or EMA [14] (Table 1).

The main area of application of liposome-based drugs is cancer therapy; however, infection treatment, anesthesia [15], photodynamic therapy [16] and vaccination have seen the use of liposomal formulations. This section will discuss more in depth the branches of nanomedicine in which liposomes application resulted in generation of marketed products approved by EMA and/or FDA as well as the future trends and limitations within this field.

2.1. Cancer treatment

The highest number (8) of liposomes-based formulation is dedicated to cancer therapy. Many anticancer agents are highly toxic and have short half-lives in vivo due to their highly hydrophobic nature leading to side effects, noncompliance and patient distress because of difficulties in administration. Within this context liposomes encapsulation represents a way to bypass/reduce side effects allowing administration and/or improving the therapeutic power of such drugs [17]. In 1995 Dolix, a PEGylated liposomal formulation loaded with the anti-cancer drug doxorubicin, was the first lipid-based formulation approved by an international agency (FDA) [18]. Since then, seven anticancer formulations have been approved while nine are undergoing clinical trials [19]. At present however all the approved formulations exploit the enhanced permeability and retention (EP&R) effect of tumor tissues, that however does not guarantee by default a sufficient degree of selective delivery [20]. An improvement would be represented by formulations exploiting a ligand-mediated mechanism to achieve selective and high yield drug delivery. Consequently, a variety of molecules, including peptides, antibodies, proteins, low molecular weight ligands, and aptamers, are being studied in conjunction with liposomes to enhance anti-cancer treatments [21].

2.2. Bacterial and Fungal infections

A serious concern in medicine is the increase of bacterial drug resistance also due to the presence of bacterial films. The latter are clusters of microorganisms characterized by higher resistance to drugs compared to isolated bacteria and against which conventional therapies have only limited effectiveness. Since drug encapsulation improves the efficacy of antibacterial drugs, liposomes are being exploited to develop formulation suited to kill even the highly drug resistant structures. For example, Arikayce®, a liposome-encapsulated amikacin formulation, has been marketed in 2018 [22].

Although less common, acute fungal infections can be extremely dangerous especially in immunocompromised patients [23]. Within this context liposomes were used to improve water solubility, drug resistance, and reduce the side effect associated with Amphotericin B, the gold standard molecule for the treatment of severe systemic fungal infections [24].

2.3. Vaccines

Particulate vaccines offer greater protection of antigens from enzymatic degradation and simultaneous delivery of molecular adjuvants with antigen to antigen presenting cells (APC), thus promoting cellular and humoral immune responses [25]. The first work reporting the ability of liposomes to induce immune responses when used as vaccine adjuvants or with associated antigens was published in the 70 s [26]. Since then, liposomes-based vaccines for Hepatitis A (Epaxal®), Influenza (Inflexal®), Malaria (Mosquirix™), shingles and post-herpetic neuralgia (Shingrix®) are well established on the market [27,28]. Traditionally, vaccines have relied on the use of whole killed or live attenuated pathogens. Today research is focused on the development of subunit vaccines that are better defined, easier to produce, and safer. Within this context, liposomes along with other lipid-based structure, represent the leading carrier choice [10]. Probably the most striking application of liposome technology in the vaccine field is the development of mRNA vaccines against COVID-19 by Pfizer/BioNTech and Moderna [29].

2.4. Other marketed applications

Liposomes have seen success in the field of anesthesia and nowadays two drugs, DepoDur® and Exparel®, have been approved and commercialized. These drugs are based on multivesicular lipid

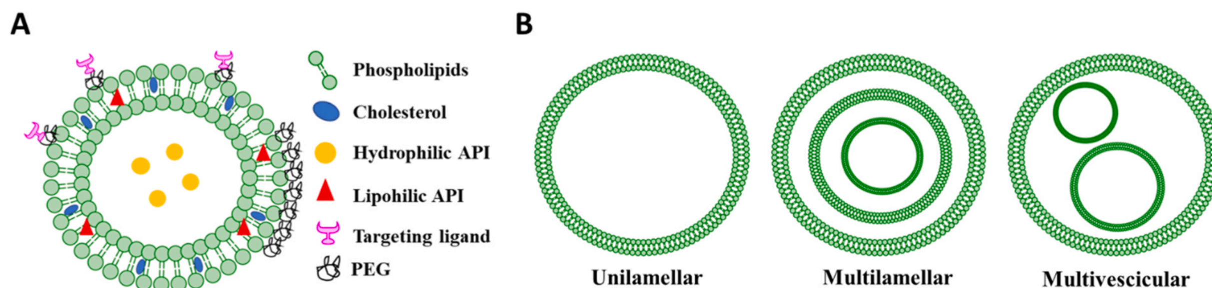

Fig. 1. A. Representative liposome structure. Phospholipids and cholesterol self-assemble into a lipid bilayer enclosing an aqueous core. Lipophilic drugs are incapsulated in the lipid bilayer while hydrophilic drugs the aqueous core. Liposomes functionalization (i.e. with targeting ligands) is usually performed after PEGylation of the particle's surface **B.** Schematization of the most common liposomes sub-types.

technology (DepoFoam) which allow delivery of the active constituent at a sufficient concentration during the entire treatment period. This guarantees the reduction of drug administration frequency, a very important factor in a prolonged treatment. DepoCyt® is another DepoFoam based liposomal drug approved by FDA and EMA for post-surgical pain management. At present other DepoFoam drugs are under development including DepoTXA (DepoTranexamic Acid) for reducing surgical bleeding and DepoMLX (DepoMeloxicam) for post-surgical analgesia [30].

Photodynamic therapy (PDT) is an interesting technology showing a series of advantages, such as minimization of damage to healthy cells, since the photosensitizers tend to build up in abnormal cells and the light is focused directly on them. This technology suffers from limitations in the lipophilic nature of most photosensitizers (PSs), short half-life of PSs in plasma, poor tissue penetration and low tumor specificity. As previously mentioned liposomes are excellent drug carriers to address such issues; at present Visudyne® is the only PDT liposomes based system available on the market while numerous multifunctional liposomes systems to enhance PDT are being researched [31].

2.5. Future research directions

Liposomes research revolves around three major areas: investigation of new API classes, development of multifunctional liposomes and overall improvement of targeting and controlled release strategies.

A very promising API class is represented by antisense oligonucleotides (ASO), single-stranded synthetic nucleic acid polymers (around 18–30 nucleotides) complementary to messenger RNA (mRNA). By binding to such RNA strands, ASOs prevent production of faulty proteins generating a therapeutic response [32,33] and at present 9 different ASOs have been approved to market [34]. The major barrier to an effective therapy/clinical translation using ASO is represented by their difficulties to cross the plasma membrane, which implies that large drug quantities have to be concentrated at the exterior of the cell to attain at least modest concentrations at the target site [33]. Liposomes, by increasing ASO stability in bodily fluids, facilitate drug distribution, improve cellular uptake, and allow ASO to bypass the endocytic process. Liposomes-ASO formulations have been successfully applied to a various array of therapeutic applications highlighting their sleeping potential. To the best of our knowledge 10 different therapeutic applications (ranging from cancer therapy to cardiac arythmia) are reported in literature [34], however at present no market approved liposomal product exists.

Nowadays the majority of liposomes systems on the market still relies on passive targeting (EP&R effect) which is often associated with low target specificity causing a limited or absent improvement of therapeutic outcomes and patient survival rates. To improve the effectiveness of therapies that rely on the EP&R effects, upgraded formulations and protocols will be needed. Theragnostic NPs, having simultaneous diagnostic and therapeutic functions, represent an improvement to such technologies. By exploiting liposomes' flexibility in surface functionalization, it is possible to obtain nano systems labelled with various imaging probes which can co-deliver therapeutic drugs while acting as imaging agents. By combining such functions theragnostic NPs allow for the monitoring of real-time drug delivery, accurate diagnosis and assessment of biological signals, easier determination of responses to a therapy, an improvement towards the use of minimally invasive procedures and better decisions concerning the end point of therapy [35,36].

Clinically available technologies such as magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET) are useful for imaging through theragnostic liposomes. Quantitative PET has emerged as the optimal technique for longitudinal imaging since it provides the best estimation of liposomal drug delivery in tumors [19]. At present however this approach is currently only in the developmental stage and only a few studies are reported exploiting such particles to study the EP&R effect in cancer models [37–39].

Another trend in liposomes research revolves around the design of delivery systems working by active targeting. The terms refer on a series of mechanisms able to release drug in a more selective and controlled way compared to EP&R. Active targeting strategies include targeting a tumor cell surface receptor or targeting a tumor micro-environment, as well as stimuli-response strategies that rely on changes in pH, temperature, redox, enzyme, light and ultrasound to trigger drug release. Antibody conjugated liposomes may represent a promising technology in the future. Currently such immunoliposomes are widely studied in numerous filed of nanomedicine such as breast cancer therapy [40,41], SARS-CoV-2 treatment [42] and many others. A key issue to assess the success and technological translation of such products concerns their characterization. Section 3 will describe the main available technologies for liposomes physicochemical characterization, and as the reader will notice the development of methods to characterize the liposomes of higher complexity is still quite lacking. This is a significant problem which hinders the control of ligand attachment to liposomes and of batch-to-batch variability [43].

Aside from improving liposomes drug delivery and imaging properties another major challenge concerns their stability and handling. Liposomes stability can be influenced by pH, size, surface charge, lipid composition, and temperature; moreover these parameters also impact drug encapsulation efficiency and the half-life in circulating blood in vivo. The development of standardized, robust and reproducible techniques/methods for their characterization is thus of the utmost importance.

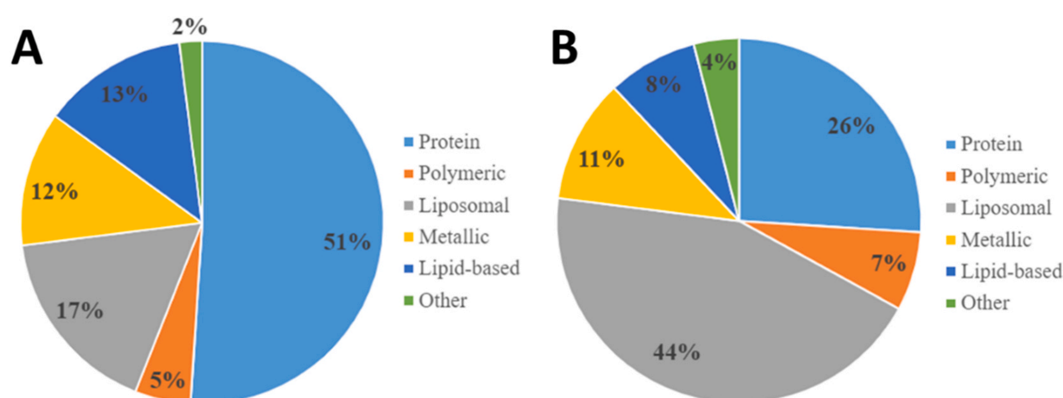

Fig. 2. A. Types of nanoparticles in clinical trials from 2002 to 2016. **B.** Types of nanoparticles in clinical trials from 2016 to 2021. The pie charts highlight the growing role of liposomal drugs (in gray) in nanomedicine.
Adapted from [10].

Table 1
Summary of liposomal products approved by FDA (US) and EMA (EU), excluding lipid-drug complexes.

Type	Name	API	Approved year/ area	Applications
Cancer therapy (Drug formulation)	Doxil®/ CaelyxTM	Doxorubicin	1995 (US) 1996 (EU)	Ovaria, breast cancer and Kaposi's sarcoma
	DaunoXome®	Daunorubicin	1996 (US, EU)	Kaposi's sarcoma
	Onivyde®	Irinotecan hydrochloride trihydrate	1996 (US) 2016 (EU)	Pancreatic adenocarcinoma
	Myocet®	Doxorubicin	2000 (EU)	Breast cancer
	Mepact®	Mifamurtide	2009 (EU)	Osteosarcoma
	Marqibo®	Vineristine	2012 (US)	Leukemia
	Vyxeos®	Daunorubicin+cytrabine	2017 (US) 2018 (EU)	Leukemia
	Zolsketil®	Doxorubicin	2022 (EU)	Breast and ovarian cancer, multiple myeloma, Kaposi's sarcoma
Other application (Drug formulation)	AmBisome®	Amphotericin B	1997 (US, EU)	Fungal infections
	DepoCyt®	Cytarabine	1999 (US) 2001 (EU)	Lymphomatous meningitis
	Visudyne®	Verteporphin	2000 (US, EU)	Age related macular degeneration
	DepoDur®	Morphine sulfate	2004 (US, EU)	Pain management
	Arikayce®	Amikacin	2018 (US, EU)	Lung infections
	Exparel®	Bupivacaine	2020 (EU)	Anesthesia
Vaccine	Epaxal®	Inactivated hepatitis A virus (RG-SB strain).	1994 (EU)	Hepatitis A
	Inflexal V®	Influenza virus surface antigens (haemagglutinin and neuraminidase), Virosomal. 3 different strains.	1997 (EU)	Influenza
	MosquirixTM	Proteins found on the surface of the Plasmodium falciparum parasites and the hepatitis B virus.	2015 (EU)	Malaria
	Shingrix®	Recombinant varicella-zoster virus glycoprotein E	2017 (US) 2018 (EU)	Shingles and port-herpetic neuralgia
	COMIRNATY™	mRNA	2021 (US, EU)	COVID-19
	SPIKEVAX™	mRNA	2022 (US, EU)	COVID-19

3. Physicochemical characterization of liposomal products for market approval

Currently published regulatory documents can be divided in two typologies: (1) Type 1, guidance-like documents, offering an overview of different methods and identification of a series of critical quality attributes (CQAs) important for market approval; and (2) Type 2 documents, i.e. specific test methods and guidelines addressing in a more detailed and practical way the procedure for the determination of certain CQAs. The Type 1 documents currently adopted for liposomes by international agencies are summarized in Table 2.

The main CQAs that should be defined/known to candidate a liposome-based drug to market approval and most common approaches used to assess them are shown in Table 3.

In the following subsections the main CQAs to be assessed for market approval will be individually discussed in a synthetic and practical way highlighting the content provided by Type 2 documents, listing available techniques and methods and providing references to the state of the art. Each subsection is dedicated to a set of CQAs. The complete list of abbreviations for each technique mentioned is in Appendix A.

Table 2
Summary of the liposome-specific regulatory guidelines documents (Type 1) developed by the main international agencies. MHLW: Ministry of Health, Labour and Welfare (JPN).

Regulatory agency	Liposomes specific regulatory guideline
FDA	Guidance for industry: liposome drug products chemistry, manufacturing, and controls; human pharmacokinetics and bioavailability; and labeling documentation.
EMA	Data requirements for intravenous liposomal products developed with reference to an innovator liposomal product.
MHLW	Guideline for the Development of Liposome Drug Products.

Table 3
Summary of the main CQAs that should be defined/known to candidate a liposome-based drug to market approval and most common approaches used to assess such CQAs. "Partially standardized": refers to methods which only check partially the requirements for a proper standardization (i.e. lack of the evaluation of intra-laboratory variability and/or the existence of formal standards).

Measured attribute	Main available related technologies	Standard method for certain CQA?
Chemical composition, structure, impurities	HPLC-Multidetection (MS, UV, dRI, ELSD, CAD), GC-MS, ^{1}H–^{13}C- and ^{31}P NMR, Raman Spectroscopy	YES
Particle size, size distribution and concentration	TEM, DLS, NTA, TRIPS, SEC/ FFF-Multidetection (UV, dRI, DLS, MALS)	YES
Surface properties (i.e. Zeta potential, hydrophobicity, surface area, surface coating)	Zeta Potential: DLS, EPR, Fluorescence labelling Hydrophobicity: Dark-field microscopy Surface Area: NMR Surface coating: Chromatography approaches	YES (Zeta potential only)
Drug loading	HPLC- (MS, UV), CE, AF4	YES (Partially standardized)
Drug release (In vitro/in vivo/in physiologically-clinically relevant media)	API separation: filtration, UF, UC, dialysis bags API quantification: LC-MS Standalone techniques: HPLC-MS, CE-ICP-MS, AF4 (dRI, UV, MALS)	YES (Partially standardized)
Chemical and physical stability, degradation paths and relative kinetics	Size/PDI: DLS, SEC, AF4, FLD assays, DCS Lipid composition: HPLC (CAD, ELSD, MS) API stability: HPLC-MS, SAXS, SANS	YES (Partially standardized)

3.1. Lipid composition

Information indicating the composition of the liposomes used for the nano system, their relative abundance, their degradation products and impurities are required for market approval by EMA [44] and FDA [45]. When synthetic and/or partially synthetic components are being used, results obtained from the application of the same procedures to reference material should be correlated. These studies work on molecular samples and they do not represent a prickly subject concerning result regimentation since numerous standardized methods on reference materials already exist. When new methods are being used, the product manufacturer should anyway provide the standardization procedure method following the rule of ICH guidelines [46].

One of the most common analytical approaches is based on the rupture of the liposome trough organic solvents (ex. chloroform, methanol, isopropanol) followed by the use of an analytical technique to quantify their composition. Liquid chromatography has been extensively applied to quantitative lipid analysis over decades using different detectors such as diode array ultraviolet (UV), refractive index (dRI), evaporative light scattering detector (ELSD), charged aerosol detector (CAD) or MS detectors [47–49]. Thin-layer chromatography (TLC) can be also used as inexpensive technology for initial separation and qualitative analysis of lipid components [50] or as an actual quantification technique coupling with densitometry analysis or mass spectrometry [51]. Gas chromatography (GC) coupled with MS detectors has been also widely exploited, however, derivatization of the lipid analytes to become volatile is often required [52–54]. Recently, supercritical fluid chromatography (SFC) has also been applied to lipidomic analysis [55].

Aside from chromatographic approaches, other methods have been applied. Nuclear magnetic resonance (^{1}H–^{13}C- and ^{31}P NMR) can be used to elucidate lipids molecular structure and to quantify them (although limited by moderate sensitivity and quantification power) [56–58]. Raman spectroscopy is also able to distinguish liposomes characterized by different lipid composition and provide insight on such composition in situ without requiring liposomes destruction [59,60]. Recently Raman microscopy has also been developed to determine the intra-particle distribution of DNA encapsulated in cationic liposomes [61]. Overall NMR and Raman spectroscopy are better suited to identify lipidic molecule structures while chromatographic approaches are better suited to separate and quantify lipid in pharmaceutical compositions.

Finally, a various array of methods based of fluorescence and colorimetric strategies have been developed [62], with the advantage of simplicity combined with high-throughput analysis.

3.2. Morphology, particle size (PS), polydispersity index (PDI) and concentration

The properties of nanosized materials such as the ability to penetrate biological barriers and clearance rate are greatly affected by their size and morphology; consequently, PS, PDI are fundamental CQAs to be evaluated in the context of regulatory approval.

Particle morphology and size can be directly visualized by several transmission electron microscopy (TEM) techniques, such as the negative stain [63], freeze structure [64], and cryogenic microscopy [65]. Negative-stain TEM has the drawback of requiring the drying of the sample before the staining process which may cause liposome shrinkage, collapse or aggregation. The other two variants instead do not require drying, and cryo-TEM possesses the advantage of requiring the least amount of work for sample's preparation. Scanning electron microscopy (SEM), although applicable, is not often exploited for lipid nanoparticles due to it being disruptive for the sample. In recent years however environmental scanning electron microscopy (ESEM) has been developed to image liposomes in their hydrated states and to investigate changes in different environments [66].

Overall, although extremely powerful for morphological studies, microscopy shows a series of drawbacks such as being time consuming and expensive, not particularly suited to calculate accurately PDI and PS, and affected by the bias of the operator.

The PDI of a dispersion and its mean hydrodynamic radius (Rh) instead can be accurately measured using dynamic light scattering (DLS) [67], nanoparticle tracking analysis (NTA) [25] and tunable resistive pulse sensing (TRPS) [68]. These single-particle sizing techniques have the advantages of being fast, simple to use and not particularly expensive, and are thus recommended for fast screening of polydisperse samples or for quality control of samples with relatively low dispersity. Since these systems usually work in batch mode a drawback is instead represented by their low relatability in measuring complex or non-homogeneous samples. Although due to historical reasons DLS is still the most diffused technique of the three, comparison studies demonstrated that NTA and TRPS can resolve significantly better multimodal nanoparticle mixtures than batch-mode DLS [69].

To obtain reliable PDI and PS results from complex samples a separation technique such as Size Exclusion Chromatography (SEC) or Asymmetrical Flow Field-Flow Fractionation (AF4) coupled with an array of concentration (UV, dRI) and size measuring (DLS, MALS) detectors is required. SEC separates lipid nanoparticles (empty or drug loaded) based on their hydrodynamic radius in a range between 1 and 100 nm and it is by far the most commonly exploited technique for the separation and characterization of liposomes [70], the device however is characterized by a series of limitations. The presence of a stationary phase can in fact induce aggregation of the sample and imposes limitations on mobile phase composition, that has to be compatible with the column hindering the possibility of native study. Moreover, it is possible to observe adsorption and/or disruption processes caused by the interaction of the sample with the stationary phase [71]. These problems are solved by AF4 [72,73], along with its miniaturized version Hollow Fiber Flow Field-Flow Fractionation (HF5) [74], that represents the most promising substitutive/complementary technique to SEC. The separation based on Rh is achieved in an empty channel by the combined action of two flows: a laminar flow (with parabolic profile) of eluent running coaxial to the channel axis and a second one, applied perpendicularly, and directed towards a channel wall called accumulation wall [75,76]. Sample components, differing by hydrodynamic size and/or other physical properties, are driven by the applied field into different velocity regions within the parabolic flow profile of the mobile phase across the channel causing their differential elution. Compared to SEC, the use of an hollow separative system eliminates the possible physical stress/interactions on the sample induced by the stationary phase and allows for flexibility in terms of mobile phase [77]. AF4/HF5 platforms are also characterized by a wider range of application (1 nm-20 μm). Overall, these features make AF4 techniques better suited to study highly complex matrices of various nature [78–81], interactions between different biological components [82,83] and to study biomedical nanodevices in a closer-to-real-life scenario [84,85]. AF4-multidetection allowed for separation and characterization (PS and PDI) of various lipid nanoparticles such liposomes [86,87], lipoproteins [88] and DNA-lipid complexes of different net charge [89].

AF4 equipped with an on-line multi-angle static light scattering (MALS/SMLS) and/or dynamic light scattering (DLS) detector can also be used gather information on the shape/morphology of the nanoparticles by evaluating the v-value [90] or shape factor [91] obtained from the calculated values of gyraton (Rg) and hydrodynamic radius. For example Parot et al. calculated in the range 0.7 – 0.9 the Rg/Rh ratios of PEGylated lipid micelles, which indicated a compact homogeneous core-shell spherical structure, while a conformation factor emerged as equal to 1 for empty unilamellar liposomes or lower when the encapsulated drug occupies significant mass fraction of the aqueous core [91]. Finally, Caputo et al. described the development of a possible standard operating procedure for MD-AF4 measurements [86].

Although particle size and shape are the CQAs that have been characterized with the highest attention, concerning their regulation at present only one Type 2 document refers specifically to liposomes

(ASTM WK54615) and overall other two (ASTM E3143 – 18b; ASTM WK54872) have been specifically designed for nano-systems of biological interest. Table 4 lists the methods and procedures for PD and PDI determination using the techniques commonly used for liposomes analysis discussed above.

Concentration is another important CQA to carefully define, impacting drug efficacy and toxicity for in vivo administration [92]. The main available techniques are based on the measurement of light absorption or scattering of the sample, or the direct counting of individual particles. UV–Vis absorbance can be applied for NPs with a characteristic absorptions if molar extinction coefficient is known, while UV-based turbidimetry can be used for non-adsorbing monodisperse particles with a known refractive index and scattering coefficient [93, 94]. Light scattering-based techniques (DLS, NTA) can also be used but are restricted to monodisperse require a calibration with a standard sample of the same size, optical properties, and dispersing solvent [95]. Counting techniques such as TEM do not require the use of standards but are affected by operational bias and by sample aggregation/deformation. Recently, approaches based on Tunable Resistive Pulse Sensing (TRPS) [96] and Laser Transmission Microscopy [97] have also been developed. At present however no standardized methods specifically developed for exosomes exist.

3.3. Morphology and Lamellarity

Although information on morphology and lamellarity can be obtained from AF4 platforms, other approaches such as microscopy (TEM, SEM) are more commonly used and regulated methodologies are available. At present 3 documents disciplining the morphological evaluation of nano systems through microscopy have been published, however none of them specifically focuses either on lipid-based particles or on biomedical nano systems (Table 5). To the best of our knowledge no standard method/ set of rules addressing lamellarity has been published.

Several microscopy techniques such as differential interference contrast microscopy [63], freeze-fracture TEM [64], and cryo-TEM [98] have also been used to directly image lipid layer, however these approaches suffer from the required sample preparation procedures which may alter the results.

Alternatively, liposome lamellarity can be determined by ^{31}P NMR with the help of paramagnetic shift reagents which are able to interact only with the external lipid bilayer [99,100], this method however is significantly influenced by the measurement conditions, including buffer types, pH, and ion concentration. ^{31}P NMR is not the only approach exploiting the behavior differences of the inner bilayers compared to the outer one. A series of spectroscopic strategies working by comparing intensities before and after a reaction restricted to the external lipid layer was developed [101–103].

Finally, Small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) can also be exploited to evaluate lamellarity as long with the crystalline state of a loaded drug [104,105].

Table 4
Summary of the Type 2 documents addressing particle size (PS) and polydispersity index (PDI) determination classified based on the technique exploited. * Method under development. † Method specifically designed for nano systems of biomedical interest. ₪ Method specifically designed liposomes.

Characterization technique	Standardized methods
TEM	ISO/WD 21363
	ISO/TS10797:2012
Cryo-TEM	ASTM WK54615₪
	ASTM E3143–18b†
SEM	ISO/WD 19749
DLS	ASTM WK54872†
	ISO 22412:2017
	ASTM E2490–09(2021)
NTA	ISO 19430:2016*
	ASTM E2834–12(2022)
SEC multidetection	ISO 16014 (2019)
FFF (AF4) multidetection	CEN ISO/DTS 21362:2021
Analytical UC	EUNCL (PCC-24)
SMLS	ISO/TS 21357:2022

Table 5
Summary of the Type 2 documents addressing particle morphology classified based on the technique exploited.

Characterization technique	Type 2 document
TEM	• ISO/WD 21363:2020 • ISO/TS10797:2012
TEM, EDX	• ISO/WD 21363:2020

3.4. Surface properties: Zeta potential, surface area, hydrophobicity, and surface coating

The Zeta-potential is a parameter associated to the surface charge of liposomes which is vital to estimate stability, in vivo performance and biological fate of colloidal systems. Since charge on the surface of liposomes governs their mobility, and that in turn changes the intensity of the scattered light, DLS is commonly used to estimate the zeta potential [106]. In particular modern DLS instruments are able to register the changes in intensity of the scattered light due to the mobility of liposomes as a result of the impact of an electric field applied on particle charges. This technique is also commonly referred as Phase Analysis Light Scattering (PALS) or Electrophoretic light scattering. Alternatively, the surface potential of liposomes can also be measured by several techniques including fluorescence labeling [107], electron paramagnetic resonance [108], and the second harmonic generation from optical analyses [109].

Since zeta-potential of liposomes significantly depends on pH, temperature, ionic strength loading and external environment [110–112], to obtain reliable, homogenous and confrontable results it is important to regulate its measurement procedure. At present only two documents regulating Zeta potential determination have been published, the first dedicated to a generical colloidal systems (ISO 13099–1:2012, −2:2012, −3:2014) and the second specifically designed for nano system of biomedical interest (ASTM E2865–12(2022)).

Currently, except for zeta-potential, no standardized methods exist for the analysis of other surface properties of liposomes such as surface area, hydrophobicity, and surface coating. The latter is typically evaluated with chromatographic approaches developed case-by-case which requires particles' dissolution [11]. Due to PEG being by far the most common coating agent, various approaches are howerer been reported to study the surface of PEG-coated NPs [113]. Methods to evaluate the hydrophobicity of soft nanoparticles such as liposomes are all in developmental stages [114]. At present the most mature method exploits conventional dark-field microscopy to measure the binding rate between the particles surface and collectors based on fluorinated hydrophobic surfaces with differential surface energy properties [115]. Concerning surface area, one method based on the measurement of the wettable surface area of organic NPs in suspension by NMR has been proposed [116].

3.5. Drug loading

A drug can be encapsulated in liposomes within the lipid bilayers (hydrophobic APIs) or inside the inner aqueous core (hydrophilic APIs). Although lipophilic drugs can be loaded with high amounts within the lipid bilayer, they are more prone to fast and uncontrolled release [117]. In contrast, the liposome core grants an overall more stable encapsulation compartment, though it is more difficult to achieve high encapsulation efficiency. Various methods exist to evaluate liposome loading, and more details can be found in a recent work [118]. Conventionally

drug loading evaluation methods are characterized by a series of common steps. At first the non-encapsulated free drug is separated from the nanocarriers by ultrafiltration, ultracentrifugation, dialysis or solid-phase extraction. The free drug amount can then be quantified and compared with the total drug amount. To fairly compare among different formulations, information concerning initial ratios between the API and lipids and/or loading capacity (%) should be reported.

HPLC coupled with a variable array of detectors such as UV–VIS, tandem molecular mass spectrometry (LC-MS/MS) or Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is the most common platform for API identification. Due to the nature of the technique these methods are extremely API specific and cannot be generally standardized. Some Type 2 guidelines have however been published (Table 6). Strictly concerning liposomes characterization, RP-HPLC silica columns showed high efficiency for simultaneous separation and characterization of free API and drug loaded nanoparticles [119–121].

Capillary electrophoresis (CE) has been used to separate and quantify loaded and not loaded APIs [122]; AF4 has also been exploited to the task as a separative step followed by API determination via HPLC [123, 124]. As a standalone technique AF4 can be instead used as a fast and economic prescreening method to qualitative observe and estimate API distribution in presence of different kinds of liposomes [86].

3.6. *Drug release in simple media, in vitro, in silico and in vivo*

Drug release in the same way as drug loading can be considered a two-step procedure involving the separation of the encapsulated API from the medium containing the free/released drug followed by the quantification of the latter. The separation technique shouldn't affect the carrier integrity or influence the concentration equilibrium of the drug between the encapsulated and the free state. While studying drug release in simple media the analytical methods are adapted from techniques conventionally used for bioanalytical purification of nanoformulations, including chromatographic methods, liquid-liquid extraction and equilibrium methods [11]. Concerning the regulatory documents EUNCL and NCI-NCL have developed and validated protocols for separation of free vs encapsulated drugs by ultrafiltration while a lipid-based nanoparticles specific document based on the use of Solid Phase Extraction has been published by Guillot et al. [125].

Table 6
Summary of the Type 2 documents addressing drug loading/release classified based on the technique exploited. These methodologies, although routinely used in relevant R&D environment, are only partially standardized since they lack the evaluation of intra-laboratory variability and/or the existence of formal standards.

Characterization technique	Parameter	Protocol	Notes
LC-MS/MS	Drug loading	EUNCL (PCC-30)	• Measure of total drug loading • Fully qualified but no formal standard exists
Ultrafiltration + HPLC, LC-MS/MS	Drug loading	EUNCL (PCC-31)	• Measure of free drug fraction • Fully qualified but no formal standard exists
RP-HPLC—ICP-MS	Drug loading	NCI-NCL (PCC-14)	• Measure of free drug fraction • Applicable to gadolinium-based contrast agent • Commonly used but not officially validated
Ultrafiltration + HPLC, LC-MS/MS	Drug release	NCI-NCL (PHA-1&2)	• Measure of drug release in plasma over time • The nanocarrier must be separable from the API trough UF and the latter must be detectable by MS. • Commonly used but not officially validated

In vitro studies suffer the lack of an acceptable and approved standard for in vitro release tests [126], and cannot fully account for two factors: (1) predicting and understanding the release mechanism; (2) evaluate the effects of blood enzymes, that could promote hydrolysis and subsequent rupture, and protein binding, that can activate macrophages uptake. In vitro-in vivo correlation (IVIVC), i.e. mathematical models correlating in vitro properties and in vivo response, do not yet exist for liposomes in the European or United States Pharmacopeia, and the FDA and EMA have not released guidelines specific for these preparations [127].

Some general rules concerning liposomes can be assumed based on the draft guidance (Type 1 document) on injectable liposomal formulation of doxorubicin hydrochloride by the FDA [128]. For example, according to this document, to conduct these tests it is recommended the use of release media that contain 50% human plasma at 37 °C with different pH values ranging between 5.5 and 7.5 to mimic drug release in various tissues (i.e. normal, cancerous).

The approaches typically used for in vitro studies can be classified in four typologies based on how sample incubation and separation from the media is performed. (1) The simplest approach is based on separation of the sample from the media containing the released API by ultracentrifugation, filtration or SEC, followed by off-line API quantification [129, 130]. Although being simple, such approaches suffers from low efficiency of the ultracentrifugation or filtration separation for submicron nanoparticles, moreover they could cause the disruption of intact liposomes altering the results. A relevant method developed by NCI-NCL involving drug release in plasma trough ultrafiltration followed by LC-MS is now under evaluation for standardization by the ASTM E56 committee [131]. (2) Alternatively, incubation can occur with the use of dialysis sacks [132] drastically simplifying the separation of the nano systems from the culture media. To obtain reliable results however attention should be paid to the experiment design; poor choice of the dialysis sack and inadequate stirring inside the latter may cause sample aggregation and obstruction of the pores leading to underestimation of drug release. To partially resolve those problems and facilitate changes of the release medium a reverse dialysis approach has been proposed [133]. This method is based on putting the sample in the bulk release media while sampling the released API inside the dialysis sack. (3) Approaches combining the dialysis sack with circulation/change of the release media have also been developed. Compared to the conventional dialysis sack methods, such improved versions allowed better discrimination release profiles from liposomes with different lipid compositions [134]. The United States Pharmacopeia, to avoid unnecessary proliferation of equipment and method designs, modifications of already existing apparatuses or the use of alternative equipment, has developed and proposed the use of two apparatus (USP I and USP IV). Further details can be found in the work by Solomon et al. [126] (4) Finally, as seen for drug loading, the use of multidetection separative platforms as standalone techniques for both sample separation and API quantification is possible. Although HPLC is still the most exploited technique of the bunch both CE [135] and AF4 [136–138] can be used. Table 7.

Performing additional in vivo studies is also highly recommended by the guidelines to achieve market approval of a liposomal drug. Mouse, rat and pig models are the most used to use to track the distribution and pharmacokinetics of liposomal drugs in the body [139–141]. A major limitation of these approaches is the lower vascularization of the cancer tissues of the xenograft models used to assess oncology therapeutics compared to human ones, that could generate suboptimal pharmacokinetics predictions [11].

Overall, the numerous difficulties and variables related to in vitro and in vivo studies of liposomes prevent the development of universal methodologies valid for all kind of formulations. A proposed solution to simplify this area of the liposomal drug approval workflow is based on the use of mathematical models, able to understand the release mechanism and predict release behavior without conducting a high number of experiments. Such *in silico* molecular modelling are becoming

Table 7
Schematization of the advantages and limitations associated with the main approaches to drug release evaluation.

Approach to drug release	Advantages	Limitations
Separation (UC or UF) + offline detection	High flexibility in terms of drug (UC) Straightforward instrument setup Uniform agitation or stirring	Liposome's damage Filter clogging/particle deformation Low efficient separation Sedimentation rate of the components
Dialysis/Reverse dialysis + API detection in the other compartment	Economic/simple Simultaneous separation and in situ monitoring of the released API Easy changes of the release medium	Underestimation of drug release caused by sample aggregation and obstruction of the sac pores
Dialysis (circulation of release media) + offline detection	Better discrimination of drug release profiles (compared to standard dialysis) Simultaneous separation and on-line monitoring of the released API Easy changes of the release media	High volume of release media Two stage diffusion Compatibility of the dialysis chamber with the apparatus
Flow separation platforms (HPLC, AF4, CE)	Separation and contemporaneous powerful characterization/ API release evaluation	Complex Optimization of experimental conditions for every system studied

increasingly accurate predictive tools during drug formulation [142–144] and nowadays various models of lipid bilayers and organs have been modeled [145,146]. However, such models still rely on data originated from in vitro and in vivo studies (not often already available) and require more validation before being adopted for regulatory purposes.

3.7. Physical and chemical stability

The main manifestation of physical instability of liposomes is represented by fusion and/or aggregation on the NPs which could also induce phase separation and leakage of the encapsulated API. Since these phenomena greatly impact size and PDI, studies exploiting DLS [147,148], SEC [70] and AF4 [149] to evaluate liposomes stability have been reported. Within this context a recent study by Bohsen et al. studied the interaction of exosomes differing in composition with bile salts which usually hinder the oral sub ministration route [150]. Additionally, the authors highlighted the accordance between AF4 and SEC results. Other approaches are based on turbidimetry [151], on fluorescence assays [152,153] or on DCS [154].

The main effects of liposomes chemical instability are represented by the hydrolysis/oxidation of phospholipids; the products of such processes are separated and detected by several chromatography approaches such as HPLC-CAD[155], HPLC-ELSD[156], TLC with colorimetric detection[157] and HPLC-MS[158]. The latter is also the most common technique exploited to evaluate the stability of the encapsulated API along with SAXS or SANS for what concerns their physical state [62].

For what concerns regimentation, a series of Type 2 documents addressing specifically the monitoring of physical stability of nanomaterials exists (Table 9). Chemical stability studies involving chromatographic measurements highly specific for each combination of NP/API are instead disciplined by well-established ICH ruling.Table 8.

4. Conclusions

The major role played by liposomes in fighting the COVID-19 pandemic, as well as their other numerous applications (ranging from cancer to pain management) suggest the importance of studying and investing on such technology. Major trends in improving liposomes performances revolve around the development of multifunctional liposomes and/or the realization of efficient selective targeting. The inner complexity of such systems however does not match the limitations associated to the characterization of even simpler liposomal systems. Many CQAs still cannot be assessed via fully standardized characterization methods while others (i.e. surface functionalization) even lack a solid determination approach. Moreover, at present no liposome standard material exists and most of the Type 2 documents published are not specifically referred to liposomes. Consequently, a sample-specific adaptation is often required raising the question on the level of specificity/flexibility that can be accepted or is required for a standardized method. Overall we believe that further effort should be focused in the development on analytical methods prone to wide applicability and the production of liposomal commercial standards exploitable to directly validate the results stemming from sample-specific modification of the standard methods and/or innovative ones.

Table 8
Summary of the Type 2 documents addressing physical and chemical stability of NPs classified based on the technique exploited. These methodologies, although routinely used in relevant R&D environment, are only "partially standardized) since lack the evaluation of intra-laboratory variability and/or the existence of formal standards.

Characterization technique	Parameter	Protocol	Notes
DLS	Physical stability (NPs size over time)	EUNCL (PCC-21)	Fully qualified but no formal standard exists.
NTA	Physical stability (NPs size over time)	EUNCL (PCC-23)	Commonly used but not officially validated.
Analytical UC	Physical stability (NPs size over time)	EUNCL (PCC-24)	Commonly used but not officially validated.
AF4-UV–VIS-MALS-DLS	Physical stability (NPs size over time)	EUNCL (PCC-22)	Must be adapted to the system studied. Fully qualified but no formal standard exists.
Chromatographic methods	Chemical stability (degradation products over time)	ICH-Q1	Has to be adapted to the system studied

Author Statement

The authors declare that the work described has not been published previously, it is not under consideration for publication elsewhere, its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright holder.

CRediT authorship contribution statement

Conceptualization, V.M., and S.G.; data curation, S.G., and V.M; methodology, S.G., V.M., A.Z., B.R.; writing original draft, S.G., V.M.; writing, review, and editing, V.M., A.Z., B.R.; supervision, V.M., A.Z., B.R., and P.R. All authors have read and agreed to the published version of the manuscript.

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Data Availability

No data was used for the research described in the article.

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Appendix A - List of Abbreviations

Abbreviation	Full Name
AF4	Asymmetrical Flow Field-Flow Fractionation
APC	Antigen Presenting Cells
APIs	Active Pharmaceutical Ingredient(s)
ASO	AntiSense Oligonucleotides
ASTM	American Society for Testing Materials
CAD	Charged Aerosol Detector
CE	Capillary Electrophoresis
Chol	Cholesterol
CQAs	Critical Quality Attribute(s)
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CT	Computed Tomography
DCS	Differential Scanning Calorimetry
DLS	Dynamic Light Scattering
DNA	DeoxyriboNucleic Acid
dRI	Refractive Index detector
EDX	Energy Dispersive X-Ray Analysis
ELSD	Evaporative Light Scattering Detector
ELSD	Evaporative Light Scattering Setector
EMA	European Medicines Agency
EP&R	Enhanced Permeability and Retention
EPR	Electron Paramagnetic Resonance
ESEM	Environmental Scanning Electron Microscopy
EUNCL	European Nanomedicine Characterization Laboratory
FDA	Food and Drug Administration
FLD	Fluorescence Detector
GC	Gas Chromatography
GP	GlyceroPhospholipids
HF5	Hollow Fiber Flow Field-Flow Fractionation
HPLC	High-Performance Liquid Chromatography
ICH	International Council for Harmonisation
ICP-MS	Inductively Coupled Plasma
ISO	International Organization for Standardization
IVIVC	In Vitro-In Vivo Correlation
LC	Liquid Cromatrography
MALS	Multi Angle Light Scattering
MD	MultiDetection
MHLW	Ministry of Health, Labour and Welfare
MRI	Magnetic Resonance Imaging
mRNA	messanger-RiboNucleic Acid
MS	Mass Spectrometry
NCI-NCL	National Cancer Institute Nanotechnology Characterization Lab
NMR	Nuclear Magnetic Resonance
NPs	NanoParticle(s)
NTA	Nanoparticle Tracking Analysis
PALS	Phase Analysis Light Scattering
PDI	PolyDispersity Index
PDT	PhotoDynamic Therapy
PET	Positron Emission Tomography
PS	Particle Size
PSs	PhotoSensitizer(s)
R&D	Research and Development
RP	Reverse Phase
SANS	Small-Angle Neutron Scattering
SAXS	Small-Angle X-Ray Scattering
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy
SFC	Supercritical Fluid Chromatography
SM	SphingoMyelin
SMLS	Static Multiple Light Scattering

(*continued on next page*)

(*continued*)

Abbreviation	Full Name
TEM	Transmission Electron Microscopy
TLC	Thin-Layer Chromatography
TRIPS	Tunable ResIstive Pulse Sensing
UC	Ultra-Centrifugation
UF	Ultra-Filtration
USP	United States Pharmacopeia
UV	UltraViolet absorption detector
Vis	Visible light absorption detector

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