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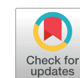
Review Paper

Analytical methods for investigating *in vivo* fate of nanoliposomes: A review



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ABSTRACT

Nanoliposomes are considered to be the most successful nanoparticle drug delivery system, but their fate in vivo has not been fully understood due to lack of reliable bioanalytical methods, which seriously limits the development of liposomal drugs. Hence, an overview of currently used bioanalytical methods is imperative to lay the groundwork for the need of developing a bioanalytical method for liposome measurements in vivo. Currently, major analytical methods for nanoliposomes measurement in vivo include fluorescence labeling, radiolabeling, magnetic resonance imaging (MRI), mass spectrometry and computed tomography. In this review, these bioanalytical methods are summarized, and the advantages and disadvantages of each are discussed. We provide insights into the applicability and limitations of these analytical methods in the application of nanoliposomes measurement in vivo, and highlight the recent development of instrumental analysis techniques. The review is devoted to providing a comprehensive overview of the investigation of nanoliposomes design and associated fate in vivo, promoting the development of bioanalytical techniques for nanoliposomes measurement, and understanding the pharmacokinetic behavior, effectiveness and potential toxicity of nanoliposomes in vivo.

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1. Introduction

In the past few decades, several kinds of drug delivery system have been widely investigated, and nanoliposomes were one of the popular species of nanoparticles potentially used as carriers of bioactive molecules [1,2]. Liposome is a colloidal union of phospholipids that assemble themselves into bilayer vesicles [1], which was first discovered by Bangham et al. in the 1960s [3]. Bangham et al. [3] found that when egg lecithin dispersed in water, it could assemble into closed bilayer structures spontaneously; subsequently, closed bilayer structures were named 'liposomes' in 1968 [4]. Liposomes can be made of natural phospholipids with various lipid chains [2]. The polar parts of phospholipids are situated at the surface of the liposomes, and the fatty acid chain parts

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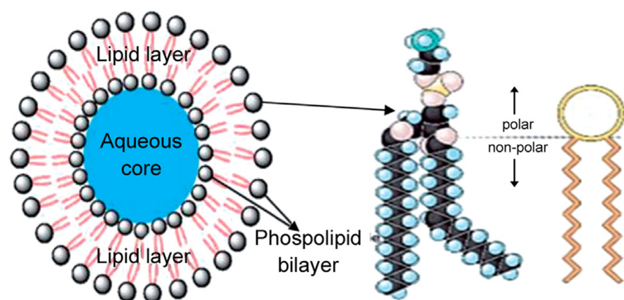

Fig. 1. Schematic representation of the structure of liposomes [2].

comprising hydrophobic core of bilayers are isolated from water (Fig. 1 [2]). Nanoliposomes are nanometric versions of liposomes, and they can provide both lipophilic and hydrophilic areas which can entrap drugs with different lipotropies in lipid bilayers, aqueous core or bilayer interface [5–7]. The size of spherical lipid vesicles can range from a few nanometers to several micrometers, and nanoliposomes applied to medical use generally range between 50 and 450 nm [8].

Nanoliposomes are deemed to be an ideal drug delivery system, because of their similar nature to cytomembrane and excellent ability to entrap diverse drugs; consequently, nanoliposomes have been extensively investigated in the past 60 years. Moreover, nanoliposomes can preferentially accumulate in tumors relying on the enhanced permeability and retention effect (EPR), which can improve efficiency and decrease the systemic side effects of anticancer drugs [9]. Due to the biological and technological superiorities of liposomes as delivery systems both in vitro and in vivo, nanoliposomes are currently considered to be the most successful drug delivery system [10]. To date, 15 liposomal drugs have been approved for clinical uses (Ambisome, Abelcet, Amphotec, DaunoXome, Doxil, Lipo-dox, Myocet, Duomeisu, Libaoduo, Visudyne, Depocyt, DepoDur, Epaxal, Inflexal V, and Lipusu) [11].

Despite their long history of development, and wide application, the in vivo fate of nanoliposomes is still not fully understood. Acquiring complete knowledge about the in vivo fate of nanoliposomes will provide useful information for designing more efficient nanoliposomes with good targeting property and a better control of undesired side effects. When designing nanoliposomes, controlling their in vivo fate is important. If designed liposomal drugs accumulate and play their therapeutic effect in healthy tissues, toxicity will generate. Moreover, only when drugs are released from nanoliposomes at the target site, can they produce expected therapeutic effects, but drugs in encapsulation state that are not released from nanoliposomes would seriously decrease their efficacy [12]. Many pharmacokinetics studies show that nanoliposomes accumulate not only in target tissues, but also in highly perfused organs like the liver and the spleen [13,14]. This would lead to new side effects such as hand-foot syndrome and serious reduction of the phagocytic activity of the liver macrophages [13,15]. Moreover, it has been reported that accumulation, distribution and retention of nanoliposomes in vivo varied in different patients, which indicates that the safety of liposomal drugs needs further investigation [16]. Therefore, investigating the fate of nanoliposomes in vivo and acquiring their pharmacokinetics information are essential to designing efficient and safe nanoliposomes for drug development. However, determining nanoliposomes in vivo remains a challenge for the current analytical methodologies due to complexity of nanoliposomes compared with the classic chemical molecules, ions, or elements. Although some existing methods have been applied to the analysis of nanoliposomes in vitro, almost none of these are fully adequate for quantitative analyses of biological samples. In addition, standardization of liposome measurement is very important in liposomal drug development. Thus, an overview of currently used bioanalytical methods is in great demand to lay the groundwork for the need of developing and standardizing a bioanalytical method for liposome measurements in blood and tissues.

The main purpose of this review is to summarize the analytical methods used for the measurement of nanoliposomes and offer critical opinions in this field. Up to now, several techniques have been reported to quantify liposomes in vivo. Major methods include radiolabeling, fluorescence labeling, magnetic resonance imaging (MRI), computed tomography (CT) and mass spectrometry. We review mechanisms of these methods in detail, and evaluate their advantages and disadvantages for various nanoliposomes in different biological materials. The review provides a comprehensive overview of the in vivo fate investigation and structural design research of nanoliposomes in the future.

2. Quantitative methods for liposomes

2.1. Fluorescence labeling

Hagtvet et al. [17] investigated nanoliposomes accumulation in tumors with fluorescence optical imaging strategy in small animals. 1,1′-dioctadectyl-3,3,3′,3′,-tetramethylindotricarbocyanine, 4-chlorobenzenesulfonat salt (DiD) was selected as a carbocyanine tracer to label liposomal doxorubicin (Caelyx®), because DiD has near infrared excitation and emission wavelengths, powerful tissue penetration and high fluorescence quantum yield. DiD has also been used in optical imaging and confocal microscopy studies to investigate nanoparticles tumor distribution [18,19]. In this study, methodology was evaluated by comparing the intensity of optical imaging signals between ex vivo and in vivo. The results indicated that liposomal doxorubicin gradually accumulated in tumors and reached plateau levels at 48 h after intravenous injection.

Rip et al. [20] have investigated the pharmacokinetics and the blood–brain barrier crossing capability of glutathione PEGylated (GSH-PEG) nanoliposomes in rats using the fluorescence method; carboxyfluorescein was used as a fluorescent tracer that autoquenched in the core of nanoliposomes. The fluorescence method established in this study successfully quantified intact liposomes in the liver, spleen, kidneys, lungs, brain, and spinal cord, as well as in plasma and brain endothelial cells; fluorescence was detected at 485/538 nm. Autoquenched carboxyfluorescein was shown to be a practical tool for the investigation of pharmacokinetics and tissue distribution of nanoliposomes. The study finally demonstrated that GSH-PEG coated nanoliposomes can observably increase the delivery of drugs to the brain compared with PEGylated nanoliposomes.

Fluorescence labeling method was also employed by Li et al. [21] to trace folate-polydiacetylene-liposomes in cells using polydiacetylenes (PDAs) as fluorescent tracer (Fig. 2 [21]). PDAs, a family of conjugated polymers, have unique optical, chromatic and fluorescent properties, and those can be applied in turn-on fluorescence and reversible fluorescence detection. Moreover, PDAs have good performance in biological systems due to their near infrared (NIR) emission. In this study, to quantify liposomes in cells, flow cytometric analysis was performed, and the internalization and distribution of folate-polydiacetylene-liposomes in Bcap-37 breast cancer cells and Hs578Bst normal cells were assessed by fluorescence cell imaging. The results of this study showed that folate-polydiacetylene-liposomes had good targeting efficiency, weak cytotoxicity and excellent biocompatibility.

Low sensitivity is undesirable and has to be carefully considered when selecting probes for in vivo fluorescence imaging. Biological matrix generally has a high photon absorbance in visible (350–700 nm) and infrared (> 900 nm) ranges, while NIR region

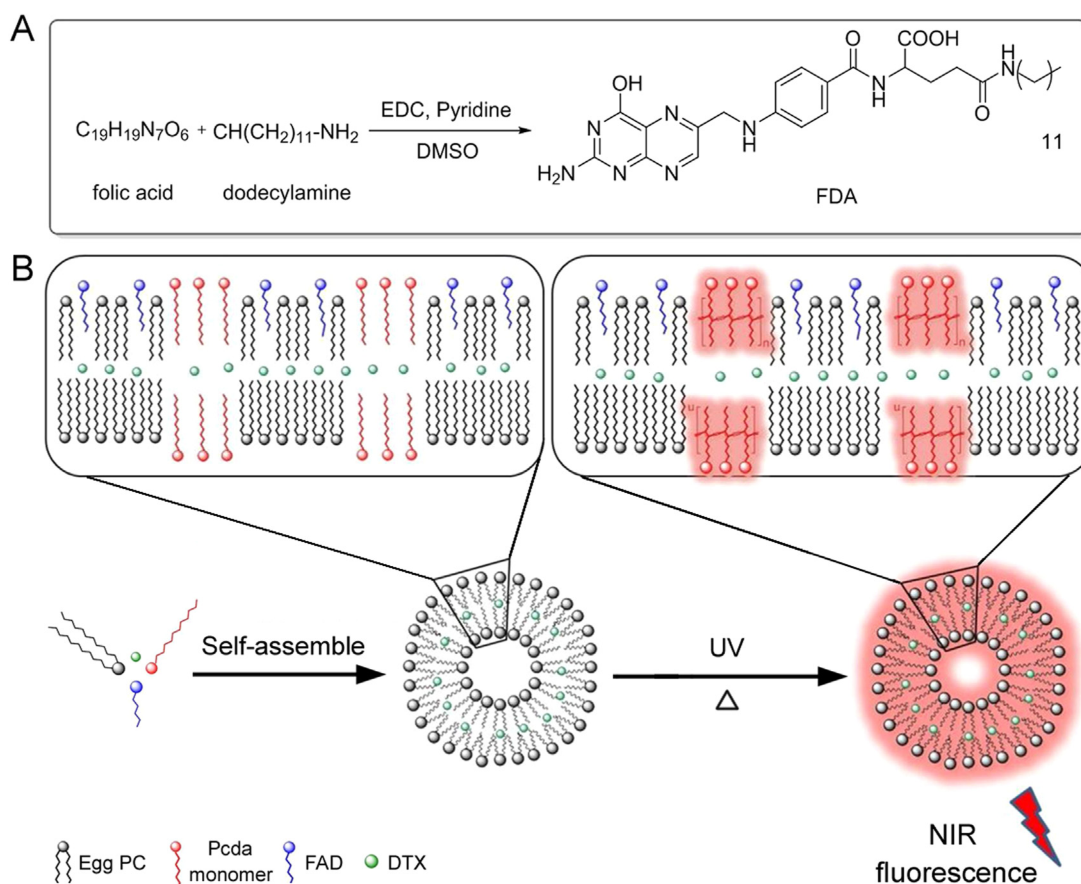

Fig. 2. (A) Synthesis of FAD. (B) Preparation of FPL [21].

(700–900 nm) absorbance spectra of bio-molecules are minimal [22]. Therefore, NIR fluorochromes can significantly improve the selectivity and resolution of fluorescence imaging, and they have acquired active development in recent years. Tansi et al. [23] selected DY-676-COOH as NIR fluorochrome to trace PEGylated nanoliposomes in mice, and DY-676-COOH is known because of its features to self-quench at very high concentrations in nanoliposomes. The study evaluated the concentration suitable for self-quenching, and entrapped DY-676-COOH in this concentration into the aqueous core of PEGylated nanoliposomes. Desu et al. [24] employed NIR fluorescence imaging using bimodal nanoliposomes to evaluate lung inflammation non-invasively, and 1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethyl indotricarbocyanine iodide was selected as the NIR fluorochrome.

Fluorescence labeling is a common method used for tracing and measuring nanoliposomes in vivo, because it has cost-effective and non-invasive properties, and can intuitively reflect the pharmacokinetic behavior of nanoliposomes. However, the fluorescence labeling method has some disadvantages that have yet to be overcome. Fluorescent reagents are usually unstable in the course of circulation in vivo, so fluorescence imaging may not provide sufficiently accurate information reflecting nanoliposomes behavior in vivo. Moreover, toxicity of fluorescent reagents to living organisms also limits the use of fluorescence imaging on nanoliposomes tracing in vivo.

2.2. Radiolabeling method

The radiolabeling method has a good performance on nanoliposomes in vivo quantitation, because of its excellent sensitivity and specificity, so it has been increasingly applied. Petersen et al. [25] loaded ^{64}Cu in the aqueous core of the nanoliposomes and quantified tissue and blood concentration of nanoliposomes by measuring ^{64}Cu with positron emission tomography (PET) (Fig. 3 [25]). PET is one of the primary molecular imaging technologies approved by the FDA for clinical application [26], due to its high sensitivity and spatial resolution [27]. Moreover, PET can determine the intensity of radioactivity specifically sequestered in a region of interest, and achieve direct quantitative analysis on the basis of differences in signal intensity [28]. ^{64}Cu has fairly long half-life as a metallic positron-emitting radionuclide, relatively low maximum positron energy (0.66 MeV) and short positron range, which provides high quality to PET images [29]. In this study, remote loading was employed to entrap ^{64}Cu into the core of nanoliposomes with 2-hydroxyquinoline as an ionophore, which achieved high loading efficiency and retention stability. Kang et al. [30] also have quantified nanoliposomes in aortic endothelial cells and various tissues by measuring ^{64}Cu with PET.

Similar to ^{64}Cu, ^{18}F is also widely used to produce high-quality PET images, and Emmetiere et al. [31] have selected ^{18}F as a tracer to measure nanoliposomes. ^{99m}Tc is a common radioisotope often used for labeling inhaled nanoliposomes [32,33]. Lee et al. [34] have investigated the deposition and clearance of inhaled nanoliposomes in the lungs by gamma scintigraphy with ^{99m}Tc radiolabeling method. Nanoliposomes ^{99m}Tc radiolabeling can be achieved by several methods: outer leaflet radiolabeling, encapsulation labeling and pH gradient loading. ^{99m}Tc encapsulation labeling was not ideal because of low radiolabeling efficiency (less than 5%), impractical manufacturing processes and diverse labeled species. Therefore, Lee et al. [34] employed the outer leaflet radiolabeling, which can provide high radiolabeling efficiency and stability. Nanoliposomes were labeled by reacting with ^{99m}Tc using

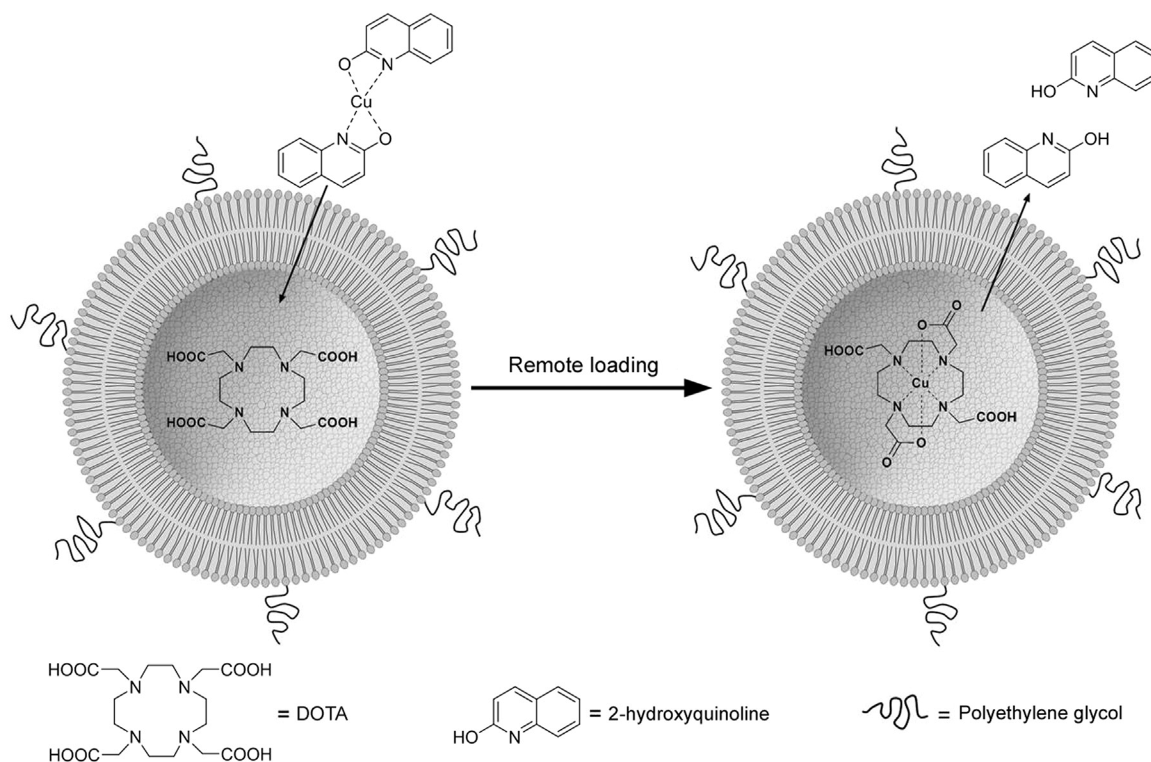

Fig. 3. Remote loading of ^{64}Cu into liposomes using 2-hydroxyquinoline [25].

$SnCl_2$ dissolved in 0.91 mM ascorbic acid as a reducing agent for 10 min at room temperature, and the labeled products were purified with anion exchange resin.

^{89}Zr was also selected to label nanoliposomes for tracing in vivo by Seo et al. [35], as the advantages of ^{89}Zr radiolabeling include relatively long decay time and facile labeling procedures. ^{89}Zr has a low fraction of gamma radiation, so PET was employed for ^{89}Zr detection in this study. Deferoxamine was used as a chelator for ^{89}Zr due to high binding affinity. Three kinds of ^{89}Zr-labeled liposomes were prepared, which were respectively labeled with ^{89}Zr on the surface, between the surface and PEG2k tip and at the end of PEG2k brush. The pharmacokinetics of ^{89}Zr-labeled nanoliposomes was successfully assessed in neu deletion tumor-bearing mice with the radiolabeling method established in this study.

Although radiolabeling is a common method for nanoliposomes quantitation, the specialty training and experience required for working with radioactivity seriously limits the use of radiolabeling techniques. Another limitation is that radiolabeling cannot simultaneously monitor multiple radioisotopes due to the poor energy resolution of radioactive material. In addition, radiolabeling may change the pharmacokinetic behavior of nanoliposomes, which would decrease the accuracy of results. Radioactive reagents can also be harmful to human and environment. These challenges remain and need to be further investigated in future research.

2.3. Magnetic resonance imaging (MRI)

MRI is a clinical detection technology with excellent spatial resolution, and it has great potential as a non-invasive tool to investigate the biodistribution of nanoparticles in vivo [36,37]. In vivo MRI has distinctive advantages over its competitors in terms of attainable anatomical resolution. The common MRI contrast agents used for nanoliposomes measuring in vivo include T1, T2,

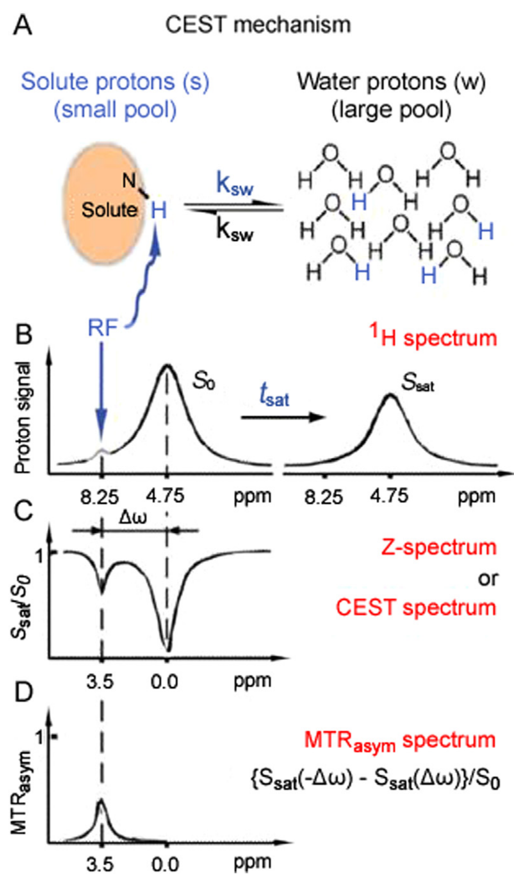

Fig. 4. Principle and measurement of chemical exchange saturation transfer [38].

and chemical exchange saturation transfer (CEST) agents (Fig. 4 [38]).

T1 contrast is generated by paramagnetic centers, which shorten the longitudinal relaxation times of water protons [39]. Ren et al. [39] determined nanoliposomes in vivo with MRI using Gd-chelate as a contrast agent. This method has been extensively applied in clinical diagnosis because of acceptable safety and positive magnetic MRI contrast [39].

T2 contrast is generated through a reduction in transverse relaxation times of water protons [38]. He et al. [40] developed an MRI method to measure nanoliposomes in breast cancer cells and mice tissues by loading nanoliposomes with magnetic iron oxide nanoparticles. Zhang et al. [41] also employed MRI to trace nanoliposomes using superparamagnetic iron oxide nanoparticles as contrast agent. Magnetic and superparamagnetic iron oxide nanoparticles are T2 contrast agents, which generate negative contrast on T2-weighted images. Superparamagnetic iron oxide nanoparticles are widely used for MRI molecular imaging since they have much higher molar relaxivity compared with paramagnetic T1 contrast [41]. However, magnetic iron oxide nanoparticles have some drawbacks seriously limiting their use in bioanalysis for nanoliposomes, including low dispersion in solvents, wide particle size distribution, toxicity and tendency to aggregate and absorb plasma proteins.

CEST is a new type of MRI contrast based on labile spins that rapidly exchange with solvent, resulting in an amplification of signal which allows detection of solute protons at millimolar to micromolar concentrations [42]. Delli Castelli et al. [43] used T1, T2 and CEST simultaneously for nanoliposomes determination in vivo, with Gd-HPDO3A and $[Tm\text{-}DOTMA]^-$ $[Na]^+$ complexes utilized to label nanoliposomes. Gd-HPDO3A complex was used as T1 and T2 MRI contrast agents, and $[Tm\text{-}DOTMA]^-$ $[Na]^+$complexes were used as T2 and CEST agents. The MRI method established in this study can also distinguish intact and broken forms of nanoliposomes in vivo. The study results indicated when the maximum of T1 contrast enhancement appeared, nanoliposomes were broken to release their content; when the maximum T2 contrast enhancement appeared, nanoliposomes maintained their intact states; and when the maximum CEST contrast occurred, intact nanoliposomes were broken in the extracellular fluids. Diamagnetic CEST (diaCEST) agents are naturally existing molecules without metal ions, and the contrast is dependent on the number and type of labile protons [38]. It acquired active development over the years, because natural organic and biodegradable compounds can provide strong CEST contrast and obviously increase the sensitivity of MRI. Chan et al. [44] developed a non-radioactive, nonmetallic, biocompatible, semi-quantitative and clinically translatable method to measure nanoliposomes concentrations in subcutaneous CT26 colon tumor-bearing mice. In this study, barbituric acid, a small, organic and biocompatible compound, was selected as a diaCEST contrast agent, because it is a heterocyclic pyridiminetrione and can produce excellent contrast at a frequency of 5 ppm away from water [45].

MRI provides a sensitive, non-invasive and high spatial resolution approach to investigate the fate of nanoliposomes in vivo. However, specificity of MRI for biological sample analysis is not fully reliable because of the high complexity of the biological matrix. Moreover, similar to other indirect analytical methods, contrast agents also change the pharmacokinetic behavior of nanoliposomes and negatively influence the accuracy of results. The safety of many contrast agents remains to be investigated.

2.4. Mass spectrometry

Mass spectrometry is a new analytical method for quantitative measurement of nanoliposomes in vivo, which is expected to have excellent selectivity, sensitivity and accuracy. Cheng et al. [46] quantified nanoliposomes and their encapsulated contents in tumors using inductively coupled plasma-mass spectroscopy (ICP-MS) (Fig. 5 [46]). In this study, nanoliposomes were first labeled with four different chelated lanthanide metals by encapsulation or surface-conjugation. Subsequently, the labeled nanoliposomes were intravenously administered to tumor-bearing mice. Finally, tumors were harvested and analyzed by ICP-MS. Crayton et al. [47] also established an ICP-MS method to investigate tumor localization, biodistribution, and blood clearance of nanoliposomes labeled with lanthanide metals. However, ICP-MS has universal shortcomings of the indirect analytical method mentioned above.

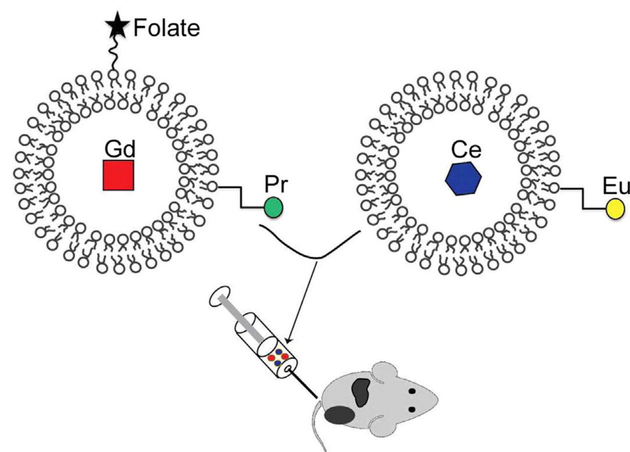

Fig. 5. Schematic of the ICP-MS based multiplex method for determining tumor uptake of liposomes and their encapsulated contents [46].

The liquid chromatography-tandem mass chromatography (LC-MS/MS) method has been developed to quantify nanoliposomes in vivo by monitoring the drugs encapsulated in nanoliposomes. Separation of encapsulated and released drugs is critical in this method because nanoliposomes are fragile. If nanoliposomes are broken during sample preparation, the measurement of released drugs will be contaminated, which results in inaccurate pharmacokinetic assessment. Deshpande et al. [48] quantified free and liposomal amphotericin B in human plasma with LC-MS/MS method, and separation between free and liposomal amphotericin B was achieved by solid phase extraction using Oasis HLB cartridges. The method was validated and successfully utilized in a pharmacokinetic study, indicating good reliability for the bioanalysis of nanoliposomes. Xie et al. [49] separately determined non-liposomal and liposomal doxorubicin in dog plasma using LC-MS/MS; the separation was also performed by solid phase extraction with Oasis HLB cartridges. It should be noted that all LC-MS/MS methods combined with solid phase extraction were reported for nanoliposomes quantitation in plasma samples, but have not been successfully used to measure nanoliposomes in tissues, primarily due to the fact that nanoliposomes are apt to be broken during the process of tissue homogenate.

Smits et al. [50] used LC-MS/MS technology to determine liposomal-encapsulated prednisolone phosphate and non-encapsulated prednisolone in whole blood and liver tissue. Prednisolone phosphate is rapidly dephosphorylated by phosphatases in vivo and converted into prednisolone. Therefore, the encapsulated drug concentration is represented by prednisolone phosphate, and prednisolone represents the free drug concentration. The method was validated and used in a pharmacokinetic study in mice. This strategy is only suitable for prodrugs like prednisolone but does not have general applicability with other liposomal drugs.

Table 1
Analytical methods for the measurement of liposomes in vivo.

Methods	Advantages	Disadvantages
Fluorescence labeling	1. Good cost-effectiveness 2. Non-invasiveness 3. Intuitional	1. Fluorescent reagents are unstable in vivo. 2. Fluorescent reagents are toxic to living organisms. 3. Fluorescent reagents may change the pharmacokinetic behavior of liposomes.
Radiolabeling method	1. High sensitivity 2. Excellent specificity	1. Requiring specialty training and experience. 2. Radiolabeling cannot simultaneously monitor multiple radioisotopes. 3. Radioactive reagents may change the pharmacokinetic behavior of liposomes. 4. Radioactive reagents are harmful to human and environment.
Magnetic resonance imaging	1. High sensitivity 2. Non-invasiveness 3. High spatial resolution	1. Unsatisfactory specificity. 2. Contrast agents may change the pharmacokinetic behavior of liposomes. 3. The safety of contrast agents remains to be investigated.
ICP-MS	1. Excellent specificity 2. High sensitivity 3. Good accuracy	1. Labeling agents may change the pharmacokinetic behavior of liposomes.
LC-MS/MS	1. Excellent specificity 2. High sensitivity 3. Good accuracy	1. The technology could not be used to measure liposomes in tissues.
Computed tomography	1. High sensitivity 2. High resolution 3. High throughput	N/A

2.5. *Computed tomography (CT)*

CT-based measurement is suitable for the investigation of long-circulating nanoparticle systems, because CT contrast agents have high atomic numbers and provide high X-ray attenuation [51]. Zheng et al. [51] quantified the distribution of nanoliposomes containing iohexol and gadoteridol in New Zealand White rabbits using CT, and the study results indicated that the CT could provide quantitative, volumetric and longitudinal assessment of the pharmacokinetic study of nanoliposomes. Sensitivity of the reported method was in the range of $\mu g/cm^3$, and the imaging speed of the method was less than one minute per scan. CT technology is also suitable for monitoring slow physiological processes, such as the passive accumulation of nanoliposomes in tumors via the EPR phenomenon. Stapleton et al. [52,53] employed the CT method to investigate the intra-tumoral accumulation of nanoliposomes in two mouse xenograft models of human cervical and breast cancers. CT imaging can perform extremely fast data acquisition with high resolution (submillimeter isotropic voxels), and it also can achieve 3D image analysis, which provides signal profiles used for volumetric quantification within organs and tissues. CT is currently the fastest and the most commonly used whole body volumetric imaging technology, which indicates its potential on high throughput biodistribution investigation.

Advantages and disadvantages of analytical methods are summarized in Table 1.

3. Conclusion

As a valuable drug delivery system, nanoliposomes have been demonstrated to be successfully used in drug development for the treatment of cancer, infections and inflammations. In this review, we summarized the analytical detection techniques used in quantification of nanoliposomes and discussed their advantages and disadvantages in their applications.

Fluorescence labeling and radiolabeling are the most commonly used methods to measure nanoliposomes in vivo. However, drawbacks of fluorescence labeling such as instability and toxicity still need to be overcome, and strict operation requirements, low monitoring efficiency and environmental pollution seriously limit the use of radiolabeling. MRI as a clinical detection technology with excellent spatial resolution also can be used for nanoliposomes tracing in vivo, but MRI contrast agents are toxic and tend to aggregate and absorb plasma proteins, which limits its use. LC-MS/MS, a sensitive, selective and accurate analytical technique, can also be used for the quantitation of nanoliposomes in biological fluid samples. Nevertheless, it is still very challenging to use LC-MS/MS methods to analyze tissue samples because nanoliposomes are apt to be broken during the tissue sample preparation procedure. Consequently, very limited information of nanoliposomes biodistribution into tissues is currently available to provide the support of liposomal drug development. We developed an LC-MS/MS method that includes a unique procedure of processing tissue samples without breaking nanoliposomes. This method would be applied to the quantitative measurement of nanoliposomes in vivo. In tissue samples preparation, ball mill with mild condition was used instead of homogenizer, which can protect nanoliposomes from fragmentation. Separation of encapsulated and non-encapsulated drug was achieved by solid phase extraction. In solid phase extraction procedure, lipophilic non-encapsulated drug retained on reversed phase cartridges whereas encapsulated drug was not because of the hydrophilic surface of nanoliposomes. Consequently, encapsulated and non-encapsulated were separated. CT method has fast imaging speed and high spatial resolution, but like other indirect analytical methods (fluorescence imaging, radiolabeling, MRI and ICP-MS), label agents may change the pharmacokinetic behavior of nanoliposomes which could influence the accuracy of the results.

Therefore, there are still many challenges to be overcome for further development of reliable analytical techniques to accurately measure nanoliposomes concentrations in vivo, which provides a fundamental basis to investigate the fate of nanoliposomes in vivo.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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