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***In Vitro* Dissolution Testing Strategies for Nanoparticulate Drug Delivery Systems: Recent Developments and Challenges**

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Abstract

Nanoparticulate systems have emerged as prevalent drug delivery systems over the past few decades. These delivery systems (such as liposomes, emulsions, nanocrystals, and polymeric nanocarriers) have been extensively used to improve bioavailability, prolong pharmacological effects, achieve targeted drug delivery, as well as reduce side effects. Considering that any unanticipated change in product performance of such systems may result in toxicity and/or change *in vivo* efficacy, it is essential to develop suitable *in vitro* dissolution/release testing methods to ensure product quality and performance, and to assist in product development. The present review provides an overview of the current *in vitro* dissolution/release testing methods such as dialysis, sample and separate, as well as continuous flow methods. Challenges and future directions in the development of standardized and biorelevant *in vitro* dissolution/release testing methods for novel nanoparticulate systems are discussed.

Keywords

In Vitro Dissolution/Release, Nanoparticulate Systems, Dialysis, Reverse Dialysis, Sample and Separate, USP Apparatus

Introduction

Over the past several decades, nanotechnology has become increasingly important in medicine. Various nanoparticulate drug delivery systems including nanosuspensions/nanocrystals, lipid nanoparticles, nanoemulsions, as well as polymeric nanocarriers have been developed to improve the pharmacokinetic and pharmacodynamic properties of therapeutics and to achieve targeted delivery to specific organs, cells and even to cellular organelles. Due to their extraordinary physical and chemical properties resulting from the nanosize effect as well as in some cases specific targeting, these nanoparticulate systems (with a size range from 10 to 1000 nm in at least one dimension) offer numerous advantages over traditional drug delivery systems. For example, sub-micron colloidal dispersions of pure drug particles, so-called nanosuspensions or nanocrystals, can overcome slow dissolution behavior associated with poorly soluble drug candidates and improve their oral bioavailability (1–3). Polymeric nanoparticles or lipid-based nanocarriers (such as liposomes and lipid nanoparticles) with particle sizes in the range of 100 nm or less can take advantage of the enhanced permeability and retention (EPR) effect which exists in areas of new blood vessel growth. For example, the EPR effect can be used to deliver anti-cancer therapeutics into tumor tissues *via* passive targeting (4–6).

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Although nanoparticulate systems are promising for the treatment of a variety of human diseases, disastrous toxicity as well as loss of efficacy or altered efficacy can occur if there is any unanticipated change in product quality or performance (7, 8). Consequently, there is an urgent need to develop standardized testing methods for these novel drug delivery systems to ensure product performance and quality, especially with the rapidly increasing number of nanotechnology-driven products that are entering the market and are in the drug development pipelines. *In vitro* dissolution testing (referred to here as “*in vitro* release testing”) is an important tool for quality control purposes as well as for prediction of the *in vivo* performance of drug delivery systems. This test has become an essential quality control test of drug development since it was officially adopted in the United State Pharmacopeia (USP) in 1970 (9, 10). However, there is no standard pharmacopeial/regulatory *in vitro* dissolution/release test currently available for nanoparticulate systems.

It has been very challenging for researchers to develop suitable *in vitro* dissolution/release testing methods for nanoparticulate systems (11, 12). The main obstacles include: 1) difficulty in separating the nanoparticles from the release medium in a rapid and efficient way; 2) the lack of understanding of drug dissolution/release mechanism(s) from such systems; and 3) the complexity of many nanoparticulate systems, some of which are designed not to release drug until uptake into the target cells. This review will summarize the commonly studied *in vitro* dissolution/release testing strategies, followed by a discussion of challenges and future directions in *in vitro* dissolution/release method development for nanoparticulate systems.

Current *in vitro* dissolution/release testing methods

In order to ensure product performance and assist in product development, extensive efforts have been made to develop suitable *in vitro* dissolution/release testing methods for nanoparticulate delivery systems (13–16). These methods can be broadly divided into three categories: *i*) membrane diffusion methods (such as the dialysis methods); *ii*) sample and separation methods; and *iii*) continuous flow methods.

Membrane diffusion methods

Membrane diffusion methods (such as the dialysis methods) are the most widely investigated methods for the *in vitro* dissolution/release testing of the nanoparticulate systems. In these methods, the nanoparticulate systems are separated from the release medium through dialysis membranes that are permeable to the free drug but impermeable to the nanoparticles. Dialysis methods have been widely used to investigate *in vitro* drug dissolution/release profiles of liposomes (17, 18), emulsions (19), polymeric nanoparticles (20, 21), as well as lipid nanocarriers (22).

Dialysis methods include: the dialysis sac method (17), the reverse dialysis sac method (18, 19, 23), and the side-by-side dialysis methods (23–25). In the dialysis sac method, the nanoparticulate systems are placed inside the dialysis sacs and these are then placed into medium reservoir containers with some agitation (often utilizing a shaking water bath) (17) (Fig. 1a). Dialysis sacs are also used in combination with official USP dissolution/release apparatus (such as USP apparatus 1 and 2). For example, a dialysis sac method was used in the USP apparatus 1 (basket type) to study brimonidine tartrate release from Eudragit long acting nanoparticles (26). This study demonstrated that brimonidine tartrate release from the nanoparticles was controlled by non-Fickian anomalous transport, which was very valuable to assist in formulation optimization to achieve the desired drug release behavior (26). However, due to the hindrance to drug diffusion through the dialysis membranes as well as the lack of adequate agitation inside the dialysis sacs themselves, violation of sink conditions has been reported (13, 19, 27, 28). Accordingly, the reverse dialysis sac method

was developed, in which the nanoparticulate systems are directly added into the release medium outside the dialysis sacs and samples of the release medium inside the dialysis sacs are taken and analyzed at different time intervals for released drug content (Fig. 1b). The reverse dialysis sac method has been successfully used to study *in vitro* drug release characteristics of emulsions and other colloidal systems (19, 28, 29). A disadvantage of this method is that high dilution of the nanoparticulate systems in the medium may result in the loss of its discriminatory ability (13, 29). Recently, a glass basket dialysis method (Fig. 1c), which is a modification of the side-by-side dialysis method, was developed for the *in vitro* release testing of lipid-based formulations (such as lipid nanocapsules and liposomes) (14, 15). In this method, a glass basket, the bottom of which is sealed by a dialysis membrane, was utilized to replace the regular basket in USP apparatus 1. This method successfully discriminated ibuprofen release from different nanoparticles (*i.e.* liposomes, lipid nanocapsules and polymeric nanoparticles). The effect of particle size on the *in vitro* drug release profiles of the polymeric nanoparticles has also been demonstrated using this method (15). However, it should be noted that violation of sink conditions may occur with this method.

Key parameters influencing drug release in the dialysis methods include agitation conditions, ratio between donor and acceptor cell volumes, and most importantly the molecular weight cutoff (MWCO) of the dialysis membrane. It is very crucial to select an ideal dialysis membrane with the largest possible pore size to allow free drug diffusion, and yet the pore size must be small enough so that the nanoparticles cannot pass through (27, 30). It has been suggested by Xu *et al.* that the MWCO of the dialysis membrane should be about 100 times the size of the drug molecule (18). In addition, it is recommended that the inside volume of the dialysis membrane should be at least 6–10 fold less than that of the outer release medium in order to provide a driving force for drug transport across the dialysis membrane in the dialysis sac method (31). Prior to starting these experiments, the potential for the drug to bind to the polymer or the dialysis membrane should be checked and a different membrane should be used if necessary and available. This method cannot be used if a suitable membrane with adequate pore size cannot be identified.

Sample and separate methods

In the sample and separate methods, the nanoparticulates are directly added into the release medium and sample separation techniques (such as ultrafiltration, ultracentrifugation, and centrifugal ultrafiltration) are used to separate the dispersed nanoparticles from the continuous phase at different time intervals. Drug content in the supernatant or filtrate is subsequently analyzed. Due to the small size of these nanoparticulate systems, it is very difficult to separate them from the media in an efficient and rapid way without influencing the drug release profile even when high external energy is applied. For example, ultracentrifugation of colistin liposomes at $300,000 \times g$ for up to 8 h at 25°C failed to sediment liposomes smaller than 100 nm (12). Furthermore, long-time and high-speed ultracentrifugation can result in destabilization of nanoparticles (such as, emulsions and liposomes), which can alter drug release. In addition, drug release continues during the separation process, which also can lead to erroneous results.

Key parameters influencing drug release in the sample and separate methods include agitation conditions, and most importantly, sample separation techniques. New sample separation techniques (such as pressure ultrafiltration) have been developed to ensure a rapid and complete sample separation. Pressure ultrafiltration can completely separate nanoparticulates from the release media within 5 min and can prevent nanoparticles from clogging the filter pores, due to the presence of continuous stirring in the ultrafiltration cells (12, 32). In some cases, the use of syringe filters with smaller pore size (0.1 μm or 0.02 μm) has been used as a simple yet efficient way for sample separation. For example, the *in vitro*

dissolution behavior of nanosized fenofibrate was investigated in USP apparatus 2 and syringe filters with small pore size ($< 0.1 \mu\text{m}$) were used for sample separation. The *in vitro* dissolution profiles obtained in this study showed good *in vitro-in silico-in vivo* correlation in both the fed and fasted states (33). Similar results were also obtained for nanosized aprepitant formulations (34).

Continuous flow method

The continuous flow method (USP apparatus 4) was originally developed for modified release oral dosage forms. The USP apparatus 4 method shows advantages in simulating the *in vivo* environment (*e.g.* subcutaneous tissue) for parenteral dosage forms as it can constantly circulate a small volume of the medium through immobilized formulations. In addition, the media volume used with the USP apparatus 4 can be adjusted to allow testing of various formulations, and this is particularly important for many low dose parenteral formulations (35). An adaptation of USP apparatus 4 has been developed to investigate drug release from parenteral sustained delivery systems such as microspheres (36–39). This method has been widely used to investigate drug release from microspheres and at various recent workshops this method has been identified as the method of choice for microspheres (40–43). Recent studies on nanosized cefuroxime axetil, a BCS II compound, showed that the USP apparatus 4 method was the most robust dissolution method for such drug nanoparticles (44). However, in some cases, when the nanoparticulate systems have very small particle size ($< 100 \text{ nm}$), it is very challenging to test their dissolution/release profiles in the USP apparatus 4 system since the nanoparticles either clog the filter leading to slow flow rates and high pressure buildup in the system or pass through the filter, thus resulting in erroneous data. Recently, a dialysis adapter that can be used with the USP apparatus 4 was developed (Fig. 2). Compared to the dialysis sac and reverse dialysis sac method, this modified USP apparatus 4 method successfully discriminated dexamethasone release from different liposome formulations (13). This combination avoided problems such as filter clogging of the apparatus 4 as well as violation of sink conditions and lack of agitation that can occur in traditional dialysis methods. This dialysis adapter based USP apparatus 4 method combines the advantages of a compendial standardized apparatus and the dialysis sac method. This dialysis adapter based USP apparatus 4 method, presents an *in vitro* release testing platform suitable for liposomes and other nanoparticulate delivery systems.

Other methods

Other *in vitro* dissolution/release testing methods such as dynamic dissolution methods and microdialysis methods have also been developed. For example, dynamic dissolution methods utilize ion-selective or drug-selective electrodes to monitor the dissolution/release profiles of electroactive drugs from varieties of nanocarriers (Fig. 3a). These methods can provide a direct, fast and reliable drug dissolution/release assessment on a real-time basis without the need for sample separation due to insensitivity to undissolved materials (30, 45). Dynamic dissolution methods have been used in combination with official USP dissolution/release apparatus (such as USP apparatus 1 or 2) (46, 47). A newly developed method based on voltammetric electrodes accurately monitored the release of chemotherapeutics (*e.g.* doxorubicin) from liposomes in serum (48). However, this method is not suitable for non-electroactive drugs.

Microdialysis is another useful method for the *in vitro* release/dissolution testing of nanoparticulate systems. In this method, microdialysis probes are placed into the dissolution vessels and continuously perfused with a medium devoid of the analyte of interest through an inner tube. The medium flows back between the inner tube and the outer dialysis membrane (Fig. 3b). The released drug in the medium is subsequently analyzed *via* common analytical methods (such as HPLC-UV system). Such microdialysis based dissolution/

release testing methods do not disturb the equilibrium of encapsulated and free drug as withdrawal of the release medium is not needed (32). Microdialysis based methods have been successfully used for the *in vitro* drug release testing of nanoemulsions, nanocapsules, and nanospheres (49, 50). However, microdialysis methods are typically not suitable when used in biorelevant media as surfactants in these media can affect recovery and hence the accuracy.

Challenges and directions in method development

With the advances in biomaterials and biotechnology, novel “smart” or multifunctional nanoparticulate delivery systems (such as targeted nanocarriers or environment-sensitive nanoparticles) have been developed to prolong the half-life of drugs in the systemic circulation, deliver drugs in a targeted manner to minimize systemic side effects, as well as release drugs at favorable rates (fast or sustained) or in an environmentally responsive manner. As a result, *in vitro* performance testing (such as dissolution/release testing) of these nanoparticulate systems is more complex and challenging than other modified release dosage forms (*e.g.* microspheres).

Understanding factors influencing drug release, from both an *in vitro* and an *in vivo* perspective, is essential to develop biorelevant *in vitro* dissolution/release testing methods. For example, there are several types of liposomes, from conventional (immediate release) liposomes to controlled release, “stealth” and immune-liposomes, as well as environmentally responsive liposomes. These liposome formulations have different release characteristics. For sustained release liposome formulations, the dialysis adapter USP apparatus 4 method (13), as discussed above, is suitable to demonstrate slow release characteristics over a period of time. However, for targeted liposome formulations, the *in vitro* release testing method should be adjusted to reflect the *in vivo* drug delivery process. This process usually includes: *i*) phase 1: the absence of drug release prior to reaching the target; and *ii*) phase 2: triggered drug release at the target site. Recently, a two-stage reverse dialysis *in vitro* release method was developed to demonstrate the two-phase drug release characteristics of these liposome formulations (Fig. 4) (18). At stage 1, the liposomes were added into the exterior release medium (pH 7.4 HEPES buffer) and release samples were taken from the interior of alternative dialysis tubes. At stage 2, surfactant (Triton X-100) was added into the exterior HEPES buffer (final concentration: 1% (v/v)) to mimic the two-phase drug release behavior of targeted liposome formulations (Fig. 4). This method was able to discriminate liposome formulations with different compositions and may be applicable for other complex nanoparticulate systems (18). Ideally an *in vitro* dissolution/release testing method should be biorelevant and have the ability to reflect or predict the real drug release characteristics.

Data interpretation is another important factor that should be taken into consideration when developing suitable *in vitro* dissolution/release methods for novel nanoparticulate systems. For example, it has been demonstrated by different researchers that the release data obtained using the dialysis sac method may not necessarily reflect the actual drug release characteristics. In fact, the permeation of released drug across the dialysis membrane was the release rate-limiting step in many cases (18, 27, 28, 51). Likewise, fast drug release obtained using normal sample and separation methods could be due to incomplete and inefficient sample separation (12). Accordingly, it is crucial to interpret drug release data with caution in order to better understand the real drug release characteristics to facilitate formulation optimization as well as quality control of novel nanoparticulate delivery systems.

Moreover, the use of mathematical modeling is very important to elucidate drug release mechanisms, and in turn assist in quality control and formulation optimization. Considering the complex processes involved in drug release from nanoparticulate systems, the reciprocal powered time and Weibull models have been suggested to be applicable for general drug release kinetic studies (52). Both these two models are generally applicable for drug release from nanoparticulate systems that occurs *via* dissolution, diffusion or a combination thereof, which is the case for most solid or semi-solid delivery systems (52). Recently, a three-parameter model that considers both diffusion-driven drug release and drug-carrier interaction has been developed to characterize drug release kinetics from various nanocarriers (such as nanocapsules, nanofibers, and nanoparticles) (53). Although limitations exist for all the kinetic models, simplified and universal models that reflect drug release characteristics are sufficient to serve as a tool for the design and development of novel nanoparticulate delivery systems. It should be noted that care should be taken when using mathematical models to use the appropriate theoretical release behavior.

Conclusions

In vitro dissolution/release tests are an important tool to not only ensure product quality and performance, but also assist in product development and regulatory processes of the nanoparticulate delivery systems. As standardized pharmacopeial/regulatory *in vitro* dissolution/release methods are not available for these complex systems, it is suggested that modification of current compendial methods should be the first option for method development. Furthermore, the development of meaningful and biorelevant *in vitro* dissolution/release testing methods should be taken into consideration.

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Fig. 1.

Schematic representation of dialysis methods. (a) dialysis sac method, the nanoparticulate systems are placed inside the dialysis sacs; (b) reverse dialysis sac method, the nanoparticulate systems are added to the medium outside the dialysis sacs and the inside medium is analyzed for released drug at different time intervals; and (c) glass basket dialysis method, a glass cylinder with its bottom sealed by a dialysis membrane is used.

Fig. 2. Schematic representation of dialysis adapter based USP 4 method in a closed loop configuration. Glass beads are added in the flow through cell to facilitate laminar flow of the media throughout the cell.

Fig. 3. Schematic representation of (a) drug-selective electrode based methods, drug release is monitored electrochemically; and (b) microdialysis based methods, where a microdialysis tube is used to monitor drug release profiles. The microdialysis tube is constructed as a concentric tube, where the perfusion fluid enters through an inner tube, and flows back between the inner tube and the outer dialysis membrane.

Fig. 4.

Schematic representation of a two stage reverse dialysis sac method (reprinted with permission) (18). Stage 1: liposomes were added to the exterior medium and samples were taken from the interior of alternative dialysis tubes; Stage 2: surfactant (Triton X-100) was added to the exterior medium and samples were taken from alternative dialysis tubes. At least two tubes are used to allow equilibration prior to the next sampling time.