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Bioadhesive properties of pegylated nanoparticles

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The design of bioadhesive nanoparticles (NPs) for targeting specific sites within the gut remains a major challenge. One possible strategy to solve this problem may be the use of pegylated NPs. In general, these carriers display different bioadhesive properties to nondecorated NPs. Thus, pegylated NPs show a higher ability to interact with the small intestine mucosa rather than with the stomach. However, the type of surface conformation of polyethylene glycol chains appears to have a great influence on the behaviour of these NPs. Theoretically, the traditional 'brush' polyethylene glycol corona would facilitate the penetration of the pegylated particles through the mucus layer and the subsequent adhesive interaction with the mucosa, which would promote their absorption by intestinal enterocytes. On the contrary, pegylated NPs with a 'loop' conformation would increase the time of residence of the adhered fraction of particles in the mucosa.

Keywords: bioadhesion, nanoparticles, oral delivery, polyethylene glycol

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1. Introduction

Over the last few decades a great effort has been made in the development of new materials and strategies that carry out methods and systems able to increase the efficacy of drugs as well as new biotechnological drugs. Devices such as nanoparticles (NPs) could provide higher concentrations of the loaded drug to the site of action, modifying the release profile of the given drug in addition to the possibility of using alternative routes of administration. This leads to higher efficiency of the therapy, a more comfortable system for patients and the avoidance of overdosing. In addition, drugs with different physicochemical properties could be included to allow an improvement of drug stability. NPs have now entered a commercial exploration period [1,2], although a number of challenges exist.

One of them is the design of a new type of particles able to reach specific areas within the body. Because of their small size, NPs are suitable for various routes of application such as oral, ocular and parenteral [3-5], with oral being the most convenient and popular way of drug administration. However, most of the NPs suffer from two major disadvantages regarding oral application: instability into the gastrointestinal fluids and low ability to develop bioadhesive interactions within the gastrointestinal tract, particularly with the epithelium of the small intestine and gut-associated lymphoid tissue (GALT). Regarding ocular administration of NPs, the problem of their rapid elimination from the precorneal area also has to be solved. On the other hand, the nasal route offers an easy way for drug administration and a huge potential for the elicitation of effective mucosal and systemic immune response. However, the small specific surface of the mucosa and the induction or presence of liquids may be the two major drawbacks for drug delivery.

It is well known that the properties of the polymer matrix as well as the NP surface could provide the solution for these drawbacks. In this view, association or coating of NPs with suitable polymers may modify their physicochemical characteristics.

One promising direction in this area is the attachment of polyethylene glycol (PEG) to polymeric NPs, referred to as pegylation. PEGs are one of the most popular polymers for surface modification of colloidal drug carriers. In fact, PEGs were found to reduce interactions of the NPs with the cells of the mononuclear phagocyte system (MPS), thereby prolonging NP circulation in the bloodstream after parenteral administration [5-7]. The enhanced resistance of pegylated NPs was explained with the steric stabilisation effect provided by specific conformation of PEG chains on the NP surface. PEG has been found to be a particularly effective steric stabiliser against opsonisation and phagocytosis, probably due to its high hydrophilicity, chain flexibility, electrical neutrality and absence of functional groups, which prevent interactions with biological components *in vivo* [7,8]. PEG chains coupled to or physically adsorbed on the surface of NPs exhibit rapid chain motion in an aqueous medium and have a large excluded volume. The steric repulsion resulting from a loss of conformational entropy of the bound PEG chains on the approach of a foreign substance and the low interfacial free energy of PEG in water contribute to the extraordinary physiological properties of NPs covered with PEG [9-11]. Similarly, PEG grafted to surfaces of biomedical devices showed an increase of their biocompatibility [12-14].

It seems that the effects of pegylated carriers are well studied regarding their parenteral administration [15], including splenic [6] and brain delivery [16]. However, there are few studies dedicated to the use of pegylated NPs for gastrointestinal, ocular or nasal delivery. In these applications, one of the most important steps of the drug absorption will be the interaction between pegylated carriers and the mucosa.

As there is a lack of data, the present paper provides an overall view of the bioadhesive properties of new pegylated NPs based on polymethyl vinyl ether-co-maleic anhydride (PVM/MA) (Gantrez® AN, ISP Corp., NJ, USA). Pegylation as a technical approach has been selected because of the opportunity to obtain surface-modified NPs with bioadhesive properties different in comparison with the naked NPs. As pegylation agents, PEGs with equal molecular weight (2000 Da) but different functional groups (hydroxyl or amino) have been selected.

2. Preparation of pegylated nanoparticles

The main difficulty of the pegylation strategy is the preparation of NPs with a stable PEG attachment [17]. Stability of PEG attachment to desorption/displacement *in vivo* is important because the configuration and conformational mobility of PEG chains could be changed. It was reported that surface density, flexibility and chain length have a great effect on the interactions of particles with the biological media [18-20]. The mode of PEG attachment to NPs is carried out mainly by physical adsorption or by covalent grafting [21,22]. The covalent binding of PEG chains is preferable because of the higher stability of the resulting PEG layer around the particle surface. Thus, covalent attachment can be obtained by either synthesis of PEG copolymer before the manufacture of NPs or by direct pegylation during the process of NP formation.

2.1 Polyethylene glycol-based nanoparticles obtained from preformed copolymer

Most of the pegylated NPs have been prepared on the base of di- or triblock copolymers of PEG with various monomers including lactide, glycolide and caprolactone [7]. The copolymers are generally synthesised by ring-opening polymerisation in organic phase (e.g., toluene, xylene) applying specific chemical conditions (i.e., controlled temperature, anhydrous media) and catalysts (i.e., stannous octoate) [23,24]. For this reason, one of the disadvantages of the copolymerisation is the possibility of toxic residues in the final product. However, this method creates copolymers with desirable hydrophobic/hydrophilic ratio of the monomers as well as low polydispersity [25]. For instance, PEG-cyanoacrylate copolymers have been synthesised by condensation of a PEG derivative with cyanoacrylate monomer in an organic solvent with further induction of the reaction with pyrimidine and carbodimide derivatives [26-28]. Gelatin NPs could also be modified by the reaction between type B gelatin and PEG-epoxide [29].

Furthermore, NPs are usually obtained by different techniques, including either precipitation or emulsification techniques followed by solvent evaporation. In the former, the preformed copolymers are dissolved in a polar solvent (i.e., acetone), and the NPs are obtained when the organic solution is added to an aqueous phase followed by elimination of the organic solvent [6,24,30]. In the latter, the copolymers are dissolved in an organic phase, which is emulsified in an aqueous phase, and the hardening of the NPs occurs by evaporation of the organic solvent [23,31,32]. The two blocks of the copolymers have a tendency for easy phase separation in the presence of water [33]. This property allows preparation of particles with a core corona structure using an oil-in-water emulsification procedure. When the aqueous phase is added, the hydrophilic PEG moieties orient themselves toward the aqueous medium forming a 'corona' layer around the hydrophobic core consisting of polylactic acid (PLA) or polylactic-co-glycolic acid (PLGA) chains [31,34]. When multiple emulsions are prepared, some PEG chains can be entrapped into the inner water phase during hardening of the particles, leading to the distribution of PEG into the NP matrix [35].

2.2 Pegylation during the preparation of the nanoparticles

Another approach for pegylation is a simultaneous polymerisation and formation of particles. This method is widely exploited for obtaining pegylated polycyanoacrylate NPs. For instance, Fontana et al. [36] prepared pegylated NPs by emulsion polymerisation of ethylcyanoacrylate monomers and PEGs with different molecular weights. The polymerisation was carried out in an acidified aqueous medium (pH 1.2) only in the presence of nonionic surfactant such as pluronic. PEG can act directly as a nucleophile initiator of

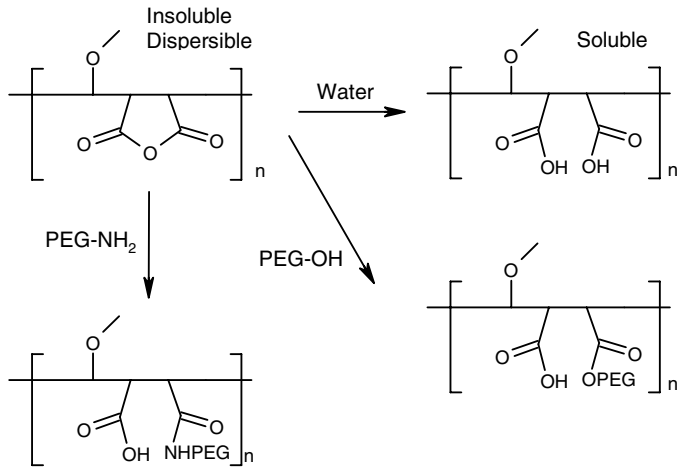

Figure 1. Schematic representation of the chemical structure of polymethyl vinyl ether-co-maleic anhydride and the possibilities to react with water or molecules possessing primary amino or hydroxyl residues.
PEG: Polyethylene glycol.

polymerisation through its C-terminal groups, thus forming a PEG-polyisobutylcyanoacrylate (PIBCA) copolymer [17].

A facile and quantitative method for the preparation of pegylated NPs has been developed based on PVM/MA. This copolymer is widely employed for pharmaceutical purposes as thickening and suspending agents, denture adhesives and as an adjuvant for the preparation of transdermal patches. The main advantages of this polyanhydride is its affordable price, low oral toxicity and the availability of functional groups, which can easily react with molecules showing hydroxyl or amino residues [37,38]. Thus, in an aqueous environment, the anhydride group is opened yielding two carboxylic acid groups. This reaction involves the dissolution of the copolymer in water [39]. However, the presence of molecules with active groups may permit the binding of ligands (i.e., PEG) to the copolymer backbone or to the surface of the just-formed NPs [38]. Similarly, this reaction can be useful to bind drugs or markers and to modulate the half-life of the resulting NPs by using a crosslinking agent [101]. **Figure 1** shows the chemical structure of PVM/MA and the possibilities to react with molecules possessing hydroxyl or amino groups.

Two different preparative procedures were developed aiming to achieve pegylated NPs. The first consisted of simultaneous incubation for 1 h of PVM/MA and PEG into acetone with the further formation of NPs by the addition of a hydroalcoholic phase. The organic solvents were then evaporated and the resulting NPs purified and freeze-dried using sucrose as cryoprotector (method 1). The second procedure included the formation of PVM/MA-NPs and their subsequent coating by incubation with PEG (method 2). Further steps such as purification and lyophylisation were similar.

The experiments clearly demonstrated that the addition of PEG in the organic phase (method 1) enabled a higher efficiency than the simple coating of the NPs to be obtained (method 2). As shown in **Figure 2**, an approximately threefold higher degree of pegylation was achieved by applying method 1 compared with method 2. In both cases a prerequisite for coupling reaction is the opening of the anhydride groups of PVM/MA. However, it is hypothesised that the absence of water during the incubation of the copolymer and PEG decreased the opening of the anhydride groups of PVM/MA and, thus, increased the possibilities for the interaction between functional groups of PEG and anhydride residues of the copolymer [40,41]. In the second procedure, the main reason for the failure could be a predominant role of hydrolysis of the anhydride moieties in water over the PEG association [42]. Hence, simple coating of preformed PVM/MA particles was also possible, although the high affinity of PEG to the water phase and the rapid hydrolysis of the polymer groups led to lower association.

3. Bioadhesion and targeting

Bioadhesion (or mucoadhesion) is generally understood to define the ability of biological or synthetic material to 'stick' to a mucosa, resulting in adhesion of the material to the tissue for a prolonged period of time [43]. This concept has received significant attention due to the potential applications in drug delivery and the improvement of drug bioavailability as a result of the prolongation of the contact time between bioadhesive dosage form (i.e., NPs) and absorbing tissue.

There are two possible ways in which a material may adhere to a mucosal surface: by binding to the tissue itself or by association with the mucus layer coating the tissue surface. Small particles (≤ 600 nm) can penetrate the mucus layer and are, therefore, protected from clearance mechanisms, extending their residence on the mucosa. However, during mucus secretion they would inevitably be pushed further from the surface of the mucosa and could be removed together with the luminal contents. In contrast, larger particles (i.e., microparticles) could only interact with the surface of the mucus layer and,

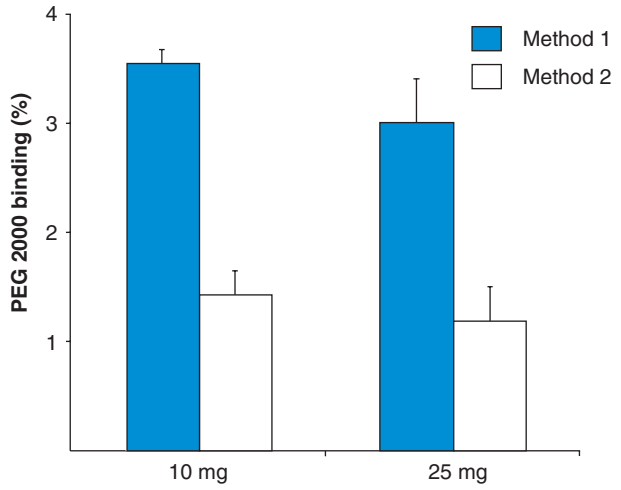

Figure 2. Influence of the preparation procedure on the association of PEG 2000 to nanoparticles evaluated at two different bulk concentrations of PEG 2000 (10 and 25 mg).
Method 1: simultaneous incubation of PEG and PVM/MA in organic phase; method 2: incubation of preformed nanoparticles into PEG-containing aqueous phase; mean values ± SD (n = 3).
PEG: Polyethylene glycol; PVM/MA: Polymethyl vinyl ether-co-maleic anhydride; SD: Standard deviation.

therefore, would be more exposed to the clearance mechanisms, including peristaltism, ciliary movements and interaction with lumen contents [44,45]. Similarly, studies using cystic fibrosis sputum demonstrated that the largest spheres studied (diameter of 560 nm) were almost completely blocked, whereas the smaller NPs (124 nm) were able to penetrate through this barrier [46]. The phenomenon of particulate transport through the mucus layer has been studied most intensively in relation to bacterial movement and their ability to invade the epithelium [47-49].

On the other hand, the direct contact of NPs with cells of the mucosa is considered as the first step before particle absorption. Historically, the absorption pathway of particulates (mainly oral) has been the subject of in-depth investigations; for example, the work of Volkheimer [50]. In the mucosa, this translocation occurs not only via the M cells in the Peyer's patches and the isolated follicles of the gut-associated lymphoid tissues [51,52], but also via the enterocytes [53,54] or other types of mucosa cells [55].

3.1 Oral delivery

The oral route constitutes the preferred route for drug delivery mainly because it is easy to dispense and safe. In addition, mucosal application of protein vaccines offers many advantages over other routes of application. Oral (or nasal) delivery can result in the induction of mucosal immune response against pathogens, which colonise and invade the mucosa. Despite some interesting results *in vivo*, bioadhesion of particulate systems through nonspecific interactions with the intestinal mucosa suffers from two major drawbacks. First, only a small fraction of the particles are the object of absorption, whereas the remaining undergo direct faecal elimination. Therefore, only a portion of the drug is concerned with the bioadhesion phenomenon, which mainly takes place in the stomach mucosa. Second, targeting to Peyer's patches for drug delivery or oral vaccination purposes appears to be unlikely so far [56].

One possible approach to design NPs able to develop specific bioadhesive interactions with the different types of mucosa may be the surface modification of NP carriers. The latter could be achieved by coupling ligands able to recognise and specifically bind receptors localised at the surface of normal and specialised cells. For this purpose different ligands including lectins [37,57,58], invasins [59] and vitamin B12 derivatives [60] have been proposed. Some of these solutions have shown a great potential for therapeutical application; however, the preparation of these functionalised NPs remains difficult, costly and time-consuming. Another option can be the modification of the NP properties by the attachment of PEGs. PEGs are hydrophilic, nonionic and nontoxic polymers, which do not possess strong bioadhesive properties compared with chitosan or carbomers [61,62]. However, some authors have reported that PEG chains may establish specific bioadhesive interactions with mucosal tissues because of their ability to interdiffuse across mucus networks [63,64]. In this view, the pegylated NPs would be able to adhere to any mucosal tissue including those presented in the gastrointestinal tract and the eye. The subsequent persistence of NP residence at the site of absorption will enhance bioavailability of the encapsulated drugs and possibly their uptake and translocation. Indeed, some studies have demonstrated a low degree of interaction between gastrointestinal enzymes and pegylated NPs, which was explained by their higher stability. *In vitro* incubation of PEG-PLA particles (~ 200 nm) in simulated gastric fluid demonstrated a ninefold increase in chemical stability over nonmodified PLA-NPs. Incubation in simulated intestinal media reduced the particle population of PEG-PLA to 57% of the applied dose, but double that of blank PLA-NPs, suggesting a partial protection by PEG groups against pancreatin mediated degradation [65].

In this context, *in vivo* behaviour of various types of pegylated NPs was investigated. PVM/MA-NPs were coupled with PEGs possessing equal molecular weight but different functional groups, namely hydroxyl groups (PEG) or amino-groups (*bis*-aminopropyl-polypropyilenglycol-polyethylenglycol-polypropilenglycol [DAP-PEG-NP]). The pegylated NPs were prepared by simultaneous incubation of the copolymer and PEG in organic phase followed by a desolvation process [41]. The method allows a yield of ~ 74% of the copolymer transformed into NPs [37,101]. **Table 1** summarises the main physicochemical properties of these formulations. The size of the resulting NPs was found to be ~ 300 nm; although, for DAP-PEG-NP, the size was slightly higher than for the other formulations. Concerning the zeta potential, all the formulations were negatively charged. For pegylated NPs, the zeta potential was significantly lower than for NPs. Regarding the association of PEG to NPs, PEG-NP displayed a lower degree

Table 1. Physicochemical characteristics of NP formulations.

Sample	Size (nm)	Zeta potential* (mV)	PEG-loading (µg/mg)	RBITC‡-loading (µg/mg)
NP	289 ± 11	-58.8 ± 4.5	-	10.3 ± 0.9
DP-NP	288 ± 4	-34.8 ± 0.5	-	10.0 ± 0.4
PEG-NP§	299 ± 22	-39.1 ± 5.2	30.2 ± 4	10.4 ± 0.1
DAP-PEG-NP§	361 ± 15	-11.5 ± 0.1	82.9 ± 2.2	8.7 ± 0.8

*Measurements were performed after dilution of samples with 0.05 M phosphate buffered saline (pH 7.4). ‡RBITC is used as fluorescent tag. §Pegylated NPs with PEG 2000 or diaminopropyl-PEG 2000, respectively. Data express the mean ± SD.

DAP-PEG-NP: *bis*-aminopropyl-polypropyilenglycol-polyethylenglycol-polypropilenglycol; DP: 1,3-Diaminopropane; NP: Nanoparticle; PEG: Polyethylene glycol; RBITC: Rhodamine B isothiocyanate; SD: Standard deviation.

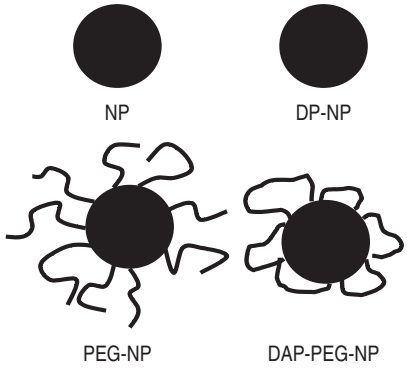

Figure 3. Schematic representation of the different NP formulations. Pegylation with PEG induces surface modified NPs with a "brush" conformation of the chains, whereas DAP-PEG forms "loop" conformation.

DAP-PEG-NP: *bis*-aminopropyl-polypropyilenglycol-polyethylenglycol-polypropilen-glycol; DP: 1,3-Diaminopropane; NP: Nanoparticles; PEG: Polyethylene glycol.

of pegylation (~ 30 µg/mg NP) than for DAP-PEG-NP (~ 80 µg/mg NP). This fact can be explained with the higher affinity of polyanhydrides to react with primary amine groups than hydroxyl residues [66].

On the other hand, depending on the PEG used for pegylation, the NPs demonstrated different surface characteristics. Pegylation with PEG (two hydroxyl ends in the polymeric chains) gave NPs with a 'brush' conformation of the chains [41], whereas for DAP-PEG (two different N-terminal groups), the NP surface was covered by double-end coupled chains forming 'loop' conformation (**Figure 3**). Another consequence of pegylation was an increased stability of the resulting NPs in an aqueous medium. This prolonged half-life was of the same order as in the case of conventional NPs crosslinked with 1,3-diaminopropane (DP-NP).

When the different NP formulations were administered by the oral route to fasted rats, their distribution within the gut, and bioadhesivity to the mucosa, appeared to be influenced by pegylation, crosslinkage and carrier shape. In all cases, 10 mg of the different NP formulations, dispersed in 1 ml water, were administered by the oral route to animals. At different times, the rats were sacrificed and the fluorescent tag extracted from the different portions of the mucosa [67]. **Figure 4** shows the amount of NPs adhered to the stomach (**Figure 4A**) and small intestine (**Figure 4B**).

The first significant observation was that pegylated NPs displayed different profiles of bioadhesion to those observed for NPs. In recent work, the bioadhesive potential of PVM/MA was demonstrated to be much more when folded in NPs than when it is administered in the form of simple aqueous solution [68]. This fact agrees with previous works suggesting that the nanoparticulate form would facilitate both the initial contact and the establishment of adhesive interactions with the components of the mucosa [69,70].

Within the stomach, pegylated NPs displayed a maximum of adhesion just after their oral administration (~ 10 – 14% of the given dose) and, after this maximum, a rapid decline in the adhered fraction over time was observed (< 5%, 3 h after the oral administration of the single dose). This profile of bioadhesion is clearly different compared with that obtained for NPs, where the maximum of adhesion was found to be 1 h post-administration and the capacity of this formulation to develop adhesive interactions with the stomach mucosa was significantly higher than for the other formulations ($p < 0.05$). For NPs, this high potential to develop adhesive interactions has been related to the generation of carboxylic groups resulting from the hydrolysis of the copolymer in an aqueous environment (**Figure 1**). In accordance with the adsorption theory of bioadhesion, these carboxylic groups would facilitate the development of hydrogen bonds with components of the mucosa, such as mucins [71,72].

Table 2 summarises the quantification of the bioadhesive properties of the different formulations tested in the stomach of animals. Area under the curve of bioadhesion (AUC_{adh}) (curves shown in **Figure 4**) estimates the intensity of the adhesive phenomenon, whereas mean residence time of the adhered fraction (MRT_{adh}) can be related with the relative duration of the adhesive interactions of NPs in the mucosa [67,68].

Pegylation decreased the intensity of the bioadhesive interactions developed by PVM/MA NPs in the stomach ($p < 0.05$; **Table 2**); however, the residence time of the adhered fraction increased by ≤ 30% of that observed for NPs.

On the other hand, the high residence time within the stomach mucosa (MRT_{adh}) and low intensity of the bioadhesive phenomenon (AUC_{adh}) observed for DP-NP may be explained

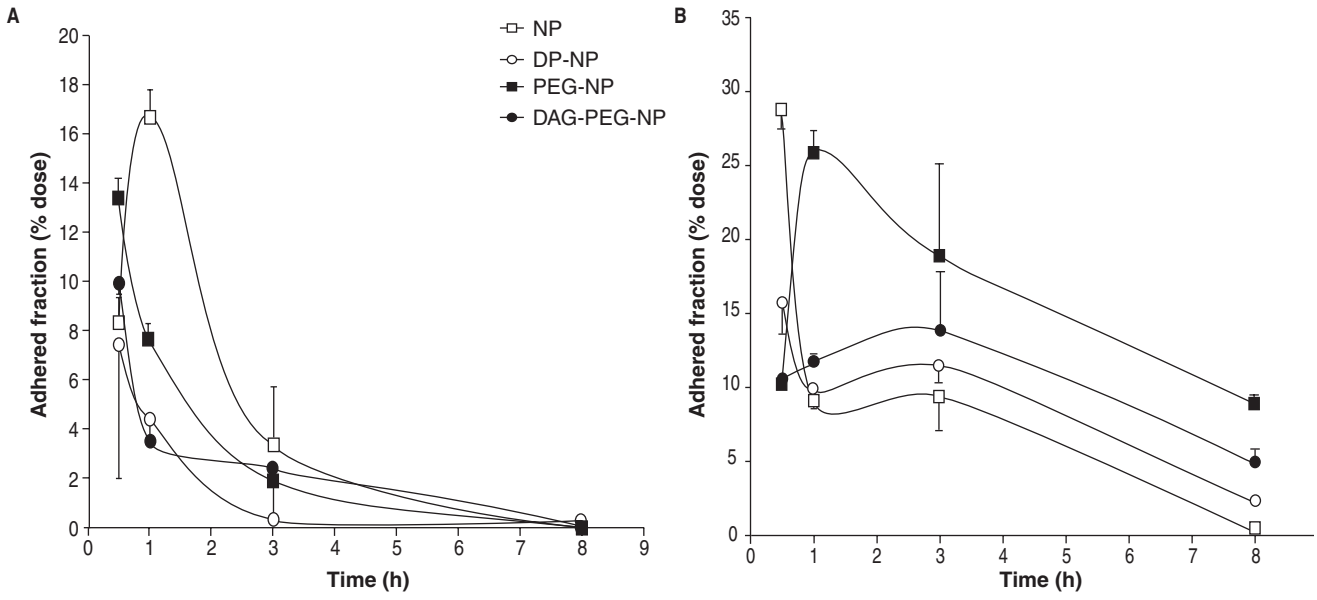

Figure 4. Evolution of the adhered fraction of NPs in the stomach (A) and small intestine (B) of animals with the time, after a single oral administration of 10-mg NPs.
DAP-PEG-NP: *bis*-aminopropyl-polypropyilenglycol-polyethylenglycol-polypropilenglycol; DP: 1,3-Diaminopropane; NP: Nanoparticle; PEG: Polyethylene glycol.

Table 2. Bioadhesion parameters in the stomach.

Sample	AUC_{adh}(mg/h)§	MRTadh (h)§
NP	2.83 ± 0.66	1.16 ± 0.15
DP-NP	1.08 ± 0.59‡	1.41 ± 0.26
PEG-NP	2.3 ± 0.26*	1.5 ± 0.22*
DAP-PEG-NP	1.18 ± 0.19‡	1.21 ± 0.35

*p < 0.05 . ‡p < 0.01 versus conventional NPs. §These parameters were calculated using the WinNonlin 1.5 software. The Mann-Whitney U-test was used to investigate statistical differences (SPSS® 6.1.2, Microsoft). In all cases, p = 0.05 was considered to be significant [67,68].

AUC_{adh}: Area under the curve of bioadhesion up to t_z;
DAP-PEG-NP: *bis*-aminopropylpolypropyilenglycol-polyethylenglycol-polypropilenglycol; DP: 1,3-Diaminopropane; MRT_{adh}: Mean residence time of the adhered fraction of NPs in the mucosa; NP: Nanoparticle;
PEG: Polyethylene glycol; tz: Last time of sampling.

by its higher physical stability and hydrophobicity (compared with NPs). In fact, crosslinkage of PVM/MA-NPs (DP-NP) would block the possibility of the generation of carboxylic groups, conferring a higher stability against dissolution [68]. However, at the same time, this reaction would also increase the hydrophobicity of the resulting carriers [73]. Hydrophobicity of NPs has been defined as one of the major hindrances for penetration into the mucus gel layer [46,74,75].

In the small intestine, for naked NPs, the highest capacity to develop adhesive interactions was found just after administration (**Figure 4**). On the contrary, pegylated NPs displayed the maximal amount of adhesion either 1 (PEG-NP) or 3 h post-administration (DAP-PEG-NP) with a plateau of adhesion for ≥ 3 h. In addition, pegylated NPs demonstrated a higher ability to develop adhesive interactions with the small intestine mucosa than with the stomach. In fact, these interactions were found to be more intensive and prolonged than those developed with conventional NPs. Similar affinity to the small intestine was demonstrated elsewhere by PEG-coated liposomes after oral administration to rats [76]. The authors reported that ~ 25% of the PEG liposomes applied were detected in the ileal segment 3 h postadministration compared with the absence of fractions in the stomach. In another study, the adhered fractions to the small intestine 3 h after oral administration were 15 – 20% depending on the type of pegylated NPs (**Figure 4B**).

Table 3 summarises the main parameters of bioadhesion in the small intestine derived from the curve of bioadhesion displayed in **Figure 4B**. As expected, PEG-NP displayed the highest AUC_{adh}. In fact, the intensity of the bioadhesive phenomenon within the small intestine was found to be twofold times higher for PEG-NP than for NP ($p < 0.01$). Comparatively, the NPs with a 'loop' layer (DAP-PEG-NP) developed weaker interaction. These results can be explained with an eventual entrapment of the particles into the mucus layer resulting from the lower mobility of the chains in the case of DAP-PEG-NP. On the other hand, according to the bioadhesion theory, the presence of functional groups is an important prerequisite for the establishment of bioadhesive forces between biological surfaces and the drug delivery systems. The latter fact could explain the low bioadhesive potential observed for DAP-PEG-NP where the chains of pegylated agent were double-end coupled to the NP surface and were not available for any interaction with the mucosa. Nevertheless, the residence time of the adhered fraction of PEG-NP was not significantly higher than for conventional NPs. In fact, the MRT_{adh} in the small intestine followed

Table 3. Bioadhesion parameters in the small intestine.

Sample	AUC_{adh} (mg/h)§	MRTadh (h)§
NP	5.96 ± 0.73	2.09 ± 0.25
DP-NP	6.58 ± 0.91	2.76 ± 0.35*
PEG-NP	10.56 ± 2.26‡	2.51 ± 0.59
DAP-PEG-NP	8.03 ± 1.58 *	3.23 ± 1.13*

*p 0.05. ‡p 0.01 versus conventional NPs. §These parameters were calculated using the WinNonlin 1.5 software. The Mann-Whitney U-test was used to investigate statistical differences (SPSS® 6.1.2, Microsoft). In all cases, p = 0.05 was considered to be significant [67,68].
AUC_{adh}: Area under the curve of bioadhesion up to t_z;
DAP-PEG-NP: *bis*-aminopropyl-polypropyilenglycol-polyethylenglycol-polypropilenglycol; DP: 1,3-Diaminopropane; MRT_{adh}: Mean residence time of the adhered fraction of NPs in the mucosa; NP: Nanoparticle; PEG: Polyethylene glycol; tz: Last time of sampling.

rank order: DAP-PEG-NP > DP-NP > PEG-NP > NP. This surprising observation suggests that additional mechanisms, other than merely mucus turnover, may also be responsible for the elimination of the adhered fraction of NPs from the mucosa. A promising explanation would be that PEG-NPs are taken up by normal absorptive cells. This is consistent with previous works, which have demonstrated the ability of pegylated NPs to cross the nasal epithelium [77,78].

Consequently, it could be assumed that the different physicochemical surface properties of the NPs determine their bioadhesive capacity. In order to confirm this idea, fluorescence studies were performed. **Figure 5** shows the distribution of fluorescence in the mucosa of the ileum after oral administration of 10 mg of either NP or PEG-NP. From this result, it is evident that both formulations displayed a different behaviour in the gut. Conventional NPs were not able to reach the enterocytes in spite of their ability to penetrate in the mucus layer (**Figure 5A**). This is consistent with Durrer and colleagues, who found that small particles can be considered as adsorbants, which penetrate into a porous adsorbent (the mucus layer) until the internal area available for adsorption is saturated [44,45,79]. However, this penetration appears to be restricted to the mucus layer and, thus, the elimination rate of the adhered fraction of NP is of the same order as that obtained for the solubilised form of the copolymer [68].

Figure 5 shows an extensive incidence of PEG-NP in the intestinal enterocytes (**Figure 5B**), suggesting that the 'brush' layer of PEG may facilitate the penetration of PEG-NP through the mucus barrier, providing an intimate contact with the intestinal wall. This phenomenon of bioadhesion would be the first step for endocytosis of PEG-NP. Numerous authors have demonstrated the uptake of NPs from the gastrointestinal tract over the past two decades [2,80-82], mainly through both Peyer's patches and enterocytes. Major attention has been given to Peyer's patches of the GALT because M cells of lymphoid tissue are adapted to absorb a

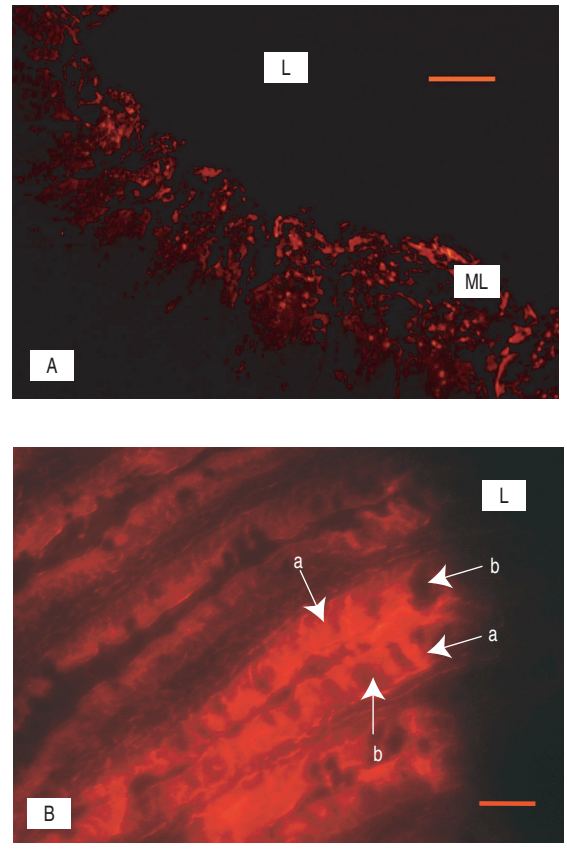

Figure 5. Visualisation by fluorescence microscopy of NPs adhered in the rat ileum 2 h after oral administration of a single dose of 10 mg. A. NP, with permission from ARBOS P, CAMPANERO MA, ARANGOA MA, RENEDO MJ, IRACHE JM: Influence of the surface characterisitics of PVM/MA nanoparticles on their bioadhesive properties. *J. Control. Release* (2003) **89**:19-30. Bar: 100 mm. **B.** PEG-NP. Bar 25 µm. Arrows marked 'a' show enterocytes; arrows marked 'b' show goblet cells.
L: Lumen; ML: Mucus layer; NP: Nanoparticle PEG: Polyethylene glycol.

of this focus: the fact that lymphoid tissue occupies a relatively small part of the total surface area of the gut; and the fact that it has been shown that many systems are able to enter through normal gut epithelial cells (enterocytes) [49].

Another interesting fact is that PEG-NP also display a higher ability to target intestinal Peyer's patches than NP. **Figure 6** shows the localisation of PEG-NP in the Peyer's patches of rat ileum.

All of these observations open opportunities for the use of these NPs as adjuvants for vaccination or immunotherapy purposes. In this context, administration of pegylated PLA-NPs loaded with radiolabelled tetanus toxoid in rats demonstrated accumulation primarily in the mesenteric lymph nodes (8% of the dose after 6 h postadministration), with barely detectable levels in liver, spleen or lung [65]. The steady radioactivity levels observed between 6 and 24 h led to the assumption that the pegylated particles remained associated with the gastroin-

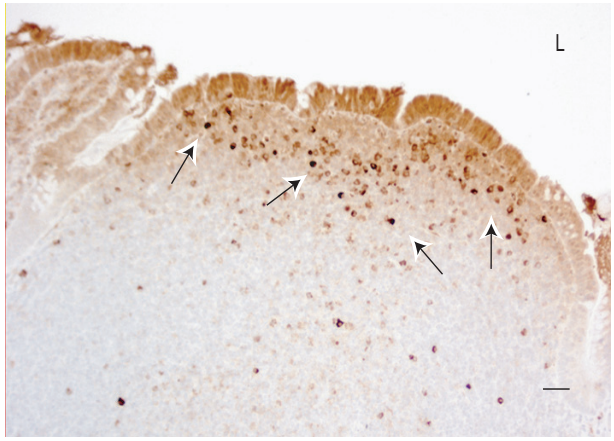

Figure 6. Visualisation of PEG-NP localised in the cells of ileal Peyer's patch of rat ileum 2 h after oral administration of a single dose of 10 mg (optic microscopy; bar: 30 µm).
Arrows show the presence of NPs in cells of Payer's patch.
L: Lumen; NP: Nanoparticle; PEG: Polyethylene glycol.

testinal mucosa, leading to a sustained drainage from the mucosa to the lymphatics and blood circulation. In addition, the higher concentration of the antigen associated with PEG-PLA-NPs in the blood were related to the oral absorption of these particles. This phenomenon could also be related to their slow clearance by the MPS due to the steric protection of the PEG-coating layer. Furthermore, Alpar *et al* described a rapid absorption process of pegylated NPs [83]. In fact, 2% of pegylated particles were found in blood as early as 5 min post oral dosing, approximately threefold greater than plain PLA-NPs.

Moreover, when PEG-NP containing ovalbumin was administered by the oral route to Balb/c mice (25 µg protein in a single dose), discrete levels of IgG_1 and IgG_{2a} against this model allergen were measured (unpublished results). Surprisingly, this pegylated formulation appeared to be able to induce important IL-10 levels and, more importantly, to sustain the levels of this cytokine for ≥ 6 weeks (**Figure 7**). These results may have a significant repercussion in immunotherapy. IL-10 is a key factor to control tolerance and other immune responses [84]. It is a potent suppressor of several effector functions of macrophages, T cells and natural killer cells. In addition, it contributes to regulate proliferation and differentiation of B cells, mast cells and thymocytes [85]. Consequently, the regulatory potential of IL-10 suggests its interest in the treatment of diseases induced by a misbalance in the T helper 1/2 response, such as autoimmune disorders [86] and allergy [87,88].

In conclusion, these data suggest that the oral administration of pegylated NPs may provide a more efficient interaction with the gastrointestinal mucosa, subsequent absorption and, finally, prolongation of the NP interaction with the immunocompetent cells.

3.2 Ocular delivery

Ocular administration is associated with the rapid elimination of drug formulations from the precorneal area due to drainage through the nasolacrimal duct and dilution by tear turnover. These processes result in a very low percentage of the drug administered (< 5%), which could penetrate through cornea and reach intraocular tissues. Many studies have demonstrated an enhanced accumulation of NPs in the conjunctival cul-de-sac and better drug bioavailability compared with traditional ophthalmic dosage forms such as solutions and ointments [89,90]. The colloidal systems can be administrated as simple eye drops, and due to their low viscosity the temporary sight hindrance will be avoided. The frequency of application could be reduced due to the sustained drug delivery from the NP matrix. However, they can also be rapidly eliminated from the ophthalmic mucosa [90]. In this respect, PEGs could be considered as coating agents permitting the modulation of NP surface properties.

Nanoparticles based on previously synthesised amphiphilic poly-ε-caprolactone-PEG copolymer were loaded with a fluorescent marker (rhodamine 6G) and their ability to interact with corneal mucosa was examined *ex vivo* on rabbit cornea [91]. It was found that PEG-coating did not enhance the interaction of the particles with corneal epithelium, which was in a discrepancy with previously reported potential of PEG-coated NPs as mucosal carriers for nasal drug delivery [65,92]. However, PEG-coated NPs were found at 50 µm depth into the corneal epithelium 1 h postincubation, whereas noncoated were mainly located at only 10 µm depth. Thus, independent of the weak interaction with the cornea, PEG coating facilitated the transcellular transport of the modified NPs.

In another study PEG-coated polycyanoacrylate NPs were loaded with an antiviral drug acyclovir and the capacity of these carriers to improve ocular bioavailability was investigated in rabbits [93]. The authors have observed a significant increase of acyclovir bioavailability after the administration of the pegylated particles and concluded that it could be caused by the improved interaction of the coated NPs with the corneal epithelium. In a subsequent study, the same antiviral drug was loaded in PEG-coated PLA nanospheres and their potential, like the ocular delivery system, was explored [94]. The pegylated particles provided higher drug bioavailability compared with nonmodified PLA nanospheres, which were explained with the improved mucoadhesion and longer residence time at the site of application. The authors have suggested that the presence of polyoxyethylene moieties on the nanosphere surface could increase permeability of cellular membranes, allowing a specific penetration enhancement for the drug molecules [95].

3.3 Nasal delivery

The nasal route is an alternative method of application, in particular for the administration of proteins and antigens. The antigens can be delivered to the immune-active tissue known as nasal-associated lymphoid tissue, and eventually

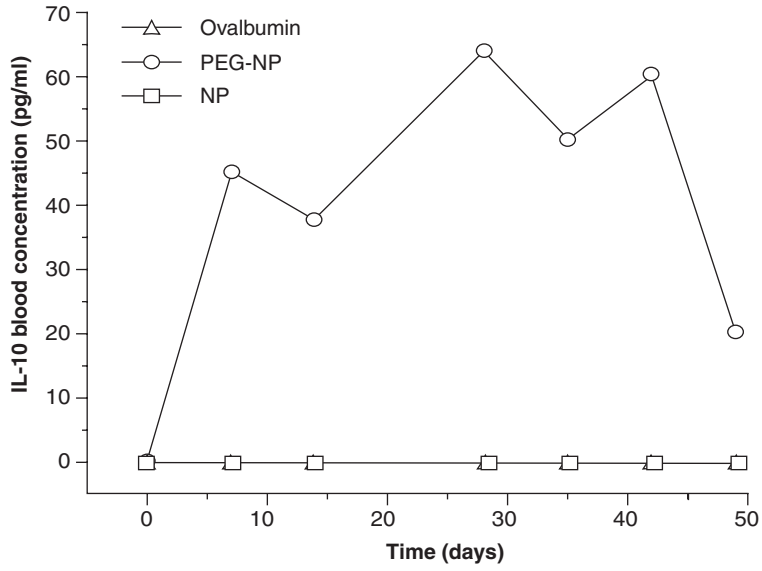

Figure 7. IL-10 plasma levels versus time after the oral administration of a single dose of NP formulations containing 25 µg ovalbumin.
NP: Nanoparticle; PEG: Polyethylene glycol.

reach the bronchus-associated lymphoid tissue. Consequently, the nasal route is considered as a very efficient way of immunisation. However, as in the case of the oral route, the surface properties of the colloidal particles can modulate the parameters that affect their interaction and transport through nasal mucosa. In this view, Tobio *et al.* [92] have explored the ability of PEG-coated PLA-NPs as protein carriers for nasal administration. Radiolabelled PEG-PLA and PLA-NPs loaded with tetanus toxoid antigen were administered intranasally to rats and radioactivity recovered in the blood and some tissues (lymph nodes, spleen, liver, lung and small bowel) was determined. The percentage of the radioactivity recovered in the blood 1 h after administration of PEG-PLA particles was found to be 10-fold higher than that observed for PLA-NPs. The authors pointed out that the higher blood-associated radioactivity was an indication for better absorption of the pegylated NPs through nasal mucosa. In another report of the same group, the antitetanus IgG levels elicited by tetanus toxoid loaded in PLA-PEG-NPs were significantly higher than those corresponding to the fluid vaccine [78]. Furthermore, this potential of pegylated NPs was also demonstrated for a model DNA nasal vaccine. In this work, a single nasal dose of DNA loaded in pegylated NPs led to a significant antibody response to the encoded protein [32]. This potential of PEG-PLA-NPs to cross the nasal epithelium has been related to a small size of NPs and a high PEG coating density [32,77].

When comparing pegylated NPs with chitosan-coated NPs, it was observed that PEG-coated NPs were more efficient than chitosan-coated particles in facilitating the transport of the associated antigen (tetanus toxoid) through the mucosa. The explanation was found in the different interaction mechanisms of both types of modified NPs with the nasal epithelium. In particular, PEG-coated NPs did not interact with the mucin, whereas those coated with chitosan were supposed to stick to the mucus layer [74,96]. Similar to the results discussed previously, it appears that the strong interaction with the mucus components decreases the diffusion ability of the carriers, and their proximal contact with the certain mucosal surface is hindered.

4. Conclusion

Pegylation can be an alternative strategy aiming to modify the bioadhesive properties of NPs, and opening new opportunities for an improvement of the efficacy and security of the therapy. In the lumen of any mucosal tract, pegylation would prevent enzymatic attack and interaction with food or mucus components, thereby improving the stability of NPs. It appears that PEG-coated NPs promote the contact with the epithelium and the absorption of NPs by a transcellular route. However, the nature of the coating can greatly affect the behaviour of NPs within the mucosa. Concerning the bioadhesive phenomenon, NPs with a 'brush' conformation (PEG-NP) appear to develop more intense adhesive interactions with the mucosa than conventional NPs. This fact can be due to the ability of these NPs to cross the mucus layer and to be taken up by enterocytes. On the other hand, NPs with a 'loop' conformation (DAP-PEG-NP) displayed the longest residence of the adhered fraction in the mucosa.

5. Expert opinion

Mucosal delivery is attractive due to ease of administration and patient convenience and compliance, thereby reducing

overall healthcare costs. In terms of mucosal immunisation via peptide, protein or plasmid DNA, the introduction of an efficient vaccine would diminish costs, patient discomfort and the need for trained personnel to administer the vaccine, which is particularly relevant in developing countries where mass vaccination is still the norm for common and emerging diseases.

The coating of NPs with polyethylene glycol may be a useful strategy to design new transmucosal devices. The mucus gel is the first obstacle encountered by any particle, or inert or live cell, to get access to the underlying microvillus surface of mucosal cells. Overall, PEG coating provides a steric barrier, which minimises the adsorption of enzymes, soluble mucins and other mucus compounds, thereby improving the stability of NPs against enzymatic attack and interaction with lumen components. As a result, PEG on NPs would prevent the physical aggregation of NPs on contact with the mucosa, thus facilitating their transport across the mucus layer.

The mucosal epitheliums (nasal, oral or ocular) represent a continuous physical barrier formed by the tightly bound cells. Within the gut, these cells can be identified morphologically by the presence of microvilli covered by membrane-associated mucins forming a glycocalyx. Thus, the villous epithelium is specialised for digestion and absorption of nutrients. In contrast, in regions of epithelium overlying Peyer's patches (lymphoid follicles) M cells are situated, which seem to be designed to promote adherence and the uptake of foreign macromolecules and particles, including microorganisms. However, although M cells account for the larger bulk of particles crossing the intestinal epithelium [51-54], alternative routes have been described, as will be discussed in this section.

The ability of PEG-NPs to avoid aggregation in the lumen and to cross the mucus layer may promote their adhesive interaction with the surface of epithelial cells and, thus, facilitate their absorption. Once in the epithelial cells, NPs are transported via blood or lymphatic vessels and accumulate in the spleen or lymph nodes. However, a direct interaction of pegylated NPs with antigen-presenting cells in the mucosa cannot be discarded. Thus, it has been shown that the release of proinflammatory cytokines and chemokines induce the migration of polymorphonuclear leucocytes [97] and/or dendritic cells [98] that can project themselves into the lumen where they can directly interact with the antigen.

In any case, pegylated NPs appear to be good transmucosal carriers with a high potential to design new adjuvants for vaccination or immunotherapy purposes.

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Patents

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