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Review Article

Toxicity evaluation of nanocarriers for the oral delivery of macromolecular drugs

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ABSTRACT

Oral administration is the most commonly used and accepted route for drug administration. However, two of the main concerns are the poor intestinal epithelium permeability and rapid degradation, which limit absorption of drugs. In this context, nanocarriers have shown great potential for oral drug delivery.

Nevertheless, special importance should be given to the possible toxic effect of these nanocarriers, such as their bioaccumulation in different tissues of the body, as well as, the different physicochemical parameters influencing their properties and so their potential toxic effect.

This review describes first some aspects related to the behavior of nanosystems within the gastrointestinal tract and then some aspects of nanotoxicology and its evaluation, including the most popular techniques and approaches used for *in vitro* and *in vivo* toxicity studies. It also reviews the physicochemical characteristics of polymeric nanoparticles that may influence the development of toxicological effects, and finally it summarizes the toxicity results that have been published regarding polymeric nanocarriers.

1. Introduction

Oral administration of pharmaceuticals has advantages over other modes of administration as a noninvasive and often safer route that does not require special training. Indeed, oral administration represents the most comfortable and convenient route for drug delivery because it is patient-friendly, painless and appropriate for self-medication [1]. Furthermore, other advantages of great interest are its low cost-effectiveness compared to most other parenteral routes, as well as the possibility of using convenient dosing schedules in the case of chronic treatments.

However, for drug delivery, the oral route also shows some cons. In many cases, particularly with macromolecular drugs, oral delivery only produces (in the best of cases) unpredictable absorption due to inactivation or degradation in the harsh conditions of the gastrointestinal tract (GIT). In fact, extreme pH conditions, the lumen components, as well as the presence of digestive enzymes and biliary salts, strongly hamper the absorption of these biologically active compounds [2,3].

One potential solution to promote the oral absorption and bioavailability of macromolecular drugs may be their encapsulation in nanocarriers [4] (e.g. polymer nanoparticles, self nanoemulsifying drug delivery systems, micelles, dendrimers). All of these nanocarriers offer protection to the loaded compound against its premature degradation during the travel from the mouth to the epithelial membrane [5]. In addition, some of them also show particular properties that allow a controlled release of the cargo. Nevertheless, all of these nanoparticles (NPs) encounter the protective mucus layer that covers the mucosal surface of the

Abbreviations: ADME/PK, absorption, distribution, metabolism, excretion, and pharmacokinetics; ATP, adenosine triphosphate; B, biocompatibility; BCS, Biopharmaceutics Classification System; CS, chitosan; CTX, ceftriaxone sodium; DCFA, 2′7′-dichlorofluorescein diacetate; EURL-ECVAM, European Union Reference Laboratory for Alternatives to Animal Testing; FAE, follicle associated epithelia; FDA, Food and Drug Administration; GALT, gut associated lymphoid tissue; GIT, gastrointestinal tract; GRAS, Generally Recognize as Safe; HPCD, cyclodextrins; ICH, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; LDH, lactate dehydrogenase; LPS, lipopolysaccharides; LSC-CS, lauryl succinyl chitosan; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dip henyl tetrazolium bromide; NB, non-biocompatibility; NCS, nanotoxicological classification system; NPs, nanoparticles; OECD, Organisation for Economic Co-operation and Development; PEG, poly(ethylene glycol); PLA, poly(D,L-lactic acid); PLAF127-PLA, poly(lactic acid)-b-pluronic-b-poly(lactic acid); PLGA, poly (lactide-co-glycolide); PLGA–PEO, poly-lactide-co-glycolic-acid–polyethylene oxide copolymer; PS, polystyrene; TJs, tight junction.

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gut, acting as a supplementary barrier that greatly influences their effectiveness as transporters [6,7]. Thus, once nanocarriers arrive at this frontier, the mucus stops and frequently traps them in the outer layers of this barrier [8]. This phenomenon, which can be of interest in some cases, is referred as mucoadhesion and is driven by physicochemical interactions between the NPs and the glycoproteins (mucins) that form the gel-like structure of the mucus [9].

However, for the delivery of macromolecular drugs (e.g. proteins, peptides, DNA) the nanocarriers must reach the absorptive membrane of the mucosal epithelium and, thus, display mucus-permeating properties. For this purpose, strategies currently pursued and investigated include the development of slippery-surfaced NPs [10] or the functionalization of nanocarriers with proteolytic enzymes [11], among others.

Once the nanocarriers have reached the surface of enterocytes and other gut cells, two different phenomena can occur. The first would be that nanocarriers remain anchored to the cells' surface by a bioadhesive phenomenon mediated by either non-specific physicochemical or ligand–receptor interactions. The second would be that NPs enter into the absorptive cell and, eventually, in the circulation. Obviously the bioadhesive phenomenon may be the first step for NP translocation.

In any case, and from a toxicological point of view, mucus-permeating NPs represent a concern. First, their transit through the mucus layer could negatively affect the mucus structure and, thus, its protective role. Second, the interaction of NPs with the absorptive cells may affect their viability and/or their normal activity. Last but not least, the possibility for the orally administered NPs of entering into the circulation by a translocation process may also induce important side effects and/or undesirable accumulation in the host.

This review will deal with the toxicological evaluation of nanocarriers including the most popular techniques and approaches used for its *in vitro* and *in vivo* analyses. It describes first some aspects related to the behavior of nanocarriers within the gastrointestinal tract. Then, it reviews such physicochemical characteristics of polymeric NPs that may influence the development of toxicological effects, and finally it also summarizes the toxicity results that have been published regarding polymeric nanocarriers.

2. Behavior of nanocarriers after oral administration

The human GIT is a structurally complex and highly dynamic barrier designed to avoid the absorption of foreign particles from the intestinal milieu to the bloodstream [12]. Therefore, oral drug delivery constitutes a great challenge. First, the lumen is a highly proteolytic environment due to its different pH conditions and the presence of digestive enzymes (e.g. pepsin, trypsin and chemotrypsin) [13]. Second, the intestinal epithelium, which is covered by the mucus layer, is permeable only to small molecules, which hinders the bioavailability of the large majority of biologically active macromolecules [14].

Nanoparticle-based systems have many advantageous features for oral drug delivery. Thus, once administered orally, NPs diffuse into the harsh environment, first to the acidic medium of the stomach and then to the intestinal milieu, protecting the cargo [13,15]. Then, depending on their physicochemical characteristics, NPs may undergo the following: (i) direct transit through the GIT, (ii) adhesion to mucus layer, or (iii) diffusion through the mucus layer and adhesion/internalization to the absorptive membrane of the intestinal epithelium [16].

In order to avoid the direct transit of the nanosystems within the GIT and subsequent elimination in feces, research has been focused on the development of NPs capable of being efficiently trapped in the protective mucus layer [17]. This phenomenon, known as mucoadhesion, increases the particle transit time across the GIT by establishing interaction forces (e.g. hydrogen bonding, van der Waals interactions, polymer chain interpenetration, hydrophobic forces, and electrostatic/ionic interactions) between the glycoproteins (mucins) that form the gel-like structure of the mucus layer and the NPs [18]. Thereby mucoadhesive NPs might enhance drug absorption by creating a drug concentration gradient between the nanoparticle matrix and the epithelial surface [16,19].

However, mucoadhesive NPs normally are immobilized in the outermost layers of the mucus barrier that are rapidly cleared, so the transit time of these nanosystems is determined by mucus clearance mechanisms [13,19]. Thus, at long times, there is almost no drug dosing to cells with mucoadhesive NPs. Furthermore, since mucoadhesive systems efficiently adhere to mucus, they are largely unable to penetrate across the adherent mucus layer and approach the underlying epithelia. Therefore, for macromolecules requiring intracellular delivery, mucoadhesive systems might appear inappropriate [14].

In light of the above, to overcome the barrier characteristics of the mucus and reach the underlying epithelia, NPs must possess mucus-penetrating properties. The development of mucus-penetrating NPs was inspired by the study conducted by Olmsted and collaborators [20]. The authors showed that many viruses are capable of penetrating cervical mucus as they are small enough to diffuse through the mesh spacing between mucin fibers and because their surfaces do not make any bond with mucin; they are highly hydrophilic and display net-neutral charge [20].

In this context, poly(ethylene glycol) (PEG) coating of NP surfaces has been demonstrated to be an effective strategy to achieve such mucus-penetrating properties [21]. Although PEG was first used to increase particle mucoadhesion and stability due to its chemical properties, PEG makes particles more hydrophilic and hence modulates their bioadhesive properties [14,22]. Thus, coating NPs with a dense layer of low molecular weight PEG have been shown to effectively minimize adhesive interactions between NPs and mucins, enabling penetration of NPs through the mucus layer [23].

Once mucus-penetrating NPs have diffused through the mucus layer, which is slowly cleared, they are in closer proximity to the intestinal epithelium. Then, cells can be exposed to a optimal dose of drugs released from the mucus-penetrating NPs by increasing their residence time at the mucosal surface. In addition, mucus-penetrating NPs can totally cross the mucus layer and reach the intestinal epithelium to enhance intracellular delivery of drugs.

Carrier digestion may occur prior to absorption; Landry et al. demonstrated that poly(D,L-lactic acid) (PLA) NPs were digested in simulated gastric and intestinal fluids [24]. The use of stabilizer played a crucial role in preventing the degradation of NPs. The intestinal epithelium is a monolayer mainly comprising absorptive enterocytes and to a lesser extent mucus-producing Goblet cells and M cells. NPs can cross the intestinal epithelium by the paracellular route and by the transcellular route. However, the paracellular route is limited because, on the one hand, it utilizes less than 1% of the mucosal surface area and, on the other hand, the tight junctions between the cells limit or completely blocks the passage of macromolecules larger than approximately 1 nm [7]. Nevertheless, although it is generally admitted that NPs do not cross the intestinal epithelium by the paracellular route, through the use of permeation enhancers (i.e. chitosan) it is possible to reversibly open the tight junctions and improve drug delivery [25].

Regarding the transcellular route, two main endocytic mechanisms have been described: phagocytosis, which can be carried out just by M cells and phagocytic immune cells, and pinocytosis [7]. The transport of NPs by the transcellular pathway depends on several factors, but it is mainly governed by the physicochemi-

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