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Evaluation of the adaptability of the analytical techniques to the specificity of nanomaterials with a focus on nanomedicines and the liposomal formulation

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Abstract

Nanomaterials are components around 100nm in size that are becoming increasingly important in our society and literature. In particular, nanoliposome-based nanomedicines are the category most present in FDA- and EMA-approved drugs. These nanometric elements pose many challenges and questions as to their properties and the ability of currently available techniques to properly analyze them. Some of the techniques most widely used in the literature are presented in this work, along with their advantages and disadvantages. These techniques cover the characterization of liposomes in terms of size and lamellarity, encapsulation efficiency and drug release, membrane composition, surface charge and in vivo fate. In conclusion, it appears that current available techniques, while enabling drug development, present numerous barriers to research in terms of reproducibility, consistency and comparison of results, time and cost. Furthermore, the lack of a gold standard slows down the development of these technologies on an even larger scale.

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1) Introduction

a. What are nanomaterials

Nanotechnology is the term generally used to designate all structures or techniques that operate at the nanometric level. This corresponds to approximately 100 nanometers or less for at least one of the dimensions. (1) Nanomaterials can take many different forms (particles, fibers, tubes, etc.) and have the same composition as their larger versions, but their physico-chemical properties sometimes vary greatly, leading to new uses. With these new properties also come potential new dangers when these nanomaterials penetrate the body. More and more of these new materials and associated technologies reach the market. They range from health to electronics and textiles. Nanomaterials can be characterized depending on their size, shape, chemical composition etc. For the OECD, the basic information's needed to identify a nanomaterial is the following:

"1. Chemical substance information: Chemical composition, known impurities, and crystallinity.

2. Particle properties: Particle size distribution of the substance and contextual particle structure/shape information (e.g., where and how constituent chemical substances are or are believed to be distributed).

3. Surface properties: Specific surface area and information on surface chemistry (general composition and intentional surface functional groups), surface ionization capacity and residual acid or base content.

4. Intended use properties: Information on intended use and applications.

5. Physical properties: e.g., solubility (although this is media specific) (2)

In addition, because of their small size, nanomaterials dissolve more quickly than the equivalent of a large mass, which can change their properties, depending on the type of liquid with which they come into contact. (1)

Nanomaterials are used in many domains in our everyday lives. In the cosmetics industry, for example, titanium oxide is used in sun cream to improve protection but also to reduce the whitening effect on the skin after application. In sport, carbon nanotubes have been used to create lighter baseball bats. (3) Nanoparticles are also used in medicines. This point will be developed below.

b. Nanomedicine

i. Lipid-based nanoparticles (liposomes)

Liposomes are self-assembling colloidal vesicles with a characteristic lipid bilayer membrane composed of amphiphilic phospholipids that not only allow the encapsulation of many hydrophilic anticancer drugs and siRNAs in their aqueous core, but can also accommodate hydrophobic cytotoxic agents in their hydrophobic membrane. The diameter of round lipid vesicles can vary from several nanometers to multiple micrometers, with nanoliposomes intended for medical purposes typically measuring between 50 and 450 nm. (4,5)

Phospholipids, primarily constituting cellular membranes, vary in composition across different cell types. Glycerophospholipids, which are frequently used in liposome preparation, are natural amphiphilic molecules. They have a glycerol backbone, a hydrophilic region determined by a unique head group, and a hydrophobic area composed of fatty acid chains. The hydrophilic head group characterizes the specific type of glycerophospholipid, dictating its zwitterionic, cationic, or anionic nature. Notably, in eukaryotic cells, prevalent phospholipids include zwitterionic phosphatidylcholine (PC) and phosphatidylethanolamine (PE), anionic phosphatidylserine (PS), and zwitterionic sphingomyelin. Less common ones comprise anionic phosphatidic acid (PA) and phosphatidylinositol (PI). Apart from naturally occurring types, synthetic or modified phospholipids are also employed in liposome creation. These liposomes are often categorized based on size, lamellarity, bilayer fluidity, and surface charge. Their size varies from small unilamellar vesicles (SUVs) with diameters of 25–100 nm, to large unilamellar vesicles (LUVs) between 100 to 1000 nm, multilamellar vesicles (MLVs) exceeding 100 nm and finally giant unilamellar vesicles, (GUVs) up to 100 μm in size. (6,7)

Liposomes, used as chemotherapeutic drug delivery systems and tools for geno- or immunotherapy, not only enhance the safety of vector systems, but also improve the expression of therapeutic proteins and suppress disease-causing genes. Thanks to their innumerable advantages, they have been studied for drug delivery to tumor tissues using two predominant targeting methods: passive and active. The prevalent strategy for active targeting involves liposomes selectively binding to cancer cells displaying specific receptors. Ligands often used for this purpose with cancer cells include small molecules such as the folic acid receptor, as well as peptides, proteins and nanoantibodies. (5,8) Liposomes, when coupled to monoclonal antibodies or antibody fragments forming immunoliposomes, can enhance the anti-tumor efficacy of the anti-cancer agent, whether free or contained in single liposomes, while

decreasing the systemic toxicity of the non-encapsulated drug. Like single liposomes, these immuno-nanotechnology vectors can carry much larger quantities of cytotoxic drugs than drug-antibody conjugates. The latter, in fact, can only be combined with a few molar equivalents of drugs without impairing their ability to bind to the antigen. What's more, immunoliposomes offer the advantage of integrating multiple antibodies and target ligands, amplifying their targeting precision. (5)

Liposomes, especially nanoliposomes, can proficiently amass in tumor microenvironments, primarily due to the enhanced permeability and retention (EPR) effect. This phenomenon is attributed to the absence of vasculature-supportive tissues in tumorous areas, resulting in the creation of porous vessels ranging from 100 nm to 2 µm. With an underdeveloped lymphatic system, this facilitates the concentration of therapeutic agents in these regions. Both external stimuli, such as heat and light, and tumor-specific internal triggers like pH, redox potential, or particular enzymes, can instigate the release of drugs from liposomes. This release often emerges from membrane instability, induced by imperfections in the bilayer, optimizing drug delivery to the target sites, thus minimizing collateral damage to healthy tissues. Owing to their biological and technological advantages, nanoliposomes are now hailed as one of the most effective drug delivery systems. However, their application has some constraints. Their drug-loading capacity for poorly soluble drugs is somewhat restricted due to limited membrane space and potential destabilization effects. Consequently, they're primarily viewed as carriers for water-soluble drugs, though with certain loading limitations. (4,5,8)

Liposomes, when modified with Polyethylene Glycol (PEG), undergo a transformation called "steric stabilization." PEG forms a hydrophilic shield around the liposome, preventing unwanted interactions with other macromolecules in its vicinity. This protective barrier enhances liposomes' blood circulation duration and minimizes their uptake by the mononuclear phagocyte system. Additionally, when liposomes are equipped with targeting ligands like antibodies or folate on the PEG chain's end, they not only circulate longer but also achieve precise delivery. However, their inherent size can pose challenges, especially in penetrating solid tumors. Coating liposomes with PEG can overcome this hurdle to some extent. What's notable about liposomes is their adaptability. Their circulation time, size, surface charge, and other properties can be tailored by incorporating various lipid molecules, sidestepping the need for complex chemical procedures that other carriers, like polymer conjugates, often require. (5,9)

Despite their extensive development and wide application, the behavior of nanoliposomes in vivo remains incompletely understood. When designing these nanovectors, it is essential to

regulate their behavior inside the body. Misdirected accumulation of liposomal drugs in healthy tissues can lead to toxicity. For drugs to have their desired therapeutic impact, they must be released at the target site. Otherwise, the efficacy of encapsulated drugs may be considerably reduced. Pharmacokinetic studies often show that, in addition to their intended destinations, nanoliposomes also tend to accumulate in organs with high blood perfusion, such as the liver and spleen. Moreover, variations in nanoliposome accumulation, distribution and retention have been observed between different patients, underlining the need for further safety assessments of liposomal drugs. (4)

Liposomes present challenges such as stability, industrial reproducibility, sterilization complications, phospholipid oxidation and imperfect control of drug release, with release profiles often too rapid. What's more, intravenous administration can trigger a reaction known as "complement activation pseudoallergy" (CARPA), leading to hypersensitivity. (5)

ii. Polymeric nanocarriers

Polymeric nanocarriers, constructed from polymers, come in various structural forms, including amphiphilic core/shell designs (like polymeric micelles), intricate hyperbranched structures (like dendrimers), and encapsulated forms (such as polymeric nanoparticles). While liposomes have received clinical validation and possess numerous benefits, they encounter issues related to stability and constrained drug release control. In contrast, polymeric nanocarriers excel by offering enhanced in vivo stability, longer drug circulation durations, greater payload capacities, and the versatility to achieve more refined and directed drug release patterns over extended durations and at preset rates (5)

1. Polymeric micelles.

Polymeric micelles, formed from amphiphilic polymers, are promising vehicles for drug delivery, especially for poorly soluble cytotoxic drugs. When these polymers come in contact with an aqueous environment, they self-assemble into nano-sized spheroidal structures. Each micelle possesses a hydrophobic core that can encapsulate poorly water-soluble anti-cancer drugs and a hydrophilic shell that can incorporate hydrophilic drugs, ensuring micellar stability. A notable advantage of polymeric micelles is their size, typically ranging between 20-80 nm. This size range permits efficient extravasation through leaky vasculature, making micelles more effective than other drug delivery systems, like liposomes and solid lipid nanoparticles.

They also can be associated with PEG like liposomes. Moreover, the surface of the micelles can be tailored using ligand-conjugated amphiphilic polymers for active targeting. Among the various amphiphilic polymers used to create micelles, combinations like poly (ethylene glycol)-phosphatidylethanolamine (PEG-PE) stand out. Linking PE to PEG is straightforward, typically involving a single conjugation step, and offers a favorable balance of lipophilic and hydrophilic properties. This synthesis also ensures chemical stability, biocompatibility, and biodegradability. Other block copolymers, though offering increased structural flexibility and drug loading capacity, have their unique challenges. Despite their advantages, the quest for the ideal amphiphilic polymer continues due to challenges in optimal drug loading, retention of drugs in circulation, and efficient drug delivery to intracellular compartments. Further studies are imperative to understand micellar kinetic stability in vivo and drug release kinetics. It is also crucial to assess the toxicity and degradation kinetics of acrylate polymers grafted with different polymeric blocks used in micellar systems. (5,10)

2. Dendrimers

Dendrimers are highly branched, three-dimensional synthetic polymer macromolecules ranging from 10-100 nm in size. They are uniquely crafted from a central core using sequential, controlled polymeric reactions, granting a high degree of architectural control. This precise synthesis means their biocompatibility and pharmacokinetics can be fine-tuned for specific uses. With their uniform properties, including a monodisperse size and defined shape, dendrimers stand out as promising vectors, especially in oncology. They boast biodegradability, excellent water solubility, and a high drug-loading capacity. Furthermore, the multiple functional groups on their surface not only modulate their toxicity but also enable the simultaneous conjugation of multiple entities, such as anti-cancer drugs, targeting motifs, and PEGs, enhancing both solubility and circulation duration of drugs in the bloodstream. (5) PAMAM (Poly(amidoamine)) dendrimers are among the most popular types used for drug delivery. They offer three distinct sites for drug encapsulation. First, the inherent empty spaces within their branched structure act as molecular traps, becoming more efficient with increasing generations. While understanding the drug and dendrimer structure is crucial for effective selection, no software currently predicts a dendrimer's trapping capacity. Secondly, their branches can form hydrogen bonds, serving as another drug retention mechanism. Lastly, the surface groups engage in charge interactions, adding another layer of drug attachment. (11)

Dendrimers serve as versatile drug delivery systems, enhancing the solubility of hydrophobic molecules due to their water-soluble nature. Moreover, they bolster drug stability. The release rate of the drug can be tailored based on the surface groups of the dendrimer. For instance, NH2 groups tend to allow slower drug release, while COOH groups facilitate quicker release. Adjusting the molar ratio between the dendrimer and the drug can either speed up or decelerate this release. Collectively, these characteristics significantly improve oral bioavailability and optimize transdermal administration. (11)

Dendrimers, with their distinct uniformity, can easily penetrate cancer cell membranes and have reduced clearance by macrophages. These carriers can be hybridized with a variety of systems such as cisplatin, antibodies, peptides, or folic acid. Though their multifaceted synthesis can be cost-intensive, dendrimers offer flexibility in drug delivery. Anti-cancer drugs can be non-covalently encapsulated in the dendrimer's core or covalently attached to its surface, tailoring drug release through controlled depolymerization. However, dendrimers with a hydrophobic core are suitable mainly for local treatments like intratumoral injections due to potential toxicity and uncontrolled drug release. In contrast, covalently attaching drugs offers better solubilization and controlled release, depending on the linkers utilized. Notably, cationic dendrimers can be cytotoxic, especially with higher generations, prompting research into safer, biocompatible dendrimer alternatives. (5,11,12)

3. Polymeric nanoparticles.

Polymeric nanoparticles (NPs) are nanoscale drug carriers ranging in size from 10 to 1000 nm, which have gained traction in pharmaceutical applications due to their enhanced drug delivery potential. These particles are classified into two primary structures: nanocapsules and nanospheres. While nanocapsules are reservoir systems featuring an oily core enveloped by a polymeric shell for controlled drug release, nanospheres are matrix systems where drugs are either retained within or adsorbed onto a continuous polymeric network.

Several advantages underscore the rising interest in polymeric NPs. They allow controlled drug release, shield biologically active molecules from environmental threats, and enhance both the bioavailability and therapeutic index of drugs. Their core-shell design typically comprises a hydrophobic interior that houses the drug, and a hydrophilic exterior made of polymers like PEG or PVP. This unique design ensures stability, lowers immunogenicity, and reduces the likelihood of the NPs being captured by the reticuloendothelial system. Biodegradability is another crucial attribute of these NPs, which break down into non-harmful byproducts.

Additionally, they enhance the solubility of active drugs, maintain stability, offer impressive pharmacokinetic control, and evade reticuloendothelial clearance without activating harmful biological responses. Polymeric NPs can be derived from both natural and synthetic sources. Natural polymers like heparin, albumin, chitosan, and gelatin are favored for delivering DNA, drugs, and proteins due to their reduced side effects and extended residence times. For instance, paclitaxel-loaded albumin nanoparticles have been employed against metastatic cancer. On the other hand, synthetic polymers approved by the FDA, such as PLGA, PLA, PEG, and PCL, are popular for their ease of production and controlled, sustained drug release. However, despite their benefits, natural polymers can present challenges in terms of rapid drug release and the need for purification before use. Like other nanocarriers, they can be tailored for targeted drug delivery with the same kind of techniques. Compared to other nanocarriers like liposomes, these NPs showcase better stability, size uniformity, and a higher drug load, making them especially promising in anti-cancer applications. (5,13,14)

c. Place in society

The first nanodrug authorized by the FDA was Doxil® in 1995. It used a nano-liposome combined with doxorubicin to treat breast cancer, ovarian cancer and solid tumors. (15) Today, there are just over fifty nanodrugs approved by the FDA using the various techniques described above and more, with just over 20% involving liposome formulations combined with a drug or biological product. (16,17) Of these fifty or so drugs, only 27 have been approved by the EMA. (18) The development of nanodrugs is particularly occupied by small and medium-sized pharmaceutical companies, whereas large companies often use their resources to focus on a single nanomedicine project. In 2005, this report showed that the EU nanomedicine industry includes 92 startups (44%), 67 SMEs (32%), and 41 large pharmaceutical or medical device companies (21%). (19) One of the biggest illustrations of the capabilities of nanomedicines and their growing development is the Pfizer Vaccine and Moderna for Covid-19, both of which are liposomal formulations. (20,21)

2) Methodology

For this search, only Pubmed was used for the scientific literature. The keywords used in the initial searches were "nanomaterials", "analytical techniques" and "liposomes". Subsequently, words associated with the various analytical techniques encountered were used in combination with "liposome". In all, over a hundred documents and sources were scanned, 70 were fully analyzed and 53 retained for the writing of this work.

3) Analytical techniques

There are a multitude of features for characterizing and studying the nanocarriers used in current research. In the context of this work, the techniques developed concern only the category most represented in nanomedicines authorized today: liposomal formations.
To ensure the proper functioning of a liposomal formulation, it is necessary to be able to characterize size and shape, lamellarity, surface charge, composition and encapsulation efficiency. (22,23)

a. Size and lamellarity

i. Dynamic light scattering (DLS)

DLS is an experiment in which a sample in a cuvette is illuminated by a monochromatic light beam. This beam interacts with the molecules in the solution, and a multitude of incident rays are scattered in all directions, depending on the shape and size of the molecules. (24) The intensity of these incident rays is captured by a detector, which can be located at 90°, 173° or 158°. Due to the Brownian motion of molecules in a solution, an incident monochromatic ray undergoes what is known as Doppler broadening. This modifies the frequency of the wavelength as a function of the molecules' movements, which also depend on temperature. These different waves can cancel each other out or add up in mutually destructive or constructive phase, enabling a signal to be detected. The detector then translates the intensity fluctuations over time to obtain a function which, using the Stokes-Einstein equation, yields a particle radius. It's important to avoid contamination such as dust, as this can alter the result. (25)

In order to perform a DLS analysis, it is important that the sample has been highly purified, for example by chromatography and centrifugation. Indeed, without this purification, high molecular weight aggregates may be present and interfere with the analysis, as shown by ***Hupfeld et al.*** The presence of larger particles causes the DLS to minimize the presence of smaller ones. In this case, DLS was used without adequate filtration and gave measurements in excess of 200 nm, as it also accentuates the larger molecules. When SEC is used, this measurement then drops to 130 nm and 100 nm with other techniques. (24,25)

The advantage of DLS is its wide range of buffers, temperatures and concentrations. It is also non-invasive, requires few samples and delivers rapid results. However, measurements are highly sensitive to temperature. In addition, DLS offers low resolution for tightly bound molecules and does not deliver highly reproducible results. This makes it a basic analysis technique that can be used as a benchmark to be complemented by other instruments to obtain the information required. (24,26)

ii. Microscopy

There are many different microscopy technologies available and they are already being used to study nanomaterials and liposomes in particular. Light microscopy is a simple and rapid way of obtaining information on size, shape, homogeneity or degree of aggregation, but only effectively for GUVs. Nano-sized SUVs are too small to obtain information on the bilayer. Polarization microscopes offer an alternative with better clarity for GUVs, but have the same limitations. Fluorescence microscopy provides an even better analysis of membrane structure, thanks to the many possible combinations of different stains. However, the interaction of lipids with dyes and fluorescent light can lead to artifacts and peroxidation. Confocal microscopy offers superior image clarity, with a 3D view and analysis of the sample's internal structure. However, quality remains limited for SUVs. These different optical techniques can exceed their physical limits, which are around 200 nm, by using "super-resolution" technologies. (23) However, these techniques will not be developed further in this work.

One of the most widely used imaging techniques is transmission electron microscopy (TEM). This uses an electron beam instead of photons as the excitation source. This beam is focused and directed by electromagnetic lenses. These electrons then interact with the sample, which must be less than 100 nm thick and placed on a grid in a vacuum chamber. Electrons can be absorbed, scattered or transmitted through the target. These interactions with the sample modify the intensity of the emitted particle. The emission then passes through other

electromagnetic lenses to be picked up by an electronic detector, which captures the electron and translates the signal into a visible image on a screen. The classical resolution of such an instrument is up to 0.2 nm, perfect for SUV liposomes. (23,27) Typically, in a negative staining analysis, the sample placed directly on the grid may have its shape and orientation altered. The sample must also be coated with an electron-dense material to ensure sufficient image contrast, such as phosphotungstic acid. These vacuum and contrast conditions can lead to dehydration and structural modification, sometimes resulting in luminous bangs that can be mistaken for a bilayer. (23) To overcome these drawbacks, several variants exist. TEM freeze-fracture uses a non-dried sample which is rapidly frozen with liquid nitrogen before being fractured in the areas of the sample with the weakest molecular interactions. A replica is then created by applying a thin layer of carbon or platinum corresponding to the sample negative. The advantage of this technique is to obtain a reusable replica showing features very close to the sample's native state, as well as providing detailed information on the 3D structure of the vesicle and lipid bilayer. It also provides information on aggregate size. However, artifacts are possible if the freezing rate is not sufficiently high, or simply due to mechanical stress. (23) Another variant, Cryo-TEM, also freezes the sample, but in solution and between two vitrified aqueous films. The part holding the assembly is continuously cooled to below -180°C to prevent the water changing state and affecting image contrast. Low-contrast samples such as liposomes can still be observed without staining, thanks to contrast-enhancing diaphragm systems and energy filters. This makes it possible to study the liposome in its state as close as possible to its native state, and to observe the characteristics of size, shape, lamellarity and interior elements such as a drug, for example. The disadvantages of this technique compared with others are, firstly, that only 2D images of a 3D element are obtained, but this can be adjusted by taking several images at different angles. Cryo-tomography can also be used. In order to avoid damage to the structures to be observed by the electron beam, it is necessary to use a lower dose and therefore reduce image resolution compared with other methods. Artifacts are also possible if the aqueous film forms ice or if shear forces are applied to the sample. (23,28)

Another microscopy technique makes it possible to study the liposome with complete freedom of environment in terms of temperature, pressure and gas. There's also no need to apply a vacuum to the sample, or any type of fixation or staining. This is Environmental Scanning Electron microscopy. This technique uses the same principles as a scanning electron microscope, analyzing secondary and backscattered electrons to form a 2D image of the sample. However, the sample chamber is separated from the electron column by a vacuum, and

contains steam to keep the sample in its original hydrated state. This technique also makes it possible to analyze drug incorporation into the bilayer, in addition to vesicle shape and size. Its limitation is that it cannot provide information on the lamellarity or interior of nanoscale structures. (23,29) A final technique that can be used is atomic force microscopy (AMF), also known as scanning force. This scanning technique uses a very fine colloidal tip mounted on a flexible lever to probe the sample surface. Interaction forces between the tip and the surface cause the lever to deflect, which is measured by a laser aimed at the back of the lever. This same reflected laser is picked up by a photodiode, providing a topographical image of the sample. The sample does not need to be under vacuum, and can provide a 3D image with sub-nanometer resolution. Precise information can be obtained not only on size and shape, but also on homogeneity, stability and even surface modification of the liposome, or the presence of ligands such as polymers or antibodies. There are two drawbacks to AMF. The first is the need for nanoparticles to be absorbed onto mica or silicone plates to ensure a uniform, atomically flat surface, and thus to modify the vesicle's size and shape. The second is that the movement of the tip can pull the liposome and thus modify its position in the sample. (23)

iii. Size-exclusion chromatography (SEC)

Size-exclusion chromatography is a benchmark technique for separating and determining elements by hydrodynamic volume. The principle consists of a column containing a stationary phase made up of a porous material. The mobile phase is the sample and a solvent, which is poured into the column. Most of the time, this material consists of agarose, dextran or polyacrylamide beads with pores of a selected size specific to the element to be analyzed, or gels. Molecules larger than the pore size of the stationary phase will be eluted first, due to their inability to fit into the beads. Molecules closer to the target size will be eluted more slowly as they penetrate the pores. Molecules small enough to pass through the pore freely, however, will not be separated as they exceed the permeation limit. (30,31)

In the case of nanosized liposomes, the stationary phase is usually a gel, as shown by ***Holzer et al*** (32). The use of gel means that some of the liposomal suspension will be absorbed by it. To prevent this, it is necessary to saturate the gel with lipids before performing the analysis. The gel is saturated when liposomes injected at constant sample loads are eluted at identical elution volumes and produce constant peak yields. It is also necessary to calibrate the column with size standards. By plotting the elution volume of these standards against their size or molecular weight, a calibration curve is obtained. The elution volumes of the standard can then

be compared with those of the sample to estimate the apparent molecular size or weight. (33,34) As this measurement is indirect, it is often necessary to combine the information obtained by SEC with that of Cryo-TEM and DLS in order to obtain a more accurate size. What's more, obtaining a fraction with a highly accurate size distribution is only possible by upstream preparation which reduces the size distribution in the sample, for example by dialysis. However, this also increases the yield of the desired particle size. (32) SEC can therefore be used to obtain liposomes close to their native state, with an approximate size, and to eliminate any impurities present in the preparation. However, the choice of buffer, pressure and flow rate must be carefully selected to avoid compression and deformation of the gel beads, osmotic shocks or shrinkage of the liposomes. This is why SEC is more appropriate for liposome preparation. (33)

The high-performance version (HPSEC) overcomes some of the shortcomings of conventional SEC. HPSEC is a version of HPLC (High-Performance Liquid Chromatography) and therefore has the advantages of shorter run times, reduced sample size, significantly increased peak resolution, analysis reproducibility and separation efficiency. This is made possible in part by the high pressure achievable and the finer columns. However, high pressure still poses a threat to liposome integrity. What's more, HPLC is more expensive and requires more experience on the part of laboratory staff. (33)

b. Encapsulation efficiency and drug release

Encapsulation efficiency (EE) is described in the literature as the ratio of encapsulated drug to the initial amount of drug in the preparation. This parameter can vary depending on drug properties, liposome size, lamellarity and the methods used to prepare the construct. Separation of liposomes from the sample is a crucial step in determining EE. (35)

i. dialysis

Dialysis techniques all use the same basic concept. The solution to be studied is contained in a donor compartment separated from a second receiving compartment with a dissolution volume by a dialysis membrane. In principle, the correct separation of free and encapsulated drug is achieved by choosing a membrane with the right cut-off. However, some of the free drug may not pass through the membrane, as it interacts with it. Some liposomes may also release their

contents during dialysis. The amount of free drug in the receiving compartment may therefore be under or overestimated in relation to the true EE of the liposome. Dialysis is still considered a good but time-consuming method. (36)

For the in vitro liposome drug release study, the drug-containing solution will be diffused according to two parameters: diffusion from the liposome to the donor compartment, and permeation of the drug through the membrane to the recipient compartment. To ensure diffusion conditions, the dissolution volume must be up to 10X the saturation volume of the drug, and the volume of the donor compartment must be up to 10X less than the dissolution volume. (37,38) Permeation is often not considered a limiting factor and is therefore neglected. This has the effect of determining the apparent kinetics as the real kinetics. However, it has been mentioned several times that dialysis membranes have an effect on these kinetics and slow down diffusion. As suggested by ***Yu et al***., This effect will depend not only on the molecular weight cut-off (MWCO) but also on the type of membrane. To compensate for this, prior calibrations and mathematical models can be applied to obtain results closer to the real kinetics of the drug. (37)

ii. Centrifugation et Ultrafiltration

Centrifuges are used to separate different particles by causing them to settle in a tube under high centrifugal force. For smaller particles such as SUVs, the use of ultracentrifuges is required to reach several hundred thousand g for several hours. Afterwards, drug concentration can be measured in the supernatant to assess EE. Centrifugation can present certain problems. Indeed, it seems that some of the liposomes smaller than 100 nm are difficult to sediment. What's more, some liposomes burst under the physical stress imposed by centrifugation into smaller liposomes. Increasing speed is possible, but also increases the chances of bursting. These effects lead to a preference for Ultrafiltration. (36,39)

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In pressurized ultrafiltration, a pressure applied to the upper surface of the dissolution subjects it to a gentle hydrostatic force, forcing the particles to separate. The results shown by ***Wallace et al.*** show better filtration results than other techniques, and also better estimates of drug release than dialysis. A similar conclusion can be observed with ***Boyd.*** (41)

iii. Gel filtration (SEC)

The technique described above can also provide information on encapsulation capacity, by separating free drug from encapsulated drug. However, the shortcomings of the classic version make it more suitable for prior purification before analysis by HPLC or centrifugation. (33,42)

iv. HPLC

High performance liquid chromatography is a benchmark technique for separating a wide range of analytes, including nanomaterials. More specifically, the SEC mode of HPLC is used to analyze liposomal formulations. However, it has the shortcomings described above, which often necessitate prior ultracentrifugation.

An adaptation of HPLC using a polymer-coated monolithic silica column to separate liposomes from the free drug named nPEC has been created by ***Itoh et al.*** (43)) This technique has been used by ***Bian et al.*** and obtained precise analytical results for free and total drug without the need for prior purification by other means. What's more, results were obtained within 30min for liposomes containing hydrophobic or hydrophilic drugs, without the need for complex manipulation. (44)

Using a customized HPLC system ***Ohmish et al.*** was able to achieve repeatability close to 95%. In terms of recovery, between 99.8 and 100% of liposome-encapsulated drugs could be recovered, indicating little or no absorption by the system - an advantage over the semi-permeable membranes of other techniques used for the same analysis. The system is also described as having a good processing capacity, as it required very little sample (5µL), little manual handling was needed to reduce any losses, and the whole process was completed in 25min, which is much faster than some ultracentrifugation or dialysis protocols. (45)

These 2 forms of HPLC seem to be interesting avenues for developing better standardization of characterization and quality control.

c. Membrane composition

The composition of a liposome membrane can be extremely varied. This can give rise to interactions that are important to understand, so as not to alter parameters such as encapsulation, surface charge or solubility.

i. Spectrometer

The Mass spectrometer measures the mass-to-charge ratio (m/z) of particles. It can be used to determine molecular weight and structural information. To do this, the sample is ionized, transforming the molecules into charged particles. Once ionized, the particles are separated according to their m/z ratio by the analyzer. Once separated, the particles hit a detector which records the abundance of the signal associated with an m/z value, enabling the composition of the sample to be determined, each component having its own ratio. In the case of a nano liposome, Electrospray Ionization (ESI) mode is more widely used, as it enables gentler ionization, keeping the molecules intact without separating them into smaller fragments. A sample requires considerable purification and concentration before it can be used by mass spectrometry (MS), particularly for complex nanostructures such as nano liposomes. This is why MS is often coupled with a high purification step such as chromatography beforehand. (46)

ii. Nuclear Magnetic Resonance

Nuclear magnetic resonance is used to determine the structure of a sample down to the atomic level. The technique is based on the property that all elements are made up of atomic nuclei with charge and spin. By applying a magnetic field, a transfer of energy is enabled, which changes the spin of the nucleus. Returning to its original position, it emits energy at a characteristic frequency, which is interpreted by a detector. (47) This technique makes it possible to analyze the sample without the need for modification, and to obtain results in line with those obtained by more conventional techniques such as HPLC. However, resolution can be problematic, as the signal obtained is proportional to the number of nuclei in the sample. Low-abundance compounds may therefore not be detected. A larger quantity of sample may then be required, which can be limiting in terms of instrument availability and capacity. A

spectrometer is often preferred, as it is faster and more accurate, despite the denaturing conditions. (48,49)

d. Surface charge

The stability of nanoparticles in suspension and the initial adsorption of nanoparticles onto the cell membrane are both largely influenced by the zeta potential, which is dependent on the surface charge. The endocytotic absorption rate after adsorption is influenced by particle size. Thus, nanoparticle toxicity is influenced by the zeta potential and size. The efficiency of nanoparticles for drug administration depends in large part on their ability to be controlled in terms of size and zeta potential, which also makes it possible to define the cellular targets for substances like liposomes. (50)

i. Doppler laser electrophoresis

LDE is one of the most widely used techniques, along with DLS, for determining the charge surface. In this configuration, a coherent laser source is split into two separate beams. These beams are directed so as to meet inside the zeta cell, where the sample dispersion is located. This intersection creates interference patterns. When a particle passes through these patterns, it generates variations that enable us to estimate its speed of travel. This technique effectively measures the frequency of particles with a certain mobility, providing a distribution of mobilities rather than a single mean value. However, due to the orientation of the zeta cell, the electrophoretic mobility of charged particles is considerably influenced by buoyancy. In the standard design of this instrument, electrophoretic mobility is deduced from particle displacements in the vertical direction, a direction in which buoyancy also acts. In the case of a liposome, the effect of buoyancy is negligible. (51) Possible disadvantages should be noted. If the sample is not homogeneous in size and charge, the measurement may be slightly distorted. The properties of pH, ionic strength or aggregation can also have this effect. As this technique is not separative, it could be coupled with a purification technique. (52,53)

ii. Capillary electrophoresis

Capillary electrophoresis (CE) is a technique based on the separation of molecules in a thin capillary, generally soaked in a buffer solution, according to their size/charge ratio. After introduction of the sample under the effect of an electric field, a voltage is applied, causing the ions to migrate to their respective electrodes. Separation is dictated by the electrophoretic mobility of the ions, which depends on their size, shape and charge. A specific feature of CE is the phenomenon of electroosmotic flow, generated by interactions between the charged walls of the capillary and the ions in the buffer, influencing the migration of molecules. CE is renowned for its ability to detail the surface charge of particles, particularly liposomes. Its major assets include low sample consumption, speed of analysis, meticulous separation and ease of automation. It is invaluable for unravelling drug-liposome interactions, revealing information on encapsulation efficiency, drug release, as well as other characteristics such as the size and integrity of the phospholipid layer. Its resource-saving and environmentally-friendly nature, thanks to limited use of samples and solvents, predicts a growing place for CE in a variety of applications. However, CE is not without its challenges. Under the influence of high voltages, liposomes may lose their stability and burst. What's more, the technique may lack sensitivity when it comes to detecting low liposome concentrations. Resolving liposomes with similar properties can also prove challenging. (7,22,52)

e. In vivo fate

In vivo fate is the final stage of nanomedicine development. Knowledge of its various interactions is essential to ensure safety and efficacy.

i. Fluorescence labeling

Nanoliposomes' accumulation in tumors can be studied using a fluorescence optical imaging technique in small animals. In a study mention in ***Su et al.*** paper, the compound 1,1′-dioctadectyl-3,3,3′,3′,-tetramethylindotricarbocyanine, 4-chlorobenzenesulfonat salt (DiD) was chosen to label liposomal doxorubicin (Caelyxs) due to its near-infrared excitation and emission wavelengths, strong tissue penetration, and high fluorescence quantum yield. This compound has also been utilized in optical imaging and confocal microscopy to analyze nanoparticles' tumor distribution. Another study was on the pharmacokinetics and the ability

of glutathione PEGylated (GSH-PEG) nanoliposomes to cross the blood-brain barrier in rats were examined using a fluorescence technique. Carboxyfluorescein served as a fluorescent tracer that self-quenched within the nanoliposomes' core. This method effectively quantified intact liposomes in organs like the liver, spleen, kidneys, lungs, brain, and spinal cord, as well as in plasma and brain endothelial cells. Carboxyfluorescein has proven valuable for studying the pharmacokinetics and tissue distribution of nanoliposomes. However, the challenge with in vivo fluorescence imaging is its low sensitivity. A solution to this has been the use of polydiacetylenes (PDAs) as tracers, given their unique optical and fluorescent characteristics, especially their near-infrared (NIR) emission. The NIR emission range (700–900 nm) is particularly advantageous as biological matrices show minimal absorbance in this spectrum, leading to enhanced imaging selectivity and resolution. While fluorescence labeling, being cost-effective and non-invasive, is a popular method for tracking nanoliposomes in vivo, it has its drawbacks. Fluorescent agents can be unstable within the body's circulation, potentially skewing imaging results, and their potential toxicity further restricts their application for in vivo nanoliposomes tracing. (4)

ii. Radiolabeling method

Radiolabeling has become an increasingly popular method for quantifying nanoliposomes in vivo due to its exceptional sensitivity and specificity. Loading nanoliposomes with a 64Cu radionuclide can use positron emission tomography (PET) to quantify its concentration in tissue and blood. The PET imaging technique is FDA-approved for its high sensitivity, spatial resolution, and ability to directly quantify radioactivity in specific regions based on signal intensity variations. The 64Cu radionuclide is ideal for PET imaging because of its longer half-life and properties that lead to high-quality images.

However, 64Cu isn't the sole radionuclide in play. Other researchers also used 18F, another radionuclide known for high-quality PET images. 99mTc is another radioisotope used for nanoliposomes, especially those inhaled. However, while radiolabeling is potent, it's not without its challenges. Handling radioactive materials requires specialized training, and the method can't simultaneously monitor multiple radioisotopes due to energy resolution constraints. Moreover, radiolabeling can potentially alter nanoliposomes' behavior in the body, compromising the accuracy of the results, and poses environmental and health concerns. These challenges underscore the need for continued research in this domain. (4)

iii. Magnetic resonance imaging (MRI)

Magnetic Resonance Imaging (MRI) stands as a prime clinical tool known for its remarkable spatial resolution. Its strength lies in its non-invasive ability to track the in vivo distribution of nanoparticles, offering superior anatomical resolution compared to other technologies.

When exploring the biodistribution of nanoliposomes in living systems, three MRI contrast agents typically come into play: T1, T2, and the newer CEST (chemical exchange saturation transfer) agents.

- T1 Contrast: Produced by paramagnetic centers, T1 contrast agents act by shortening the water protons' longitudinal relaxation times, creating clear contrasts in MRI scans.
- T2 Contrast: Here, the main players are the magnetic and superparamagnetic iron oxide nanoparticles. They influence the transverse relaxation times of water protons, yielding a negative contrast on T2-weighted images. Notably, superparamagnetic iron oxide nanoparticles are a favorite for molecular MRI imaging due to their elevated molar relaxivity compared to the paramagnetic T1 agents. Yet, they're not without challenges. Their inconsistencies in dispersion, broad particle size distribution, potential toxicity, and tendency to clump together and absorb plasma proteins curb their widespread use in nanoliposome bioanalysis.
- CEST Agents: Emerging as a groundbreaking MRI contrast type, CEST agents hinge on labile spins that swiftly interchange with a solvent. This leads to a boosted signal, making it feasible to detect solute protons even in low, millimolar to micromolar, concentrations.

Interestingly, these contrast agents can potentially be used synergistically, paving the way for enhanced and multi-dimensional imaging. MRI offers a unique capability to differentiate between intact and damaged nanoliposomes in the body. A notable advancement in this realm is the diaCEST (diamagnetic CEST) agents. These are naturally occurring molecules devoid of metal ions, and their contrasting ability hinges on the count and nature of labile protons. Due to their organic and biodegradable nature, they amplify MRI sensitivity significantly. While MRI excels in offering a non-invasive method with high spatial resolution for tracking nanoliposomes in vivo, it's not without challenges. The intricate nature of biological matrices can compromise MRI's specificity. Furthermore, while enhancing imaging, contrast agents can alter nanoliposomes' behavior in the body and potentially skew results. The safety profile of many such agents also awaits comprehensive evaluation. (4)

iv. Mass spectrometry

Mass spectrometry has emerged as a promising analytical technique for the quantitative measurement of nanoliposomes in vivo, promising exceptional selectivity, sensitivity, and accuracy. While inductively coupled plasma-mass spectroscopy (ICP-MS) offers a way to quantify both nanoliposomes and their encapsulated contents in tumors, it isn't without the typical limitations associated with indirect analytical methods. On the other hand, the liquid chromatography-tandem mass chromatography (LC-MS/MS) approach focuses on quantifying in vivo nanoliposomes by tracking the drugs they enclose. Yet, while LC-MS/MS combined with solid-phase extraction has proven effective for quantifying nanoliposomes in plasma, it hasn't been as successful with tissue samples. This is mainly because the tissue homogenate process tends to damage the nanoliposomes. (4)

v. Computed tomography (CT)

CT-based imaging is particularly apt for studying long-circulating nanoparticle systems due to the high X-ray attenuation offered by CT contrast agents with their high atomic numbers. Beyond this, CT offers a comprehensive package for the pharmacokinetic study of nanoliposomes, granting quantitative, volumetric, and longitudinal insights. It's especially beneficial for observing slower physiological events, like the passive accumulation of nanoliposomes in tumors through the EPR phenomenon. With its ability to rapidly acquire data at high resolutions, coupled with 3D image analysis, CT furnishes detailed volumetric quantifications within various organs and tissues. Given that CT stands as today's most prevalent whole-body volumetric imaging technology, it underscores its significant promise for extensive biodistribution research.

4) Conclusion

Liposomes and nanotechnologies are gaining increasing prominence in scientific publications. From the first developments in the 60s to the present day, these technologies have been improved and refined. They open up a whole new field of possibilities for the treatment of disease. This is why the need for analysis techniques is growing and becoming ever more necessary. They play an essential role in the characterization, quality control and safety assessment of nanomedicines. These nanoscopic particles have unique properties that still pose many challenges today. A wide arsenal of techniques exists and is used in the literature to characterize nanomedicines according to size and distribution (such as dynamic light scattering, electron microscopy) to those studying surface properties, molecular composition and release characteristics. As nanomedicine has progressed over the years, analytical techniques have been refined and new ones created. Today, resolution and precision have greatly improved. However, due to their unique structures and compositions, some particles cannot be fully characterized with existing techniques. Indeed, it is almost automatic to read protocols in the literature where techniques are combined or at least compared with each other in order to characterize only part of the nanomedicines under study. Although this combination enables us to obtain information close to the real thing, there is still a degree of uncertainty, particularly at liposome level, as to what exactly happens to the liposomes in vivo. The great variability of the techniques used goes beyond the scope of what has been presented in this work and represents one of the biggest black spots on the subject of analytics. Indeed, although OECD, FDE and EMA are working with the scientific community to establish a true gold standard for characterization, one does not yet exist. Although certain techniques such as DLS, Ultrafiltration, HPLC, cryo-TEM and capillary electrophoresis seem to stand out in the literature, none is perfect or self-sufficient for the study of their designated parameter. This need for multiple manipulations also leads to problems of variations in results and lack of reproducibility. Interpretation of results can therefore also be problematic, as can the choice of technique to be used. It is also clear that there is a need for a large amount of equipment and qualified personnel, which not only increases the time required for research, but above all its cost. All these problems are a barrier to the development of new nanotechnologies, but they will hopefully be overcome with time and the progress of research.

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