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ORIGINAL ARTICLE

In vitro release modulation and conformational stabilization of a model protein using swellable polyamidoamine nanosponges of β-cyclodextrin

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Abstract New swellable cyclodextrin-based poly(amidoamine) nanosponges, named PAA-NS10 and PAA-NS11, were synthesized by crosslinking β-cyclodextrin with either 2,2-bisacrylamidoacetic acid or with polyamidoamine segments deriving from 2,2-bisacrylamidoacetic acid and 2-methylpiperazine, respectively. Water uptake studies showed a tremendous swelling capacity of both nanosponges, forming hydrogels. Time dependent swelling experiments in various aqueous media showed that the nanosponge hydrogels were stable over a period of at least 72 h maintaining their integrity. Thermal analysis showed that the two nanosponges were stable up to 250 and 300 °C, respectively. Both PAA-NS10 and PAA-NS11 were converted to aqueous nanosuspensions using the High Pressure Homogenization technique. Bovine serum albumin (BSA) was used as model protein to study the encapsulating capacity of these new β-cyclodextrin-based PAA-nanosponges. High protein complexation capacity was observed, as confirmed by UV spectroscopy. BSA encapsulation efficiency was greater than 90% on w/w basis for both nanosponges. In vitro BSA release studies were carried out showing a prolonged release of albumin from the two swollen BSA loaded β-CD PAA-NS over a period of 24 h.

Keywords Swellable nanosponges · Cyclodextrin · Poly(amidoamine) nanosponges · Water uptake · Bovine serum albumin · Prolonged release

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Introduction

Long term stability is a critical point in the successful development of pharmaceuticals, including macromolecular ones like proteins [1]. As protein solutions are generally not stable, they are stored in the lyophilized state. However, proteins can reversibly (or sometimes, even irreversibly) denature upon lyophilization and consequently adopt conformations markedly distinct from the native ones. Thus a major obstacle in protein formulation development is the maintenance of the native protein structure both during the formulation process and upon the long-term storage [2]. 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 are often limited by several drawbacks, due to their insufficient stability, costly production, immunogenic and allergic potential, as well as poor bioavailability and sensitivity towards proteases [3]. An approach recently adopted is the encapsulation of proteins within polymeric

carriers, which can protect them against degradation and ensure their transport and delivery [4]. Polymeric nanoparticles constitute versatile drug delivery systems, which can potentially overcome physiological barriers and could control the release of actives to specific cells or intracellular compartments [5–8].

Polyamidoamines (PAAs) are synthetic polymers obtained by Michael type stepwise polyaddition of amines to bisacrylamides (Fig. 1) carrying ter-amino and amido groups regularly arranged along their polymer chain [9–11].

They are normally biodegradable and biocompatible, with cell toxicity two or three orders of magnitude lower than other popular polycations, such as poly(lysine) (PLL) or poly(ethyleneimine) (PEI).

Amphoteric PAAs carrying carboxyl groups as side substituents are easily prepared and nearly as biocompatible as dextran, as in the case of ISA23 (depicted in Fig. 2), studied by R. Duncan and her group in London and Cardiff [12].

Recently we have found that β-cyclodextrin (β-CD) can behave under proper conditions as monomer in stepwise polyadditions. In particular, in water at 20–25 °C and pH $\geq$11 the primary β-CD hydroxyls undergo Michael-type addition to bis-acrylamides. We determined that approximately five hydroxyl groups per β-CD molecule react. This means that β-CD acts as *multifunctional* monomer in stepwise polyadditions to bisacrylamides and can be copolymerised with bis-amines giving directly β-CD-PAA resins. This synthetic method was adopted for preparing the novel swellable PAA nanosponges reported here.

In this work preliminary studies on the characterization of these new types of β-CD-PAA nanosponges, in particular to ascertain their hydrogel nature and water uptake capacity, were carried-out. Moreover the potential of PAA nanosponges for encapsulation, prolonged release and stabilization of proteins was evaluated using bovine serum albumin as model molecule.

Materials

Bovine serum albumin (BSA) and vanadium pentoxide, catalyst used in elemental analysis, were purchased from Sigma-Aldrich (Milan, Italy). β-Cyclodextrin (β-CD) was a kind gift of Waker Chemie (Germany). Swellable β-CD-based PAA-nanosponges were synthesized at the University of Milan. All other chemicals and reagents were of analytical grade. Milli Q water (Millipore) was used throughout the studies.

Methods

Synthesis of swellable PAA nanosponges

The description of the synthetic procedure of β-CD based PAA nanosponges is beyond the scope of this paper and the details are reported in a previous patent [13]. Briefly, β-cyclodextrins were crosslinked with 2,2-bisacrylamido acetic acid (BAC) in aqueous solution at pH >11, using lithium hydroxide and under a nitrogen atmosphere. This type of PAA nanosponge was nicknamed PAA-NS10. Alternatively, 2-methyl piperazine (2-MP) was used along with BAC. This type of nanosponge was nicknamed PAA-NS11. A brief scheme of these reactions is given in Fig. 3a and b.

FTIR of PAA-NS

PAA-NS10: 701, 754 cm^{-1} (δ_w sec-amide NH), 1,028 cm^{-1} (ν aliphatic C–N), 1,082–1,327 cm^{-1} (ν C–O–C), 1,634 cm^{-1} (ν sec-amide C=O, ν C=C), 2,926 cm^{-1} (ν asymmetric CH_2), 3,422 cm^{-1} (ν OH).

PAA-NS11: 701 cm^{-1} (δ_w sec-NH), 936 cm^{-1} (δ C–O–H out of plane), 1,035 cm^{-1} (ν aliphatic C–N), 1,077–1,369 cm^{-1} (ν C–O–C), 1,525 cm^{-1} (δ sec-amide NH, ν C–N), 1,666 cm^{-1} (ν sec-amide C=O, ν C=C), 2,921 cm^{-1} (ν asymmetric CH_2, ν CH_3 symmetric C–H), 3,431 cm^{-1} (ν OH).

Nanosponge size reduction using high pressure homogenization technique

A 2% w/v aqueous suspension of the two swellable β-CD-PAA nanosponges (PAA-NS) were homogenized with an Ultra Turrax for 10 min at 24,000 rpm. These homogenized suspensions were then transferred into a high

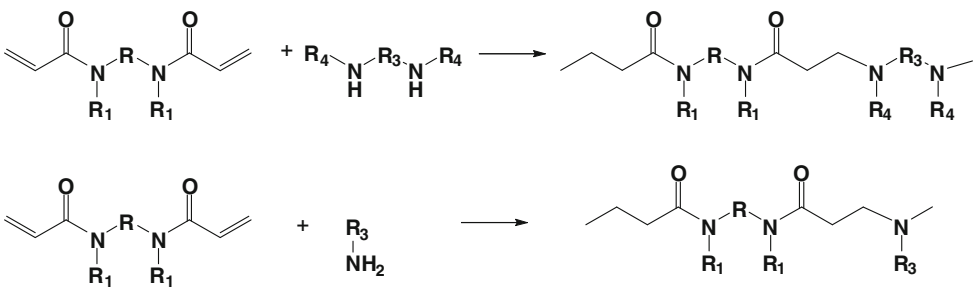


Fig. 1 Scheme of the synthesis of Polyamidoamines (PAAs)

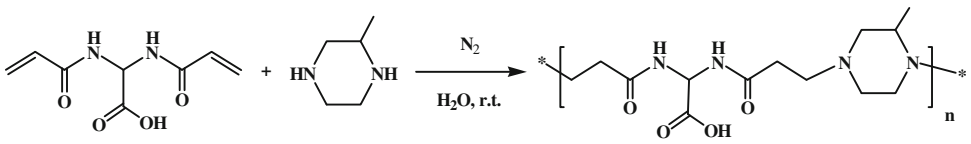


Fig. 2 Synthesis and structure of the PAA named ISA23

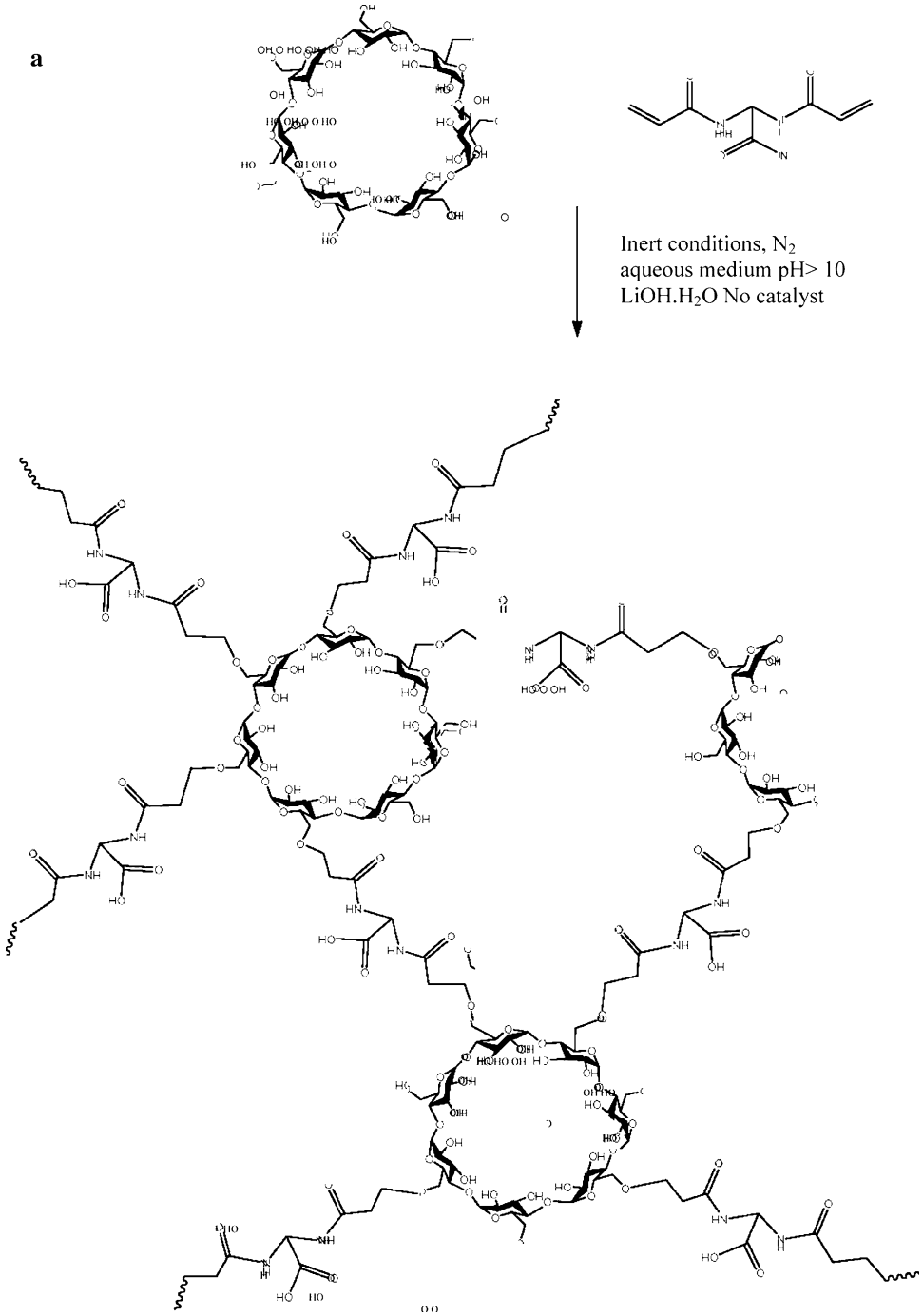
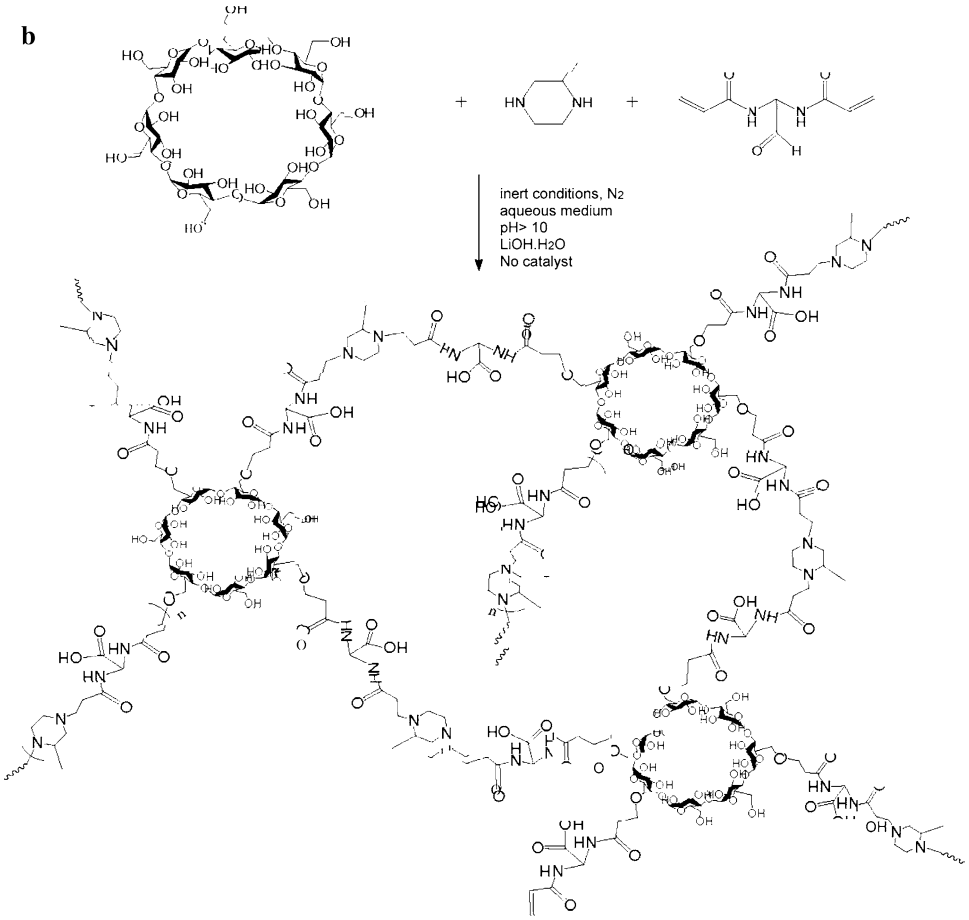


Fig. 3 **a** Scheme for synthesis of PAA-NS10. **b** Scheme for synthesis of PAA-NS11

pressure homogenizer (Emulsiflex C5, Avestin) and subjected to 12 cycles of homogenization, that is, 5 cycles at 5,000 psi for 5 min, followed by 5 cycles at 7,000 psi for 5 min and 2 cycles at 5,000 psi in a recirculation mode. The PAA-NS aqueous nanosuspensions obtained were used for characterization and protein complexation studies. They could be safely stored in a refrigerator at 4 °C without aggregation phenomena.

Fig. 3 continued



Characterization of swellable PAA-NS

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) analysis was performed, using a Perkin Elmer system 2000 spectrophotometer, to understand if there are some interaction between BSA and NS and also to characterize NS. The spectra were obtained using KBr pellets in the region from 4,000 to 650 cm^{-1}.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) was carried out using a Perkin Elmer DSC/7 differential scanning calorimeter (Perkin-Elmer, CT, USA) equipped with a TAC 7/DX instrument controller. The instrument was calibrated with indium for melting point and heat of fusion. A heating rate of 10 °C/min was employed in the 25–300 °C temperature range. Standard aluminum sample pans (Perkin-Elmer) were used; an empty pan was used as reference standard. Analyses were performed in triplicate on 5 mg samples under nitrogen purge.

Transmission electron microscopy

Transmission electron microscopy (TEM) analysis was employed to evaluate the particle shape and sizes of PAA-NS. A Philips CM 10 transmission electron microscope was used and the particle size was measured using the NIH image software. The nanosponge aqueous suspensions were sprayed on Formwar-coated copper grid and air-dried before observation.

Elemental analysis

Elemental analyses were performed on Flash EA 1112 series CHNS-O analyser (Thermo electron corporation). The samples were weighed on high precision balance (Sartorius CP2P-least count 0.001 mg) in order to get high reproducibility. The samples were weighed in special tin containers for CHNS analysis and silver containers for

oxygen estimation. Vanadium pentoxide was used as a catalyst.

Particle size analysis and zeta potential measurements of PAA-NS

PAA-NS sizes and polydispersity indices (P.I.) in aqueous suspensions were measured by Dynamic Light Scattering using a 90 Plus particle sizer (Brookhaven Instruments Corporation, USA) equipped with MAS OPTION particle sizing software. The measurements were made at a fixed angle of 90° and at 25 °C for all samples. The NS samples were suitably diluted with filtered distilled water before analysis. Zeta potential measurements were carried out using an additional electrode in the same instrument. For zeta potential determination, samples of the PAA-NS formulations were diluted with 0.1 mM KCl and placed in the electrophoretic cell, where an electric field of about 15 V/cm was applied.

Swelling and water uptake studies

PAA-NS10 and PAA-NS11 were soaked in water and in various media, i.e. saline solution, pH 1.2, pH 4.5 and pH 7.4 buffers, in a graduated cylinder at a concentration of 0.5% w/v. The PAA-NS hydrogels formed were then observed at the following time points: 1, 3, 5, 8, 20, 24, 32, 48, and 72 h. The marking of the hydrogel in the graduated cylinder is noted at these time points and the percentage of swelling (w/v) is calculated as follows:

$$\frac{\text{Marking of the cylinder at specified time point} \times 100}{\text{Initial marking before soaking}} = \text{percentage of swelling}$$

After 72 h, the PAA-NS hydrogels were filtered from the aqueous media and excess fluid gently removed by dry filter paper before weighing of the PAA-NS. The following equation was used to calculate the percent water uptake:

$$\frac{\text{Weight of the hydrogel after 72 h} \times 100}{\text{Initial weight of the dry polymer}} = \text{percentage of water uptake}$$

PAA nanosponge loading with BSA

An aqueous solution of the model protein BSA (5 mg/mL) was incubated at room temperature with the two types of PAA-NS nanosuspensions for 24 h under stirring The PAA-NS aqueous nanosuspensions were then centrifuged at 20,000 rpm to obtain a polymeric residue encapsulating the BSA. The PAA-NS residues were resuspended in water, washed with water and stored at 4 °C for 6 months to determine the stability of the systems over time.

After washing a volume of the two BSA-loaded aqueous nanosuspensions were freeze-dried to obtain dried nanocomplexes for protein loading evaluation. BSA loading in PAA-NS was determined extracting the dry complexes with water by the use of sonication for 30 min at room temperature and measuring the BSA concentration in the supernatant spectrophotometrically at 278 nm using a DU 730 instrument (Beckman).

BSA complexation capacity of PAA-nanosponges

Increasing amounts of PAA-NS (between 0 and 1% w/v) were incubated for 24 h with a fixed solution of BSA (1 mg/mL) and the supernatants obtained after centrifugation at 5,000 rpm for 15 min were analyzed spectrophotometrically at 278 nm using the instrument previously described. The experiments were repeated three times.

In vitro BSA release studies

In vitro BSA release studies were carried out using a dialysis bag containing 2 mL of loaded NS nanosuspension in a beaker equipped with a magnetic stirrer. For the experiments 25 mL were used as the receiving medium stirred at 50 rpm and at a temperature of 37 ± 0.5 °C. At fixed times 500 µl of the receiving medium was withdrawn

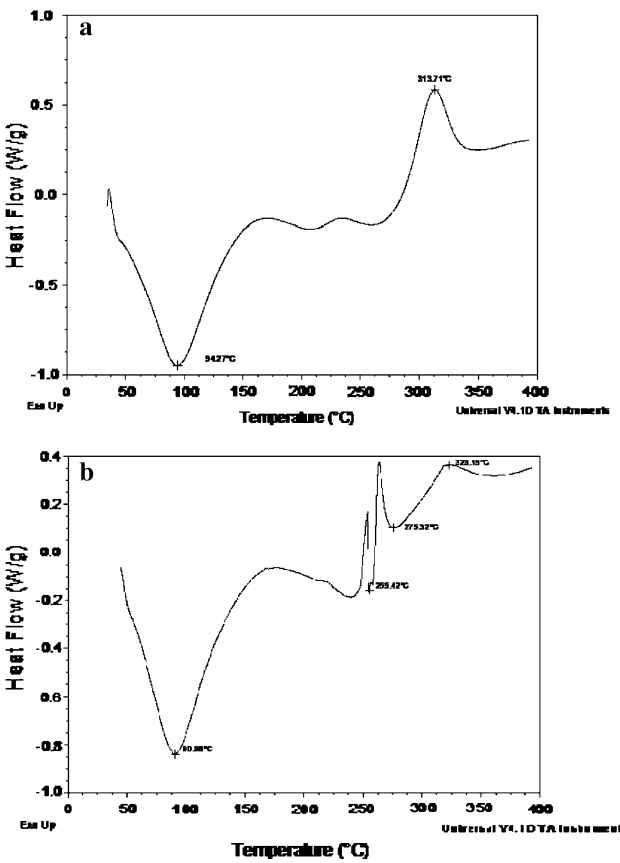


Fig. 4 DSC of **a** PAA-NS10 and **b** PAA-NS11

and analyzed using the UV spectrophotometer at 278 nm with minimal dilution using a narrow walled quartz cuvette with a capacity of 500 µl. The release studies were performed in triplicate both in water and in phosphate buffer pH 7.4 as receiving phases.

BSA conformational stability assessment using sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS gels were used for the assessment of the protein samples using an electrophoretic system consisted of a stacking gel, separation gel, electrode buffer (running buffer) and electrodes. The stacking gel (4% v/v) and the separation gel (10% v/v) were composed of 0.5 M Tris hydrochloride, sodium dodecyl sulphate (SDS) (10%), acrylamide to *N*,*N*′-methylene bisacrylamide ratio of 37.5:1 w/w, ammonium persulphate (10%) and TEMED in a certain proportion. The electrode buffer (500 mL) was prepared using Tris buffer (125 mM), glycine (960 mM) and SDS (0.5%). The pH of the buffer was around 8.3. A constant voltage of 200 mV and a current of 500 mA was applied during the separation course for 30 min. Then the gels were transferred to a container with Blue Comassie (0.004% v/v) in methanol:acetic acid:water (40:10:50, v/v) in a mechanical shaker for 2 h. The gels were carefully removed and treated with a mixture of 30% methanol, 10% acetic acid and 60% water overnight and then added with glycerol before drying.

Samples of BSA-loaded PAA-NS and BSA were prepared at a concentration of 1.5 µg/mL of buffer [Tris HCl (62.5 mM), pH 6.8, SDS (2%), glycerol (10%), 2-mercaptoethanol (5%), blue bromophenol (0.0025%)]. The sample bands were compared with a standard marker protein band.

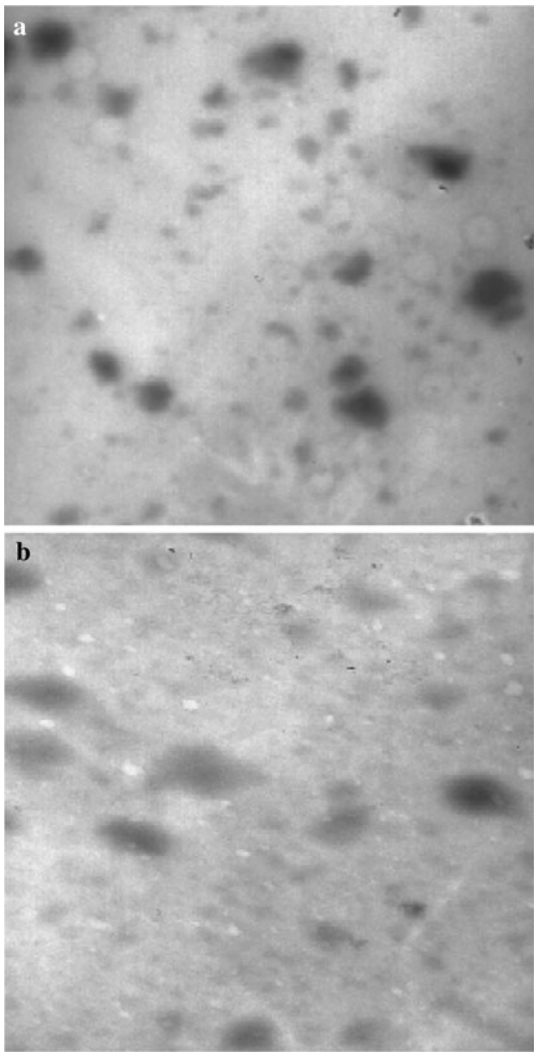


Fig. 5 TEM photomicrographs of **a** PAA-NS10 and **b** PAA-NS11 (magnification ×46,000)

Results and discussion

In this work, new β-CD based PAA nanosponges have been synthesized by crosslinking β-cyclodextrin with either 2,2-bisacrylamidoacetic acid or polyamidoamine segments deriving from 2,2-bisacrylamidoacetic acid and 2-methyl-piperazine (Fig. 3a and b).

These type of β-CD PAA nanosponges belong to a new family of swellable polymers and they are structurally different from the previously reported β-CD based nanosponges obtained using carbonate bonds [14–18].

FTIR studies, whose details are reported in the "Methods" section, confirmed the structure of both NS polymers evidencing the presence of amide groups. The DSC profiles of the two PAA-NS are reported in Fig. 4 showing a different thermal behaviour.

NS10 is thermostable up to 300 °C, as evidenced in the thermograms by the exotherm peak besides the water evaporation peak, while NS11, having a different cross-linking, showed a stability after heating only up to 250 °C.

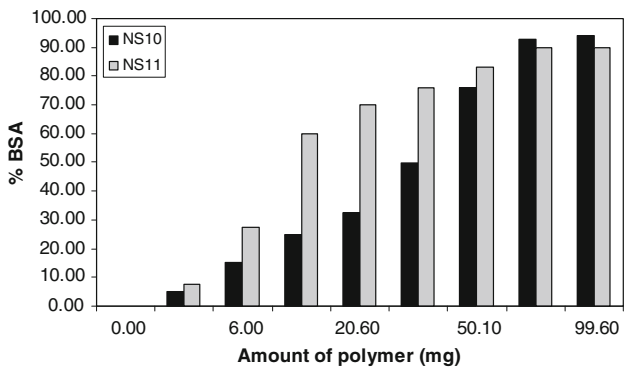


Fig. 6 Complexation studies of BSA with the two types of swellable nanosponges (PAA-NS)

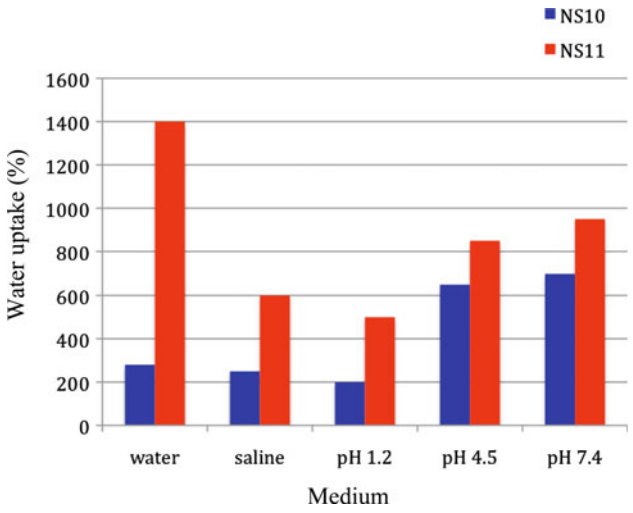


Fig. 7 Water uptake of the two types of PAA-NS in various media

The presence of nitrogen in the PAA-NS was determined by elemental analysis. It was observed that NS11 had more nitrogen content than NS10 due to the presence of 2-methylpiperazine-deriving units in the crosslinking segments.

Both PAA-NS could be reduced to a nanometric size using the high pressure homogenization (HPH) technique, a very effective tool for nanosizing and for producing nanosuspensions in the pharmaceutical field. Twelve cycles of HPH produced colloidal PAA-NS aqueous suspensions with a mean particle size of 502.13 ± 11.2 nm (P.I. = 0.12) and 410.18 ± 14.5 nm (P.I. = 0.10) for PAA-NS10 and PAA-NS11, respectively. The low PI values confirmed

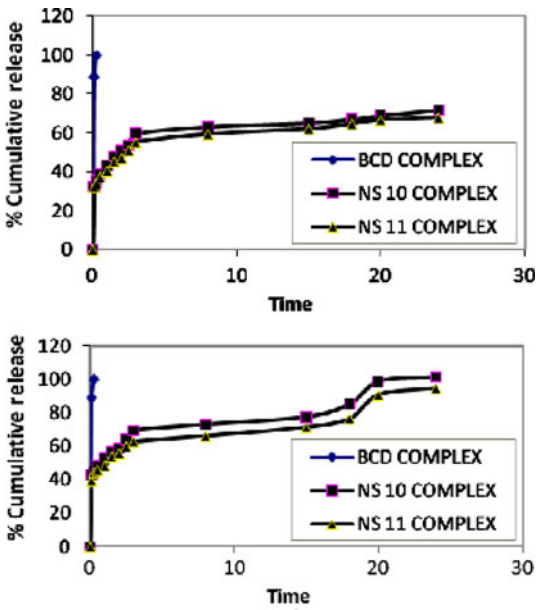


Fig. 8 In vitro BSA release profiles from the two PAA-NS complexes in **a** water and **b** phosphate buffer pH 7.4 compared to the BSA *β*-cyclodextrin complex over time (h)

the narrow size distribution of the two nanosuspensions. TEM analyses (Fig. 5) showed that the homogenized NS10 and NS11 were roughly spherical with an average particle size between about 350 and 500 nm.

The zeta potential values of the two PAA-NS in aqueous suspension were −31.70 ± 1.9 and −35.35 ± 1.4 mV, respectively, which are sufficiently high to avoid aggregation phenomena during storage. Preliminary studies showed that the two types of nanosponges are not hemolytic up to a 2% w/v concentration after incubation for 2 h with erythrocytes (data not shown).

A BSA loading as high as 90 and 92% w/w was achieved after washing for PAA-NS10 and PAA-NS11, respectively. Interaction of cyclodextrins or their polymer derivatives with proteins like BSA have been reported in literature [17], but the BSA high encapsulation efficiency of PAA-NS might be ascribed to the peculiar nanosponge structure.

The sponge-like matrix of PAA-NS with cyclodextrin cavities besides the cross-linked network might favour the BSA loading due to the presence of various binding sites.

BSA complexation studies carried out with the addition of increasing concentrations of PAA-NS were in agreement with the loading results. As the amount of PAA-NS increased from 0 to 1% w/v, the amount of BSA encapsulated increased proportionally reaching a maximum around 0.3% w/v of nanosponge concentration (Fig. 6).

PAA-NS11 in particular shows a greater complexation capacity as compared to PAA-NS10, probably due to the additional nitrogen atom of 2-methylpiperazine and to the presence of a different cross-linked network.

For hydrogels water uptake, hence the swelling degree, is of paramount importance. Various media viz saline, pH 1.2, pH 4.5 and pH 7.4 buffers were used for studying the water uptake capacity of PAA-NS's. The results are shown in Fig. 7.

It may be observed that both samples absorb water to a remarkable extent, but do not exhibit the same behaviour in this respect. In particular, PAA-NS10 shows a lower water uptake than PAA-NS11 under all conditions, which is probably related with its tighter structure with shorter connecting arms between the CD moieties. Moreover, both nanosponges show a marked dependence of water uptake on pH. The water uptake of both PAA-NS is remarkably higher at pH 4.5 and 7.4 than at pH 1.2, probably because the carboxyl group of the BAC moiety, being fairly strongly acidic (pKa 2.5 or whereabouts) [12], is fully ionized above pH 4, but poorly ionized at pH 1.2. This effect is less pronounced for PAA-NS11 because masked, in part, by the ionization at acidic pH of the tert-amine groups of the 2-methylpiperazine moieties. The water uptake capacity of both PAA-NS decreases in saline compared with plain water.

In vitro release studies showed that the BSA release kinetics, after an initial burst effect, are prolonged over time for both the PAA nanosponges (Fig. 8a, b).

After 24 h the BSA release reached about 70% in the experiments in water, while about 100% in phosphate buffer at pH 7.4. The difference in the BSA release profiles might be due to a different stability of PAA-NS with the pH. Poly(amidoamines) can degrade at pH greater than 7.0. Studies are in progress to confirm this hypothesis.

A complex of BSA with parent β-cyclodextrin was prepared for comparison purposes. It was found that the loaded β-cyclodextrin complex had a very fast release rate as compared to the PAA-NS complexes: all the BSA was released in 15 min. The in vitro slow release of BSA incorporated in nanosponges is probably to be ascribed to the cooperation of the cyclodextrin cavities and the PAA network. The chemical and physical nature of the PAA-NS hydrogels obviously governs the diffusion rate of the protein in the surrounding medium. In fact, the release rate of BSA is slower from PAA-NS11 compared with PAA-NS10, possibly due to the more relapsed structure of the former when swollen in water allowing easier adaptation of the bulky protein inside the gel, coupled with the zwitterionic nature of the crosslinking segments, possibly leading to ionic interactions.

Conformational stability of proteins is very important for maintaining their native structure and thus their activity. The BSA bands observed in the polyacrylamide gel for both BSA-loaded PAA-NS samples are very similar and also match with that of the standard BSA. If albumin underwent degradation, its band would be vanished indicating loss of native conformation of the protein. No conformational change of BSA was observed after formulation in PAA-NS and after 6 month storage at 4 °C in nanosponges. During storage BSA could undergo to moisture-induced solid state aggregation leading to conformational changes, due to intermolecular thiol-dilsulfide exchange [19]. It is also known that BSA can degrade over time due to moisture-induced aggregation. PAA-NS encapsulation seems to protect BSA from these types of degradation.

Conclusions

Two types of new swellable β-CD PAA nanosponges have been synthesized and used to produce stable aqueous nanosuspensions by HPH technique. The PAA-NS nanoparticles had a spherical shape, sizes of about 500 nm and a narrow size distribution. Both PAA-NS showed a very high water uptake and a marked swelling capacity forming stable nanohydrogels.

BSA was successfully incorporated in the two swellable PAA-nanosponges and in vitro studies showed that the BSA was released from the PAA-NS with prolonged release kinetics. Moreover, BSA encapsulation in PAA nanosponges protected and stabilized the protein during storage.

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