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Cytotoxicity and biocompatibility evaluation of a poly(magnesium acrylate) hydrogel synthesized for drug delivery

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ABSTRACT

We report the synthesis and characterization as well as cytotoxicity and biocompatibility studies of a poly(magnesium acrylate) hydrogel (PAMgA) developed for drug delivery applications. Two hydrogels with different mesh sizes, large and short, were synthesized (L-C PAMgA and S-C PAMgA). The hydrogels were characterized through swelling, FT-IR and DSC. Cytotoxicity *in vitro* was evaluated on cell line NIH-3T3 fibroblasts via direct contact and two indirect contact methods (MTT and flow citometry). Both PAMgA hydrogels exhibited low cytotoxicity with survival rates higher than 90%. To select their administration route, biocompatibility was evaluated after intraperitoneal, subcutaneous, and oral administration to mice of both hydrogels at different dose ranges. Swelling percentages obtained were $33.3 \pm 4.2\%$ and $166.7 \pm 8.3\%$ for L-C PAMgA and S-C PAMgA respectively, showing a great difference in both hydrogels. Among the administration routes assayed, the hydrogels were well tolerated after oral administration of a wide dose range (10–500 mg/kg), thereby indicating that both PAMgA hydrogels are excellent candidates for oral administration due to their *in vitro* biocompatibility and oral non-toxicity. These results together with the fact that their synthesis is simple and inexpensive make them good candidates for the design of oral drug delivery devices.

1. Introduction

The field of controlled release has evolved towards the use of biomaterials that could improve treatment efficacy (Langer and Tirrel, 2004). Thus, polymers have gained importance in the pharmaceutical industry as drug carriers. A group of polymers of special interest in biomedical applications is constituted by hydrogels. The design and preparation of hydrogels have attracted a great deal of interest in biomedical engineering, pharmaceutical applications, and biomaterials science because of their tunable chemical and three-dimensional (3D) physical structure, good mechanical properties, high water content, and biocompatibility. These unique properties offer great potential for the utilization of hydrogels in tissue engineering, biomedical implants, drug delivery, and bionanotechnology (Drury and Mooney, 2003; Mabilleau et al., 2006; Tomic et al., 2006; Jina et al., 2009; Tan et al., 2009). Furthermore, the increasing efforts devoted to controlled molecule release forecast a continuous growth of hydrogels applications in the future as carriers for the delivery of drugs, peptides and proteins, as targeting agents for site-specific delivery, or as components for the preparation of protein or enzyme conjugates (Peppas et al., 2000; Lin and Metters, 2006).

Acrylic-based hydrogels are easy to manufacture being regarded as safe excipients. Moreover, the mucoadhesive properties of these polymers make them suitable materials for increasing the residence time of the drug device at the absorbing tissue and thereby increasing the drug bioavailability (Bromberga et al., 2004; Peppas et al., 2009). It is well known that the GI tract is the most popular route of drug delivery because of the facility of administration of drugs for compliant therapy, and its large surface area for systemic absorption. It is, however, the most complex route, so that versatile approaches are needed to deliver drugs for effective therapy (Peppas et al., 2000; Fulzele et al., 2003). Among these strategies mucoadhesive hydrogels can be very valuable when used as carriers that interact with the mucosa lining in the GI tract.

When designing materials for drug delivery purposes it is of utmost importance to characterize them in terms of biocompatibility. *In vitro* cell culture studies are very useful in assessing cytotoxicity. The effects of cells exposure to chemical agents at different concentrations and times can be determined by assessing

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cytotoxicity. In the biocompatibility evaluation of any biomaterial, *in vitro* tests should be conducted prior to *in vivo* tests to minimize animal suffering. However, cytotoxicity assays measure only effects on cells during the first 12–48 h after exposure to the test substances and many biological reactions *in vivo* propagated beyond that exposure time. Moreover, differences in sensitivities have been observed between primary and established cell lines (Lovschall et al., 2002; Fernández-Carballido et al., 2008). Thus i*n vitro* tests may not fully capture the whole *in vivo* situation thereby indicating that cell culture methods cannot replace whole animal testing.

The objective of the present work was to synthesize and characterize polymagnesium acrylate hydrogels (PAMgA). Their biocompatibility was evaluated both *in vitro* and *in vivo*. *In vitro* cytotoxicity studies were performed by different methods (direct contact, MTT and flow citometry analyses) in cell line NIH-3T3 fibroblasts. In order to select the administration route of the material biocompatibilty was assessed *in vivo* after administration of different doses of both hydrogels to mice by several routes (intraperitoneal, subcutaneous and oral).

2. Materials and methods

2.1. Materials

The monomer precursors (acrylic acid and magnesium hydroxide) and the initiator (ammonium persulfate, PSA) were purchased from Sigma–Aldrich Chemical (USA). All other solvents and reagents (analytical grade) were purchased from Merck (Spain) and used as supplied.

2.2. Synthesis of magnesium acrylate monomer (AMgA)

The preparation of the monomer was carried out by a neutralization reaction. Acrylic acid (2 mol) was added dropwise to a magnesium hydroxide solution (1 mol) under constant stirring and a slow stream of nitrogen. After 48 h of stirring under nitrogen atmosphere, the milky dispersion became transparent, indicating the end of the neutralization process. Subsequently, the solution was concentrated at 25 wt.% (w/w, %; per gram of monomer) in a rotator evaporator (Rubio-Retama et al., 2007).

2.3. Synthesis of poly(magnesium acrylate) (PAMgA)

Synthesis was carried out following the free radical polymerization method using ammonium persulfate (PSA) as initiator (McNeill and Sadegui, 1990) Fir this, two different aqueous solutions of PSA (5 mM and 40 mM), containing 25 wt.% of monomer were prepared to obtain hydrogels with different mesh sizes. Polymerization was induced by thermolysis of the initiator at 60 °C. At the end of the polymerization reaction, the hydrogels were extracted from the reactor and maintained in distilled water at 25 °C for 12 days. To extract all unreacted monomer, removal and replacement of fresh water was done daily until the medium showed constant water conductivity. Subsequently, the hydrogels were freeze–dried using a freeze-dryer (LIOALFA-6, TELSTAR, Spain) and sterilized by autoclave. Two different hydrogels were obtained: (i) networks prepared with 5 mM PSA with long segments of polymer between crosslinking points (L-C PAMgA), and (ii) networks prepared with 40 mM PSA resulting in short polymer chains between crosslinking points (S-C PAMgA).

2.4. Differential scanning calorimetry (DSC)

The water content, glass transition and degradation temperatures of the hydrogels were determined by thermal analysis in a Mettler 820 DSC (Mettler Toledo, Switzerland). The temperature scale was calibrated using the melting temperatures of indium and zinc. The DSC cell was used for heating the samples (15 mg) from −100 °C to 500 °C with heating rate of 10 °C/min in a dry nitrogen gas flow. Samples of both hydrogels were assayed. All samples were double scanned. An empty aluminium pan was used as reference.

2.5. Infrared spectroscopy (FT-IR)

Fourier transform infrared spectra (FTIR) were recorded on a Nicolet IR200 spectrometer (Thermo Fisher Scientific, USA) within the range of 4000–400 cm^{-1}. The FTIR spectra were obtained for both native PAMgA hydrogels at room temperature and, pellets prepared with KBr and freeze–dried PAMgA hydrogels at a ratio of KBr to polymer powder around 40 (w/w).

2.6. Swelling studies

The hydrogel swelling capacity was investigated by the gravimetric method. Disks of 1 cm-diameter of both lyophilised hydrogels (15 ± 1 g) were placed in petri dishes containing distilled water at 25 °C. To ensure complete equilibration, samples were allowed to swell and equilibrate for 48 h. After equilibrium was reached the discs were blotted free of surface water with filter paper, and the wet weight of the gel sample was measured at room temperature. All swelling tests were performed in triplicate. The swelling percentage was calculated using the following equation:

$$S_w = \frac{W_f - W_0}{W_0} \times 100 \quad (1)$$

where W_f is the mass of the gel at equilibrium and W_0 is the dry mass of the gel at time 0.

The percentage equilibrium water-content (EWC) of the hydrogels was determined in water at 25 °C. Excess surface liquid was wiped and swollen samples were weighed. Equilibrium water content was determined using the equation:

$$\mathrm{EWC} = \frac{W_f - W_0}{W_f} \times 100 \quad (2)$$

2.7. In vitro cytotoxicity studies

Cytotoxicity of the hydrogels was studied by a direct contact method and two indirect techniques (MTT colorimetric assay and flow cytrometry analysis) (Smith et al., 2006). Cytotoxicity was tested on mouse embryonic fibroblast NIH-3T3 cells obtained from the American Type Culture Collection (ATCC). Cultures were maintained at 37 °C under 5% CO_2 and 95% air atmosphere in RPMI-1640 medium (Sigma–Aldrich Chemical, USA). Samples of both hydrogels were previously sterilized by autoclave.

2.7.1. Direct contact method

NIH-3T3 fibroblast cells were suspended at a density of 1×105 cells/ml (500,000 cells/petri dish) in RPMI-1640 medium and directly incubated at 37 °C with the hydrogel samples. After 48 h, the number of fibroblast cells was counted using a Cell Counter System CASY (Model DT, Innovatis Bielefeld, Germany). Moreover, inverted compound microscopy (model Diaphot 300, Nikon, Spain) was used to inspect physical cell appearance.

2.7.2. Indirect method: MTT colorimetric assay

Cell viability was also determined by the MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay (Sigma–Aldrich). The MTT assay measures the ability of metabolic active cells to form blue formazan crystals through cleavage of the tetrazolium ring of MTT. For this, NIH-3T3 fibroblast cells were seeded onto well plates. Fibroblasts (reference

cells) and fibroblasts with different concentrations of L-C PAMgA and S-C PAMgA dispersed in PBS were added to a complete RPMI-1640 medium and incubated at 37 °C in 5% CO_2, 95% air-humidified atmosphere, for 24 h. To account for cell viability the absorbance was measured at 570/630 nm. Untreated cells were used as control (100% viability). The decrease in cell viability caused by the hydrogels was estimated in terms of percentage of cell viability. Relative cell viability (%) was calculated by means of [abs]test/[abs]control × 100. All assays were performed in triplicate.

2.7.3. Indirect method: flow cytometry analysis

Flow cytometry has become a method of choice for analysis of cell death in a variety of cell systems with many methods being developed, and modifications of established assays being introduced at a rapid pace. There are several methods that can be used to quantitatively determine viability of cells. These methods typically use non-permeant dyes (e.g. propidium iodide, 7-Amino Actinomycin D) that do not enter cells with intact cell membranes or active cell metabolism. Cells with damaged plasma membranes or with impaired/no cell metabolism are unable to prevent the dye from entering the cell. Once inside the cell, the dyes bind to intracellular structures producing highly fluorescent adducts which identify the cells as "non-viable". In order to evaluate the effect of the direct interaction between L-C PAMgA and S-C PAMgA hydrogels and cell membranes a flow cytometry analysis was performed. To determine cytotoxicity of the hydrogels, NIH 3T3 cells were detached from flasks and placed into petri dishes at a density of 500,000/petri dish in RPMI-1640 medium. Cells were treated with 1 mg/ml L-C PAMgA or S-C PAMgA dispersed in PBS. Moreover, a blank test was performed with the addition of PBS to control cells. After 5 days of treatment cell viability was evaluated using propidium iodide (PI) (Invitrogen, Spain) for 30 min and samples were analyzed by a flow cytometer (model FACSCalibur, Becton Dickinson, USA) equipped with 488 nm Argon and 635 nm helium–neon lasers that emitted fluorescence (585/42 nm). In this experiment, cells with damaged cytoplasm membranes were coloured with PI. Viability was expressed as percentage of uncoloured cells.

2.8. In vivo biocompatibility studies

Animal care and animal studies were carried out in accordance with guidelines for investigations and experiments in conscious animals and were approved by the Animal Ethics Committee of the Complutense University of Madrid (Spain). Adult male Swiss mice weighing 28–32 g were used for the biocompatibility studies. Mice were kept under standard laboratory conditions and had access to food and water ad libitum. After sterilization of the hydrogels, several dose ranges were administered by different routes: intraperitoneal (i.p.), subcutaneous (s.c.) and, oral administration (p.o.).

Animals were daily weighted until the end of the experiments. In all cases a clinical behaviour study was performed in which restlessness, mobility, chirping, vomiting, diarrhoea and death were evaluated according to a 0–2 scale (0 = normal response or no response, 1 = mild response or one episode and, 2 = severe response or more than one episode). Finally the animals were sacrificed and thereafter, visual organ examination was performed (liver, spleen, lungs, kidney, heart and intestine). Negative control animals injected with PBS were also used.

2.8.1. Intraperitoneal administration

Animals were divided into several groups and injected intraperitoneally with L-C or S-C PAMgA dispersed in PBS at doses ranging from 5 to 80 mg/kg. Sterilized hydrogels (0.1 ml) were injected into the peritoneum while the mice were restrained but not anaesthetized. The animals were closely observed the first 72 h post-injection and daily thereafter. Animals were euthanized after two months or in case they presented inability to ambulate or lost of more than 20% weight. The weight of each animal was recorded immediately before intraperitoneal injection, daily after injection, and at the time of euthanasia.

Animals were divided into six groups (A–F) being each group composed of 5 animals. The negative control group was injected with PBS (n = 5/group; Group 0-P). Animal groups are as follows:

Group A/P: L-C PAMgA (5 mg/kg)
Group B/P: L-C PAMgA (20 mg/kg)
Group C/P: L-C PAMgA (80 mg/kg)
Group D/P: S-C PAMgA (5 mg/kg)
Group E/P: S-C PAMgA (20 mg/kg)
Group F/P: S-C PAMgA (80 mg/kg)

2.8.2. Subcutaneous administration

The study was performed by s.c. administration of 0.1 ml of L-C PAMgA or S-C PAMgA dispersed in PBS at doses ranging from 5 to 20 mg/kg. Animals were divided into four groups of five animals each (A, B, C, and D). A negative control group was injected with PBS (n = 5/group; Group 0-S). Animal groups are as follows:

Group A/S: L-C PAMgA (5 mg/kg)
Group B/S: L-C PAMgA (20 mg/kg)
Group C/S: S-C PAMgA (5 mg/kg)
Group D/S: S-C PAMgA (20 mg/kg)

Animals were injected subcutaneously with the formulations into the right plantar surface of one hind paw of each mouse using a 25-G hypodermal needle. In addition, 0.1 ml of PBS solution was injected into the left hind paw of each mouse. Paw oedema was determined at various times by measuring paw volume by means of a plethysmometer (Water plethysmometer Letica model LI7500, Spain). The right back paw volume local inflammatory response was evaluated in two phases: acute inflammation (3, 5, 7, 24 and 48 h) and, chronic inflammation at 2–3 day intervals for 29 days. The increase in paw volume due to oedema was calculated. After 29 days, animals were euthanized.

2.8.3. Oral administration

Oral administration of the PAMgA hydrogels was performed at a wide dose range for which animals were divided into the following experimental groups (n = 5/group):

Group A/O: L-C PAMgA (10 mg/kg)
Group B/O: L-C PAMgA (100 mg/kg)
Group C/O: L-C PAMgA (500 mg/kg)
Group D/O: S-C PAMgA (10 mg/kg)
Group E/O: S-C PAMgA (100 mg/kg)
Group F/O: S-C PAMgA (500 mg/kg)

The clinical behaviour of the animals (restlessness, chirping, mobility, vomiting, diarrhoea and death) was continuously monitored the first hour after oral administration, intermittently for the next 4 h, at 24 h and thereafter animals were followed up for 14 days. After 14 days animals were euthanized and visual organ analysis was performed.

3. Results and discussion

With ongoing research in advanced drug delivery systems, research into hydrogel delivery systems has focused primarily on systems containing polyacrylic acid backbones. These hydrogels are known for their super-absorbency and ability to form extended polymer networks through hydrogen bonding. In addition, they are excellent bioadhesives, which means that they can adhere to mucosal linings within the gastrointestinal tract for extended periods, releasing their encapsulated medications slowly over time.

In our study the synthesis of the hydrogels was carried out following the free radical polymerization method using ammonium persulfate (PSA) as initiator and without the use of a cross-linker agent. In the synthesis cross-linking occurred by electrostatic interaction between Mg^{2+} ions and COO^- groups on different polymer chains (McNeill and Sadegui, 1990).

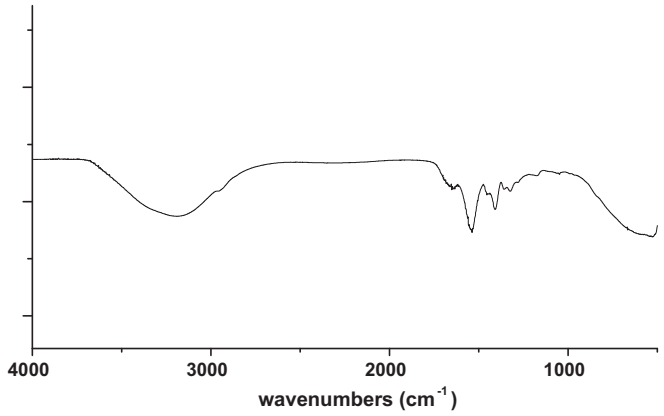

Fig. 1. FT-IR spectrum of PAMgA hydrogel.

L-C PAMgA and S-C PAMgA hydrogels were double scanned by DSC (data not shown). On the first heating, both thermograms showed one broad endothermic peak at around 100 °C which corresponds to the evaporation of the solvent. The glass transition temperature appeared at around 75 ± 1 °C and 77 ± 1 °C for L-C-PAMgA and S-C-PAMgA respectively indicating that the transition temperature scarcely depends on the network mesh size. A second peak appearing at 358.32 ± 3 °C is associated with the beginning of the decomposition of PAMgA and, the third broad peak obtained at 452.27 ± 2 °C which indicates the end stages of polymer decomposition.

In the IR spectrum of PAMgA hydrogel (Fig. 1), the presence of characteristic peaks in the region 1650–1300 cm^{-1} is ascribed to COOH stretching vibrations. Thus, the peaks at 1407 cm^{-1} and 1308 cm^{-1} are attributed to COOH stretching vibrations of non-ionized and ionized carboxylic groups. The carboxylate group is associated with specific absorption bands: one at approximately 1547 cm^{-1} (asymmetric stretching vibration), and one at approximately 1456 cm^{-1} (symmetric stretching vibration). The FT-IR patterns obtained for both hydrogels can be superimposed and the same behaviour occurs when comparing the patterns obtained before and after freeze–drying the material. Unreacted acrylate monomer was absent since it was completely removed by dialysis until the medium showed constant water conductivity.

Water uptake by the hydrogels was monitored for an extended period of time until equilibrium was reached. Swelling percentages of both S-C PAMgA and L-C PAMgA hydrogels at 25 °C were determined by means of Eq. (1). In all cases hydrogel disks swelled rapidly when immersed in water with the equilibrium being reached after 48 h. The swelling percentages obtained were $33.3 \pm 4.2\%$ and $166.7 \pm 8.3\%$ for L-C PAMgA and S-C PAMgA respectively, showing a great difference in both hydrogels. This result could be explained by the formation of polymer entanglements in the L-C PAMGA system which would hinder the water penetration and thus swelling of the gel. The equilibrium water content (EWC), estimated by means of Eq. (2), was $25 \pm 3.6\%$ and $62.5 \pm 6.7\%$ for L-C PAMgA and S-C PAMgA, respectively.

Cell culture methods, also known as cytotoxicity tests, can be used to evaluate the toxicity of hydrogels. Among them, extract dilution and direct contact methods are recommended by ISO guidelines and are the most frequently used (ISO/EN, 10993-5). Regarding toxicity most of the problems associated with hydrogel carriers depend on the unreacted monomers, oligomers and other chemical agents (reticulating or catalyzing agents) that leach out during application. Several studies have evaluated the formation of hydrogels with less chemical agents in order to eliminate the problem of residual chemicals which could compromise biocompatibility (Peppas et al., 1999; Hao et al., 2009; Rivarola et al., 2009). In our case, hydrogels of PAMgA were synthesized by thermal polymerization without the presence of a catalyzing agent.

Cytotoxicity of the hydrogels was evaluated *in vitro* by direct and indirect methods, using NIH 3T3 fibroblasts as model cell line (Smith et al., 2006). In the direct contact method, optical microscopy showed that cells possess normal morphology after 48-h incubation with L-C PAMgA or S-C PAMgA (Fig. 2). A decrease of 20–25% was observed in the number of fibroblasts after exposure to the hydrogels. The fact that cell morphology was not altered after incubation makes us think that this decrease in cell counts could be related to the high viscosity of PAMgA hydrogels which would hinder cell growth.

Cell viability was measured by using the MTT colorimetric assay (indirect method), which is based on the conversion of MTT to dark blue formazan crystals by mitochondrial dehydrogenase. Cell viability was assessed after exposing NIH 3T3 cells to increasing concentrations of L-C PAMgA and S-C PAMgA hydrogels (0.033–0.666 mg/ml) (Fig. 3).

A dose-dependent decrease pattern in fibroblast cell viability was observed for L-C PAMgA hydrogel. This effect was most pronounced at a concentration of 0.67 mg/ml at which a reduction of approximately 25% occurred when compared to untreated control cells. Within the concentration range assayed (0.03–0.53 mg/ml) maximum decreases of 15% in cell viability resulted after 24 h exposure. The results show the higher concentration of L-C PAMgA hydrogel the greater decrease in cell viability (Fig. 3). By contrast, S-C PAMgA hydrogel at the concentration range tested (0.03–0.67 mg/ml) resulted in no significant modification of cell viability with respect to untreated cells. The utilization of the highest amount of initiator (40 μM) during the synthesis of S-C PAMgA led to better results in cell viability, which would be attributed to differences in hydrogel viscosity. It seems that the differences in cell viability would not be related to the swelling capacity of the hydrogels neither to the initiator agent used during their synthesis.

In order to evaluate the effect of the direct interaction between the hydrogels and cell membranes, a flow cytometry analysis was also performed (indirect method). In this case, a high concentration of L-C PAMgA or S-C PAMgA (1 mg/ml) was tested for cell viability after exposure for 5 days. Exposure time was prolonged up to 5 days to determine if the differences found in the MTT assay between both hydrogels could be related to viscosity or chemical interaction. Flow cytometry is a very sensitive technique in which only cells with damaged cytoplasm membranes are coloured with the dye (propidium iodide), whereas in other techniques normal death cell could interfere in the viability results. Viability was expressed as a percentage of the uncoloured cells (Fig. 4).

Results from flow cytometry analysis revealed that for both hydrogels the cells remained more than 90% viable when compared to control cells indicating that the hydrogels possess very low cytotoxicity. Therefore, under our experimental conditions the hydrogels did not significantly interact and/or damaged fibroblast membranes. From all the cytotoxicity studies, it can therefore be concluded that S-C PAMgA exhibited good cell viability regardless the method employed (direct contact or indirect methods). Cell viability results obtained for L-C PAMgA were slightly poorer, which could be attributed to differences in viscosity between both hydrogels. In summary, L-C PAMgA and S-C PAMgA hydrogels exhibited low cytotoxicity since no potentially toxic auxiliary agents (reticulating and/or catalyzing agents) other than the initiator (PSA) have been added during the synthesis process.

It is well known that results obtained from cell line studies are reproducible and have the advantage of allowing to reduce the number of animals needed for experimentation. However, low specificity of cell lines and the fact that *in vitro* tests may not fully capture the *in vivo* situation led us to perform a biocompatibility

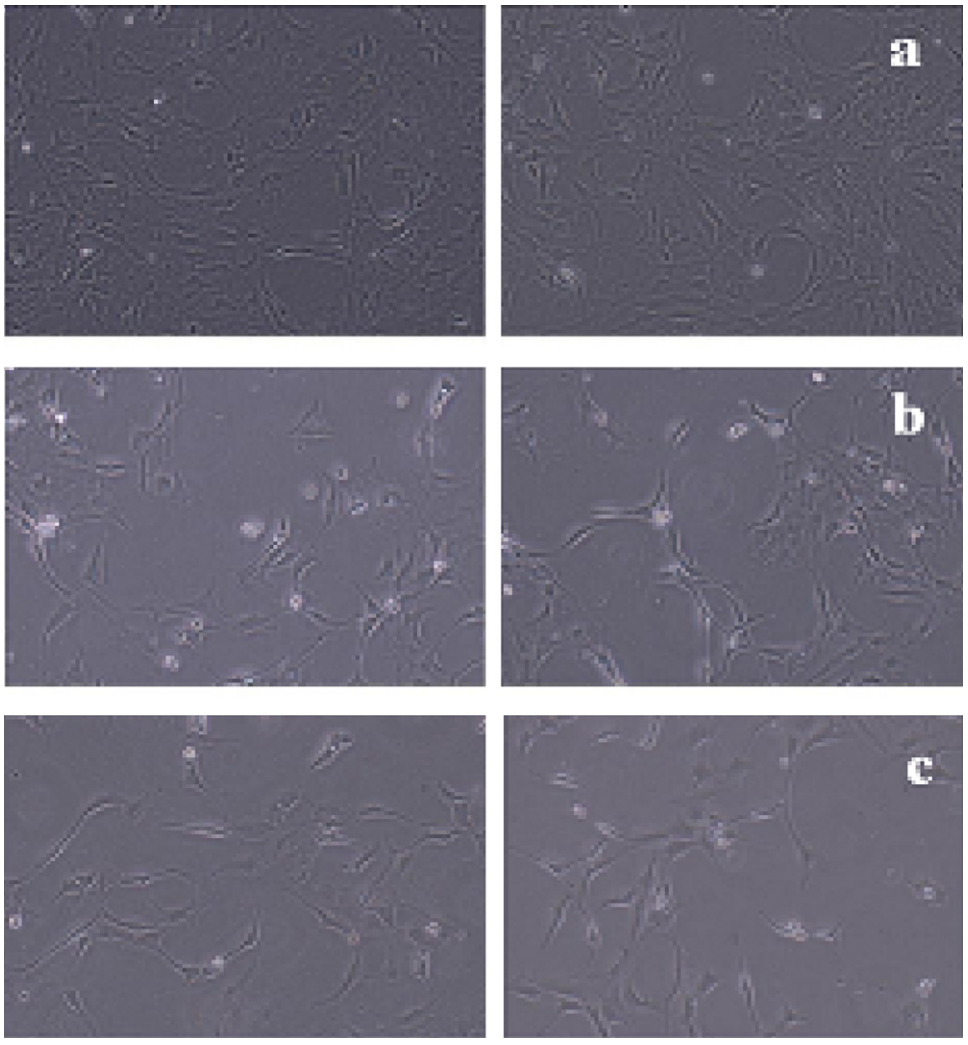

Fig. 2. Optical microphotographs obtained after 48 h incubation of control NIH 3T3 fibroblasts (a), cells in direct contact with L-C PAMgA (b) and, cells in direct contact with S-C PAMgA (c).

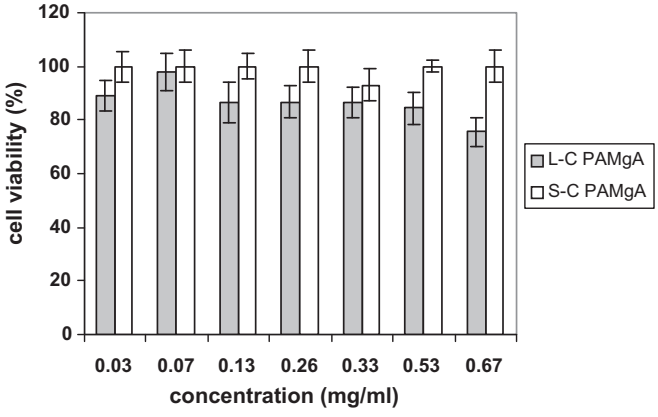

Fig. 3. Mean NIH 3T3 fibroblast viability results obtained by the MTT colorimetric assay (indirect method) after incubation for 24 h with different concentrations of L-C PAMgA or S-C PAMgA (0.033–0.666 mg/ml).

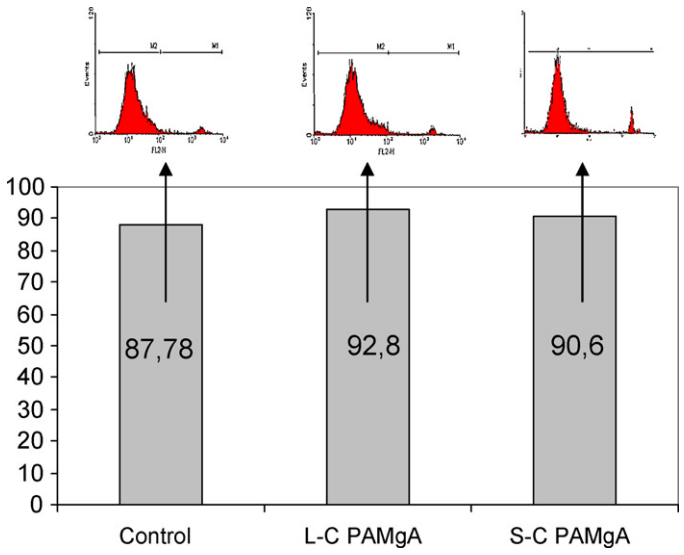

Fig. 4. Mean NIH 3T3 fibroblast viability results obtained by flow cytometry obtained for control cells and cells exposed to 1 mg/ml of L-C PAMgA and S-C PAMgA for 5 days.

Table 1
Clinical responses obtained after administration of L-C PAMgA to mice by different administration routes. Scales 0–2 (0 = normal response or no response, 1 = mild response or one episode and 2 = severe response or more than one episodes).

Dose (mg/Kg)	i.p.			s.c.		p.o.
	5	20	80	5	20	10–500
Restlessness	1	1	1	1	1	1
Chirping	0	0	0	0	0	0
Mobility	1	2	2	1	2	0
Vomiting	0	0	0	0	0	0
Diarrhoea	0	0	0	0	0	0
Death	0	1	2	0	0	0

study in animals (mice) from which results with a more physiological meaning would be expected (Chen and Singh, 2008).

Biocompatibility was studied after administration of the hydrogels by different routes: i.p., s.c. and p.o. In all cases clinical responses such as restlessness, chirping, mobility, vomiting, diarrhoea and death were evaluated according to a 0–2 scale (Tables 1 and 2).

In addition, body weight changes were monitored throughout the study. It was found that less than 10% weight decreases occurred 48 h after administration of the hydrogels by s.c. and i.p. routes. The same decreases were obtained in control animals and are attributed to assay-related initial stress more than to the invasive routes of administration. After 72 h normal weight increases occurred until the animals were euthanized. Oral administration of the hydrogels did not result in initial weight decreases with a normal weight increase pattern thereafter.

Administration of the hydrogels by s.c. and i.p. routes resulted in a dose-dependent decrease in animal mobility (Tables 1 and 2) regardless the hydrogel used. However, this response did not experience any modification when the hydrogels were given p.o. A highly dose-dependent death-related response occurred when animals received both hydrogels by i.p. administration, with death rates of 20% and 60% obtained for doses of 20 and 80 mg/kg, respectively. In all cases deaths occurred within the first 48 h after administration. Upon death, macroscopic visual organ examination (liver, spleen, lungs, kidney, stomach and intestine) was performed. Observation showed widespread haemorrhages in the liver and intestine with normal appearances of the other organs.

The death-related response obtained after i.p. administration could be attributed to hypocalcaemia produced by an ionic exchange reaction occurring between calcium and magnesium ions. Hicks et al. (1989) found the same behaviour after i.p., i.v. and s.c. administration of a sodium polyacrylate polymer at doses ranging from 5 to 100 mg/kg, suggesting that localization of the viscous, high-molecular-weight material in vascularized compartments, together with its known ability to form insoluble precipitates with calcium, led to the operation of a calcium sink resulting in hypocalcaemia which would explain the haemorrhages as well as neuromuscular and cardiac malfunctions observed in the said study.

Table 2
Clinical responses obtained after administration of S-C PAMgA to mice by different administration routes. Scales 0–2 (0 = normal response or no response, 1 = mild response or one episode and 2 = severe response or more than one episodes).

Dose (mg/kg)	i.p.			s.c.		p.o.
	5	20	80	5	20	10–500
Restlessness	1	1	1	1	1	1
Chirping	0	0	0	0	0	0
Mobility	0	1	2	1	2	0
Vomiting	0	0	0	0	0	0
Diarrhoea	0	0	0	0	0	0
Death	0	1	2	0	0	0

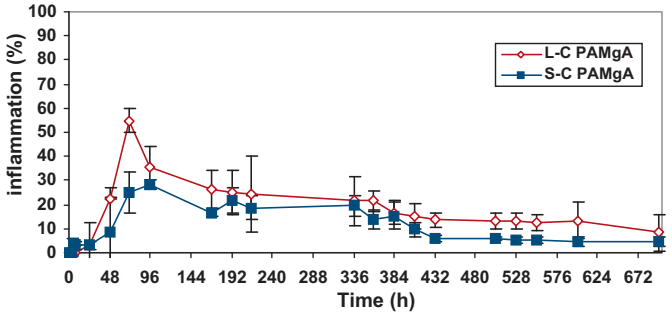

Fig. 5. Inflammatory responses obtained after s.c. administration of 5 mg/kg of L-C or S-C PAMgA hydrogels to mice.

In our study, s.c. administration of the hydrogels resulted in mild haemorrhage episodes but at the dose range tested deaths did not occur. Nevertheless, a further dose increase will probably lead to a death dose-related increase. Administration of a biomaterial by the s.c. route can be used to evaluate its biocompatibility through local inflammatory responses. For this, L-C PAMgA and S-C PAMgA hydrogels were injected subcutaneously at two doses (5 mg/kg and 20 mg/kg) into the left hind paw of each mouse. The local inflammatory response was evaluated in two phases: acute inflammation (3, 5, 7, 24 and 48 h) and, chronic inflammation at 2–3 day intervals for 29 days (Figs. 5 and 6). It can be noted that after s.c. administration, a long lasting inflammatory reaction occurred in all cases.

Following the implantation of a biomaterial, an inflammatory process is initiated consisting of a complex series of reactions tending to prevent the in progress tissue damage, isolate and destroy the foreign material and activate the repair processes. The early events constitute the acute phase response, which can destroy the implanted system or lead to a chronic inflammatory phase and/or granulation tissue development (Fournier et al., 2003). In our case, acute inflammatory responses were developed after injection which were less pronounced and of shorter duration when the hydrogels were assayed at a low dose level (5 mg/kg).

After this acute process, baseline values were practically reached. Thereafter, a chronic inflammatory response occurred during which inflammatory reactions up to 10-fold higher those of the acute phase were observed. This chronic phase response is characterized by the proliferation of fibroblasts and macrophages. It is known that acrylic polymers are not biodegradable and if the material persists as a permanent stimulus, it may result in granuloma with intense active inflammation, accumulation of activated macrophages and lymphocytes (Fournier et al., 2003). In our case, L-C PAMgA gave a higher chronic inflammatory response which could be related to its higher viscosity. These characteristics will result in a stronger foreign body reaction due to the increased difficulty of the body to eliminate the material.

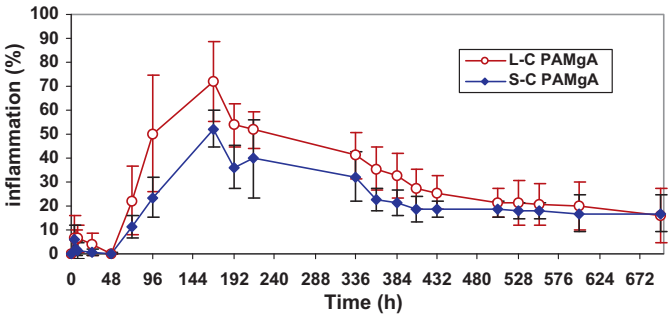

Fig. 6. Inflammatory responses obtained after s.c. administration of 20 mg/kg of L-C or S-C PAMgA hydrogels to mice.

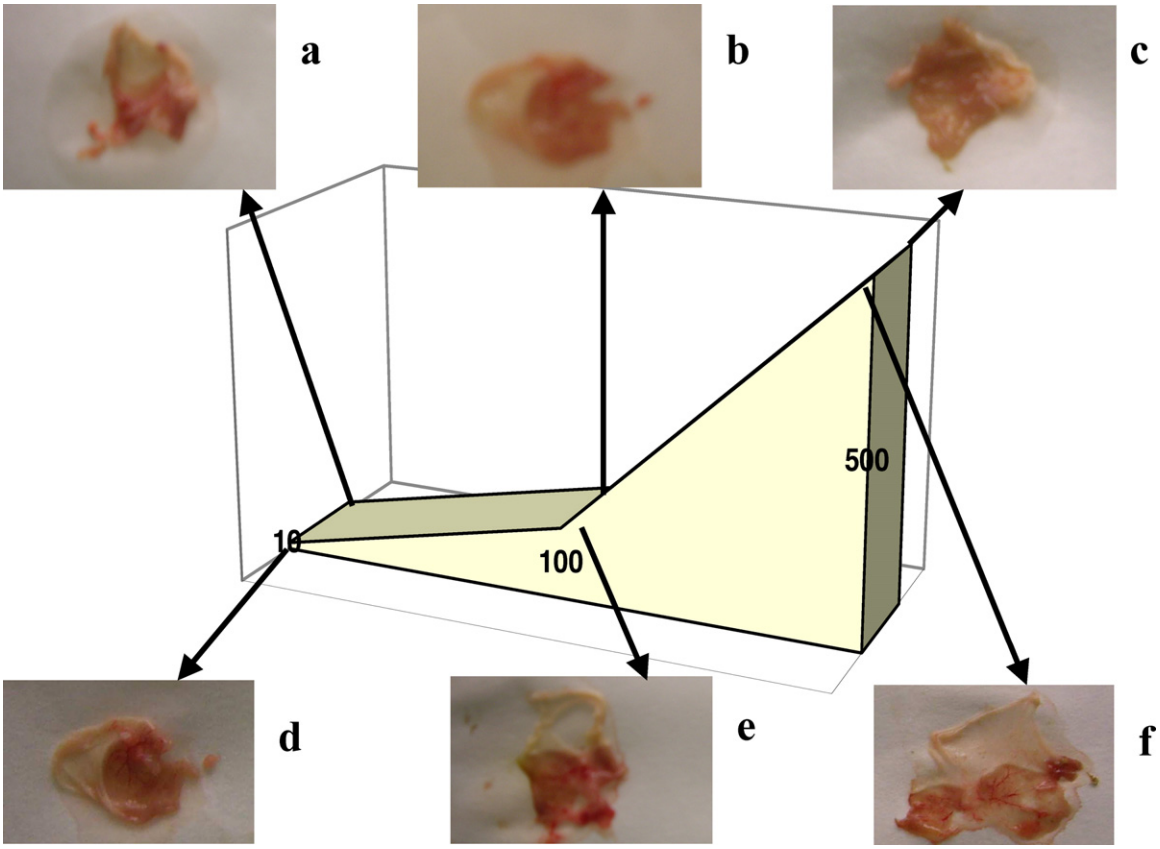

Fig. 7. Optical photographs of stomach samples corresponding to oral administration of (a) 10 mg/kg, (b) 100 mg/kg and, (c) 500 mg/kg of L-C PAMgA, and (d) 10 mg/kg, (e) 100 mg/kg and, (f) 500 mg/kg of S-C PAMgA hydrogels.

The results obtained after s.c. administration of both L-C and S-C PAMgA hydrogels showed a dramatically low local parenteral biocompatibility with a significant dose–response relationship found, which was more pronounced for L-C PAMgA. Regarding parenteral biocompatibility of acrylic polymers, controversial results have been described. Some studies have shown good biocompatibility after s.c. administration of different acrylic polymers to rats (Kruft et al., 1997; Rihova et al., 1997; Yan and Gemeinhart, 2005); however, other authors have indicated that acrylic polymers might not be biocompatible by parenteral routes of administration (Emmler et al., 2008).

Biocompatibility of both hydrogels was also evaluated after oral administration at a wide dose range (10–500 mg/kg). At all doses tested no signs of toxicity were observed with all animals surviving the duration of the study. Moreover, there were no significant changes in body weight, food consumption or visual organ examination (liver, spleen, lungs, kidney, stomach and intestine). The same behaviour was obtained by Hicks et al. (1989) after oral administration of a 100 mg/kg dose of sodium polyacrylate polymer to rats. It must be noted that the dose ranges tested in our study are much wider than the one reported by Hicks et al. (1989). Fig. 7 shows optical photographs of stomach samples obtained after oral administration 10, 100 and 500 mg/kg doses of both PAMgA hydrogels. It is well known that polyacrylate polymers exhibit gastric mucoadhesiveness (Peppas et al., 2000). In our case observation of stomach samples after oral administration of such wide dose range shows intact mucosa and normal organ appearances thereby confirming their good oral biocompatibility. It must be noted that this behaviour is obtained also at the highest dose assayed (500 mg/kg) which will imply that even an oral dose of 35 g given to humans will be well tolerated.

These promising results lead us to conclude that both PAMgA hydrogels are non-toxic and biocompatible following oral administration to mice of wide dose ranges, with further biocompatibility studies needed to be conducted in other animal species. Monomers most often used for the synthesis of mucoadhesive polymers include acrylic and methacrylic acids; therefore, the development of non-invasive delivery systems, especially for the oral route, is of utmost importance.

4. Conclusion

We have developed a novel hydrogel of polymagnesium acrylate for controlled drug delivery with two different mesh sizes. S-C PAMgA exhibited good cell viability whereas the slightly poorer results obtained for L-C PAMgA were attributed to its different viscosity. Both hydrogels exhibited low cytotoxicity in cell line NIH-3T3 fibroblasts and interesting properties since no toxic auxiliary agents other than the initiator were added during their synthesis.

Dose-dependent death-related responses occurred for both hydrogels after i.p. administration (5–80 mg/kg). Inflammatory reactions occurred after s.c. administration (5–20 mg/kg), whereas both hydrogels were well tolerated after oral administration of a wide dose range (10–500 mg/kg). These results reveal that both PAMgA hydrogels are good candidates for oral administration due to their *in vitro* biocompatibility and oral non-toxicity at doses up to 500 mg/kg. The promising results obtained after oral administration of the hydrogels together with the fact that their synthesis is simple and inexpensive make them valuable carriers for the development of novel oral pharmaceutical formulations.

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