Synthesis of Poly(acrylic acid) Nanogels and Application in Loading and Release of an Oligothiophene Fluorophore and Its Bovine Serum Albumin Conjugate

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Summary: Nanometer-sized poly(acrylic acid) (PAA) hydrogels were synthesized by emulsion polymerization of methyl acrylate and subsequent acid hydrolysis, and their pH-dependent swelling behaviour was studied by dynamic light scattering. To determine the suitability of PAA nanogels as pH-sensitive carriers for biomedical applications, loading and release of an oligothiophene fluorophore and its albumin conjugate onto the PAA nanogels were investigated as a function of pH by absorption and photoluminescence measurements. It was observed that loading and release processes of both the oligothiophene and its conjugate could be controlled by changing pH of their solutions.

Keywords: loading and release; nanoparticles; oligothiophene; poly(acrylic acid); stimuli-sensitive polymers

Introduction

The controlled release technology promises revolutionary changes in many fields and in particular in medicine and biotechnology, allowing improvements in tissue engineering and *in vivo* imaging[1–3] as well as temporal and distribution control over drug delivery.[4] The development of ''smart'' carriers is based on molecular level engineering in order to optimize their properties, such as solubility and stability in water or physiological fluids and responsiveness to local pathology-associated changes.[5–8]

Several smart carriers have been developed for targeted delivery of diagnostic and therapeutic agents.[6,9] They include water-soluble polymers, ceramics, polymeric micelles, dendrimers and liposomes.[10–12] However, despite the widespread use in different fields, many of them either lack the desired control of their functional properties to interface with biological system or have a limited mechanical stability, thus preventing many possible applications.[13]

Crosslinked hydrogel networks, characterized by high water content and carbon-based network structure, represent the closest synthetic approximation to biological tissue and materials.[14] As their chemical and physical properties can be tailored with a high degree of control, these polymers have been widely investigated as drug delivery systems.[15,16] Ionizable functional groups bound to the polymeric chains give pH-responsivity to these materials. In

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particular, acrylate-based polymers exhibit a swelling behaviour depending on pH and ionic strength of solution, due to their carboxylic groups.[17,18] Hence, the pH changes within the body allow to direct the response towards a certain tissue or cellular compartment.[19–20]

Nanogels were recently developed for drug delivery and imaging,[1,2,21] as they present a number of advantages. Firstly, they can travel not only in the bloodstream, but they can also overcome anatomic barriers (blood brain barrier and the branching pathways of the pulmonary system) and reach tumor tissues, due to their ''leaky'' microvasculature.[1] Secondly, nanogels can be transported into the cell by some cellular uptake mechanisms (clathrin- and caveoli-mediated endocytosis, pinocytosis, and phagocytosis), and achieve the cytosolic drug delivery by pH-induced endosomal disruption.[21] Finally, if the polymer is non-degradable (e.g. polyacrylates), the nanometer size, below the renal threshold, ensures that it is not accumulated in the body.[22]

However, unlike in the case of microgels,[23,24] few studies have been conducted about loading and release processes of drugs and proteins with nanogels. Cationic nanogels have been employed as nonviral vectors for the targeted gene delivery and poly(*N*-isopropylacrylamide) (NIPAM) nanogel particles were studied in order to evaluate the driving forces in the loading and release of bovine serum albumine (BSA) and γ-globulins (γG).[25–27] To the best of our knowledge, no loading and release experiments of drugs/proteins with PAA nanogels are reported in literature.

In this paper the preparation of PAA nanogels by a two-step synthesis involving emulsion polymerization of methyl acrylate and subsequent acid hydrolysis is described. Nanogels were suspended in borate buffer and their swelling at different pH levels was analysed by dynamic light-scattering (DLS). The PAA nanogels were successfully employed for the pH-controlled loading and subsequent release of labelled protein systems. As a prototype system we used BSA protein labelled with an oligothiophene fluorophore (OTF). Indeed, OTFs are characterized by remarkable photostability, fluorescence intensity and colour tunability,[28] prerequisites for multicolour experiments and simultaneous tracking of various cell events.[28b]

Materials and Methods

Methyl acrylate (MA), sodium dodecyl sulfate (SDS), ammonium persulfate (APS) and sodium thiosulfate (STS) were purchased from Sigma Aldrich and used without further purification. Deionized water with a resistivity of $\geq 18.0\,M\Omega/cm$ was employed in the experiments. 2,5-dioxopyrrolidin-1-yl, 5″-(methylsulfanyl) 2,2′,5′,2″-terthiophene-5-carboxylate (OTF, Figure 1b),[28b] and its bovine serum albumin conjugate (BSA–OTF)[29] were employed as received, and their chemical and optical properties, as well as their synthesis were reported elsewhere.[28b,29]

Synthesis and Characterization of Poly(methyl acrylate) (PMA) Nanogels

The poly(methyl acrylate) (PMA) nanogel was synthesized by emulsion polymerization.[18] Small unilamellar vesicles were prepared by mixing SDS (250 mg, 0.867 mmol) and water (50 mL) at room temperature and sonicating them for 15 min. This dispersion was heated to ca. 65 °C in nitrogen atmosphere. The initiating agents APS (10 mg, 0.0438 mmol), STS

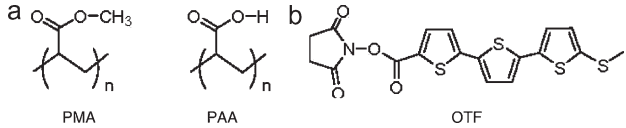

Figure 1.
Structure of PMA, PAA (a) and OTF (b).

(10 mg, 0.0403 mmol) and MA monomer (12.5 g, 0.145 mol) were added to the SDS vesicles dispersion. The solution turned turbid within 10 min, indicating the successful start of the radical polymerization. The polymerization was carried out for 2 h and stopped by cooling in an ice bath. The reaction mixture was transferred to centrifugal filter devices (Amicon Ultra-15, 10 kD, Millipore Bedford, MA) and centrifuged at 5000 rpm for 1 h, to concentrate the sample by ultrafiltration. The chemical structure of PMA is shown in Figure 1 (a).

To induce the hydrolysis of the methyl ester groups, which are less cleavable than other monomer (i.e. poly(*tert*-butyl acrylate)),[30] the crosslinked PMA nanogels were dissolved in THF and irradiated in a microwave oven (input power 600 W, 78 °C) in the presence of a catalytic amount of trifluoroacetic acid.[31] After 1 h, the pale yellow precipitate was resuspended in deionized water, indicating that PAA (Figure 1a) nanogels were obtained.

Characterization of PMA and PAA Nanogels

The PMA and PAA nanogels were freeze-dried using a LIO-5P apparatus (Cinquepascal s.r.l., MI, Italy). The FTIR spectra were obtained (KBr) by means of a Jasco FT/IR-6300. ^{1}H NMR spectra of PMA and PAA nanogel were obtained on a Bruker spectrometer operating at frequency 400 MHz. The chemical shifts are reported in parts per million downfield (δ) from TMS. PMA nanogel (D_2O in water, 10%, v/v): δ 1.60 – 2.50 ppm (m, 3H, CH_2CH), δ 3.67 ppm (s, 3H, CH_3). PAA nanogel ($CDCl_3$): δ 3.38 – 3.68 ppm (m, 3H, CH_2CH).

Dynamic Light Scattering Measurements

Hydrodynamic diameter of PMA and PAA nanoparticles was measured with a Zetasizer Nano Series (Malvern Instrument) at a fixed angle of 90°. Nanogel was diluted in borate buffer and the pH was fixed at various values by adding submicroliter amounts of 0.5 M NaOH and 0.5 M HCl. The suspension was filtered through a Millipore membrane filter (0.22 μm pore size) to remove dust particles. All the measurements were carried out at room temperature and every sample was measured five times. Polystyrene nanospheres (60 ± 5 nm; Duke Scientific Corporation) were employed to calibrate the LS instrument.

Absorption and Photoluminescence Measurements

Absorbance and fluorescence spectra were recorded with a Cary 5000 UV-Vis-NIR spectrophotometer and Cary Eclipse fluorescence spectrophotometer (Varian), respectively.

Loading and Release of OTF into PAA Nanogels

To evaluate the nanogel-loading behaviour in two different expansion states, two incubation solutions were prepared at different pH values (solution **a,** pH 4.5 and solution **b**, pH 7.0). The incubation solution was prepared by mixing nanogel (0.1 g/L), OTF (3.66×10^{-3} g/L) and Tween 20 (0.1%, w/v)[29] in a 1 mM borate buffer. The volume for each sample was 0.5 mL. After incubating for 2 h, the OTF-loaded nanogels were purified by removing OTF molecules by gel filtration on Sephadex G-25 column (NAP-25, GE-Helthcare).[32] Two borate buffers 1 mM, pH 4.0 and pH 5.0, were employed for the elution of OTF / nanogel mixture at pH 4.5 and pH 7.0, respectively.

Absorbance and fluorescence spectra of the loaded nanogels ($\lambda_{ex} = 373$ nm and $\lambda_{em} = 560$ nm) were measured in borate buffered solution in triplicate for every sample. Control experiments on OTF and nanogel were performed to assess the suitability of NAP-25 column for purification of nanogel/OTF solutions. The uptake of OTF by the nanogel (milligram of OTF per milligram of nanogel) can be calculated according to the formula:

$$\text{mg of OTF/mg of nanogel} = A_n A_c^{-1} V_{sys} C_i/\text{mg of nanogel} \quad (1)$$

where A_c and A_n are respectively the absorbance of the control and of the nanogel after the uptake process, V_{sys} is the total volume of the system, and C_i is the initial concentration of OTF.[23,24]

Release experiments were carried out at pH 9.0, as at this pH the nanogels are swollen, allowing the release of OTF through the enlarged meshes of their polymeric network.[15,33] The mixture was shaken for 2 h at room temperature and then purified by gel filtration on a NAP-25 column (borate buffer 1 mM, pH 9.0). The spectral properties of the nanogels after release ($\lambda_{ex} = 373$ nm and $\lambda_{em} = 560$ nm) were measured in borate buffered solution.

Loading and Release of BSA-OTF in Nanogels

Loading experiments of BSA-OTF were carried out similarly to the procedure reported for OTF. Two different solutions were prepared at pH 4.5 (solution **c**) and pH 7.0 (solution **d**). Nanogel (0.1 g/L) and BSA-OTF (3.66×10^{-3} g/L) were mixed in borate buffer (1 mM). After an incubation time of 2 h, unloaded bioconjugates were removed from the BSA-OTF-loaded nanogel by gel filtration on a NAP-25 column. Two borate buffers (1 mM), pH 4.0 and pH 5.0 were employed for the elution of BSA-OTF/nanogel mixture at pH 4.5 and pH 7.0, respectively. Two reference solutions of BSA-OTF and nanogel were eluted separately by gel filtration, demonstrating the suitability of the separation conditions. Absorbance and fluorescence spectra of the BSA-OTF loaded nanogels ($\lambda_{ex} = 373$ nm and $\lambda_{em} = 485$ nm) were measured in borate buffered solution in triplicate for every sample.

For the release experiment, a suspension of nanogels loaded with BSA-OTF was equilibrated at pH 9.0, shaken for 2 h at room temperature and then eluted in a NAP-25 column (borate buffer 1 mM, pH 9.0). The spectral properties of the nanogels after release were recorded in borate buffered solution ($\lambda_{ex} = 373$ nm and $\lambda_{em} = 485$ nm).

Results and Discussion

Emulsion polymerization involves the propagation reaction of free radicals with monomer molecules in the micelles, which behave like separate microcontinuous reactors.[34] In the PMA synthetic procedure the SDS concentration was above its critical micelle concentration (CMC) in water,[35] to ensure that the particles were almost all formed via micellar nucleation.[34b] Further, sonic radiation was applied to the system to induce acoustic degasification.[36]

Sawai and co-workers prepared microgels by emulsion polymerization demonstrating that the extent of pH-dependent swelling of the crosslinked gel was much less than that of the non-crosslinked one. According to these results, any crosslinker agent has been used in the reported synthetic procedure.

The hydrolysis of PMA nanogels was monitored by FT-IR and ^{1}H NMR. A comparison of the FT-IR spectra of PMA and PAA nanogels revealed the disappearance of a peak at 1738 cm^{-1} - characteristic of the carbonyl stretching of the methyl ester groups - and the appearance of two new peaks at 1650 and 1416 cm^{-1}, arising from, respectively, the asymmetric and symmetric carbonyl stretching of the carboxylate groups (Figure 2).

The successful outcome of the hydrolysis reaction was confirmed by considering the NMR spectra of PMA and PAA nanogels, as the peak at δ 3.67 ppm, due to the methoxy protons of PMA, disappears in the ^{1}H NMR spectrum of PAA nanogel. Hydrogels containing weakly acid carboxylic groups change their swelling and permeability properties in response to pH. The dynamic light scattering (DLS) technique was selected to check the hydrodynamic radius of nanogels in solution over a wide range of pH.[17,18] The purified PMA precursor nanogel was measured; its average diameter was 70 ± 5 nm. Subsequently, a selective surface hydrolysis was performed and DLS measurements were repeated at different pH values. Figure 3

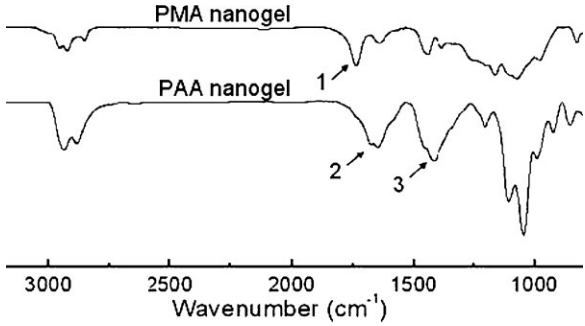

Figure 2.
FT-IR spectra of PMA and PAA nanogel. Peak 1: ester C=O stretching; peak 2: asymmetric carboxylate stretching; peak 3: symmetric carboxylate stretching.

shows the plot of the hydrodynamic diameter versus pH. Thus, the higher the pH of solution, the larger is the nanogel diameter, reaching the maximum value 120 ± 5 nm at pH 9.0.

Nanogel Loading and Release Behaviour

As the nanogel undergoes swelling from 70 nm to 120 nm in response to an increase in solution pH, the pore size of the network becomes larger, allowing the flow of water-soluble compounds or proteins.[15] When the pH is reduced, the permeability of the nanohydrogel decreases, inducing the entrapment of solutes inside the nanogel network. Differently, at alkaline pH values the volume and permeability of the nanohydrogel increased once again, allowing the release of the previously trapped substances from the polymer matrix and their diffusion in the external solution.[15]

Loading experiments were performed at pH 4.5 and 7.0 with both the oligothiophene fluorophore (OTF, Figure 1b) and its albumin conjugate (BSA-OTF).[28b,29] OTF is hydrophobic and pH-insensitive, while its conjugate with BSA is pH-sensitive and its charge correlates with the isoelectric point of the protein (p*I* 4.7).[37] The maximum emission wavelength of the BSA-OTF conjugate was reported to be blue-shifted by about 50 nm compared with that of free OTF.[29]

Figure 4 reports the fluorescence measurements of OTF encapsulated in the nanogels. The spectra were obtained after purification performed by gel filtration chromatography in order to remove the excess of free oligothiophenes and their spectral contribution. It was observed that running the loading experiment either at acid pH of 4.5 or at neutral pH of 7.0, the uptake of OTF was practically the same, indicating that the loading mechanism is pH-independent. According to the formula (1), the uptake was 0.014 and 0.017 mg OTF/mg nanogel for the experiments run at the pH values 4.5 and 7, respectively. These data could be explained by assuming that loading and release processes for nonpolar, hydrophobic solutes such as OTF proceed through hydrophobic interactions of the solutes and the lipophilic domain of the nanogel.[38]

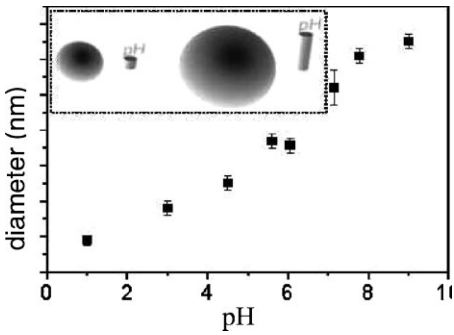

Figure 3.
Plot of the hydrodynamic diameter of nanogel particles versus pH of the surrounding solution. **Inset**. Schematic representation of PAA nanogel swelling as a function of pH.

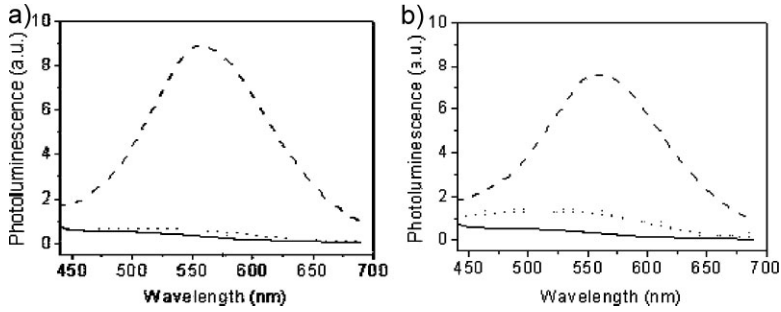

Figure 4.
a) Photoluminescence spectra of the PAA nanogel (straight line) at the same concentration (0.1 g/L), after the uptake of OTF at pH 4.5 (dash line) and the release experiment (dot line). $\lambda_{ex} = 373$ nm. b) PL spectra of the nanogel (0.1 g/L, straight line), after the OTF uptake at pH 7.0 (dash line) and the release experiment (dot line). $\lambda_{ex} = 373$ nm.

A comparison of loading and release data clearly demonstrate the diffusion of the oligothiophene in the external solution (Figure 4).

In the experiment with BSA-OTF at pH 4.5, the albumin conjugate had a positive charge (pH < p*I*); nearly 50% of carboxylic groups on the nanogel surface were dissociated (pH ~ pK_a of the nanogel).[39] When the BSA-OTF loading was run at pH 7, both the BSA probe and nanogel were negatively charged (pH > p*I*). In Figure 5a the photoluminescence spectra of the loaded BSA-OTF at pH 4.5 and pH 7 are reported. In particular, the BSA-OTF loading occurred at pH 4.5 with an uptake of 0.036 mg of BSA-OTF per mg of nanogel, whereas at pH 7 the loaded amount of the conjugate was much lower (0.008 mg of BSA-OTF/mg nanogel).

These data could be explained by assuming that at pH 4.5 the net positive charge of the BSA conjugate provides a driving force for its loading into the negatively charged gel via ion-exchange.[40] The uptake process via ion-exchange was absent when running the experiment at pH 7, as both BSA-OTF and the nanogel had a negative charge. As recent studies on acrylate-based microgels employed the ion-exchange theory, it should be deduced that nanogels behave similarly to microgels.[15,23,24] In fact, in PAA nanogel the counter-ions bound to the carboxylate groups can be exchanged with positive charges from the external solution accord-

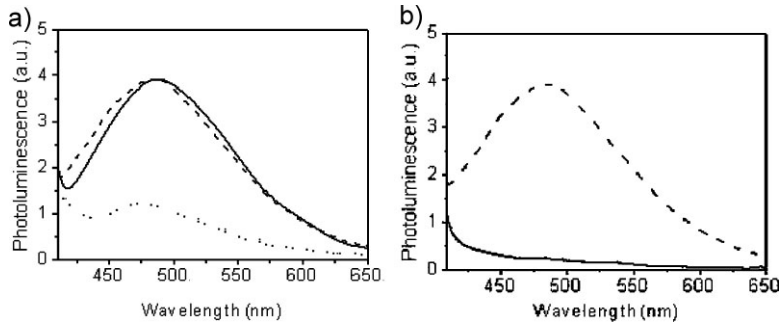

Figure 5.
a) Photoluminescence spectrum of BSA-OTF encapsulated in PAA nanogel after incubation at pH 4.5 (dash line) and at pH 7 (dot line). The fluorescence spectra of the BSA-OTF at the concentration used in the experiment ($C = 3.66 \times 10^{-3}$ g/L, line). b) Photoluminescence spectrum of BSA-OTF encapsulated in PAA nanogel after incubation at pH 4.5 (dash line) and after the release experiment (straight line). $\lambda_{ex} = 373$ nm.

ing to the Donnan equilibrium.[15,41] After the absorption phase, the cations can be included into the nanogel network by physical inclusion if there is a dimensional compatibility between the average pore size and the molecule diameter.[15a] Thus, it can be estimated that the mesh size of the PAA nanogel should be larger than 4 nm, which corresponds to the diameter of BSA.[42] An exact study on the evaluation of the mesh size is still in progress.

Figure 5b shows the release experiment run with the nanogels previously incubated in the presence of BSA-OTF at pH 4.5. The nanogels were separated from the released BSA-OTF by SEC. The absorbance and fluorescence spectra indicate the complete release of the oligothiophene-BSA derivative from the nanohydrogel network.

Figure 6 summarizes the uptake data of OTF and BSA-OTF into the nanogel, running the loading experiment at pH 4.5 and 7. It is evident that with pH-insensitive OTF loading occurs in quite a pH-insensitive way. Vice versa, employing a pH-dependent bioconjugate such as BSA-OTF, loading seems to be strictly pH-dependent. This result can be ascribed to the different uptake mechanisms described above.

These results can be expressed in terms of encapsulation efficiency (E.E.) which is the weight ratio of encapsulated to total OTF (or BSA-OTF).[43] The encapsulation efficiencies obtained running the OTF and BSA-OTF loading experiments at pH of 4.5 and 7 are reported in Table 1.

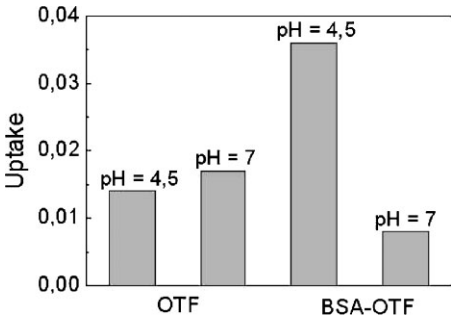

Figure 6.
Plot of the uptake data of nanogels (mg fluorophore/mg nanogel) for the loading experiments run with OTF and BSA-OTF.

Table 1.
Encapsulation efficiency (E.E.) values calculated according to the following formula: EE (%) = (Wen/Wtot) *100.

Sample	E.E. (%)
OTF loading, pH 4.5	38.2
OTF loading, pH 7	46.4
BSA-OTF loading, pH 4.5	98.4
BSA-OTF loading, pH 7	21.8

The encapsulation efficiency values are comparable to those mentioned in literature both for poly(acrylic acid) as well as for other polymeric nanoparticles.[44]

Conclusion

Loading and release of an oligothiophene fluorophore OTF and its bovine serum albumin conjugate, BSA-OTF, were induced and studied for the first time on the same PAA gel nanoparticles in suspension. First hydrogel nanoparticles were synthesized by a novel two-step procedure. The studies on the volume response as a function of pH by dynamic light scattering technique are in agreement with the reported data.[18] The swollen state of nanohydrogel corresponds to increased permeability of the polymer network, which favours the loading or release of small molecules or proteins through the nanometer-sized meshes of PAA gel.[15]

It was observed that loading and release processes are driven by electrostatic and/or hydrophobic interactions depending on the chemical nature of solutes and on pH of the external solution. In the case of hydrophobic and non-polar oligothiophene fluorophore (OTF)[28] loading and release proceed throughout hydrophobic interactions of the solutes and the lipophilic domain of the nanogel. Conversely, for the conjugated albumin (BSA-OTF), hydrophilic and pH-sensitive depending on the protein isoelectric point,[29] the same processes occur mainly via the ion-exchange mechanism. Hence, it was possible to exert the control over loading and release processes of oligothiophene-based

fluorophores by changing pH of the external solution. As the pH was varied in quite a physiological range, PAA nanohydrogel is a suitable carrier for BSA labelled with a fluorescent oligothiophene derivative. These results suggest that nanohydrogels bearing either free OTFs or their conjugates with proteins could be carried through organs and tissues for site-specific staining.

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