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# Quantification of the bioadhesive properties of protein-coated PVM/MA nanoparticles

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**Abstract**

This work describes the bioadhesive properties of poly(methyl vinyl ether-co-maleic anhydride) (PVM/MA) nanoparticles fluorescently-labelled with rhodamine B isothiocyanate, and coated with either *Sambucus nigra* lectin (SNA-NP) or bovine serum albumin (BSA-NP). The different formulations (10 mg) were administered to animals by the oral route and the fraction of adhered particles to the mucosa was estimated by measuring the fluorescent marker after the digestion of the tissue. Plotting the amount of adhered particles in the whole gut versus time enabled us to determine the affinity of the formulation for the biological support (expressed as $Q_{max}$), the intensity and relative duration of the bioadhesive phenomenon ($AUC_{adh}$ and $MRT_{adh}$, respectively), and the elimination rate of the adhered particles ($k_{adh}$). SNA-NP displayed a similar adhesive affinity and adhesive intensity for the gut mucosa than the control particles; although, its maximum of interaction with the mucosa was observed 1 h post-administration, whereas control and BSA-NP took place only 30 min post-administration. On the other hand, the coating of nanoparticles with SNA significantly reduced the $k_{adh}$ ($P < 0.01$) and, thus, $MRT_{adh}$ was 35 min longer for the lectin-conjugate than for the control. BSA-NP displayed a highest initial affinity for the gut mucosa and $AUC_{adh}$ was calculated to be 1.5 fold higher than for the control or SNA-NP. However, BSA-NP were eliminated more rapidly from the mucosa than SNA-NP and, thus, the $MRT_{adh}$ was only 27 min longer than control. In summary, the parameters describing the bioadhesive profile of a given formulation may be useful to quantify the potential of colloidal particulates to interact with a mucosa and to evaluate the influence of different ligands on the bioadhesive properties of the resulting drug carriers. 

*Keywords:* Nanoparticles; Poly(methyl vinyl ether-co-maleic anhydride); Bioadhesion; Conjugates; *Sambucus nigra* lectin; Bovine serum albumin

## 1. Introduction

The oral route is one of the preferred ways for drug delivery. However, a large amount of drugs remain poorly available when administered by this route. Among other reasons, this fact can be

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related to (i) a too short gastric residence time of the pharmaceutical dosage form, (ii) a low permeability and/or solubility of the drug within the gastro-intestinal tract, (iii) or a lack of stability within the gut.

One possibility to enhance drug absorption may be the use of biodegradable nanoparticulate systems with bioadhesive properties. Within the gut, these pharmaceutical dosage forms have been proposed to target particular regions, such as the stomach (Arangoa et al., 2001) and colon (Lamprecht et al., 2001), and increase the drug absorption. Drugs whose oral bioavailability was improved by their association to nanoparticles include vincamine (Maincent et al., 1986), dicumarol (Chickering et al., 1997), furosemide (Akiyama et al., 1998), salmon calcitonin (Sakuma et al., 1999), avarol (Beck et al., 1994) and azidodeoxythymidine (Dembri et al., 2001).

The immobilisation of the particles at the intestinal surface is the basis to improve the drug absorption and may be obtained by the development of either non-specific or specific bioadhesive interactions. In the former, the intensity and the fraction of particles interacting with the biological mucosa appear to be mainly dependent on the physico-chemical properties of the colloidal drug delivery system. Among other characteristics, the influence of the size, density, and modifications in the surface of the resulting carriers on the transit and adhesion within the gastrointestinal have been deeply evaluated. Tuleu et al. demonstrated that high-density pellets displayed a higher delay in the gut transit, compared with low-density particulates, related to a significant decrease of the gastric emptying rates (Tuleu et al., 1999). Similarly, a small particle size may dramatically prolong the residence time of the pharmaceutical dosage form in the gastrointestinal tract, due to an important decrease on the influence of the intestinal clearance mechanisms and the high increase on the surface able to interact with the biological support (Desai et al., 1996; Duchêne and Ponchel, 1997). Another attempt to modify the non-specific bioadhesive profile of nanoparticles may be their coating with different macromolecules and polymers. In this context, the coating of poly(methacrylate) nanoparticles with non-ionic surfactants (i.e. polysorbate 80 and poloxamine 908) significantly increased the mucoadhesive capacity of these carriers within the gut (Araujo et al., 1999). Similarly, a prolongation on the gastrointestinal transit time was found when poly(acrylic acid) microspheres were coated with polyglycerol esters of fatty acids (Akiyama et al., 1995).

In the latter, a biomimetic approach based on the covalent binding of lectins to the surface of colloidal carriers has been proposed to specifically recognised receptors located within the gastrointestinal tract. Lectins are proteins able to provide specific binding to biological surfaces bearing sugar residues located at the surface of epithelial cells (Goldstein et al., 1980). In the literature, very few works described the preparation and evaluation of biodegradable lectin conjugates. Among them, the conjugates obtained from tomato lectin (TL) coupled to particles have been more deeply studied (Lehr et al., 1992; Irache et al., 1996; Florence, 1997; Hussain et al., 1997; Montisci et al., 2001).

Recently, the copolymer between methyl vinyl ether and maleic anhydride has been proposed as a new material to prepare bioadhesive nanoparticles for oral drug delivery (Arbos et al., 2002). This copolymer (Gantrez™ AN from ISP, USA), which is widely employed in pharmacy and cosmetics, may be used to easily prepare lectin conjugates by simple incubation and without the need of chemical reagents.

The aim of this work was to investigate the bioadhesive behaviour of nanoparticles from poly(-methyl vinyl ether-co-maleic anhydride) (PVM/MA) coated with different ligands after their oral administration to laboratory animals. For this purpose, two different formulations were prepared: PVM/MA nanoparticles coated with bovine serum albumin (BSA) and a lectin-conjugate obtained by the covalent linkage of *Sambucus nigra* agglutinin (SNA) to the surface of PVM/MA nanoparticles.

## 2. Materials and methods

### 2.1. Chemicals

PVM/MA Gantrez® AN 119 (MW 200,000) was kindly gifted by ISP (Barcelona, Spain). Rho-

damine isothiocyanate (RBITC), 1,3-diaminopropane (DP) and BSA were supplied by SIGMA (St. Louis, USA). *S. nigra* agglutinin (SNA) was purchased from Vector Laboratories (Burlingame, USA). All other chemicals used were of reagent grade and obtained from Merck (Darmstadt, Germany).

### 2.2. Preparation of nanoparticles and ligand conjugates

PVM/MA nanoparticles were prepared by a solvent displacement method (Arbos et al., 2002). In brief, 100 mg PVM/MA copolymer was dissolved in 5 ml acetone and poured into 10 ml of an ethanol:water phase (1:1 by volume) under magnetic stirring. The organic solvents were eliminated under reduced pressure (Büchi R-144, Switzerland) and the freshly prepared PVM/MA carriers were incubated with 1.25 mg RBITC for 5 min at room temperature.

For the preparation of BSA-NP, the resulting fluorescently labelled nanoparticles were incubated with 3 mg BSA per mg bulk polymer at room temperature for 2 h (BSA-NP). On the other hand, lectin-PVM/MA nanoparticle conjugates were obtained by incubating nanoparticles with 0.3 μg DP per mg polymer for 5 min and, then, with 40 μg SNA per mg polymer (SNA-NP) for 1 h. Nanoparticles only treated with 0.3 μg DP per mg bulk polymer (D30-NP) were used as control.

The resulting nanoparticles and conjugates were purified by centrifugation at 17 000 rpm for 15 min (Rotor 3336, Biofuge Heraeus, Hanau, Germany). The supernatants were removed and the pellets resuspended in water and the resulting suspension centrifuged again at the same conditions as described above. Then, they were lyophilised in a Genesis 12EL apparatus (Virtis, USA) using sucrose (5%) as cryoprotector.

### 2.3. Characterisation of PVM/MA formulation

The particle size and the zeta potential of nanoparticles were determined by photon correlation spectroscopy (PCS) and electrophoretic laser doppler anemometry, respectively, using a Zetamaster analyser system (Malvern Instruments, UK). Samples were always diluted with 0.05 mM phosphate buffered saline (PBS, pH 7.4) and measured at 25 °C with a scattering angle of 90°.

The amount of PVM/MA transformed into nanoparticles was determined by gravimetry from freeze-dried samples. The yield was calculated from the difference between the initial amount of the polymer used to prepare nanoparticles and the weight of the freeze-dried samples.

The quantification of the amount of SNA bound to nanoparticles was performed by high performance liquid chromatography (HPLC) (Arbos et al., 2002). The system consisted of an isocratic solvent delivery pump (Hewlett Packard, HP 1050 quaternary pump), with a HP 1050 diode-array detector set at 280 nm. A Tosho-Haas (Barcelona, Spain) TSK-Gel QC-PAK GFC 300 column (5 μm, $7.8 \times 15$ mm I.D.) was used at room temperature. The mobile phase was 0.05 M sodium phosphate buffer pH 6.8 containing 0.05 M sodium sulphate and 0.5% sodium azide. The flow-rate was 0.6 ml/min and the analysis ran for 10 min. Aliquots of the clear supernatants obtained from centrifugation during the removal of the unbound lectin were taken and 100 μl injected. The quantity of SNA bound to the particles was calculated as the difference between the initial amount added and the quantity of ligand determined in the supernatants.

The amount of loaded RBITC in the carriers was estimated by spectrofluorimetry (Perkin Elmer, USA) at $\lambda_{ex}$: 554 nm and $\lambda_{em}$: 575 nm), as the difference between the initially marker added and the RBITC content determined in the supernatants obtained during the purification step.

Finally, the amount of albumin coating PVM/MA nanoparticles was calculated by the difference between the total amount of BSA used to prepare the batch and the amount of protein quantified in the aqueous supernatants, and determined using the microbichinchoninic acid (Mi-

croBCA) protein assay (Pierce®, Rockford, USA). Calibration curves were made in the supernatants of blank nanoparticles. Each sample was assayed in triplicate and results were expressed as the amount of albumin per mg nanoparticle.

### 2.4. Bioadhesive profile studies

Animal protocols (026/99) were performed in compliance with the regulations of the responsible committee of the University of Navarra in line with the European legislation on animal experiments (86/609/EU).

Male Wistar rats, average weight 225 g (CIFA, Universidad de Navarra, Pamplona, Spain), were housed under normal conditions with free access to food and water. The animals were placed in metabolic cages and fasted overnight to prevent coprophagia but allowing for free access to water.

About 1 ml suspensions of the different formulations, containing 10 mg nanoparticles (around 45 mg particles per kg body weight), was administered to rats. At different times, animals were sacrificed, the entire GI tract removed and cut in six segments: stomach, four portions of small intestine and caecum. These segments were opened lengthwise along the mesentery and rinsed gently with saline. Each rinsed mucosa segments was cut into portions of 2 cm length and treated with 1 ml NaOH 3 M for 24 h. RBITC was extracted with 2 ml methanol, vortexed for 1 min and centrifuged at 4000 rpm for 10 min. Aliquots (1 ml) of the obtained supernatants were diluted with water (3 ml) and assayed for RBITC content by spectrofluorimetry to estimate the fraction of adhered particles to the mucosa.

The developed method was fully validated. The parameters evaluated were linearity, sensitivity and accuracy. In addition, standard curves were prepared daily in biological samples.

### 2.5. Quantification of the bioadhesive phenomenon

The total adhered fraction of the different PVM/MA formulations in the whole gastrointestinal tract was plotted versus time. From these curves, describing the bioadhesive profile of the different formulations tested, the adhesive interactions between the carriers and the mucosa were quantified by means of the following parameters: $Q_{max}$, $AUC_{adh}$, $k_{adh}$ and $MRT_{adh}$.

$Q_{max}$ (expressed in mg) was defined as the maximal amount of nanoparticles adhered to the gut surface. $k_{adh}$ was defined as the terminal elimination rate of the adhered fraction with the gastrointestinal mucosa and calculated using the WINNONLIN 1.5 software. $AUC_{adh}$ or the area under the curve of bioadhesion was evaluated by means of the trapezoidal rule upto $t_z$, which denoted the last sampling point. Finally, $MRT_{adh}$ was the mean residence time of the adhered fraction of nanoparticles in the mucosa and calculated as follows:

$$MRT(h) = \frac{AUMC_{adh}\ (mg\ h)}{AUC_{adh}\ (mg)} \quad (1)$$

where $AUMC_{adh}$ (Area Under the first Moment Curve) is approximated by the trapezoidal rule and extrapolated to infinity. However, $AUC_{adh}$ was accepted if at least 80% of its value was incorporated by $AUC_{adh}(0 - t_z)$.

### 2.6. Statistical methods

The Mann–Whitney $U$-test was performed on the bioadhesion parameters of PVM/MA conjugates to determine statistical significance. $P < 0.01$ was considered to be significant.

## 3. Results and discussion

### 3.1. Characterisation of PVM/MA nanoparticles

PVM/MA nanoparticles were obtained by a solvent displacement method and a subsequent cross-linkage with DP or albumin. In aqueous mediums, the maleic anhydride residues of the polymer backbone may react with water molecules (Mo et al., 1977), resulting in the dissolution of the carriers. Therefore, the treatment of PVM/MA nanoparticles with molecules showing nucleophile residues (i.e. hydroxyl or amine groups) is recommended to minimise the interaction with water and, thus, stabilise and prolong the half-life of the resulting carriers. This reaction

is the basis to link proteins to the surface of PVM/MA nanoparticles; although, a previous cross-linkage of particles with DP reduces the amount of ligand which can be bound to the surface of nanoparticles.

The size, zeta potential, RBITC content and the amount of ligand bound to the surface of PVM/MA nanoparticles are summarised in Table 1. The yield of the process was calculated to be 73.8 ± 2.6%, and the mean size of the resulting carriers was always close to 300 nm. However, the coating of PVM/MA nanoparticles with either SNA or BSA significantly increased the negative zeta potential of control particles.

On the other hand, the RBITC loading in D30-NP and SNA-NP was much lower than in BSA-NP. These results may be explained by the previous cross-linkage of nanoparticles with DP, which would minimise the possibilities for interaction between the isothiocyanate groups of the marker and the anhydride residues on the nanoparticles. Indeed, this reason is also consistent with the relatively low amount of SNA bound to the nanoparticles cross-linked with DP, when compared with BSA-NP that was prepared by the incubation between albumin and non-hardened nanoparticles. Another reason explaining the high capacity of BSA-NP to incorporate RBITC is the fact that isothiocyanate groups may strongly interact with proteins in aqueous mediums (Schreiber and Haimovich, 1983).

### 3.2. Validation of the RBITC analytical method in mucosa segments

The first step was to validate the analytical method and, under the experimental conditions described here, no quenching phenomena were found. Linearity was determined by plotting the fluorescence data versus the particle concentration. The curves were linear on 3 different days over the range of 25–500 μg particle per $cm^2$ gut segment. In addition, linear regression displayed coefficients greater than 0.99. The sensitivity of the curves was around 25 μg particles per $cm^2$ gut segment. The intra-day accuracy studies were within the acceptable limits ( < 15%).

### 3.3. Bioadhesive profile of PVM/MA nanoparticles in the GI tract

Fig. 1 shows the bioadhesive profiles of the three formulations tested by representing the fraction of nanoparticles adhered to the different gastrointestinal segments (stomach; small intestine: I1–I4; caecum) at 30 min, 1, 3 and 8 h post-administration.

For the three formulations tested, the mucosa portions where the nanoparticles appeared to interact in a high extent were the stomach and the intermediate sections of the small intestine (represented by I2 and I3 in Fig. 1). Within the stomach, the adhered fraction of nanoparticles remained constant for at least 1 h, and represented around 17 and 10% of the given dose for BSA-NP and SNA-NP, respectively. The high tropism of BSA-NP for the stomach mucosa can be related to the fact that albumin may strongly interact with mucins, especially under acidic conditions (Hassan and Gallo, 1990).

On the other hand, the adhesive interactions developed by SNA conjugates within the stomach are consistent with the reported specificity of this lectin for glycosyl residues of sialomucin located within the stomach (Kodaira et al., 2000). However, contrary to BSA-NP and D30-NP, the max-

Table 1
Physico-chemical characteristics of PVM/MA nanoparticles ($n = 6$)

| | Size (nm) | Zeta potential[a] (mV) | RBITC content (μg/mg) | Ligand bound (μg/mg) |
|---|---|---|---|---|
| D30-NP | 307 ± 9 | −28.8 ± 1.8 | 3.60 ± 0.03 | – |
| SNA-NP | 300 ± 6 | −31.0 ± 1.0 | 3.75 ± 0.19 | 45.0 ± 2.50 |
| BSA-NP | 315 ± 7 | −40.7 ± 0.5 | 13.77 ± 0.10 | 336.5 ± 53.0 |

[a] Determined in buffer phosphate (pH 7.4; 0.05 M).

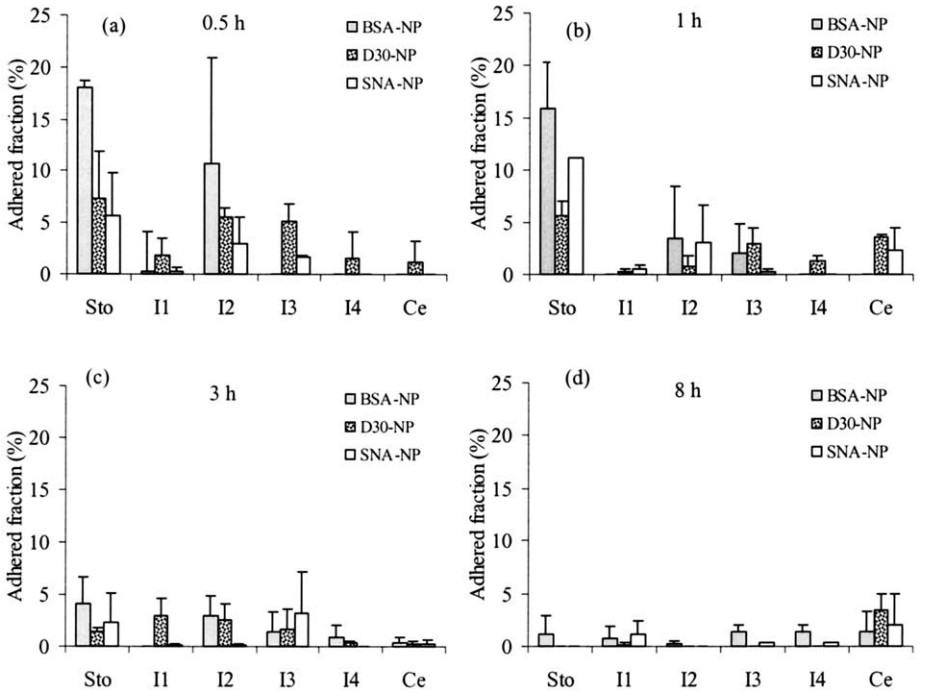


Fig. 1. Adhesion of PVM/MA nanoparticle formulations (D30-NP, SNA-NP and BSA-NP) in the gastrointestinal mucosa after the oral administration of 1 ml aqueous dispersion containing 10 mg nanoparticles. Data are expressed as the percentage of the given dose and each value represents the mean of four experiments. (a) 0.5 h, (b) 1 h, (c) 3 h and (d) 8 h post-administration. Sto, stomach; I1, I2, I3, I4, intestinal portions; Ce, caecum.

imum of adhesion of SNA conjugates with the stomach mucosa was found 1 h post-administration. This fact is in consistence with Irache et al., who also observed that the maximum of interaction between several lectin conjugates and mucins required around 70 min of incubation. This delay has also been suggested in different studies concerning cell cultures (Ertl et al., 2000) and everted gut sacs (Carreno-Gomez et al., 1999).

Anyway, 3 h post-administration, the fraction of nanoparticles adhered to the first portions of the gut dramatically decreased and the remaining carriers tended to accumulated in the gut distal regions.

### 3.4. Bioadhesive properties of particulates

Fig. 2 shows the evolution of the cumulative adhered fraction (expressed in mg) of the different formulations tested on the whole gastrointestinal tract along the time.

From these curves it was clear that the bioadhesive profile developed by SNA-NP within the gastrointestinal tract was different than that ob-

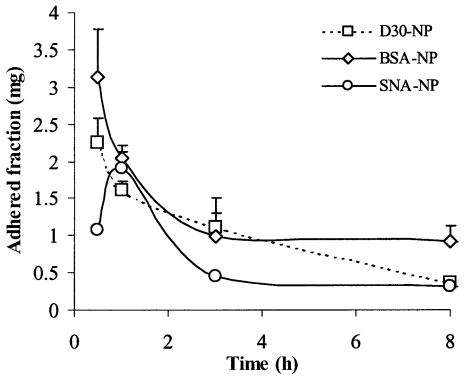


Fig. 2. Evolution of the adhered fraction of nanoparticles in the whole gastrointestinal tract with the time, for the three formulations tested, after single oral administration of 10 mg of particles. Data are expressed as the mean ± S.D. ($n = 4$).

Table 2
Bioadhesion parameters for the different formulations tested

| | $AUC_{adh}$ (mg h) | MRT (h) | $k_{adh}$ (per h) | $Q_{max}$ (mg) |
|---|---|---|---|---|
| D30-NP | 5.58 | 4.58 | $0.88 \pm 0.24$ | $2.25 \pm 0.06$ |
| SNA-NP | 5.55 | 5.16 | $0.56^{**} \pm 0.07$ | $2.06 \pm 0.24$ |
| BSA-NP | 8.25 | 5.03 | $0.72 \pm 0.21$ | $3.14 \pm 0.94$ |

**, $P < 0.01$ vs. D30-NP.

served for either BSA-NP or D30-NP. While for these two formulations, the maximum of adhesion appeared only 30 min post-administration, for the SNA-nanoparticle conjugates the maximal adhesivity within the gut occurred 1 h post-administration. As described before, this fact may be due to a much more rapid process to develop non-specific or physico-chemical interactions (based on weak bonds) than specific-receptor mediated interactions.

These curves, describing the bioadhesive profile of the different formulations tested in the whole gastrointestinal tract, can be analysed to estimate some parameters able to quantify the intensity, extent and duration of the interactions between the drug delivery system and the mucosa. These parameters are: $Q_{max}$, $AUC_{adh}$, $MRT_{adh}$ and $k_{adh}$. In principle, $Q_{max}$ can be related with the capacity or affinity of the material to develop bioadhesive interactions with the biological support, while $AUC_{adh}$ would describe the intensity of the adhesive phenomenon and $k_{adh}$ represent the rate of elimination of the adhered fraction to the mucosa. Finally, $MRT_{adh}$ would be able to estimate the relative duration of the adhesive interactions.

The examination in detail of these parameters illustrated the influence of the nature of the ligand in the bioadhesive properties of PVM/MA nanoparticles (see Table 2). From these data, it was clear that both the capacity to adhere and the intensity of the interactions between SNA-NP and the gastrointestinal mucosa were of the same order than the control formulation (D30-NP). However, control particles were eliminated from the mucosa more rapidly than SNA conjugates. In fact, $k_{adh}$ was significantly higher for D30-NP than for SNA conjugates ($P < 0.01$) and, therefore, the relative duration or residence time of the adhered fraction of SNA-coated particles in the mucosa was 35 min longer than for control nanoparticles. For BSA-NP nanoparticles, the intensity of the adhesive phenomenon within the gut was found to be around 1.5 fold higher than for D30-NP or SNA-NP. This fact may be related to the highest initial capacity of BSA-nanoparticle conjugate to interact with the mucosa. Nevertheless, like BSA-NP were removed more rapidly from the mucosa than SNA-NP, the $MRT_{adh}$ was only 27 min longer than for control particles.

## 4. Conclusion

The analysis of the adhesion curves (cumulative amount of adhered particles in the whole gut vs. time) permits to quantify (i) the affinity of the material for the biological support ($Q_{max}$), (ii) the intensity of the bioadhesive phenomena ($AUC_{adh}$), (iii) the relative duration of the adhesive bonds ($MRT_{adh}$) and (iv) the elimination rate of the adhered particles ($k_{adh}$). All of these parameters may be useful to both estimate the bioadhesive potential of a given drug delivery system and make easy comparisons between different formulations.

The modification of the surface properties deeply modified the adhesive properties of PVM/MA nanoparticles. Concerning the formulations tested here, the covalent binding of SNA to the surface of PVM/MA nanoparticles permitted to increase the residence time of the carriers to the gut mucosa, mainly due to a significant decrease of $k_{adh}$, inspite of the maximal adhesivity took more time than for the control. On the contrary, BSA-coated nanoparticles displayed a high capacity to interact with the mucosa, specially in the stomach and, therefore, the intensity of the bioadhesive phenomena was significantly higher than for SNA-NP or D30-NP formulations.

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