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RESEARCH PAPER

Toxicity Studies of Poly(Anhydride) Nanoparticles as Carriers for Oral Drug Delivery

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ABSTRACT

Purpose To evaluate the acute and subacute toxicity of poly (anhydride) nanoparticles as carriers for oral drug/antigen delivery.

Methods Three types of poly(anhydride) nanoparticles were assayed: conventional (NP), nanoparticles containing 2-hydroxypropyl-β-cyclodextrin (NP-HPCD) and nanoparticles coated with poly(ethylene glycol) 6000 (PEG-NP). Nanoparticles were prepared by a desolvation method and characterized in terms of size, zeta potential and morphology. For *in vivo* oral studies, acute and sub-acute toxicity studies were performed in rats in accordance to the OECD 425 and 407 guidelines respectively. Finally, biodistribution studies were carried out after radiolabelling nanoparticles with ^{99m}technetium.

Results Nanoparticle formulations displayed a homogeneous size of about 180 nm and a negative zeta potential. The LD_{50} for all the nanoparticles tested was established to be higher than 2000 mg/kg bw. In the sub-chronic oral toxicity studies at two different doses (30 and 300 mg/kg bw), no evident signs of toxicity were found. Lastly, biodistribution studies demonstrated that these carriers remained in the gut with no evidences of particle translocation or distribution to other organs.

Conclusions Poly(anhydride) nanoparticles (either conventional or modified with HPCD or PEG6000) showed no toxic effects, indicating that these carriers might be a safe strategy for oral delivery of therapeutics.

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ABBREVIATIONS

%ID/g	percentage of injected dose per gram
^{99m}Tc	technetium-99m
ALT	alanine transaminase
AST	aspartate transaminase
Bw	body weight
CT	computed tomography
Hb	hemoglobin
HCT	hematocrit
HPCD	2-hydroxipropyl-β-cyclodextrin
ITLC	instant thin layer chromatography
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
NP	conventional poly(anhydride) nanoparticles
NP-HPCD	nanoparticles containing 2-hydroxypropyl-β-cyclodextrin
NP-PEG	pegylated poly(anhydride) nanoparticles
PEG	poly(ethylene glycol) 6000
PLT	platelet count
PVM/MA	copolymer of methyl vinyl ether and maleic anhydride
RBC	red blood corpuscles count
SPECT-CT	single-photon emission computed tomography
WBC	white blood corpuscles count

INTRODUCTION

In the last decades, the development of nanoparticles for medical and pharmaceutical applications has received a great interest (1–3). Among other, polymeric nanoparticles offer interesting advantages for drug delivery purposes (4–6). As pharmaceutical dosage forms, polymeric nanoparticles

may protect the loaded therapeutic agent against extreme pH conditions and/or enzymatic degradation. Their surface modification with ligands permits to drive their distribution *in vivo* and, thus, improve their targeting properties for a specific tissue or groups of cells within the body (5,7). This functionalization of nanoparticles would be useful to promote the arrival of the encapsulated drug to its ideal site for action or absorption and, thus, reach a particular target inside the cell or improve its bioavailability (8). Last but not least, it is also important to remember the capability of polymeric nanoparticles to control the release of the loaded drug (5).

Although numerous efforts have been carried out to exploit desirable properties of polymeric nanoparticles, attempts to evaluate potentially undesirable effects are limited in comparison (9). The same properties as its small size and large surface area, which make nanoparticles so attractive for medical intentions, may provoke undesirable tissue accumulation and subsequent long-term toxicity (10). Therefore there is a pressing need for careful consideration of nanoparticles toxicity. Hence, nanotoxicology as a new science is becoming a trending topic.

Nevertheless, to date, the first challenge that nanotoxicology has to face is the lack of special regulation to deal with potential risks of nanoparticles. In order to solve this problem, different organizations are developing new regulatory initiatives, such as thus from both the European Committee for Standardization (CEN/TC 352) and the International Organization for Standardization (ISO/TC 229), in order to ensure that products derived from nanomedicine are safe without hindering innovation (11) and, thus, support commercialisation and market development (12). In parallel, since 2006, activities related to the development or revisions of test guidelines to assess nanotoxicology have been performing by the Working Party on Manufactured Nanomaterials (WPNM) within the Organisation for European Economic Co-operation (13). In this way, WPMN has recommended the guidelines 425 and 407 for the evaluation of oral acute and sub-acute toxicity of nanomaterials, respectively (14).

In the last years, promising poly(anhydride) based nanoparticles have been developed from the copolymer of methyl vinyl ether and maleic anhydride (PVM/MA). This polymer shows a great potential for oral drug/antigen delivery due to its well-studied bioadhesive properties when formulated as nanoparticles (15–17). This capability to establish bioadhesive interactions can be modulated by the modification of poly (anhydride) nanoparticles with different compounds such as poly(ethylene glycol)s or cyclodextrins (17,18). In this regard, a study conducted by Yoncheva and collaborators demonstrated that pegylated nanoparticles possessed high affinity to adhere to the small intestine compared to conventional nanoparticles (19) and these nanoparticles may be suitable carriers for DNA (20). In another study it was corroborated the synergistic effect of the combination between bioadhesive nanoparticles and cyclodextrins on the oral bioavailability of paclitaxel and other drugs ascribed to the groups II and IV of the biopharmaceutical classification system (21,22).

The aim of this study was to evaluate the toxicological profile of different types of poly(anhydride) nanoparticles: conventional (NP), nanoparticles containing 2-hydroxypropyl-β-cyclodextrin (NP-HPCD) and nanoparticles coated with poly (ethylene glycol) 6000 (PEG-NP). More particularly, in this work we report their safety through acute and sub-acute toxicity studies. Additionally, *in vivo* biodistribution of these nanoparticles after oral administration was also investigated.

MATERIALS AND METHODS

Chemicals

Poly(methyl vinyl ether-co-maleic anhydride) or poly(anhydride) (Gantrez® AN 119; Mw 200,000) was provided by ISPcorp. (Waarwijk, The Netherlands). Poly(ethylene glycol) 6000 (PEG) was provided by Fluka (Switzerland). 2-hydroxypropyl-β-cyclodextrin (HPCD) was provided by Sigma–Aldrich (Steinheim, Germany). Acetone was obtained from VWR Prolabo (Fontenay-sous-Bois, France). Deionized water (18.2MΩ resistivity) was prepared by a water purification system (Wasserlab, Pamplona, Spain). ^{99}Mo-^{99m}Tc generator (Drytec; GE Healthcare Bio-science, UK) was eluted with 0.9% NaCl following the manufacturer's instructions. $SnCl_2{\cdot}2H_2O$ and HCl were from Panreac (Barcelona, Spain); 0.9% NaCl was purchased from Braun (Barcelona, Spain) and NaOH from Fluka (Switzerland). The anaesthetic isoflurane (Isoflo™) was from Esteve, (Barcelona, Spain) and the euthanasic T-61 from Intervet (Madrid, Spain). All other chemicals and solvents used were of analytical grade.

Preparation of Poly(Anhydride) Nanoparticles

Poly(anhydride) nanoparticles were prepared from a dissolution of Gantrez® AN 119 in acetone by a simple desolvation method previously described. The resulting suspensions were always dried in a Mini Spray-dryer Büchi B290 (Büchi Labortechnik AG, Switzerland) as described previously (23). Three different types of nanoparticles were evaluated: conventional nanoparticles (NP), nanoparticles containing HPCD (NP-HPCD) and pegylated nanoparticles with PEG 6000 (PEG-NP).

Conventional Poly(Anhydride) Nanoparticles (NP)

Briefly, 500 mg of the copolymer of methyl vinyl ether and maleic anhydride (Gantrez® AN 119) were dissolved and stirred in 30 ml acetone. Then, the desolvation of the

polymer was induced by the addition of 15 ml purified water under magnetic stirring to the organic phase. In parallel, 1 g of lactose was dissolved in 10 ml purified water and added to the nanoparticle suspension under agitation for 5 min at room temperature. Finally, the suspension was dried in the Mini Spray-dryer Büchi B290. The recovered powder was stored in closed vials at room temperature.

Poly(Anhydride) Nanoparticles Containing 2-Hydroxypropyl-β-cyclodextrin (NP-HPCD)

Briefly, 500 mg of the copolymer of methyl vinyl ether and maleic anhydride were dissolved and stirred in 20 ml acetone. Then, 10 ml acetone containing 125 mg HPCD were added to the polymer solution under magnetic stirring and incubated for 30 min. Nanoparticles were obtained by the addition of 15 ml purified water under magnetic stirring to the organic phase. In parallel, 1 g of lactose was dissolved in 10 ml purified water and added to the nanoparticle suspension under agitation for 5 min at room temperature. Finally, the suspension was dried in the Mini Spray-dryer Büchi B290. The recovered powder was stored in closed vials at room temperature.

Pegylated Poly(Anhydride) Nanoparticles (PEG-NP)

In this case, 62.5 mg PEG were dissolved in 15 ml acetone and mixed with 15 ml acetone containing 500 mg of the copolymer of methyl vinyl ether and maleic anhydride and incubated under magnetic stirring for 1 h. Then, nanoparticles were obtained by the addition of 15 ml purified water under magnetic stirring to the organic phase. The solvents were eliminated under reduced pressure (Büchi R-144, Switzerland) and nanoparticles were purified by double centrifugation at 17,000 rpm for 20 min (Sigma 3K30, Germany). The pellet was resuspended in 25 ml purified water containing 1 g of lactose. Finally, the suspension was dried in the Mini Spray-dryer Büchi B191. The recovered powder was stored in closed vials at room temperature.

Characterization of the Nanoparticles

Size and Zeta Potential

The mean hydrodynamic diameter and the zeta potential of nanoparticles were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zetamaster analyzer system (Malvern Instruments, UK) and a ZetaPlus zeta potential analyzer (Brookhaven Instruments Corp., Holtsville, NY). The diameter of the nanoparticles was determined after dispersion in purified water (1/10) and measured at 25°C by dynamic light scattering angle of 90°C. The zeta potential was determined by diluting the samples in a 0.1 mM KCl solution adjusted to pH 7.4. All measurements were performed in triplicate.

The morphological examination of the nanoparticles was obtained by scanning electron microscopy (SEM) in a Zeiss DSM 940A scanning electron microscope (Oberkochen, Germany) coupled with a digital image system (DISS) Point Electronic GmBh. Previously, a small amount of the spray-dried powders was diluted with purified water and the resulting suspension was centrifuged at 17,000 rpm (Sigma 3K30, Germany) for 20 min in order to eliminate sugars. Finally, the pellet was shaded with a 12 nm gold layer in a Hemitech K 550 Sputter-Coater.

Quantification of HPCD and PEG

The amount of HPCD and PEG associated to the nanoparticles as well as the amount of the polymer transformed into nanoparticles were estimated using two different HPLC methods previously described (24,25). Specifically the apparatus used for the HPLC analysis was a model 1100 series Liquid Chromatography, Agilent (Waldbronn, Germany) coupled with an evaporative light scattering detector, ELSD 2000 Alltech (Illinois, USA). An ELSD nitrogen generator Alltech was used as the source for the nitrogen gas. Data acquisition and analysis were performed with a Hewlett-Packard computer using the ChemStation G2171 AA program.

On one hand, PEG separation was carried out at 40°C on a PL aquagel-OH 30 column (300 mm × 7.5 mm; particle size 8 μm) obtained from Agilent Technologies (GB, United Kingdom). The mobile phase composition was a mixture of methanol (A) and water (B) in a gradient elution at a flow rate of 1 ml/min. On the other hand, HPCD separation was carried out at 50°C on a reversed-phase Zorbax Eclipse XDB-Phenyl column (2.1 mm × 150 mm; particle size 5 μm) obtained from Agilent Technologies (Waldbronn, Germany). This column was protected by a 0.45 μm filter (Teknokroma, Spain). The mobile phase composition was a mixture of acetonitrile (A) and water (B) in a gradient elution at a flow-rate of 0.25 ml/min. In all cases, for ELSD quantifications, the drift tube temperature was set at 115°C, the nitrogen flow was maintained at 3.2 l/min and the gain was fixed to 1.

The amount of HPCD and PEG associated to nanoparticles was calculated as the difference between the initial HPCD and PEG and the amount of those recovered in the supernatants. Similarly, the amount of the poly(anhydride) copolymer was estimated by difference in the same way.

Labelling of Nanoparticles with ^{99m}Tc

Poly(anhydride) nanoparticles were labeled with ^{99m}Tc by reduction with tin chloride (26). Briefly, 1 mCi of freshly

eluted ^{99m}Tc pertechnetate was reduced with 0.03 mg/ml stannous chloride and the pH was adjusted to 4 with 0.1N HCl. Then, nanoparticles (NP, NP-HPCD or PEG-NP) in water were added to the pre-reduced ^{99m}Tc mixture. The suspension was vortexed for 30 s and incubated at room temperature for 10 min. The overall procedure was carried out in helium-purged vials using helium-purged solutions to minimize oxygen content and avoid oxidation of tin chloride. The radiochemical purity was examined by a double-solvent instant thin layer chromatography (ITLC) system using silica gel coated fiber sheets (Polygram® sil N-RH, Macherey-Nagel, Düren, Germany) with methyl ethyl ketone (first solvent) and 18% sodium acetate (second solvent) as mobile phases. After labeling, nanoparticles were mixed with non-labelled ones to adjust the required dose (either 30 mg/kg or 300 mg/kg) and the volume was adjusted to 1 ml.

Animals

The experimental protocols involving animals were carefully reviewed and approved by the Ethical Committee for Animal Experimentation of the University of Navarra (Spain). Eight week old male and female Wistar rats were purchased from Harlan (Horst, The Netherlands) and employed for acute and sub-acute toxicity studies as well as biodistribution studies. On the day of arrival, the animals were weighed in order to assure that body weight (bw) variation did not exceed ±20% (27,28). They were randomly housed in groups in polycarbonate cages with stainless steel covers to allow acclimatization to the environmental conditions (12 h day/night cycle, temperature 22±2°C, relative humidity 55±10%, standard diet "2014 Teklad Global 14% Protein Rodent Maintenance Diet" from Harlan Iberica Spain and water *ad libitum*).

Acute-Toxicity Study

This study was designed according to the OECD guideline 425 for the Testing of Chemicals (27). The procedure of the limit test was applied because no toxicity was expected after a single oral dose: a limit dose of 2000 mg/kg bw was selected. Twenty female Wistar rats were divided into four groups (n=5): Group I (Control), Group II (NP), Group III (NP-HPCD) and Group IV (PEG-NP). A single dose of 2000 mg/kg bw was administered by gavage to each animal in 2 ml/100 g bw of purified water, except for the control group, that received purified water only (vehicle). After the administration each animal was observed for a period of 48 h for signs of toxicity or mortality. If no such signs were observed after 48 h, then the same dose was administered to animal number 2. This procedure was sequentially followed until a total of five animals were administered. The animals were fasted 4 h prior to dosing and 3 h after the nanoparticles were administered. Individual animal weights were registered on day 0 and weekly during 14 days. Animals were observed individually for any clinical signs or any symptoms of toxicity at different time intervals after administration (10 min, 30 min, 1 h, 3 h, 6 h) and daily during 14 days. On completion of the study, on day 14, blood samples were extracted from the retro-orbital sinus under isoflurane anesthesia. The following hematological parameters were analyzed: hemoglobin (Hb; g/dl), hematocrit (HCT, %), red blood corpuscles count (RBC), white blood corpuscles count (WBC), absolute erythrocyte indices and differential WBC. Biochemical analyses of plasma samples were performed with a Hitachi 911™ (Roche Diagnostics) analyzer using the protocols obtained from Roche for the determination of the standard parameters: total protein (g/dl), albumin (g/dl), glucose (mg/dl), aspartate transaminase (AST; U/l), alanine transaminase (ALT; U/l), cholesterol , creatinine, urea (mg/dl) and total bilirrubin.

Thereafter, the animals were sacrificed in CO_2 chamber, subjected to necropsy and various organs were collected and fixed for further histopathological examination.

Sub-acute Toxicity Study

This study was designed according to the OECD guideline 407 for the Testing of Chemicals (28). The dose selected of each nanoparticle type was based on the expected therapeutic dose according to previous studies (22), 30 mg/kg bw, and ten times the reported value, 300 mg/kg bw. Animals were randomly divided into seven groups, containing five male and five female rats per group. Group I served as the control. Groups II and III received NP, 30 mg/kg bw and 300 mg/kg bw, respectively. Groups IV and V were treated with NP-HPCD, 30 mg/kg bw and 300 mg/kg bw, respectively. Finally, Groups VI and VII received PEG-NP, 30 mg/kg bw and 300 mg/kg bw, respectively.

The animals were administered the respective doses for a period of 28 days, once daily, by oral gavage. During this period they were observed for any clinical signs of toxicity, mortality or changes in body weight. Blood samples were collected before the first administration, on day 15, and at the end of the study from the retro-orbital sinus under anesthesia and hematological and biochemical parameters were analyzed (see Acute Toxicity Study). On day 28, animals were sacrificed in CO_2 chamber, subjected to necropsy and various organs were collected, weighed and fixed for further histopathological examination.

Histophatological Studies

Tissue samples from different body organs (including thymus, kidney, liver, pulmonary, spleen, stomach, liver and intestines) were taken during necropsy, fixed in 4% formaldehyde solution, dehydrated and embedded in paraffin. Paraffin sections (3 μm) were cut, mounted onto glass slides, and dewaxed and stained with haematoxylin and eosin (H&E) for the subsequent histopathological examination.

In Vivo and *Ex Vivo* Biodistribution Studies with Radiolabelled Nanoparticles

For this study we only used female rats which were divided in groups of three animals each. The groups were the same as described in the "Sub-acute toxicity study". The radiolabelled dose (1 mCi) was administered on days 1, 15 and 28 of the study. After the administration of nanoparticles, animals were anesthetized with 2% isoflurane and placed in prone position on the gammacamera. The gammagraphic studies were performed in an SPECT–CT (Symbia; Siemens Medical System, USA). A high-resolution low-energy parallel-hole collimator was used. The scan parameters for CT were 130 kV, 30 mA s, 1 mm slices and Flash 3D iterative reconstruction with a Gaussian filter with a full-width at half maximum of 8.4.. For image acquisition the gammacamera was programmed to reach 500.000 events with a static program. The images were acquired 8 h after the administration of the radiolabelled nanoparticles.

For *ex vivo* studies, 24 h after the administration of nanoparticles, animals were euthanized with T-61 (after anesthesia with 2% isoflurane gas). Then blood, urine and different organs (lungs, heart, spleen, pancreas, liver, kidneys, bone, gut and muscle) were collected and the radioactivity of each organ measured in a gamma counter (Compugamma CS, RIA; LKB Pharmacia, Finland) calibrated for ^{99m}Tc energy. For practical reasons, the gastrointestinal tract of animals was divided as follows: stomach, small intestine, caecum and rectum. All of these sections were first washed by careful gentle injection of 0.9% NaCl through the lumen. The washing liquids were recovered, weighed and also measured in the gamma counter. Finally, results were expressed as the percentage of injected dose per gram (%ID/g).

Statistical Analysis

Data were expressed as the mean ± SD of at least three experiments. For the nanoparticles characterization the Student t test was used. Other statistical significance analysis were carried out using the non-parametric Mann–Whitney U test. P values of <0.05 were considered as statistically significant. All calculations were performed using SPSS® statistical software program (SPSS® 15.0, Microsoft, USA).

RESULTS

Nanoparticles Characterization

The main physicochemical characteristics of poly(anhydride) nanoparticles formulations are summarized in Table I. Overall the different nanoparticle formulations exhibited a homogeneous mean size of about 170–190 nm, with a low polydispersity index. Conventional nanoparticles (NP) displayed a mean size slightly smaller than that observed for NP-HPCD and PEG-NP. These observations were confirmed by scanning electron microscopy (Fig. 1). The morphological analysis of the three types of nanoparticles revealed that NP and PEG-NP displayed a round shape while NP-HPCD were characterized by an irregular aspect. In addition, NP-HPCD showed rough surface in comparison with the smooth shape observed for NP.

On the other hand, the incorporation of either HPCD or PEG in the nanoparticles decreased the negative zeta potential of the resulting carriers.

Concerning the yield of the process, in all cases, this parameter was calculated to be very high and close to 100%. Finally, the amount of HPCD associated to nanoparticles was calculated to be about 87 μg/mg. For pegylated nanoparticles, the amount of PEG linked to nanoparticles was 46 μg/mg.

Acute Toxicity Study

Throughout all the observation period, the animals dosed with the nanoparticles displayed neither any sign of toxicity

Table I Data Expressed as the Mean ± SD (n=6). NP: Poly(Anhydride) Nanoparticles; NP-HPCD: Hydroxypropyl-β-cyclodextrin-Poly(Anhydride) Nanoparticles; PEG-NP: Pegylated Nanoparticles. PDI: Polydispersity

	Size (nm)	PDI	Zeta potential (mV)	Yield (%)	HPCD or PEG content μg /mg
NP	170 ± 2	0.15	−45.3 ± 2.5	98 ± 3.1	
NP-HPCD	189 ± 2	0.14	−35.9 ± 3.5	96 ± 4.2	87.7 ± 2.2
PEG-NP	182 ± 2	0.25	−34.6 ± 3.7	97 ± 2.3	45.7 ± 1.9

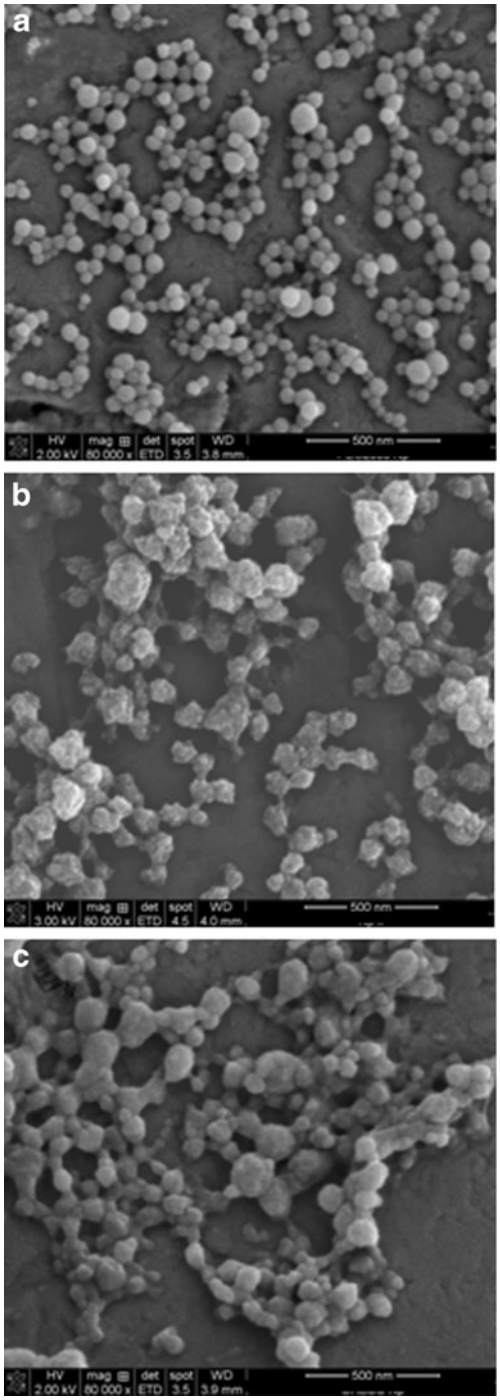

Fig. 1 SEM photographs of nanoparticles: (**a**) NP, (**b**) NP-HPCD, (**c**) PEG-NP.

nor any abnormal behavior. Similarly, in all cases, the hematological and biochemical parameters analyzed showed normal values that did not differ from the control group. Finally, gross and histological pathological examination of the vital organs did not exhibit any evidence of toxicity during the animal necropsies. Thus, the different poly(anhydride) nanoparticles were found to be orally safe at the single limit dose of 2000 mg/kg bw.

Sub-acute Toxicity

During the sub-acute toxicity study no mortality was observed in the different treatment groups. Detailed physical examinations conducted weekly did not demonstrate any unusual change in behavior and no signs of toxicity were observed throughout the study. Thus, long-term administration of the poly(anhydride) nanoparticles had no adverse effects on the general health of animals. No significant differences were observed in body weights of the animals of treatment groups compared with control ones (Fig. 2) All the hematologic and biochemical values were found to be within the normal range with no difference between the control and the treatment groups for both male and female animals (Figs. 3 and 4). The relative organ weight of heart, liver, kidneys, adrenals, thymus, spleen, ovaries and testis were also unaffected by the treatments (data not shown). Regarding histology, the gross and histopathology of various organs revealed that the natural architecture remained normal. No dose related toxicity lesions were observed. Histological findings of a female dosed with the highest dose 300 mg/kg bw are presented in Fig. 5.

In Vivo Biodistribution Studies

In order to evaluate the biodistribution of orally administered nanoparticles, the different types of poly(anhydride) nanoparticles were labelled with ^{99m}Tc. The labelling yield was always over 90%. In this study, the animals (as in the sub-acute toxicity study) received orally every day a dose of nanoparticles (either 30 mg/kg or 300 mg/kg bw) during 28 days On days 1, 15 and 28, the dose of nanoparticles included 1 mCi of technetium labelled carriers. Figure 6 shows the localization of radiolabelled nanoparticles 8-h post administration of the dose corresponding to day 15. The images revealed that nanoparticles were located in the stomach and distal parts of the gastrointestinal tract with no evidences of distribution in other organs or nanoparticle translocation. This distribution was found to be similar for all the formulations and doses tested. Similarly, the distribution patterns of nanoparticles (NP, NP-HPCD and PEG-NP) within the animals were found to be similar on the images captured on days 1, 15 or 28 (data not shown).

Ex Vivo Biodistribution Studies

According to the previously obtained images, the highest accumulation of radioactivity was found in the stomach, small intestine, caecum and large intestine

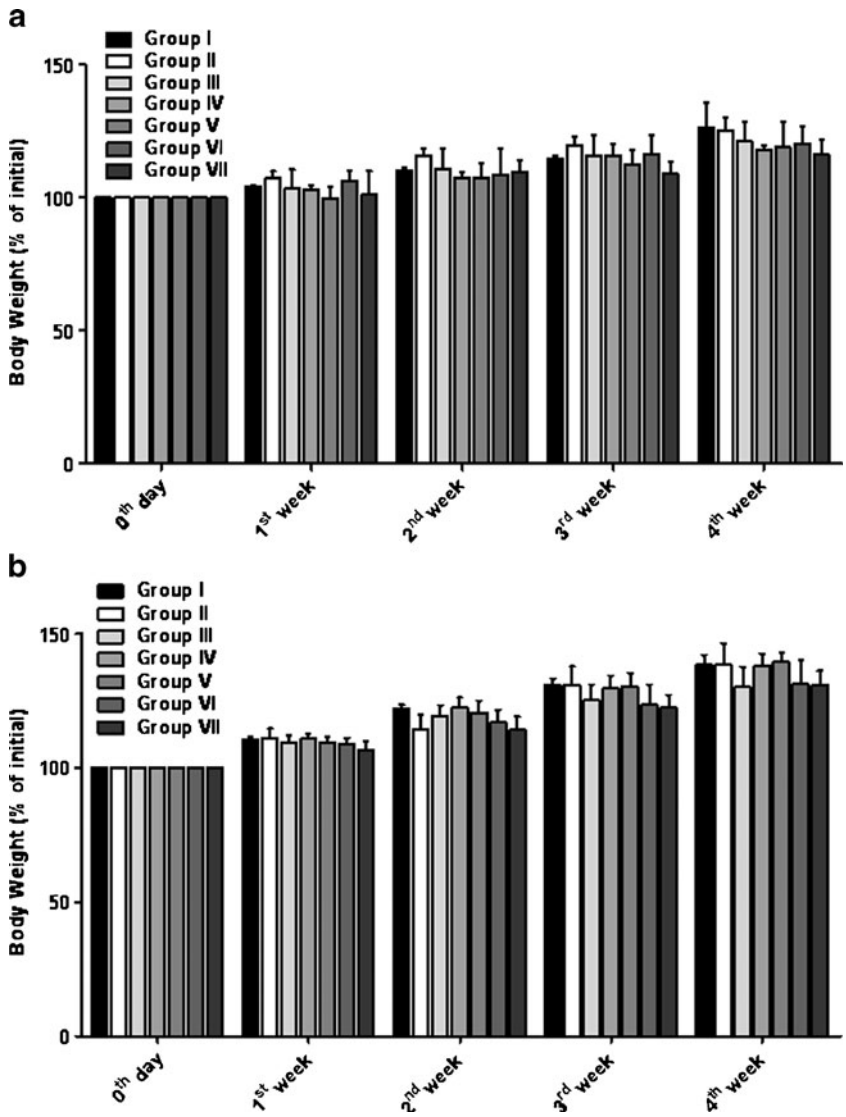

Fig. 2 Body weight registered of female (**a**) and male rats (**b**) through sub-acute toxicity study.

The percentage of injected dose per gram (%ID/g) of radiolabelled nanoparticles (NP, NP-HPCD and PEG-NP) in each one of these organs 24 h after oral administration of the dose corresponding to day 28 is represented in Fig. 7. A relative low concentration of radioactivity was also found in the liver and the kidneys, but this represented less than 0.05% ID/g in all the cases (data not shown).

Comparing the different types of formulations, the radioactivity in the stomach and the large intestine was significantly higher for PEG-NP than for NP and NP-HPCD ($p<0.05$). Similarly, the radioactivity found in the washing liquids of the gastrointestinal tract of animals was found to be significantly higher for animals treated with PEG-NP than with NP or NP-HPCD.

Despite NP and NP-HPCD showed similar behavior, NP-HPCD seemed to pass slower through the stomach and small intestine. In fact, slightly higher radioactivity concentration was found in these organs for NP-HPCD but this difference was not statistically significant.

DISCUSSION

From a general point of view, *in vivo* toxicity studies are more desirable since direct verification of the effect of nanoparticles toward the human body is achieved. Nevertheless, to date, the reported toxicity studies have mainly focused on *in-vitro* examinations rather than in *in-vivo* experiments. This fact can be due to the ease in execution as well as in the control and interpretation of the experiments compared with *in-vivo* tests.

The copolymers of methyl vinyl ether and maleic anhydride and their ether and salt derivatives (Gantrez® series) are widely employed in a diverse range of topical and oral pharmaceutical formulations as thickening and suspending

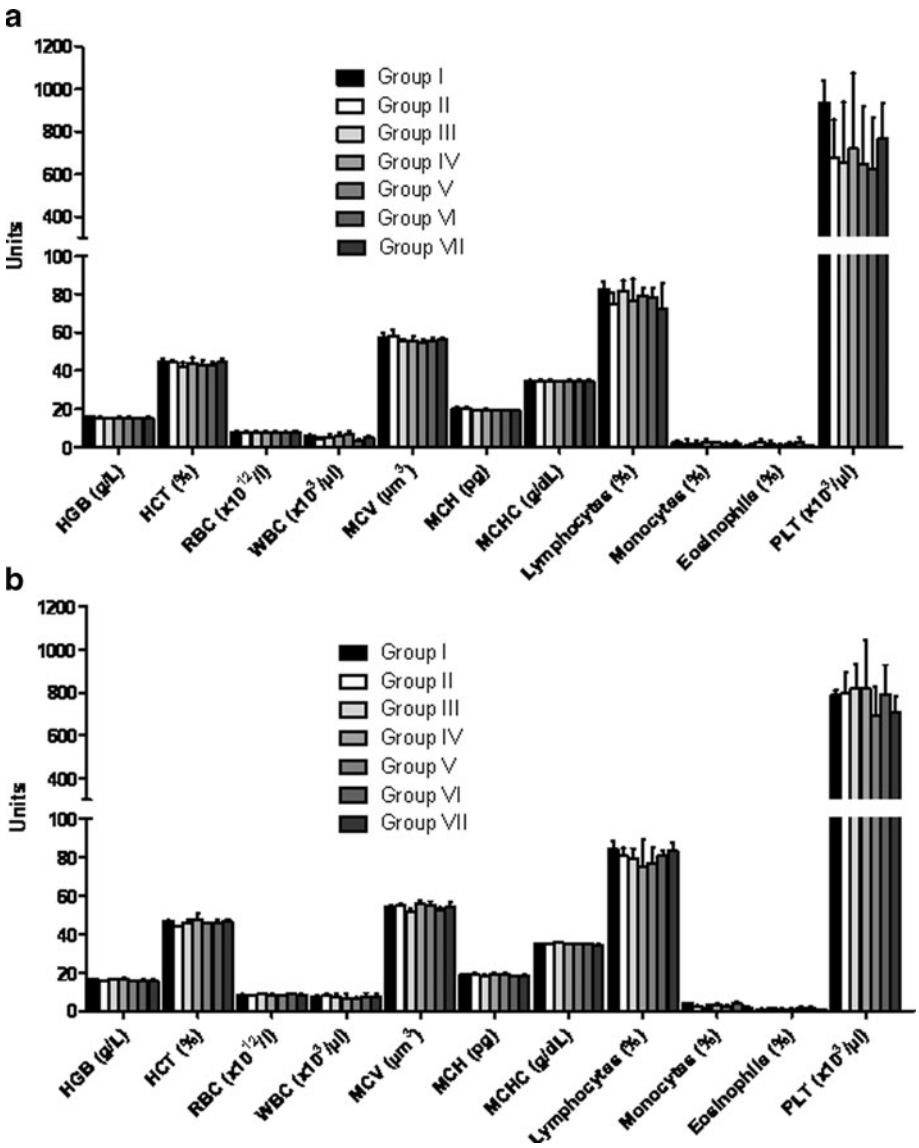

Fig. 3 Hematological parameters in female (**a**) and male rats (**b**) evaluated for sub-acute toxicity.

agents, denture adhesives, film-coating agents and adjuvants for transdermal patches (29,30). In the last years, these copolymers have also been employed to prepare nanoparticles with bioadhesive properties as vehicles for oral drug/antigen delivery (15–17,21–25). These carriers offer a high versatility derived from the presence of anhydride residues which can easily react (under mild conditions) with different ligands and excipients and, thus, yielding "decorated" nanoparticles with improved targeting or controlled release properties. As a consequence these nanoparticles may facilitate the interaction and presentation of the loaded antigen or allergen with the APCs (16) and/or improve the oral bioavailability of different drugs such as fluorouridine (15), paclitaxel (21) or atovaquone (22). In spite of these interesting results, no information about the toxicological profile of these nanoparticles was still known.

Although Gantrez®polymers alone are generally regarded as non-toxic and non-irritant for oral administration (31), their properties may change completely upon transformation in a nanoparticulate system. This is because the size, charge and surface modifications of the nanoparticles often decide their fate *in vivo* (32,33). In this context, the main objective of this work was to evaluate the toxicity of the following types of poly(anhydride) nanoparticles when orally administered: conventional nanoparticles (NP), nanoparticles containing 2-hydroxypropyl-β-cyclodextrin (NP-HPCD) and pegylated nanoparticles (PEG-NP).

For this purpose we decided to follow the standard procedures and guidelines proposed by the Organization for Economic Cooperation and Development (OECD) which are currently used to test and assess chemicals. For the acute toxicity study, a limit test based on the oral administration to laboratory animals of a single oral dose of 2 g nanoparticles per kg bw in water was applied. This dose was around 200-times higher than the usual dose employed in previous studies with these nanoparticles (16,18,21). In all cases, the

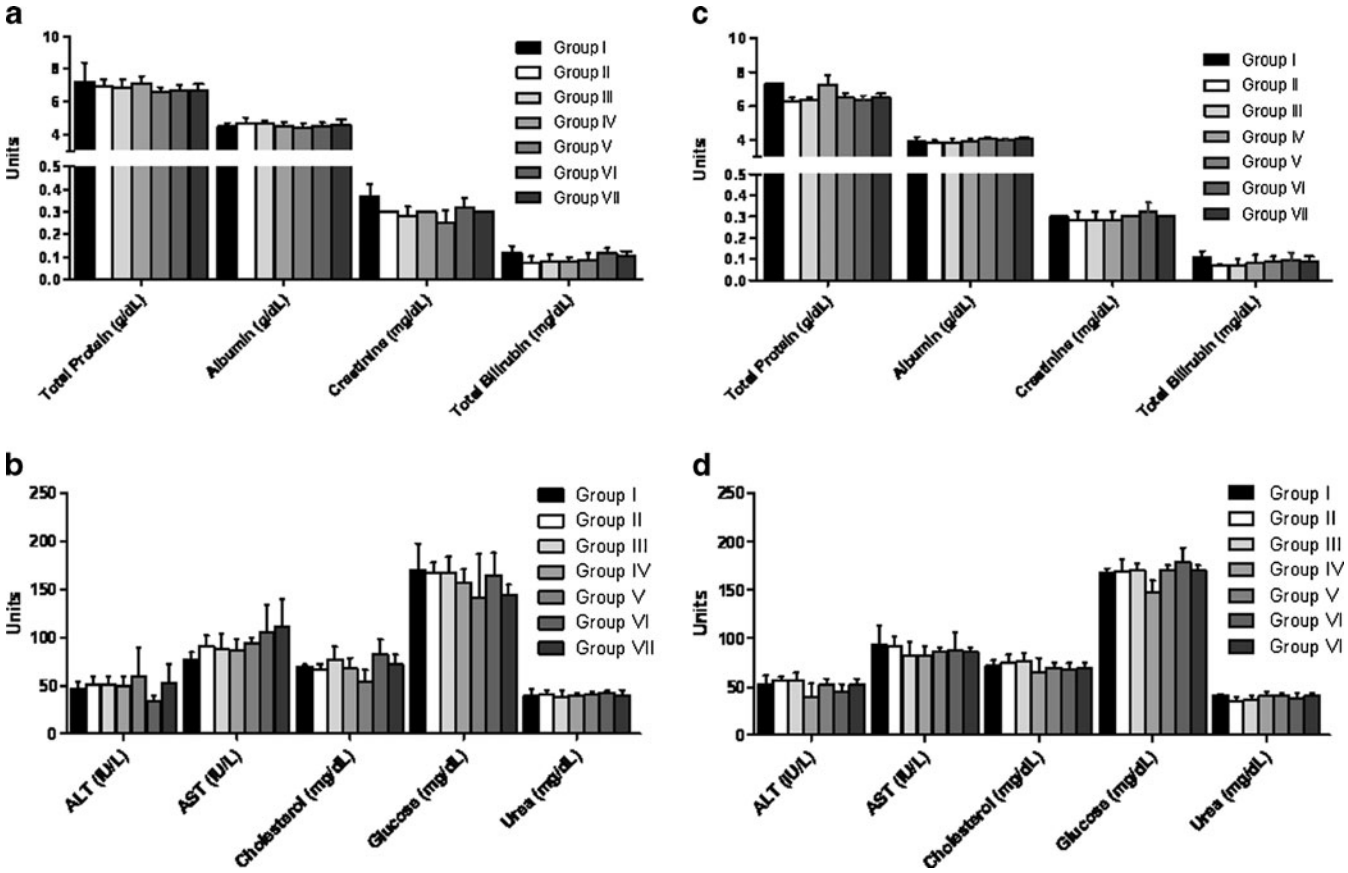

Fig. 4 Biochemical parameters in female (**a** and **b**) and male rats (**c** and **d**) evaluated for sub-acute toxicity.

administered dose had no toxic effect. In fact, neither any death occurred nor abnormal or toxic responses were observed in the rats during the 14-day observation period. In addition no macroscopic pathological alterations attributed to nanoparticles were found in the necropsies. These findings were consistent with previous results with nanoparticles containing chitosan. Thus, Sonaje and collaborators (34) reported an apparent absence of toxicity in mice treated with 100 mg/kg chitosan nanoparticles with a mean size of about 220 nm. In the same way, the DL50 of gold nanoparticles coated with chitosan (10–50 nm) was found to be greater than 2000 mg/kg (35). On the contrary other types of nanoparticles, such as zinc or titanium oxide particles, appear to induce some toxicological problems. Thus, Wang and collaborators reported that the oral administration to mice of a large dose of 5 g/kg bw of titanium oxide nanoparticles (25, 80 or 155 nm) showed no obvious acute toxicity. However, animals treated with very fine nanoparticles (25 or 80 nm) displayed important changes of serum biochemical parameters associated to liver injury and nephrotoxicity (36). In another interesting report, it was demonstrated that the oral administration of 5 g/kg bw of zinc microparticles (1080 nm) induced more severe liver damage than zinc nanoparticles (60 nm), while these small ones could induce heavier renal damage and anemia (37). This different toxicological profile between polymeric and metallic particles may be ascribed, at least in part, to the capabilities of chitosan and poly(anhydride) to yield carriers able to develop sustained muco- bioadhesive interactions with components of the mucus layer and/or the membrane epithelia (19,38,39). This fact would minimize the possibilities for their "translocation" and entry into the circulation compared with "non-adhesive" ones such as metallic nanoparticles.

Interestingly, an absence of toxicological effects were also observed during the sub-acute toxicity studies with the three types of poly(anhydride) nanoparticles. All the animals survived the duration of the study, with no significant changes in clinical signs, food consumption or body weight. In these experiments, the hematological parameters of all the animals were found to be within the normal ranges with no differences between the control and experimental groups. These results suggested that poly(anhydride) nanoparticles are nontoxic when administered orally as they did not affect the circulating red cells, hematopoiesis or leucopoiesis that could otherwise have caused hematological disorders (40). With regard to biochemical parameters analyzed, which may reflect alterations in blood enzymes and are used to

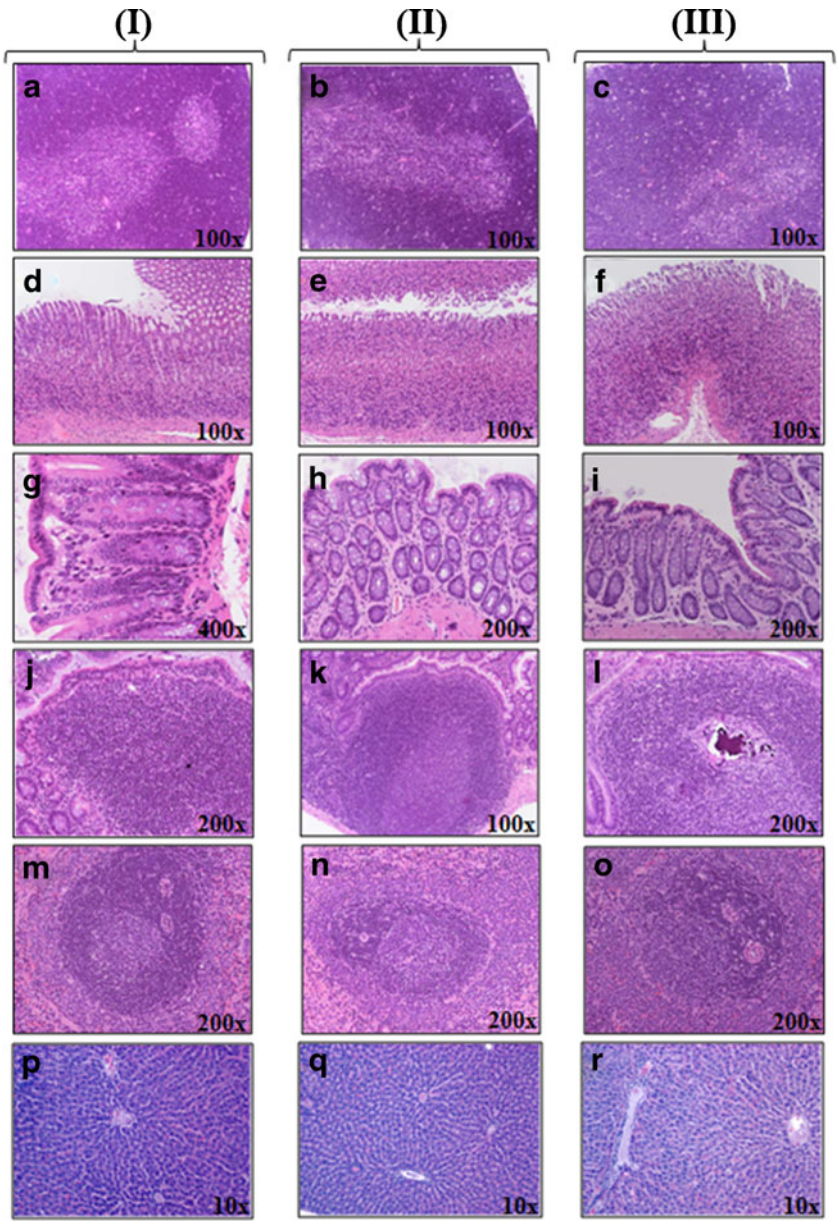

Fig. 5 Light photomicrographs of organ tissues after 28 days administration of poly (anhydride) nanoparticles: (I) NP; (II) NP-HPCD and (III) PEG-NP. The images presented here are from the females highest dose group, 300 mg/kg bw. The images are from thymus (**a**-**c**), stomach (**d**-**f**), intestine (**g**-**i**), Peyer patches (**j**-**l**), spleen (m-o and liver (**p**-**r**).

diagnose organ diseases (i.e. from heart, liver or kidney), no significant differences ($p > 0.05$) between control and treated groups of both male and female animals were also observed. Again, this fact confirmed that poly(anhydride) nanoparticles did not generate cumulative and latent biochemical changes following multiple administrations even using doses 30-times higher than those reported in other studies (15–21). Moreover, all the outcomes aforementioned were corroborated with the macroscopic and histological analysis of the target organs. Indeed, no toxic lesions were evident in any of the organs evaluated and all the organs tested showed unaffected natural architecture. In spite of the number of subacute oral toxicity studies with nanoparticles is very scarce, the presented results appears to be in line with other previously reported involving gold nanoparticles or hydrogel nanoparticles. In the former, Dhar and co-workers reported the absence of any hematological or biochemical abnormalities observed with gellam gum-reduced gold nanoparticles (14 nm) orally administered daily at a dose of 300 mg/kg during 28 days (41). In the latter, nanoparticles obtained by the combination of hydroxyl propyl methyl cellulose and polyvinyl pyrrolidone (100 nm) were 28-day daily administered to rats and none of the treated animals evidenced toxic effects on vital organs or metabolic abnormalities (42).

In order to gain insight about the fate of poly(anhydride) nanoparticles in the body, the carriers were radiolabelled with technetium and orally administered to rats. Neither imaging nor gamma counter data showed radioactivity concentration in liver, kidneys or spleen, confirming that nanoparticles were in no way absorbed through the intestine and transported to

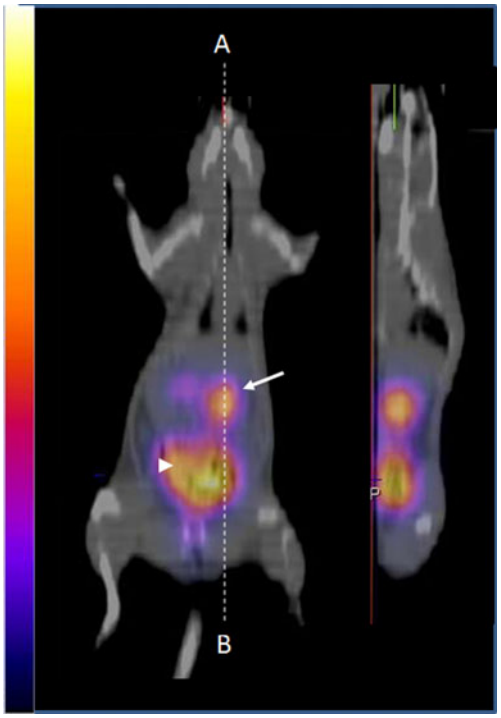

Fig. 6 Localization of ^{99m}Tc labelled nanoparticles (NP) 8 h post administration of the dose (300 mg/kg bw) corresponding to day 28th. Coronal (left) and sagittal (right) SPECT-CT fused 6 mm-thick images. Sagittal cut was made following the A-B line. Color bar indicates relative radioactivity concentration. Arrow: stomach; arrowhead: small intestine and caecum.

the blood circulation. These findings are different to that observed for other types of nanoparticles such as zinc (36) or silver nanoparticles (43). In this last report, describing a sub-acute study in rats, it was demonstrated that the toxicity of silver nanoparticles (60 nm) is related with their accumulation in the kidneys after absorption through the gut (43). Therefore, the safety of poly(anhydride) nanoparticles would be directly related to the absence of a "translocation" phenomenon.

Comparing the three types of poly(anhydride) nanoparticles, it was observed that the radioactivity values quantified in the gastrointestinal tract of animals treated with PEG-NP were three times higher than for animals dosed with NP-HPCD or NP values (Fig. 7). This slow transit through the gut for PEG-NP is consistent with previous observations reported by Yoncheva and collaborators, who demonstrated that pegylation of nanoparticles increased the bioadhesive capability of the resulting carriers (18,19). In these studies it was postulated that pegylated poly(anhydride) nanoparticles would display a PEG-surface layer in a "brush" conformation. Due to this morphology, pegylated nanoparticles would be capable of diffuse across the mucus protective layer and reach the surface of the enterocytes. As a consequence, the residence time of these nanoparticles in the gut would be longer than for other types of carriers (i.e NP or NP-HPCD).

CONCLUSION

Acute and sub-acute toxicity studies of poly(anhydride) nanoparticles administered by the oral route to rats demonstrated the absence of adverse effects related to either the treatment or the sex. The distribution of the three different nanoparticle formulations appeared to be restricted to the gastrointestinal tract of animals and no evidences of "translocation" or absorption of particulates was found. However, the residence within the gut of PEG-NP was found to be significantly higher than for conventional nanoparticles or NP-HPCD. Overall, the current findings confirms that poly(anhydride) nanoparticles are safe for both oral short-term and prolonged administrations.

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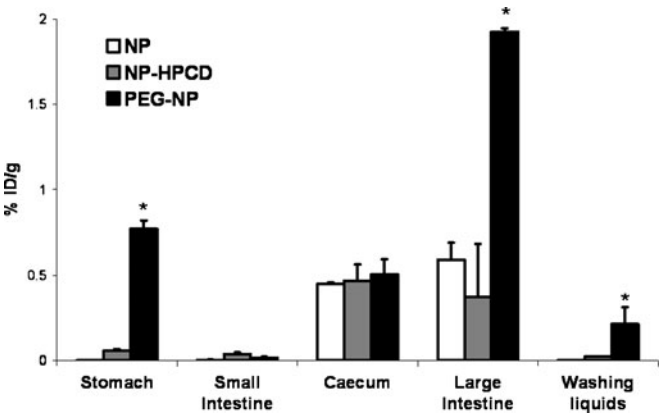

Fig. 7 *Ex vivo* biodistribution studies of ^{99m}Tc-labelled poly (anhydride) nanoparticles. Animals were sacrificed 24 h post administration of the dose (300 mg/kg bw) corresponding to day 28th. * Statistically significant difference ($p < 0.05$). Results expressed as the mean ± SD ($n=3$).

REFERENCES

1. Singh R, Nalwa HS. Medical applications of nanoparticles in biological imaging, cell labeling, antimicrobial agents, and anticancer nanodrugs. J Biomed Nanotechnol. 2011;7(4):489–503.
2. De Jong WH, Borm PJ. Drug delivery and nanoparticles: applications and hazards. Int J Nanomed. 2008;3(2):133–49.
3. Lanone S, Boczkowski J. Biomedical applications and potential health risks of nanomaterials: molecular mechanisms. Curr Mol Med. 2006;6(6):651–63.
4. Moghimi SM, Hunter AC, Murray JC. Nanomedicine: current status and future prospects. FASEB J. 2005;19(3):311–30.
5. Vauthier C, Labarre D, Ponchel G. Design aspects of poly (alkylcyanoacrylate) nanoparticles for drug delivery. J Drug Target. 2007;15(10):641–63.
6. Chan JM, Valencia PM, Zhang L, Langer R, Farokhzad OC. Polymeric nanoparticles for drug delivery. Methods Mol Biol. 2010;624:163–75.
7. Kumari A, Yadav SK, Yadav SC. Biodegradable polymeric nanoparticles based drug delivery systems. Colloid Surf B: Biointerf. 2010;75(1):1–18.
8. des Rieux A, Fievez V, Garinot M, Schneider YJ, Préat V. Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. J Control Release. 2006;116 (1):1–27.
9. Medina C, Santos-Martinez MJ, Radomski A, Corrigan OI, Radomski MW. Nanoparticles: pharmacological and toxicological significance. Br J Pharmacol. 2007;150(5):552–8.
10. Rivera Gil P, Oberdörster G, Elder A, Puntes V, Parak WJ. Correlating physico-chemical with toxicological properties of nanoparticles: the present and the future. ACS Nano. 2010;4 (10):5527–31.
11. Rivière G. European and international standardisation progress in the field of engineered nanoparticles. Inhal Toxicol. 2009;21 Suppl 1:2–7.
12. Clift MJD, Gehr P, Rothen-Rutishauser B. Nanotoxicology: a perspective and discussion of whether or not *in vitro* testing is a valid alternative. Arch Toxicol. 2011;85:723–31.
13. Organization for Economic Cooperation and Development. Nanosafety at the OECD: the First Five Years 2006–2010. Paris: OECD Publishing; 2011. Available from: http://www.oecd.org/dataoecd/6/25/47104296.pdf.
14. Organization for Economic Cooperation and Development. Preliminary review of OECD test guidelines for their applicability to manufactured nanomaterials. Paris: OECD; 2009. Vol. No. 15 - ENV/JM/MONO (2009)21.
15. Arbós P, Campanero MA, Arangoa MA, Irache JM. Nanoparticles with specific bioadhesive properties to circumvent the pre-systemic degradation of fluorinated pyrimidines. J Control Release. 2004;96 (1):55–65.
16. Gómez S, Gamazo C, Roman BS, Ferrer M, Sanz ML, Irache JM. Gantrez AN nanoparticles as an adjuvant for oral immunotherapy with allergens. Vaccine. 2007;25(29):5263–71.
17. Agüeros M, Ruiz-Gatón L, Vauthier C, Bouchemal K, Espuelas S, Ponchel G, *et al.* Combined hydroxypropyl-beta-cyclodextrin and poly(anhydride) nanoparticles improve the oral permeability of paclitaxel. Eur J Pharm Sci. 2009;38(4):405–13.
18. Yoncheva K, Lizarraga E, Irache JM. Pegylated nanoparticles based on poly(methyl vinyl ether-co-maleic anhydride): preparation and evaluation of their bioadhesive properties. Eur J Pharm Sci. 2005;24(5):411–9.
19. Yoncheva K, Guembe L, Campanero MA, Irache JM. Evaluation of bioadhesive potential and intestinal transport of pegylated poly(anhydride) nanoparticles. Int J Pharm. 2007;334(1–2):156–65.
20. Yoncheva K, Centelles MN, Irache JM. Development of bioadhesive amino-pegylated poly(anhydride) nanoparticles designed for oral DNA delivery. J Microencapsul. 2008;25(2):82–9.
21. Agüeros M, Zabaleta V, Espuelas S, Campanero MA, Irache JM. Increased oral bioavailability of paclitaxel by its encapsulation through complex formation with cyclodextrins in poly(anhydride) nanoparticles. J Control Release. 2010;145(1):2–8.
22. Calvo J, Lavandera JL, Agüeros M, Irache JM. Cyclodextrin/ poly (anhydride) nanoparticles as drug carriers for the oral delivery of atovaquone. Biomed Microdevices. 2011;13(6):1015–25.
23. Ojer P, Salman H, Da Costa Martins R, Calvo J, López de Cerain A, Gamazo C, *et al.* Spray-drying of poly(anhydride) nanoparticles for drug/antigen delivery. J Drug Del Sci Tech. 2010;20(5):353–9.
24. Agüeros M, Campanero MA, Lrache JM. Simultaneous quantification of different cyclodextrins and Gantrez by HPLC with evaporative light scattering detection. J Pharm Biomed Anal. 2005;39 (3–4):495–502.
25. Zabaleta V, Campanero MA, Irache JM. An HPLC with evaporative light scattering detection method for the quantification of PEGs and Gantrez in PEGylated nanoparticles. J Pharm Biomed Anal. 2007;44(5):1072–8.
26. Areses P, Agüeros MT, Quincoces G, Collantes M, Richter JÁ, López-Sánchez LM, *et al.* Molecular imaging techniques to study the biodistribution of orally administered (99m)Tc-labelled naive and ligand-tagged nanoparticles. Mol Imaging Biol. 2011;13 (6):1215–23.
27. Organization for Economic Cooperation and Development. Test No. 425: acute oral toxicity: up-and-down procedure. Guidelines for the testing of chemicals. Paris: OECD; 2006.
28. Organization for Economic Cooperation and Development. Test No. 407: repeated dose 28-day oral toxicity study in rodents. Guidelines for the testing of chemicals. Paris: OECD; 2001.
29. Arbós P, Wirth M, Arangoa MA, Gabor F, Irache JM. Gantrez AN as a new polymer for the preparation of ligand-nanoparticle conjugates. J Control Release. 2002;83(3):321–30.
30. Sharma NC, Galustians HJ, Qaquish J, Galustians A, Rustogi KN, Petrone ME, *et al.* The clinical effectiveness of a dentifrice containing triclosan and a copolymer for controlling breath odor measured organoleptically twelve hours after toothbrushing. J Clin Dent. 1999;10(4):131–4.
31. Andrews GP, Jones DS. Poly(methyl vinyl ether/maleic anhydride). In: Rowe RC, Sheskey PJ, Quinn ME, editors. Handbook of pharmaceutical excipients. 6th ed. Washington DC: American Pharmacists Association; 2009. p. 534–5. London: Pharmaceutical Press.
32. Chiu GN, Wong MY, Ling LU, Shaikh IM, Tan KB, Chaudhury A, *et al.* Lipid-based nanoparticulate systems for the delivery of anti-cancer drug cocktails: implications on pharmacokinetics and drug toxicities. Curr Drug Metab. 2009;10(8):861–74.
33. Kean T, Thanou M. Biodegradation, biodistribution and toxicity of chitosan. Adv Drug Deliv Rev. 2010;62(1):3–11.
34. Sonaje K, Lin YH, Juang JH, Wey SP, Chen CT, Sung HW. *In vivo* evaluation of safety and efficacy of self-assembled nanoparticles for oral insulin delivery. Biomaterials. 2009;30:2329–39.
35. Pokhartar V, Dhar S, Bhumkar D, Mali V, Bodhankar S, Prasad BLV. Acute and subacute toxicity studies of chitosan reduced gold nanoparticles: a novel carrier for therapeutic agents. J Biomed Nanotecnol. 2009;5:233–9.
36. Wang B, Feng WY, Wang TC, Jia G, Wang M, Shi J-W, *et al.* Acute toxicity of nano- and micro-scale zinc powder in healthy adult mice. Toxicol Let. 2006;161:115–23.
37. Wang J, Zhou G, Chen C, Yu H, Wang T, Ma Y, *et al.* Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. Toxicol Lett. 2007;168:176–85.
38. Takeuchi H, Yamamoto H, Niwa T, Hino T, Kawashima Y. Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. Pharm Res. 1996;13:896–901.

39. das Neves J, Bahia MF, Amiji MM, Sarmento B. Mucoadhesive nanomedicines: characterization and modulation of mucoadhesion at the nanoscale. Expert Opin Drug Deliv. 2011;8(8):1085–104.
40. Hayes AW. Principles and methods of toxicology. 4th ed. Philadelphia: Taylor & Francis; 2001.
41. Dhar S, Mali V, Bodhankar S, Shiras A, Prasad BLV, Pokharkar V. Biocompatible gellan gum-reduced gold nanoparticles: cellular uptake and subacute oral toxicity studies. J Appl Toxicol. 2011;31:411–20.
42. Dandekar PP, Jain R, Patil S, Dhumal R, Tiwari D, Sharma S, *et al.* Curcumin-loaded hydrogel nanoparticles: application in anti-malarial therapy and toxicological evaluation. J Pharm Sci. 2010;99:4992–5010.
43. Kim WY, Kim J, Park JD, Ryu HY, Yu IJ. Histological study of gender differences in accumulation of silver nanoparticles in kidneys of Fischer 344 rats. J Toxicol Envirom Health A. 2009;72:1279–84.