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Bioeliminable polymeric nanoparticles for proteic drug delivery

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Abstract

Bioeliminable co-polymers based on poly(methacryloylglycylglycine-OH_x-*co*-hydroxypropylmethacrylamide$_y$) were successfully converted into nanoparticles by using the co-precipitation technique. Human serum albumin (HSA) and a modified (β-cyclodextrin were used, respectively, as model protein drug and stabilizer. Nanoparticles were characterized from a dimensional and morphological point of view by means of laser diffraction granulometry and scanning electron microscopy (SEM). The prepared nanoparticles displayed a monomodal diameter distribution in the range of 130 nm, confirmed by SEM micrographs. Protein loading efficiency and drug release kinetics investigations, carried out on bioeliminable nanoparticles loaded with fluoresceinated HSA (HSA-FITC), showed that protein loading is in the range of 60% with a typical time controlled release profile.

In vitro cytotoxicity investigations of the polymer matrices and resulting nanoparticles were carried out by using different assays aimed at the evaluation of the interactions of the materials with cell metabolism and the cell membrane. On the whole, bioeliminable polymers and nanoparticles resulted in high cytocompatibility thus suggesting their suitability for biomedical applications.

Keywords: Bioeliminable polymers; Nanoparticles; Protein delivery

1. Introduction

Therapeutically relevant proteins such as antibodies, cytokines, growth factors and enzymes are playing an increasing role in the treatment of viral, malignant and autoimmune diseases. However, the development and successful application of therapeutic proteins is often impeded by several difficulties, bound to their insufficient stability and shelf life, costly production, immunogenic and allergic potential, as well as poor bioavailability and sensitivity towards proteases (Haag and Kratz, 2006). Nowadays, controlled delivery systems represent the best approach to protein drug administration. In particular, protein drugs can be encapsulated within injectable colloidal polymeric carriers, which can protect them against degradation and ensure their transport and delivery to the specific site of action at a controlled rate (Kubik et al., 2005).

In recent years, the strategy of utilizing nanoparticles as a carrier system for cell specific targeting and delivery of drugs and active agents has gained increasing interest.

Nanoparticles are sub-micron sized polymeric colloidal particles with a therapeutic agent of interest encapsulated within their polymeric matrix or adsorbed or conjugated onto the surface (Brigger et al., 2002; Bala et al., 2004; Labhasetwar, 1997). Polymeric nanoparticles constitute a versatile drug delivery system, which can potentially overcome physiological barriers and guide the drugs to specific cells or intracellular compartments. It also allows for controlling the release pattern of drug and sustaining drug levels for a long time by appropriately selecting the polymeric carrier (Vasir and Labhasetwar, 2005).

Water-soluble polymers such as *N*-(2-hydroxypropyl)-methacrylamide (HPMA) co-polymers are frequently employed as drug carriers because of their ability to improve the solubility of hydrophobic compounds, reduce non-specific toxicity, and increase the therapeutic index of low molecular weight

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anticancer drugs (Putnam and Kopecek, 1995; Kopecek et al., 2000). HPMA is however not biodegradable and consequently the molecular masses of HPMA co-polymers have been limited to 40 kDa or below to ensure eventual renal elimination (Vincent and Duncan, 2006).

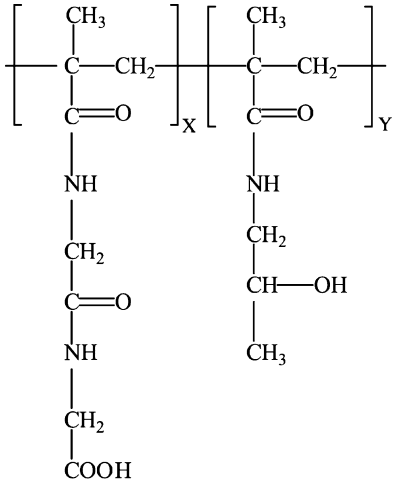
Anticancer drugs chemically bound to water-soluble polymeric carriers such as HPMA co-polymers (polymeric prodrugs) have exhibited decreased systemic toxicity, a result of the altered biodistribution of polymer-bound drugs as compared to free drugs (Nori et al., 2003). Several drug-polymer conjugates based on HPMA co-polymers have been studied clinically. A doxorubicin-(HPMA co-polymer) conjugate, known as PK1, was the first drug-polymer conjugate to enter clinical trials (Vasey et al., 1999). Targeted HPMA co-polymer-bound doxorubicin conjugates have previously been shown to have a significant anti-tumor effect *in vitro* and *in vivo* (Jelinkova et al., 1998; Ulbrich et al., 1996) and have shown greater potency than free doxorubicin in the treatment of ovarian cancer *in vivo* and *in vitro* (Malugin et al., 2006). Recently authors have reported the first endocrine-chemotherapy combination in the form of the model compound HPMA co-polymer-aminoglutethimide-doxorubicin (Duncan et al., 2005).

The objective of the present study was to investigate the suitability of a set of bioeliminable co-polymers based on poly (methacryloylglycylglycine-OH_x-*co*-hydroxypropylmethacrylamide$_y$) (Scheme 1) as polymer matrices for nanoparticle preparation. The co-precipitation technique was chosen as the method of nanoparticle formulation and opportunely adjusted for this type of materials. Human serum albumin (HSA) was used as protein model drug and both its loading efficiency and release profile were carefully investigated using the fluorescein isothiocyanate conjugate (HSA-FITC). Moreover, a careful *in vitro* biological investigation of the cytotoxicity of co-polymers and resulting nanoparticles was carried out by mean of different types of assay.

2. Materials and methods

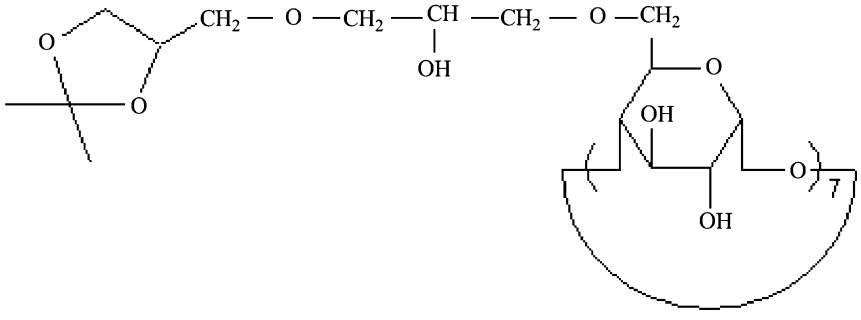
2.1. Materials

Four co-polymers poly (methacry l oy lgly cylgly cine-OH_x-*co*-hydroxypropylmethacrylamide$_y$) [poly(Gly_x-*co*-$HPMA_y$)] containing different proportion of the two comonomers with



poly(Gly_x-*co*-$HPMA_y$)

poly(methacryloylglycylglycine-OH_x-*co*-hydroxypropylmethacrylamide$_y$)



GIG-βCDx

O-Glycidyl-*O*-*iso*propylidenglycerol grafted β-cyclodextrin

Scheme 1. Structures of the materials used in the formulation of nanoparticles.

a molecular weight in the range of 40 kDa were supplied by Polymer Laboratories Ltd. (UK).

l-*O*-glycidyl-2,3-*O*-isopropylidenglycerol-(β-cyclodextrin (GIG-(βCD) was prepared at Polymer Laboratories Ltd. according to a formerly established synthetic protocol (Solaro et al., 1993; Chiellini et al., 1992).

Human serum albumin was kindly provided by Kedrion SpA (Castelvecchio Pascoli, Italy).

Fluoresceinated human serum albumin containing nine fluorescein isothiocyanate groups per HSA molecule was purchased from Sigma–Aldrich.

Cell line 3T3/BALB-c Clone A31 mouse embryo fibroblast (CCL163) was purchased from American Type Culture Collection (ATCC) and propagated as indicated by the supplier.

Dulbecco's Modified Eagles Medium (DMEM), 0.01 M pH 7.4 phosphate buffer saline without Ca^{2+} and Mg^{2+} (PBS), bovine calf serum (BCS), glutamine and antibiotics (penicillin/streptomycin) were purchase from GIBCO Brl. Cell proliferation reagent WST-1 and Lactate dehydrogenase cytotoxicity detection kit (LDH) were purchased from Roche Diagnostic. *In vitro* toxicology assay kit Neutral Red based was purchased from Sigma Chemicals.

For the *in vitro* release studies, dialysis membranes (Spectra/Por, Float-A-Lyzer, diameter 10 mm, M_W cut-off 100 kDa) were purchased from Spectrum Laboratories Inc. (USA).

2.2. Preparation of nanoparticles (NPs)

2.2.1. HSA loaded NPs

The preparation of protein-containing NPs was performed by controlled co-precipitation according to a general procedure (Chiellini et al., 2001). Data relevant to individual experiments are summarized in Table 1, whereas a typical experiment is described as follows. A solution of 12.5 mg of poly (Gly_{25}-*co*-$HPMA_{75}$) in 2 ml of 1:4 water/ethanol mixture was added by a syringe equipped with 22G needle to a solution of 5 mg HSA and 7 mg of GIG-(βCD in 5 ml of bi-distilled water kept under magnetic stirring at room temperature. The nanoparticle suspension was stored at 4 °C.

2.2.2. HSA-FITC loaded NPs

Nanoparticle dispersions containing a fluorescent marker were prepared as reported in Table 1, whereas a typical experiment is described as follows: a solution of 12.5 mg of poly (Gly_{25}/$HPMA_{75}$) in 2 ml of 1:4 water/ethanol mixture was added to 5 ml of an aqueous solution of 4.5 mg HSA, 0.5 mg HSA-FITC and 7 mg of GIG-βCD under magnetic stirring at room temperature. In order to guarantee for an uniform marker distribution within the nanoparticles, HSA-FITC was mixed with HSA aqueous solution prior to the addition of the polymer solution. Preparation was carried out in the dark and nanoparticle suspension was stored at 4 °C.

2.3. Purification of NPs

The nanoparticle suspension was placed in a polypropylene conical tube and centrifuged at 8000 × *g* for 30 min at 4 °C by using an ALC PK121R refrigerated centrifuge. The resulting pellet was suspended either in distilled water, pH 7.4 phosphate buffer saline (PBS) or cell growth medium (DMEM).

2.4. Evaluation of HSA-FITC incorporation into NPs.

The extent of HSA-FITC incorporated into NPs was evaluated by spectrofluorometrical analysis based on the standard curve obtained from HSA-FITC alone, with excitation and emission wavelengths of 485 and 535 nm, respectively.

HSA loaded nanoparticle suspensions and HSA-FITC loaded nanoparticle suspensions were purified by centrifugation at 8000 × *g* for 30 min at 4 °C. The resulting pellets were frozen at −20 °C and then freeze-dried in a 5Pascal Lio 5P lyophilizator. Nanoparticle powder was re-dispersed in an appropriate vol-

Table 1
Formulations of HSA and HSA-FITC loaded nanoparticles

Sample	Polymer[a]		GIG-βCD[b] (mg)	HSA[b] (mg)	HSA-FITC (mg)
	Type	(mg)			
A	p(Gly_{25}-*co*-$HPMA_{75}$)	12.5	15	5	–
B	p(Gly_{25}-*co*-$HPMA_{75}$)	12.5	7	5	–
B_{FITC}	p(Gly_{25}-*co*-$HPMA_{75}$)	12.5	7	4.5	0.5
C	p(Gly_{25}-*co*-$HPMA_{75}$)	12.5	15	10	–
D	p(Gly_{25}-*co*-$HPMA_{75}$)	12.5	7	10	–
E	p(Gly_{50}-*co*-$HPMA_{50}$)	25	30	10	–
F	p(Gly_{50}-*co*-$HPMA_{50}$)	25	30	5	–
G	p(Gly_{50}-*co*-$HPMA_{50}$)	12.5	15	5	–
H	p(Gly_{50}-*co*-$HPMA_{50}$)	12.5	7	5	–
HFITC	p(Gly_{50}-*co*-$HPMA_{50}$)	12.5	7	4.5	0.5
I	p(Gly_{50}-*co*-$HPMA_{50}$)	12.5	15	10	–
L	p(Gly_{50}-*co*-$HPMA_{50}$)	12.5	7	10	–
M	p(Gly_{100})[c]	12.5	7	5	–
N	p(Gly_{100})[c]	12.5	7	10	–

[a] Dissolved in 2 ml, 80% ethanol solution.
[b] Dissolved in 5 ml of water.
[c] Dissolved in 2 ml, 50% ethanol solution.

ume of PBS and maintained under magnetic stirring at 4 °C until complete dissolution. The samples (100 μl) and the standards of HSA-FITC were placed into a 96 well polystyrene microplate and analyzed with Multilabel Plate Readers (Victor^3, Perkin-Elmer). All measurements were performed in triplicate.

2.5. In vitro HSA-FITC release studies

Release kinetics studies were performed on HSA-FITC loaded NPs by using Spectrapor cellulose ester membrane with a MWCO of 100,000 Da.

HSA loaded and HSA-FITC loaded lyophilized NPs were suspended in an appropriate volume of PBS and then placed into dialysis tubes. The samples were dialysed against 200 ml of pH 7.4 0.01M PBS at 37 °C. At specific time intervals whole external medium was replaced with the same volume of fresh PBS. The concentration of the released HSA-FITC into PBS was determined by spectrofluorometrical analysis with excitation and emission wavelengths of 485 and 535 nm, respectively. All measurements were performed in triplicate.

2.6. Biological tests

2.6.1. Cell line

Cytotoxicity evaluations of polymers and relative NPs were carried out using the 3T3/BALB-C Clone A31 cell line. Cells were grown in DMEM containing 10% Bovine Calf Serum BCS, 4 mM glutamine and 100 U/ml:100 μg/ml penicillin:streptomycin (complete DMEM).

2.6.2. Subculturing

A 25-ml flask containing exponentially growing 3T3 cells was observed under an inverted microscope for cell confluence. The complete DMEM media was then removed, and cells were rinsed for a few minutes with PBS. The buffer solution was removed, and cells were incubated with 0.5 ml of trypsin/EDTA solution at 37 °C in 5% CO_2 incubator for 5 min or until the monolayer started to detach from the flask. Cells were suspended in an appropriate volume of DMEM at a split ratio of 1:6 or 1:10 in a 75 ml flask.

2.6.3. Determination of IC_{50} of soluble polymers and NPs

For the determination of the IC_{50} (50% inhibitory concentration, that is the material concentration at which 50% of cell death in respect to the control is observed) of bioeliminable polymers and relative NPs a subconfluent monolayer of 3T3 fibroblast was trypsinized using a 0.25% trypsin, 1 mM EDTA solution, centrifuged at 200 × *g* for 5 min, re-suspended in growth medium and counted. Appropriate dilution was made in order to obtain 3×10^3 cells per 100 μl of medium, the final volume present in each well of a 96 well plate. Cells were incubated at 37 °C, 5% CO_2 for 24 h until 60–70% confluence was reached. The medium from each well was then removed and replaced with medium containing a different concentration (1–10 mg/ml) of polymeric materials or nanoparticles. Control cells were incubated with fresh growth medium and wells containing only growth medium were used as blanks. After 24 h of incubation with medium containing the polymeric sample, cells were analyzed for viability with Cell Proliferation Reagent WST-1, Neutral Red Uptake and Lactate Dehydrogenase Release (LDH) assays, respectively.

2.6.4. Cell proliferation assays

Quantitative proliferation of cells exposed to polymers and NPs was evaluated by means of three different assays.

2.6.4.1. WST-1 cell proliferation assay. Cells were incubated with WST-1 reagent diluted 1:10 (as indicated by the manufacturer) for 4 h at 37 °C, 5% CO_2. Plates were then analyzed with a Biorad Microplate Reader. Measurements of formazan dye absorbance were carried out at 450 nm, with the reference wavelength at 620 nm.

2.6.4.2. Neutral Red Uptake. Cells were incubated with Neutral Red diluted 1:10 (as indicated by the manufacturer) for 2 h at 37 °C 5% CO_2. Medium containing the vital dye was then removed and cells were briefly washed with PBS. The incorporated dye was then liberated from the cells in an acidified ethanol solution. An increase or decrease in the number of cells or their physiological state results in a concomitant change in the amount of dye incorporated by the cells in the culture. Plates were then analyzed with a Biorad Microplate Reader. Measurements of Neutral Red absorbance were carried out at 540 nm, with the reference wavelength at 620 nm.

2.6.4.3. Lactate Dehydrogenase Release. Briefly, 100 ml of polymer and nanoparticle solution samples were re-suspended in 1% serum DMEM medium, added with 100 μl of fresh 1% serum DMEM medium and the resulting solution was placed in contact with cells for 24 h. Then 100 μl of reagent solution (diaphorase/NAD+ catalyst, tetrazolium salt INT and sodium lactate) was added to each well and the plate was incubated at room temperature for 30 min. The plate was then analyzed with a Biorad Microplate Reader. LDH quantification was performed by measuring formazan formation at 490 nm and using 620 nm as reference wavelength. A 1% serum DMEM medium was used as blank (200 μl per well) whereas DMEM containing 2% Triton X-100 (200 μl per well) was used as the positive control.

All measurements were performed in triplicate.

2.7. Methods

2.7.1. Granulometry in suspension

Dimensional analyses were carried out using a Coulter LS230 Laser Diffraction Particle Size Analyzer, equipped with *small volume module plus*. Nanoparticle suspensions were added into the cell until 30–50% obscuration of PIDS detector was reached. Deionised water was used as background and diameter distribution was processed using the Fraunhofer optical model. Three runs were performed on each sample.

2.7.2. Morphological analysis

Nanoparticle morphology was investigated by means of scanning electron microscopy (SEM), using a JEOL LSM5600LV scanning electron microscope. The nanoparticle samples were

purified by centrifugation and the resulting pellets were re-suspended in deionized water and lyophilised. Gold sputtering was performed before SEM analysis.

2.7.3. Microscopy

For routine culturing and qualitative evaluation of morphology, cells were analyzed under an inverted microscope, Nikon Eclipse TE2000-U.

3. Results and discussion

3.1. Preparation of nanoparticle suspensions

Hydroxypropyl methacrylamide based co-polymers are a class of biomedical polymers obtainable through straightforward synthetic pathways. They can be tailored to their final specific applications by modulating the choice of co-monomers and are amenable to bio-functionalization with biological targeting moieties thanks to the presence of reactive functional groups.

One of the main interests of the present research was to investigate the possibility of preparing nanoparticles based on poly (methacryloylglycylglycine-OH_x-*co*-hydroxypropylmethacrylamidey) [poly(Gly_x-*co*-$HPMA_y$)] bioeliminable co-polymers by using the co-precipitation technique.

Four different poly(Gly_x-*co*-$HPMA_y$) co-polymers characterized by different content of the two monomeric units, 1-*O*-glycidyl-2,3-*O*-isopropylidenglycerol-(β-cyclodextrin (GIG-pCD) and human serum albumin were used as synthetic polymer, stabilizer and proteic components, respectively (Scheme 1).

As reported in Table 2, by an appropriate tuning of the formulation ratio between the synthetic polymer, the stabilizer and the protein, it was possible to obtain nanoparticle suspensions using three of the four poly(Gly_x-*co*-$HPMA_y$) co-polymers. In particular co-polymers containing 25, 50 and 100% of methacryloylglycylglycine-OH monomer units resulted in the suitable preparation of nanoparticle by the co-precipitation technique, while the preparation of nanoparticles based on poly(Gly_{10}-*co*-$HPMA_{90}$) failed due to the high aqueous solubility of the polymer. This characteristic does not allow colloid formation during the interaction of the polymer solution with the protein/cyclodextrin aqueous solution.

Table 2
Size characterization and yield of HSA and HSA-FITC loaded nanoparticle suspensions

Sample	Yield[a] (%)	Diameter ± S.D. (nm)
A	30	139 ± 18
B	37	132 ± 16
B_{FITC}	43	132 ± 18
C	52	156 ± 21
D	71	133 ± 17
E	39	110 ± 16
F	22	149 ± 19
G	21	132 ± 16
H	24	134 ± 17
H_{FITC}	28	123 ± 17
I	61	158 ± 22
L	63	138 ± 18
M	25	128 ± 17
N	22	132 ± 18

[a] After purification by centrifugation.

Co-precipitation is an original technique developed in our laboratory and optimized for a set of bioerodible hemiesters of alternating co-polymers of maleic anhydride and alkyl vinyl ethers (Chiellini et al., 2001, 2006) that relies on the use of mixtures of readily available proteins, such as albumin, with synthetic polymers that are sparingly soluble in water and contain carboxylic groups able to form ionic bonds with proteins. Briefly, co-precipitation is based on the dropwise addition of an organic polymer solution to an aqueous protein solution under gentle magnetic stirring; the progressive interaction between the two components giving rise to the formation of nanoparticles suspension. In comparison to similar methods for nanoparticle preparation (Fessi et al., 1998), the co-precipitation method does not apply the use of chlorinated solvents or energetic mixing, which are both known to cause appreciable protein denaturation. This method turned out to be particularly suitable for the preparation of nanocarriers for protein drugs or bioactive agents (Chiellini et al., 2006; Pawar et al., 2004).

Nanoparticle dispersions containing a fluorescent marker were prepared by using a mixture of HSA with human serum albumin fluorescein isothiocyanate, a modified HSA characterized by an intense green fluorescence. The introduction of the fluorescent marker did not alter the formation of nanoparticles (Table 2) and it was employed for the evaluation of protein release kinetics as well as for the determination of drug content and encapsulation efficiency of the nanoparticles.

Nanoparticle suspensions were purified by centrifugation at $8000 \times g$ for 30 min in order to remove the polymeric material and stabilizer not incorporated into nanoparticles and the obtained pellets were lyophilized. This procedure afforded 30–35% yield of purified nanoparticles having the same composition as the formulation feed. Scanning electron microscopy of the purified and lyophilized solid pellet showed a homogeneous distribution of spherical nanoparticles (Fig. 1), thus confirming, as reported in previous studies, that the use of functionalized cyclodextrins in nanoparticle preparations help to maintain their integrity during the purification procedure (Chiellini et al., 2006).

Dimensional analysis carried out by light scattering evidenced that, independent of the type of polymer matrices employed, nanoparticle dispersions containing either HSA or HSA-FITC were constituted by monodispersed nanoparticles having 120–160 nm average diameter (Fig. 2).

3.2. Determination of HSA-FITC loading, encapsulation efficiency and release kinetics

Studies aimed at setting up a procedure for the preparation of bioeliminable polymeric nanoparticles loaded with therapeutically active proteins (protein drugs) were carried out using fluoresceinated HSA as the model biomolecule. Evaluation of

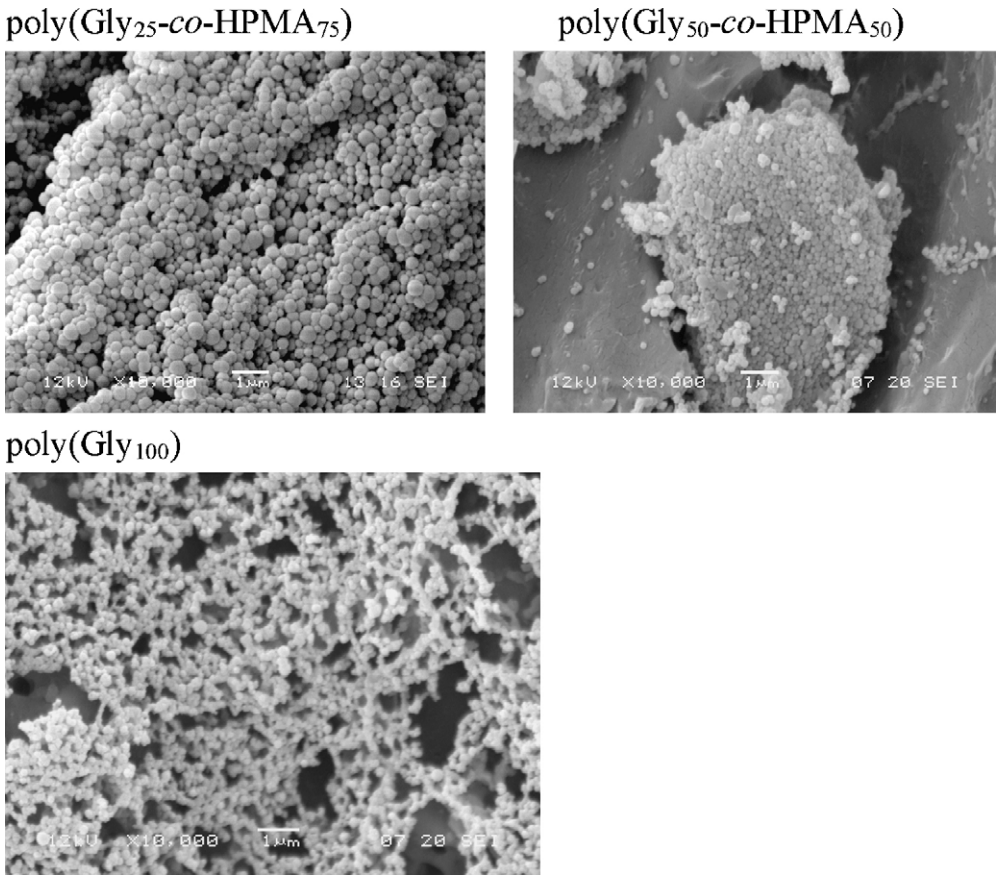


Fig. 1. Scanning electron micrograph of poly(Gly_x-*co*-$HPMA_y$) based nanoparticles.

loading and encapsulation efficiency of the above mentioned model protein, was carried out on NPs based on p(Gly_{25}-*co*-$HPMA_{75}$) as the bioeliminable polymer matrix using the formulation conditions reported in Table 1. This preparation was chosen since, on the basis of the cytotoxicity experiments, p(Gly_{25}-*co*-$HPMA_{75}$) showed the lowest toxicity among the polymers used for NPs preparation (Table 3). Results indicate that the employment of co-precipitation techniques allows for the preparation of bioeliminable polymeric nanoparticles with a protein loading in the range of 10% and an encapsulation efficiency of 60%.

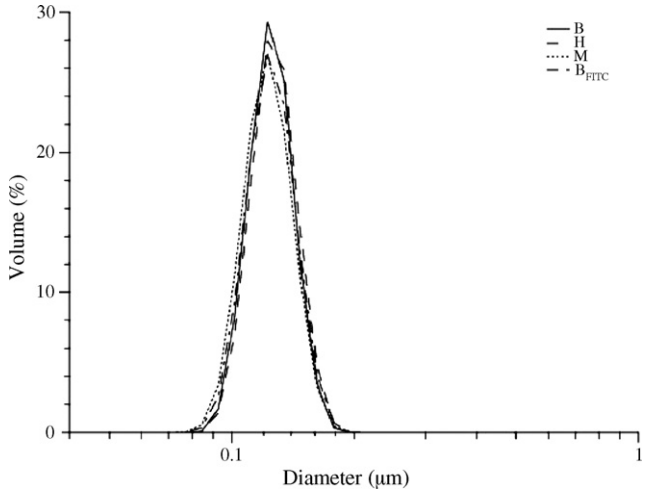


Fig. 2. Diameter distribution of HSA and HSA-FITC loaded nanoparticles based on poly(Gly_{25}-*co*-$HPMA_{75}$) [B], poly(Gly_{50}-*co*-$HPMA_{50}$) [H], poly(Gly_{100}) [M] and poly(Gly_{25}-*co*-$HPMA_{75}$) [B_{FITC}].

The release kinetics studies were performed by using the dialysis bag diffusion technique on nanoparticles based on poly(Gly_{25}-*co*-$HPMA_{75}$) loaded with HSA-FITC and on HSA-FITC alone, used as the reference. The experimental conditions were set up in order to reproduce the physiological environment in terms of temperature, pH and salts concentration. A 0.01 M PBS pH 7.4 was chosen as dialysis medium because of its buffer strength, which compensates for the reduction in pH during NP erosion and subsequent dissolution.

The *in vitro* drug release profile of HSA-FITC from NPs and the diffusion of the protein through the dialysis membrane are

Table 3
IC_{50} values of bioeliminable polymers

Polymer	$IC_{50} \pm$ S.D. (mg/ml)		
	WST-1	NR	LDH
Poly(Gly_{10}-*co*-$HPMA_{90}$)	7.38 ± 0.41	5.30 ± 0.29	ND[a]
Poly(Gly_{25}-*co*-$HPMA_{75}$)	7.24 ± 0.40	4.40 ± 0.25	ND
Poly(Gly_{50}-*co*-$HPMA_{50}$)	3.24 ± 0.21	3.06 ± 0.17	8.35 ± 0.53
Poly(Gly_{100})	4.36 ± 0.24	5.06 ± 0.30	8.30 ± 0.49

IC_{50} values were calculated by probit analysis ($P < 0.05$, χ^2-test).
[a] ND, not detected.

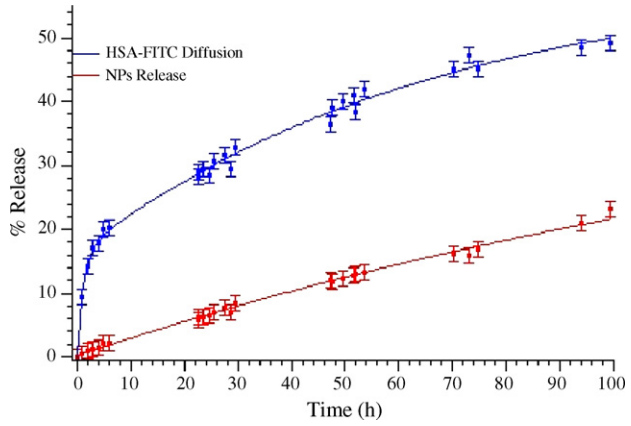


Fig. 3. HSA-FITC release from nanoparticles and HSA-FITC diffusion through the dialysis membrane.

shown in Fig. 3. HSA-FITC release from NPs was constant during the first 100 h during which up to 20% of total drug content is released. It is evident that the diffusion step is faster than the release process and it does not affect the release kinetics profile. Satisfactorily as expected for a controlled drug release system, the release of protein from the prepared bioeliminable NPs was time-controlled and did not display an initial burst step.

3.3. *In vitro* biological assays

In order to evaluate the biocompatibility of bioeliminable polymers and their resulting nanoparticles, *in vitro* experiments were carried out using the balb 3T3 mouse embryo fibroblasts clone A31 cell line, following the procedures of ISO 10993 – Part 5: "Test for cytotoxicity – *in vitro* methods".

Cytotoxicity, defined as the "in *vitro* evaluation of toxicological risks using cell culture", is a rapid, standardized, sensitive and inexpensive way to assess the *in vitro* biocompatibility of materials to be used in biomedical applications. Assays deal with the assessment of various aspects of cellular function such as cell viability and proliferation, loss of membrane integrity, reduced cell adhesion, biosynthetic activity and altered cell morphology (Chiellini et al., 2006). Moreover, the information gained from these types of investigations may be used in the design of further *in vivo* experiments.

All of the four poly(Gly_x-*co*-$HPMA_y$) polymers displayed a suitable solubility in Dulbecco's Modified Eagles Medium, thus allowing for the investigation of their IC_{50}.

Poly(Gly_x-*co*-$HPMA_y$) were dissolved in DMEM at different concentrations ranging between 1 and 10 mg/ml. Cells were incubated for 24 h with polymer solutions and then tested for cell viability and proliferation using WST-1 tetrazolium salt, Neutral Red Uptake and LDH assays. The obtained results, reported in Table 3, predominantly indicate a high level of cytocompatibility for all of the four investigated polymers. Co-polymers containing 10 and 25% of methacryloylglycylglycine-OH monomer displayed high IC_{50} values evaluated both with WST-1 and with Neutral Red assays. Moreover, data obtained from the LDH assay showed that further to the incubation of cells with poly (Gly_{10}-*co*-$HPMA_{90}$) and (Gly_{25}-*co*-$HPMA_{50}$) no membrane-damage is detected.

Nanoparticles prepared with poly(Gly_{25}/$HPMA_{50}$), (Gly_{50}/$HPMA_{50}$) and poly(Gly_{100}) were also submitted to cytotoxicity evaluation using WST-1 assay at a final concentration of 5 mg/ml. In accordance with the results obtained for the bioeliminable polymeric materials, all the prepared nanoparticle samples resulted to be fully cytocompatible, with values of cell viability in the range of 100% in respect to the control.

4. Conclusions

Bioeliminable co-polymers poly(methacryloylglycylglycine-OH_x-*co*-hydroxypropylmethacrylamide$_y$) can be successfully converted into nanoparticles by an appropriate tuning of the co-precipitation technique, a versatile method that appears advantageous for the preparation of nanocarriers loaded with non-conventional drugs such as proteins and peptides. HSA loaded nanoparticles display a monomodal distribution with an average size of 130 nm and a significant encapsulation efficiency. Protein release kinetics performed in physiological conditions indicate that with the employment of the prepared nanostructured systems, it is possible to achieve a controlled release of the loaded protein model drug. A careful *in vitro* investigation of the cytotoxicity of polymeric matrices and the resulting nanoparticles shows their high cytocompatibilities, thus indicating the suitability of the prepared nanosystems as carriers for controlled release of protein drugs. The *in vitro* evaluation of cell fate of the prepared nanoparticles performed by confocal laser scanning microscopy will constitute the subject of a forthcoming paper.

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