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Review paper

Current status of *in vivo* bioanalysis of nano drug delivery systems

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ABSTRACT

The development of nano drug delivery systems (NDDSs) provides new approaches to fighting against diseases. The NDDSs are specially designed to serve as carriers for the delivery of active pharmaceutical ingredients (APIs) to their target sites, which would certainly extend the benefit of their unique physicochemical characteristics, such as prolonged circulation time, improved targeting and avoiding of drug-resistance. Despite the remarkable progress achieved over the last three decades, the understanding of the relationships between the in vivo pharmacokinetics of NDDSs and their safety profiles is insufficient. Analysis of NDDSs is far more complicated than the monitoring of small molecular drugs in terms of structure, composition and aggregation state, whereby almost all of the conventional techniques are inadequate for accurate profiling their pharmacokinetic behavior in vivo. Herein, the advanced bioanalysis for tracing the in vivo fate of NDDSs is summarized, including liquid chromatography tandem-mass spectrometry (LC-MS/MS), Förster resonance energy transfer (FRET), aggregation-caused quenching (ACQ) fluorophore, aggregation-induced emission (AIE) fluorophores, enzyme-linked immunosorbent assay (ELISA), magnetic resonance imaging (MRI), radiolabeling, fluorescence spectroscopy, laser ablation inductively coupled plasma MS (LA-ICP-MS), and size-exclusion chromatography (SEC). Based on these technologies, a comprehensive survey of monitoring the dynamic changes of NDDSs in structure, composition and existing form in system (i.e. carrier polymers, released and encapsulated drug) with recent progress is provided. We hope that this review will be helpful in appropriate application methodology for investigating the pharmacokinetics and evaluating the efficacy and safety profiles of NDDSs.

1. Introduction

In the past 30 years, the development of nanotechnology was fruitful and many novel technologies have been applied to disease diagnosis, pharmaceutical discovery and tissue engineering [1–3]. NDDS is a rapidly developing and most remarkable nanomedicine technique. Doxil®, liposomal doxorubicin (DOX), was the first approved NDDS in 1995 for the treatment of AIDS-related Kaposi's and ovarian cancer with reduced side effects and passive tumor targeting effect [4]. In recent years, many types of NDDSs, such as liposomes, micelles, polymer-based nanoparticles (NPs), nanoemulsions, nanogels, inorganic NPs and inorganic/organic (core/shell) NPs, have been subjected to preclinical and clinical studies [5].

In NDDSs, small molecular drugs or biotherapeutics are entrapped or chemically bonded onto nanoparticles. Different from traditional pharmaceuticals, NDDSs exhibit material physicochemical characteristics related to drug delivering properties after administration [6,7]. The NDDS enhances pharmacological and pharmaceutical properties of the parent drugs by prolonging circulation time, improving efficacy and targeting, overcoming drug-resistance, reducing immunogenicity and toxicity [8,9].

The superiority of NDDSs attracted global investment, so the research funding in nanomedicine from the US National Institutes of Health (NIH) from 2011 to 2019 is around 623 million US dollars

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[10,11]. However, only 51 nanomedical products have been approved by the Food and Drug Administration (FDA) till recently [12]. The low clinical transition ratio was partially due to the incomplete understanding of the pharmacokinetic properties of NDDSs [13]. The biological fate of NDDSs remains elusive. The conventional pharmacokinetic studying methods, such as fluorescence labeling, could not track or distinguish in vivo nanocarriers and payloads simultaneously.

Herein, we review the recent advances of the bioanalytical techniques for pharmacokinetic research on NDDSs for the first time. The measurement strategies and results for the released and encapsulated drug as to the carrier polymer of NDDSs and their biodistribution are enumerated, and obstacles and perspectives of these technologies are discussed.

2. Classical NDDS

NDDSs such as liposomes, micelles, polymer-based NPs, nanoemulsions, nanogels, inorganic NPs and inorganic/organic (core/shell) NPs have long been developed for delivering APIs to the specific site of action. Nowadays increased number of NDDSs appear in preclinical and clinical phases [14].

2.1. Liposomes

Liposomes are lipid-based concentric bilayer vesicles (particle size ≈ 400 nm) comprising either synthetic or natural phospholipids [15]. The phospholipid molecules typically consist of a polar phosphate group and two hydrophobic fatty acid chains, which can spontaneously form a closed, bilayer structure by self-assembling in an aqueous environment [16]. The hydrophilic phosphate groups are exposed on the outer and inner surfaces of the liposomes and the hydrophobic fatty chains are packed and piled up in between [17]. Hence, liposomes are utilized for carrying water-soluble APIs in the cavity or lipophile APIs within the lipid bilayer (Fig. 1A). Because of their size, hydrophobic character and surface electrical charge, liposomes can be rapidly recognized and cleared by the mononuclear phagocyte system (MPS) [18]. One of the solutions to overcoming this issue is to modify the surface of liposome with amphiphilic polymers, such as poly (ethylene glycol) (PEG). The PEG coating can protect the liposome from enzymatic and immunologic clearance, hence called “stealth properties”. Therefore, modifying with PEG (PEGylation) has become a widely applied strategy to reduce opsonization and prolong circulation time of liposomes [19]. The approved Doxil® in 1995 has adopted this surface-bound methoxy polyethylene glycol liposomal formulation (STEALTH®), and a half-life of approximately 55 h in humans has been achieved.

2.2. Polymeric micelles

Polymeric micelles are solid spherical aggregates with size ranging from 10 nm to 100 nm, composed of amphiphilic copolymers containing hydrophobic and hydrophilic blocks [20]. The micelles can be formed spontaneously by self-assembly when the concentration of amphiphilic copolymers exceeds the critical micelle concentration (CMC). With a hydrophobic core and a hydrophilic shell layer or corona, polymeric micelles have been used as carriers for various lipophilic drugs, which significantly increase the drug concentration in an aqueous medium (Fig. 1B) [21]. After injection, polymeric micelles are susceptible to being diluted below the CMC in the blood. This may lead to the dissociation of micelles into unimers [22]. The CMC is dependent on the factors such as chemical structure of the polymer as well as the molecular weight (MW) of each polymeric block. The marketed Nanoxel-PM® is docetaxel provided in an mPEG-PDLLA micellar formulation [23]. Furthermore, Genexol®-PM is paclitaxel loaded PEG-PLA micelle.

2.3. Polymeric NPs

Polymeric particles in size of 40–400 nm are capable of carrying APIs in the polymeric matrix or on the surface of the particle by absorption or conjugation [24]. Polymeric NPs are solid systems classified into nanospheres and nanocapsules depending on the

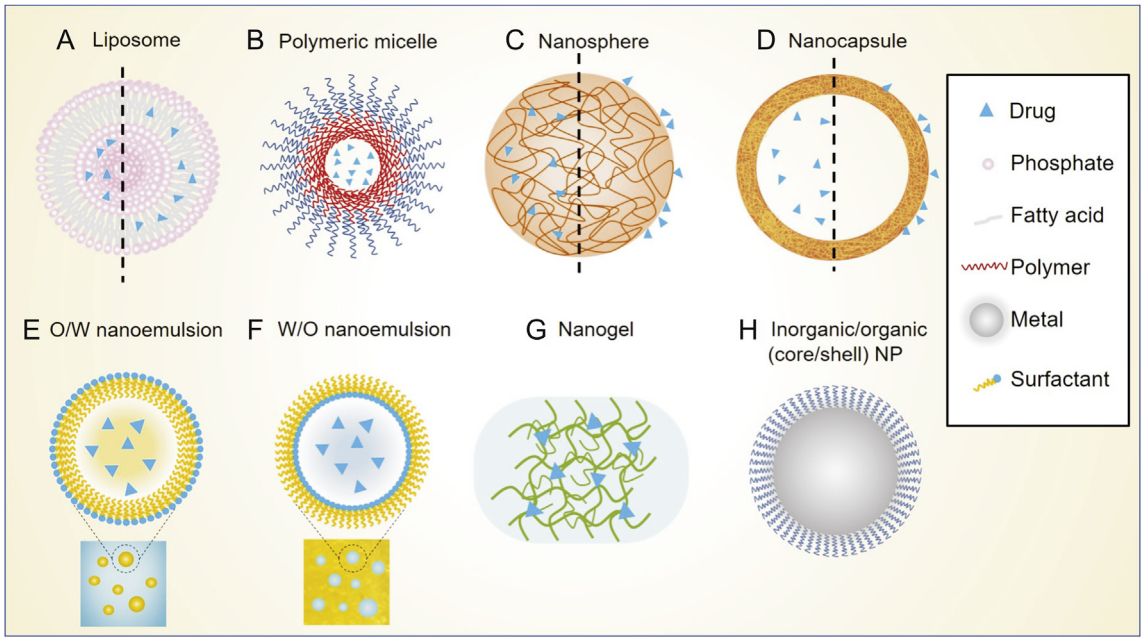

Fig. 1. Classical nano drug delivery system (NDDSs) used in drug delivery, including: (A) liposome, (B) polymeric micelle, (C) nanosphere, (D) nanocapsule, (E) O/W nanoemulsion, (F) W/O nanoemulsion, (G) nanogel, and (H) inorganic/organic (core/shell) nanoparticle (NP).

type of polymer, the localization of APIs and the production procedure [25,26]. Nanospheres are essentially monolithic systems having a solid matrix, whereby the APIs can be either loaded onto the surface or dissolved within it (Fig. 1C). In contrast, nanocapsules are reservoir systems composed of a polymeric shell, and the APIs are confined in the inner liquid core or adsorbed on the surface (Fig. 1D) [27]. Polymeric NPs can be produced from natural polymers such as chitosan or dextran, as well as from synthetic polymers, such as poly(lactide) (PLA), poly (lactide-co-glycolide) (PLGA), poly(alkylcyanoacrylates) or poly(epsilon-caprolactone) [28–30]. Synthetic polymers such as PEG, PLA and PLGA have been approved by FDA for human use over decades [31]. The 2018 approved lipid NP drug Onpattro® contains two kinds of lipids: heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino) butanoate (DLin-MC3-DMA) and α-(3'-{[1,2-di(myristyloxy)proponoxy] carbonylamino} propyl)-ω-methoxy, polyoxyethylene (PEG_{2000}-C-DMG) [32].

2.4. Nanoemulsions

Nanoemulsions are oil-in-water (O/W) or water-in-oil (W/O) dispersion of two kinds of immiscible liquids. One is dispersed as droplets in the other one and stabilized by an amphiphilic surfactant [33] (Fig. 1 E and F). The diameter of attained droplet is usually of 10–600 nm in size. This nanocarrier is adequate for the delivery of both hydrophilic and hydrophobic drugs, which is ideal for improving the solubility of hydrophobic drugs in aqueous media and protecting them from hydrolysis and enzymatic degradation. Drug release process from nanoemulsion involves solute partitioning from droplets into surfactant layer and then diffusing into surrounding phase. Nanoemulsions prepared by low-energy emulsification frequently require large amounts of surfactants for stabilization. High dose of surfactant will cause cell membrane fluidization. Therefore, the major disadvantages of nanoemulsions are their limited stability and low adhesivity. The oil-in-water nanoemulsion Restasis®, cyclosporine A in castor oil droplets with polysorbate 80 as the emulsifying agent, has been approved by FDA in 2002 for the treatment of keratoconjunctivitis sicca [34].

2.5. Nanogels

Nanogels are nanoscale hydrogel particles with size ranging from 100 nm to 200 nm, composed of cross-linked hydrophilic polymer network [35]. Nanogels are capable of absorbing water up to a thousand-fold of their dry weight. The swollen hydrogel forms a gigantic 3D framework, which can be utilized for entrapping drugs, polymers and dispersed phase of liquid (Fig. 1G). Nanogels are mainly used as drug carriers for delivery of both hydrophilic and hydrophobic drugs [36]. Owing to their swelling/shrinking property, flexibility in form, large surface area and highly water content, a controlled and sustained release manner can be achieved. Many nanogels formulations are available on the market; most of them are cosmetic remedies, a number of them are toothpaste formulation and several formulations are personal skin care products [37,38].

2.6. Inorganic NPs and inorganic/organic (core/shell) nanoparticles

Inorganic NPs include metal and metal oxides, such as gold (Au), silver (Ag), platinum (Pt), iron oxide (Fe_3O_4), titanium oxide (TiO_2), copper oxide (CuO) and zinc oxide (ZnO) [39]. Many of the inorganic NPs have long been used in clinic for various therapeutic applications, such as platinum compounds for cancer therapy and silver as antibacterial agents. Similar to organic pharmaceuticals, inorganic pharmaceuticals can also benefit from NDDSs by improving their pharmacokinetic performance, such as enhanced targeting, drug loading, and immune system evasion.

Inorganic/organic (core/shell) nanoparticles are complexation of inorganic NPs with an organic polymer shell (Fig. 1H) [40]. The polymeric protective shell can promote purely steric repulsions so as to reduce the range and strength of electrostatic and van der Waals interactions between the colloids. The hybrid aggregates are typically in the size around 100 nm and have remarkable colloidal stability even against ionic strength variations. Ferumoxytol (Feraheme®) are iron oxide nanoparticles coated with poly-glucose sorbitol carboxy-methyl-ether, which have been approved by FDA for treatment of iron deficiency in adults with chronic kidney disease in 2009 [41].

3. Quantification of the in vivo trafficking of NDDSs

To exert the desired biologically effect, the encapsulated/embedded APIs must be released from NPs and reach their target site. Although NDDSs have been applied as drug carriers for over twenty years, pharmacokinetics studies have always been focused on the total drug concentrations and the polymers have often been overlooked. Furthermore, the characterization of the systematic release profile for the drug loaded particles remains incomplete. Different from the traditional pharmacokinetic studies of APIs, beside the released drug, the key aspects of the pharmacokinetic studies on NDDSs further include encapsulated drug and carrier polymers. The polymer quantitation and differentiation between released and encapsulated drug were the main technical difficulties bothering the bioanalysis of NDDS. With the technology development, methods have been merged for distinguishing the released and encapsulated drugs in vivo, such as LC-MS/MS, FRET, ACQ and AIE fluorophores and ELISA (Table S1).

3.1. Quantitation methods for the released and encapsulated drug in vivo

The NDDS encapsulation preserves the payloads drugs and endows them with prolonged circulation time and solubilization, which is therefore often referred to as a circulating "reservoir" of drug. To date, numerous efforts have been dedicated to reveal the release profiling of NDDSs.

3.1.1. LC-MS/MS

LC-MS/MS is a standard bioanalysis method for small molecule drugs, which recently is also considered as the preferred choice for profiling the drug release process of NDDSs in vivo. Distinguishing the released and encapsulated drug by LC-MS/MS is a critical challenge. Smits et al. [42] were the first to report an LC-MS/MS method for the differential quantification of released and encapsulated drug of prednisolone phosphate loaded liposomes in whole blood and liver tissue. Since released prednisolone phosphate will be immediately dephosphorylated by phosphatases in vivo, the prednisolone was utilized as surrogate analyte for the released drug. Similarly, the determination of released drug from polymeric micelles was reported in Braal et al.'s study [43]. CriPec® is a docetaxel-temporarily covalently conjugated micelle (approximately 65 nm). This conjugate is stable at pH 5.0 at room temperature. The quantitation of the released docetaxel can be therefore measured free from the interference of the conjugated drug. For the quantitation of the conjugated docetaxel, the drug needs to be detached from micelles at 37 °C and pH 7.4 for three days. Since the LC-MS/MS method alone cannot differentiate the detached drug from the previously released drug, the drug-loaded micelles should be separated first.

Solid phase extraction (SPE) is widely applied to separate the

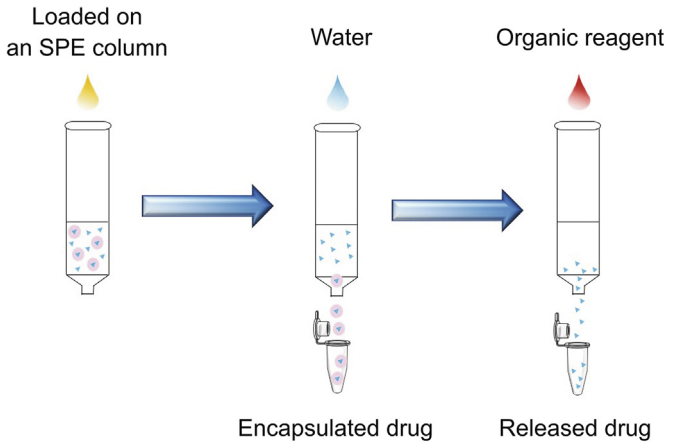

Fig. 2. Procedure for the separation of released and encapsulated drug in liposomes by solid phase extraction (SPE) in plasma sample.

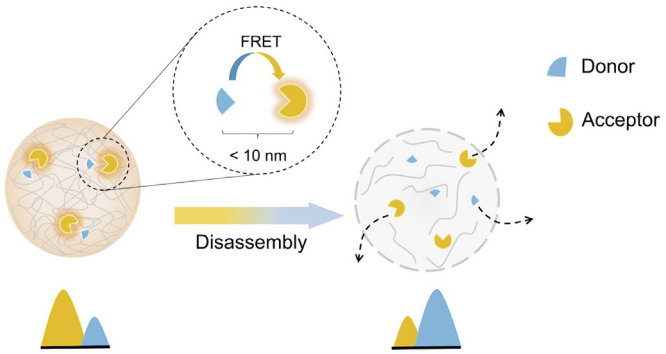

Fig. 3. Illustration of the Förster resonance energy transfer (FRET) that is being developed for tracking the bio-distribution of nano drug delivery system (NDDSs).

released and encapsulated drug. A common SPE procedure is as follows: 1) sample loading; 2) water washing (liposomes with hydrophilic surface will not be retained on SPE column); and 3) hydrophobic solvent washing (the adsorbed released drug will be then eluted) (Fig. 2). Deshpande et al. [44], Su et al. [45] and Xie et al. [46] have utilized SPE separation method for profiling the pharmacokinetic behavior of amphotericin B and DOX liposomes in vivo. A similar approach was adopted by Wang et al., whereby the release and uptake processes of DOX liposome in health tissues and in tumors were profiled [47]. This SPE separation method has also been conditionally applied to separate polymeric NPs. Song et al. [48] were the first to utilize a similar separation method for the quantitation of gedatolisib released from PLA-PEG NPs. The concentration of encapsulated gedatolisib was calculated by subtracting the released gedatolisib values from the total values. In consideration of the properties of the payload drug, here an MCX SPE column was employed instead of an HLB SPE column.

Ultrafiltration was also considered as a separation method for liposomal released and encapsulated drug. Xie et al. [46] investigated the accuracy of this separation method using DOX liposomes as test sample. The recovery rate of DOX separated by ultrafiltrate was no more than 10%, which may be related to the adsorption of DOX to device and plasma proteins.

A new class of separation techniques was developed by Chen et al. [49]. Based on the specific binding of biotin and streptavidin, streptavidin-Fe_3O_4@PDA was utilized as the separation nanoprobes to separate biotin-DTX-liposomes from plasma in the presence of a magnetic field, and 75% recovery efficiency has been achieved.

3.1.2. *FRET*

FRET is a new tool to reveal the biological fate of NDDSs in vivo. Its principle involves energy transfer between a donor fluorophore in its electronic excited state and an adjacent acceptor fluorophore (1–10 nm) (Fig. 3) [50]. A spectrum overlap between the donor and acceptor greater than 30% is necessary for an efficiently energy transfer [51]. In the FRET-based strategies for investigating the stability and drug release of NDDSs, a FRET pair (donor and acceptor) should be co-encapsulated in the core of micelles [52,53]. FRET signal decays rapidly regarding exceeding of the energy transfer distance, which corresponds to the payload release [50].

However, the payload release can be made either by FRET pair diffusion or by micelles dissociation. In a recent study by Sun et al., the in vivo stability of the micelles has been investigated by immobilizing the FRET pair on the hydrophobic end of the carrier polymers. The FRET pair loaded polymers were then transformed into micelle. Therefore, the signal decreasing in vivo is merely correlated to micelles dissociation (Fig. 4). [54]. To minimize the impact of FRET fluorophore on micelle properties, only a small percentage of the hydrophobic ends were loaded with the FRET pair (1% for both), and the loading of drug is not influenced.

The FRET method has some restrictions of its own: 1) Re-illumination caused by repartitioning into hydrophobic constructs. The FRET pairs are generally highly hydrophobic compounds, prefer to repartition into hydrophobic constructs such as membranes, hydrophobic cavities of biomacromolecules or hydrophobic cores of physiological micelles. 2) Low sensitivity. Since the acceptor can be only excited by the donor indirectly, generally the fluorescence intensity of the FRET system is relatively weak.

3.1.3. *ACQ fluorophores*

ACQ fluorophores possess excellent fluorescent properties when dispersed in solution. The fluorescence can be turned off by fluorescent quenching, in case the fluorophores aggregated and formed stable π-π stack [55]. ACQ fluorophores are usually conjugated aromatic systems with strong hydrophobicity, which is prone to aggregate in a hydrophilic solvent, such as water. Based on this characteristic, ACQ probes are encapsulated in the hydrophobic core of nanocarriers. The fluorescence emission indicates a dispersed state of ACQ, which represents intact nanocarriers (Fig. 5A). Once the nanocarriers are dissociated, the fluorescence is quenched immediately accompanying with the ACQ probes aggregating in the aqueous medium. The tiny aggregates can be dispersed in the solvent and appear as homogenous solutions without precipitation. This aggregating process is reversible. When new micelles are introduced, the aggregates can dissolve or disperse into monomers again in the hydrophobic domains and regain their fluorescence. Although the ACQ probes are originally designed for imaging, He et al. applied this strategy for quantitation of mPEG–PDLLA polymeric micelles in vivo. The near-infrared aza-BODIPY fluorescent probe P2 was encapsulated in the hydrophobic core of the polymeric micelles. Excellent linearity of fluorescent response versus polymeric micelle concentration over the range of 9.77–625 μg/mL was observed [56]. The result of pharmacokinetic study in rat indicates that ACQ can be an alternative for the bioanalysis of NDDSs. The major drawback of ACQ method is re-illumination. Additionally, the application of ACQ is restricted in hydrophobic NDDSs.

3.1.4. *AIE fluorophores*

In contrast to ACQ, AIE fluorophores exhibit hardly emission in dilute solvent. In aggregate state, the free rotation of dye molecules is restricted, which dramatically boosts emission (Fig. 5B). In the

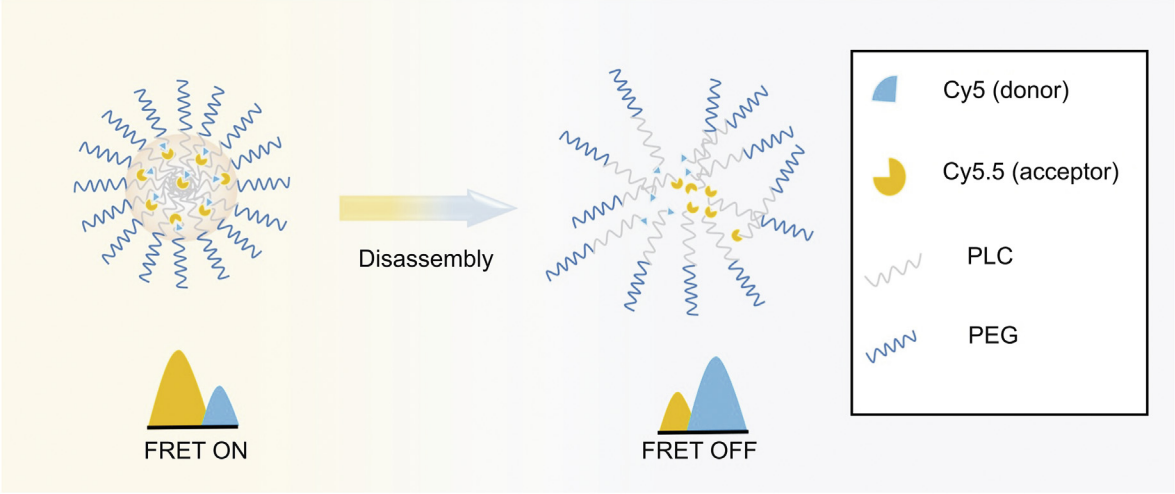

Fig. 4. Design and working principle of the Förster resonance energy transfer (FRET) micelles.

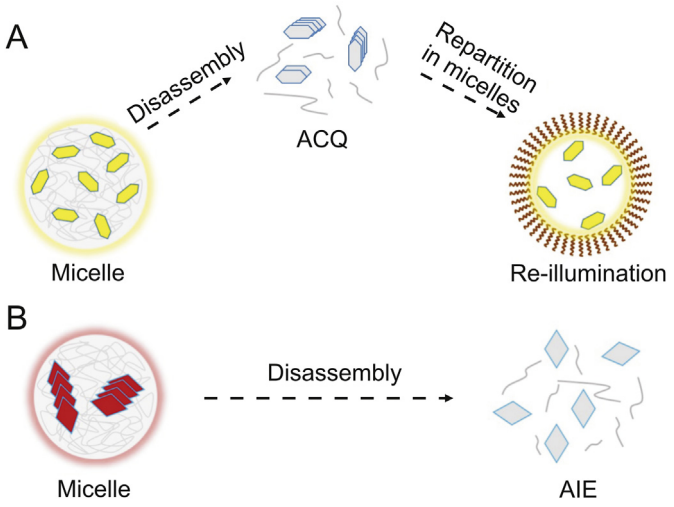

Fig. 5. Illustration of (A) aggregation-caused quenching (ACQ) and (B) aggregation-induced emission (AIE) that are being developed for tracking the drug encapsulated in nano drug delivery system (NDDSs).

case of nanocarrier dissociation, the fluorescence of leaked AIE probes is extremely weak in the environment. Therefore, the AIE probes can be employed to indicate the state of nanoparticles in vivo. Till recently, not many reports about monitoring the in vivo fate of NDDSs with AIE strategy are available. Conventional short-wavelength one-photon excited AIE material has some drawbacks in application, such as toxicity, short penetration depth (<100 mm), interference of tissue autofluorescence and photobleaching phenomenon. Furthermore, AIE is hindered by scattering, which relates to the long-wavelengths of the exciting light and high signal-noise ratio at a deep focal plane. By comparison, two-photon excited AIE has the advantages of low biological damage, low-energy irradiation, high-energy fluorescence, reduction of autofluorescence and excellent penetration depth. Most recently, Zhuang et al. employed a two-photon excitable AIE fluorescence probe to compare the pharmacokinetic behaviors of DOX and DOX-loaded micelles in vivo [57]. The doped TBIS fluorophore endowed mPEG-SS-Poly (AEMA-co-TBIS) (mPEATss) micelles with great AIE feature without influencing the drug loading.

3.1.5. *ELISA*

The ELISA is an immunological assay for detection of biological molecules such as proteins, antibodies, hormones and cytokines. ELISA has been also applied in investigating the release profile of the biomacromolecule loaded nanoparticles. The most prevalent ELISA method is known as “sandwich” ELISA, for the analyst antigen is stuck between two kinds of antibodies. Wherein, a primary antibody is first immobilized to the surface of the plate for capturing the analyst antigen in the serum; therefore, the primary antibody is also known as capture antibody. The captured antigens can be followed and recognized by an enzyme-linked antigen-specific antibody, which is referred as detecting antibody. The coupled enzyme serves here for an optical detection, signal amplification and quantitation of the captured analyst antigen [58].

In a study by Wang et al., the sandwich ELISA method was employed for quantifying the released payloads stromal cell-derived factor 1 (SDF-1) and bone morphogenetic protein 2 (BMP-2) to evaluate the encapsulation efficiency and release kinetics of the chitosan oligosaccharide/heparin (CSO/H) NPs in vitro [59]. In the work of Azie et al., latent transforming growth-factor beta (TGF-β) was conjugated to superparamagnetic iron oxide nanoparticles (SPIONs). The release profile of the active TGF-β from the SPIONs was subsequently monitored by ELISA [60]. However, the ELISA has the advantage in sensitivity, but it also has certain limitations, such as limited varieties of commercial ELISA kits, narrow linear ranges and cross-reactivity issue.

3.2. *Quantitation methods for polymer*

After the administration, the polymeric material of NDDSs may subject to disassembly, distribution, metabolism and excretion. In contrast to the payload drug, the knowledge to the in vivo fate of the polymeric materials is insufficient. The pharmaceutical polymer excipients are the main component of NDDS and are generally considered to be inert ingredients. However, the polymer related adverse drug reaction (ADR) reports on iatrogenic illnesses keep increasing, such as hypersensitivity reactions [61], cell vacuolation [62] or splenomegaly [63]. The accumulation of polymer may have potential toxicity and has aroused attention of regulatory authorities. Several analytical techniques have been developed for the bioanalysis of the polymeric material in NPs. Nuclear magnetic resonance (NMR) [64], colorimetric methods [65,66], SEC [67] and high-performance liquid chromatography (HPLC) [68–71]

technologies are inadequate in sensitivity. The LC-MS/MS and ELISA are currently employed techniques for in vivo polymer quantitation (Table S1).

3.2.1. LC-MS/MS

LC-MS/MS is a common analysis technique in the small molecular drug, which has recently made a great improvement in bioanalysis of polymers. The major challenge for the quantitative analysis of polymers by LC-MS/MS lies primarily in their polydispersity, which includes series of homologues with different degrees of polymerization and MWs. To overcome this challenge, several mass spectroscopy data acquisition strategies, such as selected ion monitoring (SIM), multiple reaction monitoring (MRM), in-source collision induced dissociation (CID) and MS^{ALL}, were applied to the polymer analysis in biological matrices. In this review, bioanalytical strategies for detecting several important pharmaceutical polymers are enumerated, including PEG, PLA, hyaluronan (HA), chitosan, and cyclodextrin (CD).

3.2.1.1. PEG. PEG is one of the most meaningful synthetic polymers in the pharmaceutical industry, which are produced commercially from ethylene oxide monomers (Fig. 6A) [72]. It is widely used as solubilizer, stabilizer, release-modifier or conjugated with drug molecules (PEGylation) and drug delivery vehicles (liposomes, micelle and nanoparticles). PEGylation, which can prolong drug half-life, enhance bioavailability, and reduce immunogenicity of the vehicle, has been approved by FDA for human use over a decade [73].

In 2004, Zhang et al. [74] developed a flow injection MS method for the quantitation of PEG300 in drug formulations under SIM mode. SIM is a variation of an MRM scanning mode, in which only the selected precursor ions will be transmitted through the quadrupole mass analyzer Q1 and Q3, and the collision energy (CE) of Q2 should be set to the level without causing obvious fragmentation (Fig. 7A). This method provided a better sensitivity in lower limits of quantitation (LLOQ, 136 ng/mL) than previously published methods: gas chromatography-mass spectrometry (GC-MS) (1 mg/mL) [75], semi-preparative HPLC (0.73 mg/mL) [69], HPLC (50 μg/mL) [68] and SEC (1.15 mg/mL) [67]. SIM methods are not routinely utilized to analyze biological samples because of the reduced selectivity and high background noise. Ashiru et al. [76] developed the first specific LC-MS/MS method for the quantitation of PEG400 in biological samples. Due to the limited selectivity of SIM, this method was prone to interference from endogenous substances, and the obtained LLOQ (2.5 μg/mL) was higher than that of the previously flow injection MS method.

MRM is a sensitive and selective scan mode, which is commonly used in the LC-MS/MS bioanalysis of small-molecular drugs. Under MRM mode, the precursor ions of the analyte are selected by the first mass spectrometer Q1 and effectively fragmented in Q2. Form the multiple product ions, a highly specific product ion is selected by the second mass spectrometer Q3 for detection. With the aids of the analyte-specific product ion, the selectivity and the signal-noise ratio could be greatly improved (Fig. 7B). A bioanalytical assay for PEG400 using MRM analysis in plasma has been exploited by Bhaskar et al. [77]. In this study, the nine most abundant oligomers and their common product ion (at *m/z* 89) were monitored in Q1 and Q3, respectively. Analyte peaks were then summed up to estimate the total amount of PEG400 in plasma with an LLOQ of 1.01 μg/mL. This approach may be appropriate for low MW PEGs. High MW PEGs contain a wide range of homologues and multicharged ions, and only a fraction of the ions can be monitored by MRM, which is inadequate for quantitation.

Warrack et al. [78] reported a combined strategy for the quantitation of high MW PEG (1.4-40 kDa) in biological samples. The polymers first undergo in-source CID, which generates fragment ions by the declustering potential (DP) in the ion source (Fig. 7C). The generated fragment ions are subjected to the following MRM as surrogate precursor ions. However, detection is still limited by insufficient fragmentation under DP in the ion source, which ultimately limits the sensitivity of the following MRM scan. The LLOQ with in-source CID is 300 ng/mL for PEG.

To improve the fragmentation efficiency, Zhou et al. [79] developed an MS^{ALL} based approach for the quantitative analysis of PEG by liquid chromatography triple-quadrupole/time-of-flight mass spectrometry (LC-Q-TOF MS). Q-TOF MS is a hybrid MS consisting of Q1, Q2 and a high-resolution TOF mass analyzer. MS^{ALL} scan mode allows all precursor ions to pass through Q1, being fragmented in Q2. Consequentially, all the product ions were scanned by the high-resolution TOF analyzer (Fig. 7D). Compared to previous approaches, MS^{ALL} is an effective strategy for quantitation of PEGs in biological samples. Therefore, this approach is also applied in quantitative analysis of PEG and PEGylated drug simultaneously, such as PEGylated DOX, paclitaxel and gemcitabine [80–82].

3.2.1.2. PLA. Benefiting from its biocompatibility and low toxicity, PLA is one of the most widely used biodegradable polymers (Fig. 6B) [83,84]. Various types of PLA, such as poly-L-lactic acid (PLLA), poly-D-lactic acid (PDLA), and poly-DL-lactic acid (PDLLA), are commercially available for medical applications. PLA usually copolymerized with PEG to produce amphiphilic copolymer, which can self-assemble into micelles for encapsulating drugs in their hydrophobic cores. Based on in-source CID technique, Shi et al. [85] developed an analytical method for quantitation of PEG-PLA in plasma. The PLA-specific fragment ions were generated in source, consequentially further fragmented into specific product ions in Q2 (*m/z* 505.0 → 217.0). Due to their higher sensitivity, these PLA-specific product ions were selected for the quantitation of PEG-PLA. The PEG-specific fragment ions were MRM transition monitored for PEG-PLA. This approach was successfully applied to the pharmacokinetic study of mPEG2000-PDLLA2500-COOH in rats.

3.2.1.3. HA. HA is a linear polysaccharide made up of D-glucuronic acid and N-acetyl-D-glucosamine, which is abundant in different types of vertebrate tissues, including connective tissues and extracellular matrix (Fig. 6C) [86,87]. This polymer is very promising due to its hydrophilicity, biocompatible, biodegradable, nontoxic and non-immunogenic features. HA usually interacts with proteins strongly and exhibits a low ionization efficiency, which challenges the biological sample preparation and quantitative analysis by LC-MS/MS. Šimek et al. [88] developed an LC-MS/MS method for the detection of DOX and oleyl hyaluronan (HA-C18:1) in plasma and tissue homogenates. The sample preparation for HA-C18:1 involved two enzymatic work-up procedures by protease and hyaluronate lyase, respectively. Digestion by a protease can release HA from protein-binding in the biological samples. Shortening HA chain by hyaluronate lyase is to achieve a sufficient ionization efficiency. The developed method was applied to the pharmacokinetic studies of DOX and HA-C18:1 after i.v. administration of DOX loaded HA-C18:1 polymeric micelle. The different pharmacokinetic profiles of DOX and HA-C18:1 indicated a premature disruption of HA micelles in vivo.

3.2.1.4. Chitosan. Chitosan is a linear polysaccharide composed of β-1,4-linked D-glucosamine and N-acetyl-D-glucosamine. Chitosan is produced by deacetylation of chitin under alkaline or enzymatic conditions (Fig. 6D) [89,90]. High MW chitosan generally exhibits less solubility, lower degradation rate and higher toxicity than low MW chitosan [89,91]. Chitosan with an MW less than 3.9 kDa has a

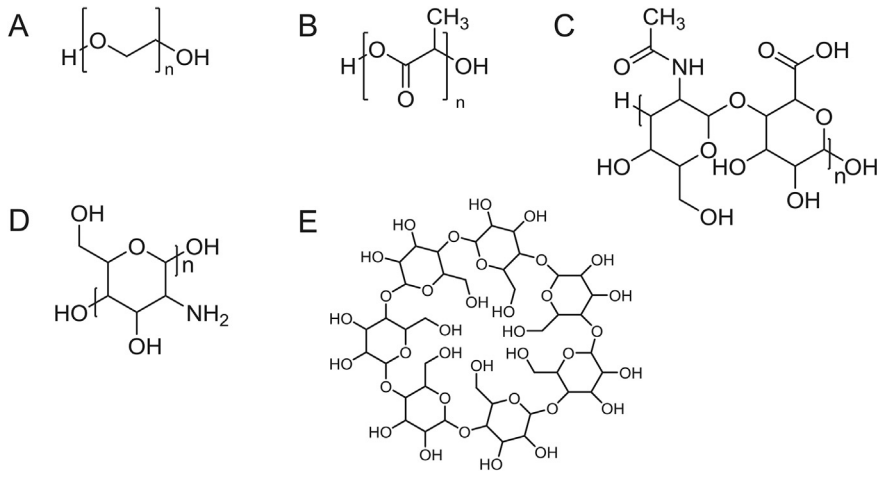

Fig. 6. Structures of (A) poly (ethylene glycol) (PEG), (B) poly(lactide) (PLA), (C) hyaluronan (HA), (D) Chitosan, and (E) β-cyclodextrin (β-CD).

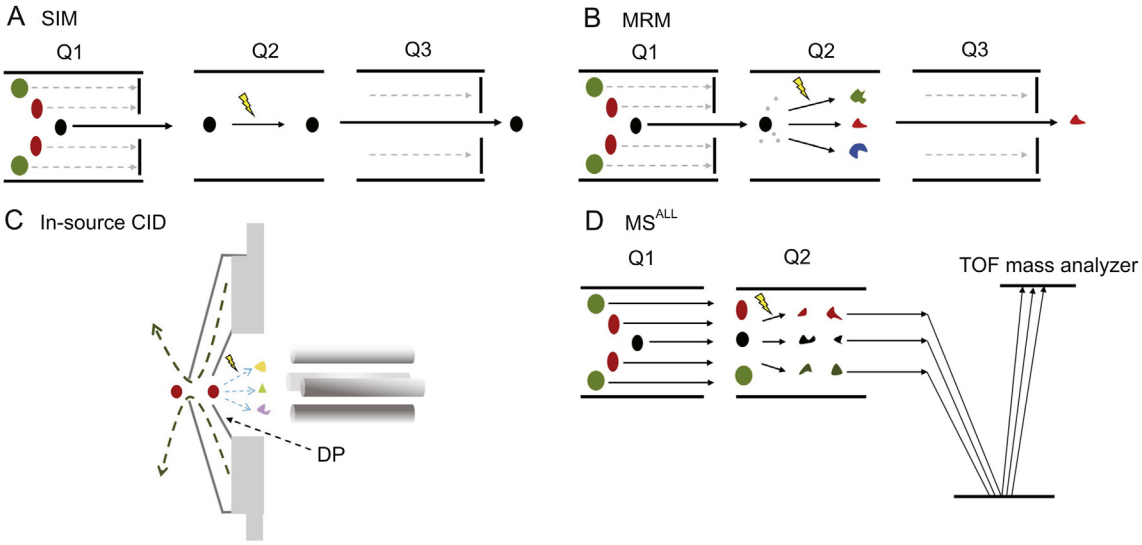

Fig. 7. Description of variety scan modes for quantitative analysis of polymers, including: (A) selected ion monitoring (SIM), (B) multiple reaction monitoring (MRM), (C) In-source collision induced dissociation (CID), and (D) MS^{ALL}.

common name called chitooligosaccharide (COS). Nowadays, the studies for investigating chitosan by LC-MS/MS are mainly focused on the characterizing of COS oligomers. Li et al. [92] reported an MRM method for the simultaneous determination of COS oligomers (D-glucosamine monomer to heptamer) in the chitosan samples. Since chitosan was susceptible to fragmentation in an ESI source, an in-source CID method (without fragment in Q2) was developed for detection of chitosan (139.7 ± 6.0 kDa), which showed excellent linearity ($r > 0.99$) with the LC concentration in a range of 20–10,000 ng/mL.

3.2.1.5. CD. CDs are cyclic oligosaccharides formed by six (αCD), seven (βCD) or eight (γCD) α-1,4-linked glucose units. The hydrophobic cone-like cavity of CD was found capable of loading drugs. Therefore, formed CD-drug complex is water-soluble and can improve the physicochemical properties of the loaded drug (Fig. 6E) [93]. The 2-hydroxypropyl-β-CD (HP-β-CD), a hydroxyalkyl derivative of βCD, is widely used as pharmaceutical excipient. HP-β-CD is a mixture of series of homologues and isomers with 2-hydroxypropyl groups randomly in position and amount (2,097,151 possible homologues) [94]. Jiang et al. [95] developed a 2D-LC-IF-MS/MS method and an RP-UPLC-MS/MS method for the detection of HP-β-CD in human plasma and CSF. HP-β-CD prefers to form sodium adducts with poor fragmentation efficiency. In this study, ammonium salt was added into the mobile phase to suppress the formation of sodium adducts. In the UPLC-MS/MS method, the MRM transition at m/z 1326.5 → 383 was selected for the quantification of HP-β-CD. An LLOQ of 50 ng/mL and 5 μg/mL was achieved for HP-β-CD in human plasma and CSF, respectively. In the 2D-LC-MS/MS method, the detection is based on in-source CID and MRM with the LLOQ in human plasma and CSF of 10 ng/mL and 100 ng/mL, respectively. The HP-β-CD undergoes an in-source CID and generates 2-hydroxypropyl substituted dihydro-pyrylium fragment at m/z 203. With this ion as surrogate precursor ion, a fragmentation transition to the product ion of 4-hydroxypyrylium at m/z 97 was identified and monitored by MRM. The separation of HP-β-CD was first performed on a HILIC column. However, it was found that the glycerophosphocholine species in plasma suppressed the detection of HP-β-CD in plasma samples [96]. To overcome this matrix interference, the chromatography was

sequentially performed on a C_{18} guard column and a HILIC column for the 1D and 2D separation. The 2D-LC-MS/MS method was more sensitive, while the UPLC-MS/MS method with shorter runtime could improve the throughput. Both these methods were successfully applied to the pharmacokinetic study of HP-β-CD in humans.

3.2.2. ELISA

The development of antibodies that specifically bind to PEG-conjugates enables the application of ELISA method in PEGylated drugs detection. The binding affinity of the anti-PEG antibodies to free PEGs is much weaker than to PEG-conjugates [97]. Richter and Akerblom were the first scientists who proposed generating antibodies against PEG by immunizing rabbits. The prepared polyclonal antibodies provide an LLOQ of PEGylated drugs down to about 1 μg/mL [98]. To further improve the sensitivity, the mouse monoclonal antibodies were prepared later, which were capable of binding PEG-conjugates specifically without differentiating their conjugates [99–102]. The concentrations of PEG-conjugates in complex biological samples can be therefore determined via sandwich ELISA. With the help of anti-PEG sandwich ELISA, detection of PEG at a concentration as low as 1.2 ng/mL has been achieved.

Danika et al. [103] developed a sensitive LC-MS/MS method (LLOQ 0.125 μg/mL) for the determination of PCK3145 in mouse plasma. Although LC-MS/MS has been utilized for quantitative bioanalysis of peptides, this technique still faces significant challenges for analysis of PEGylated peptides, such as high polydispersity of PEG, high MW and poor ionization efficiency. They developed an indirect ELISA method for the detection of PEG-PCK3145 by PEGylated protein ELISA kit (Enzo Life Sciences). The concentration of PCK3145 was obtained from the quantification of PEG-PCK3145. Anti-PEG sandwich ELISA achieved a higher sensitivity (LLOQ 0.132 ng/mL) and widely applications for PEG-conjugates. The specificity of this technology remains to be confirmed. Moreover, ELISA method is not suitable for the analysis of free PEGs, which is a limitation for its application in monitoring the biological fate of PEG-based NDDSs.

3.3. Quantitation methods for the NPs

The NDDSs delivery-related activity and their uptake mechanism are still unclear, but an increased cell uptake of nanostructures has been verified by several in vitro and in vivo studies. The enhanced cell uptake may affect the biodistribution of payload drugs and therefore has gained great attention in the field of NDDSs. The MRI, radiolabeling, fluorescence spectroscopy and LA-ICP-MS are currently employed techniques for in vivo NPs quantitation (Table S1).

3.3.1. MRI

MRI, one of the most commonly used medical diagnostic techniques, possesses unique features including noninvasiveness, no exposure to ionizing radiation, high contrast in soft tissues and high spatial resolution [104]. MRI scanner utilizes pulses of radio waves for exciting hydrogen nuclear and records the emitted radio frequency during the relaxation processes from the excited hydrogen atoms. Since hydrogen atoms in form of water are abundant in human tissues and they are different in location, amount and bonding status, spatially localized spectra of the tissues in terms of the hydrogen nuclear density can be generated. According to the different relaxation properties between the hydrogen atoms in different body fluids and tissues, different contrasts will be generated. MRI often requires the use of contrast agents for better image quality. Gd-based contrast agents are the only FDA approved contrast agents for MRI to be used on patients with all types of cancers [105].

The intrinsic/background signals will interfere drug distribution signals, so Gd MRI is not suitable for quantitative clinical measures of NPs. ^{19}F-MRI offers a quantifiable signal, but the sensitivity is far from ideal. Magnetic particle imaging (MPI) is a new non-invasive imaging modality. Employing superparamagnetic nanoparticles (SPNs) as contrast agents, MPI has been applied to monitor the biodistribution of NDDSs. MPI can provide a wide range of imaging depths, linearly quantifiable signals, high sensitivity, and real-time imaging. Zhu et al. [106] designed a superparamagnetic Fe_3O_4 nanocluster@poly (lactide-co-glycolide acid) core-shell nanocomposite loaded with DOX, which serves not only as a drug delivery system but also as an MPI quantification tracer. The nanocomposite can be degraded under a mild acidic microenvironment (pH = 6.5), which leads to a sustained release of DOX and gradual decomposition of the Fe_3O_4 nanocluster. The decomposition-induced MPI signal decay is proportional to the release rate of DOX over time ($R^2 = 0.99$). A quantitative monitoring procedure of the drug release process in cell culture has been successfully established.

3.3.2. Radiolabeling

Labeling nanoparticles with radionuclides allows tracing the nanoparticles in vivo and investigating their biodistribution, drug targeting and clearance quantitatively. Classical methods for radiolabeling NPs generally involve functionalizing the particle surface, core or coating with radio-tag [107]. A main concern of the classical radiolabeling methods is the introduction of a bulky lipophilic prosthetic tag or charged metal ion chelate-tag into the system, which may affect the pharmacokinetic and toxicity profiles of the original NPs [108]. Efforts have been made to explore alternative radiolabeling methods for NPs in order to avoid alerting their surface properties. The newly emerging labeling methods include radiochemical doping, physisorption, direct chemisorption, isotope exchange, cation exchange, particle beam or reactor activation and cavity encapsulation [109]. The in vivo biodistribution of radiolabeled NDDSs can be obtained by modern imaging techniques, such as positron emission tomography (PET). In Engudar et al.'s study, liposomes were remote loaded ^{124}I and evaluated by PET/CT imaging in vivo. A prolonged blood circulation half-life of 19.5 h was observed for the radiolabeled liposomes. Lower accumulation of radiolabeled liposomes in the spleen, liver, kidney and tumors was observed than usually long-circulating liposomes [110].

3.3.3. Fluorescence spectroscopy

Recently, semiconductor NPs, also known as quantum dots (QDs), have been extensively applied in fluorescence spectroscopy [111,112]. Fluorescence technology is an efficient approach to studying the biodistributions of nanostructures in cells and tissues. Compared with the conventional organic dyes, QDs have optical transitions in the near-infrared region, where the tissue absorption is minimal [113]. Kenesei et al. [114] applied spectral imaging fluorescence microscopy to monitor the distribution of fluorescent polystyrene nanoparticles modified with PEG or carboxylic acid groups in male and pregnant female mice. Spectral imaging combined with post hoc spectrum analysis allowed visualizing nanoparticles in various tissues and helped to overcome the limitations caused by the high autofluorescence of native tissues.

3.3.4. LA-ICP-MS

ICP-MS has been regarded as a sensitive analytical method for the determination of ultra-trace levels of metals and metalloids [115]. LA-ICP-MS is a derived technology of ICP-MS, which is equipped with a laser ablation system for vaporizing the sample [116]. By rastering a laser beam across the surface of a cryosectioning tissue sample, an LA-ICP-MS imaging is performed, which can provide a high spatial resolution of the absorbed metallic NPs in different tissues. Elci et al. [117] developed an LA-ICP-MS method to quantitatively image the biodistributions of PEGylated AuNPs. This imaging approach will provide important tissue/organ distribution data, which will greatly facilitate the design and study of nanomaterials for biomedical applications.

3.4. Metabolite profiling methods for NDDSs

The metabolism and elimination of the polymer material are very important features of NDDSs. The accumulation of the polymers or their metabolites in tissue or in organs such as liver, spleen or kidneys, is a potential source of iatrogenic illness. Polymers such as PLA and HA are biodegradable, which can be degraded into low MW monomers or oligomers and be relative rapidly eliminated from the body. For non-biodegradable polymers like PEG, the MW and shape have critical influences on the glomerular filtration and thereby affect the elimination routes and rate. Copolymer, consisting of biodegradable and non-biodegradable polymer blocks, is a compromise solution for polymer excipients to balance their performance and excretion. For example, PEG-PLA block copolymer consists of low MW PEG and PLA polymers alternate in sequence, which can be degraded in vivo to non-biodegradable PEG-segments below the renal excretion cut-off [118]. Therefore, monitoring the synthetic polymers according to ADME concept is of great significance for early detection of progressive accumulation and for prevention of iatrogenic illnesses.

Determining the excretion routes and identifying metabolites are two important features of the polymer mass balance study. Hereby, the eliminated polymers in urine, bile or feces should be analyzed regarding their MW and quantity. These bio-samples can be subjected to solid-phase or liquid extraction techniques for recovering and enriching of polymer components. Subsequently, SEC or LC-MS techniques are generally used to analyze the MW distribution of the eliminated polymers (Table S1). The aforementioned fluorescence and radiometric techniques are also applicable for the quantitation of polymers in excreta samples. Since both the fluorescence- and radio-labeling techniques are surrogate analysis methods, they are generally used in conjugation with SEC for the quantitation of polymers. With the help of radio labeling, the degradation of poloxamer 188 [119], and HA [120] were investigated. Meanwhile, the degradation of chitosan [121] and PVA [122] were studied by means of fluorescence. Polymers usually do not have strong chromophores for UV detection. Refractive index (RI) is an alternative detection for the quantitation of polymers at high concentration and of high purity. The quantitation of the degraded PLGA [123], PLA [124], chitosan [121,125] and HA [126] were performed by SEC or HPLC in combination with RI.

4. Conclusion and perspectives

The ideal NDDSs should provide APIs with the properties of sustained release, prolonged circulation time, improved stability, solubility and targeting. Annually, a great deal of pharmacokinetic information about drug-loaded NDDSs has been reported. However, the approved nanotechnology-based products are limited. The low drug pass-through rate may partially attribute to the inadequate understanding of their pharmacokinetic properties. The present review discusses the recent advances in the bioanalysis of NDDSs, including technological progress in the analysis of the released and encapsulated drug respectively. Besides identifying their pharmacokinetics activities, the bioanalysis of the polymer material of NDDSs is also discussed. Among the enumerated analytical methods, LC-MS/MS is the most comprehensive approach for either profiling the pharmacokinetic behavior of NDDSs in clinical trial or for polymer quantitation in vivo.

Because of the huge gap between the released (in-)active ingredients and NPs in their pharmacokinetics, a comprehensive understanding of the in vivo fate of NDDSs is necessary to ensure their safe clinical applications. There has been a continued demand for developing efficient bioanalytical methods toward this goal. We hope this review will contribute to critical implications in the evaluation of NDDSs in vivo.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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