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Research paper

Stimuli-responsive hydrogels for controlled pilocarpine ocular delivery

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ABSTRACT

A series of vinyl hydrogels containing α-aminoacid (L-histidine, L-valine) residues was synthesized and their swelling properties evaluated at different pHs and temperatures. Unlike the zwitterionic compound containing only the L-histidine, a dual-stimuli responsiveness was improved in the carboxyl acid hydrogels carrying the L-valine residues (HVa). Besides the COOH functionality, the presence of either isopropyl and amido groups in the monomer structure renders the hydrogel also temperature-responsive, in a similar manner as the well-known poly(*N*-isopropylacrylamide) (pNIPAAm). The three HVa hydrogels (cross-linked with 1, 2, and 5 mol% of *N,N′*-ethylene-bisacrylamide, EBA) show a phase separation at the same critical pH 4, although a different swelling was improved by the amount of EBA. In buffered solutions, the effect of increasing temperature led to decrease the swelling and, as the pH is close to the critical one, a further and sharper collapse of the hydrogel may be tuned. The release study of pilocarpine in physiological conditions showed a burst effect within the first few hours, followed by a sustained release for a week. The initial burst effect was strongly dependent on the kind of hydrogel investigated. As the pilocarpine is a basic molecule (pK_a 7.2), it may interact more strongly with the free carboxyl groups in the ionized state of the HVa hydrogels than the zwitterionic species of the histidine compounds. The releasing profile shows a three time greater release of the pilocarpine loaded in the HVa hydrogels. The hydrogels were found to be non-toxic against the mouse fibroblast NIH3T3 cells. The presence of pilocarpine strongly increased the cell proliferation even after 2 days.

1. Introduction

Synthetic polymers are of increasing interest in drug delivery as therapeutic agents [1]. In the cross-linked (hydrogel) form, they become water-swollen networks like solids, containing a significant amount of water. The most intensively studied biomedical application for hydrogels is as vehicles for the delivery of pharmaceuticals [2]. Hydrogels can be used to release drugs slowly over time or to trigger release in response to a wide variety of chemical and physical stimuli [3,4]. In these gel applications, it is often the transport of solute through the network which determines the performance of the resulting product. Recently, multiple stimuli-responsive hydrogels have attracted significant research interest because the most pH- and temperature-sensitive dual functional systems have a great importance in biological applications and can mimic the responsive macromolecules found in nature [5–7]. The swelling properties of these dual-stimuli-responsive hydrogels can abruptly and reversibly change in response to pH and temperature variations. Hence, these materials have great potential applications in the controlled drug delivery and release.

Recently, polymeric systems are developing to find a suitable carrier for the ocular drug therapy of most glaucoma patients [8–12] and to increase the efficacy and the bioavailability of the drug. Pilocarpine, as a natural parasympathomimetic alkaloid, is an imidazole derivative with a basicity constant pK_a of 7.2 [13,14] and exhibiting pharmacological activity [15] (Scheme 1).

The drug has been used in the treatment of chronic open-angle glaucoma and acute angle-closure glaucoma for over 100 years. Among other things, in ophthalmology, pilocarpine is also used to reduce the possibility of glare at night from lights if the patient underwent implantation of phakic intraocular lenses; the use of pilocarpine would reduce the size of the pupils, relieving these symptoms. The most common concentration for this use is pilocarpine 1%, the weakest concentration. Since the drug has been widely used topically in the eye, because of the good water solubility, it suffers from the low ocular bioavailability because of its low lipophilicity and the short residence time of aqueous solution in the eye.

Several papers, based on hydrogels as a platform for new ocular drug delivery, appeared in the literature [8–11]. More recently, Sinko reported the interesting evaluation for the controlled ocular delivery of pilocarpine and its subsequent pupillary constriction of

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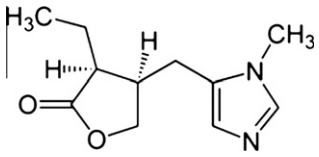
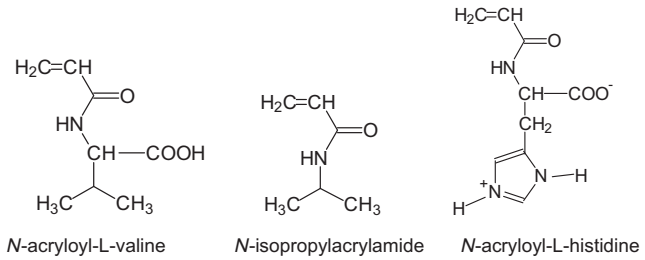


Scheme 1. Structure of the pilocarpine.

fast forming hydrogels containing thiol groups [8]. The presence of the imidazole nucleus renders the pilocarpine an effective target for metal binding [16] and, at physiological pH, a suitable positively ionized molecule to allow ionic interaction with negatively charged polymers [17]. The purpose of the present research is to investigate on the delivery of pilocarpine-loaded hydrogels as a platform for the glaucoma therapy [9,10,18]. We believe that the novel proposed hydrogels may have a useful application as ophthalmic inserts, like the Ocusert-pilocarpine device [19], due to their non-toxic effect and for their good transparency. These inserts should increase the contact time between the preparation and the conjunctival tissue, to ensure a sustained release suited to systemic treatment. In comparison with the other ophthalmic polymeric devices, the proposed platforms may have improved advantages as phase transition systems. Changes in external stimuli, like temperature, may trigger the release of the ionically complexed drug, enhancing its bioavailability [20].

In previous papers, we reported the research activity concerning the potential applications of some polyelectrolyte hydrogels proposed for cisplatin chemotherapy [21,22]. The interaction between the platinum(II) species and the hydrogel allows a chemical-controlled, along with the diffusion-controlled, mechanism. In the present investigation, pilocarpine-loaded stimuli-sensitive-hydrogels were prepared and the ocular drug delivery monitored in the physiological medium and at different temperatures. The several hydrogels considered in this study are based on vinyl polymers carrying α-aminoacid residues, like L-histidine [17,23] and L-valine [24,25]; they are obtained from the monomers reported in Scheme 2.

The valine moiety contains, besides the carboxyl group, the amido and the isopropyl groups in a structure closer to that of the well-known temperature-sensitive poly(*N*-isopropylacrylamide) (pNIPAAm) having a lower critical solution temperature (LCST) of 32 °C, close to that of the human body [26]. The LCST can be tuned by changing the pH. Any increase of the pH leads to increase the charge density and thus the LCST. This will render the material pH- and temperature-responsive in aqueous solution, like the soluble free polymers already reported in our previous paper [25]. Among the three acrylic hydrogels incorporating the L-valine moiety, in this study, we wish to focus a wider characterization of the hydrogel HVa2, i.e. cross-linked with 2 mol% of *N*,*N*′-ethylene-bis-acrylamide because of its improved mechanical and swelling properties.



Scheme 2. Structures of vinyl monomers used for the hydrogels.

2. Materials and methods

2.1. Instruments and chemicals

Spectrophotometric measurements were carried out with a Specord 210 (Analytikjena) equipped with 10 mm quartz cuvettes. A TitraLab 90 titration system (Radiometer Analytical), supported mainly by the TIM900 Titration Manager and connected to the TimTalk 9 (a Windows-based software, for remote control), was used to measure the pH and the temperature by means of the combined pH electrode Red Rod and the temperature sensor type T201. Before performing pH measurements, the Tim900 was calibrated against two buffer solutions of known pH value. The system was used to study the hydrogels swelling at different pHs and temperatures. The monomer *N*-acryloyl-L-valine (AVA) was synthesized as previously reported [24,27]. The cross-linking agent *N*,*N*′-ethylene-bisacrylamide (EBA, 98%), the triethylamine (TEA, 99.5%), and the ammonium peroxo-disulfate (APS, 98%) were purchased from Fluka Co. The pilocarpine hydrochloride (99%) was purchased from Sigma–Aldrich. Tris, PBS, and acetate buffer solutions (0.01 M and 0.05 M) were prepared in 0.15 M NaCl and in twice-distilled water. All the remaining chemicals and solvents, from Fluka and Sigma–Aldrich, were used without a further purification. Dulbecco's Modified Eagle's Medium (DMEM), trypsin solution, and all the solvents used for cell culture were purchased from Lonza (Switzerland). Mouse immortalized fibroblasts NIH3T3 were purchased from American Type Culture Collection (USA). High-density polyethylene (HDPE) was supplied from US Pharmacopeia, Rockville-Maryland (USA), and organo-tin stabilized poly(vinylchloride) from Gradko International Limited (UK).

2.2. Synthesis

The two hydrogels containing the L-histidine residues, namely H5 and CH1, and cross-linked with 5 and 1 mol% of EBA, respectively, were obtained as reported in previous papers [17,23]. While the gel H5 was synthesized only from the monomer *N*-acryloyl-L-histidine (Hist) in aqueous media (in the presence of a weighed quantity of cross-linker EBA and the radical APS initiator), the hydrogel CH1 was obtained from a mixture of the monomers Hist and NIPAAm at a NIPAAm/Hist molar ratio of 10. The three cross-linked hydrogels, named HVa1, HVa2, and HVa5, were synthesized by the free radical polymerization of the *N*-acryloyl-L-valine monomer according to a previously reported procedure [22]. The monomers were dissolved in water, and then the desired amount of EBA (1, 2, or 5 mol% of the moles of monomers) and TEA (10 mol% of the moles of monomers) were added. Unlike the monomer EBA, the monomer AVA was dissolved in a 3 M NaOH solution. The feed composition of the three hydrogels is reported in Table 1.

Briefly, their preparation was carried out in a glass tube, under nitrogen atmosphere, by the following procedure. The solution of the monomers was treated under vacuum for 30 min and flushed with nitrogen; then, a freshly prepared and degassed aqueous solution (6.0 mg/mL) of APS (0.25 mol% of the moles of monomers) was added under nitrogen. The reaction mixture was kept at room temperature for 24 h. Afterwards, the gels were gently removed and daily washed with twice-distilled water (500 mL each) for 1 week. The resulting swollen and transparent gels were treated with an hydrochloric acid solution, whereupon the voluminous material collapsed to a small volume of hard consistency. The so-obtained white product was isolated and further washed with twice-distilled water for three days; then, it was cut in small discs and dried at r.t. up to a constant weight.

Table 1
Feed composition of the cross-linked HVa polymers (Hydrogels).

Hydrogel	Monomer AVA [a] (mmol)	Monomer EBA [b] (mmol)	TEA [c] (μL)	APS [d] (μL)	V_T [e] (mL)	Gelation time (h)
HVa1	3.20	0.032	45	314	4.36	3.0
HVa2	6.32	0.125	90	625	5.92	0.5
HVa5	6.49	0.323	96	660	9.16	0.5

[a] Amount of monomer dissolved in the stoichiometric quantity of 3 M NaOH solution.
[b] Amount of cross-linking agent.
[c] Amount of TEA (10 mol%) solution.
[d] Amount of APS (0.25 mol%) aqueous solution (6.0 mg/mL).
[e] Total volume of the mixture.

2.3. Optical transmission

Samples of hydrogels (HVa1, 36 mg; HVa2, 42 mg; HVa5, 83 mg) were swollen in a slightly basic PBS solution. When the equilibrium degree of swelling was reached, they were washed with fresh PBS (pH 7.40) and immersed in a pilocarpine solution (3.2 wt%) for loading. The swollen gel samples were transferred in a quartz cuvette, filled with PBS, and the transmission of light was measured at 480 nm [28]. Two replicates were in good agreement and averaged.

2.4. Swelling measurements

The swelling of the hydrogels was studied at different pHs and temperatures, following a previously reported procedure [17,22,29,30] in a thermostatted glass cell (100 mL) connected to a temperature probe and a glass electrode. The TimTalk 9 software was used to control the measurements performed by the TitraLab 90 titration system. A weighed amount of dry gel in the form of slabs (20–40 mg) contained in a Strainer cell (70 μm pore size) was suspended in the buffer solution of desired pH. The degree of swelling (DS) and the equilibrium degree of swelling (EDS) was evaluated as a function of time by removing the Strainer cell, blotting it with a tissue paper for removing any surface water, and weighing it (W_{wet}) on an analytical balance (Mettler AC100). The procedure was repeated at intervals, and the EDS value was daily calculated (every 24 h) by the relation: $EDS = (W_{wet} - W_{dry})/W_{dry}$, where W_{dry} is the weight of the dry gel before swelling.

2.5. Loading of pilocarpine

The drug loading into the hydrogels was obtained by soaking a weighed amount of the dry gel (40–80 mg) in a 3 wt% of pilocarpine hydrochloride stock solution. The deswelling of the hydrogels was improved in the dark at room temperature for 2 weeks, and with occasional stirring. Then, the solid compounds were filtered through a Strainer cell and washed with distilled water for three times (3×20 mL each). They were dried at room temperature and then under vacuum to a constant weight. To evaluate the effect of the drug on the EDS of the network, aliquots of pilocarpine hydrochloride (as a stock solution 1.54 wt%, or as solid material to reach a greater concentration) were added daily to a water solution (70 mL) containing a weighed amount of the swollen gel HVa2 (10.2 mg of dry gel). Hence, the gel incorporating the complexed pilocarpine was blotted with a tissue paper and weighed. The EDS was evaluated in the same way as that reported in Section 2.4.

2.6. In vitro pilocarpine release

A weighed quantity (2–6 mg) of dry pilocarpine-loaded hydrogel contained in a Strainer cell was suspended in 60 mL of PBS solution at pH 7.40. At intervals, sample solutions were analyzed by spectrophotometric measurements at 216 nm. The temperature was controlled at 25.0 ± 0.1 °C and 36.0 ± 0.1 °C by the TitraLab 90 apparatus and with the glass cell thermostatted with a Haake D8 thermostat within ±0.1 °C. The cumulative amount of pilocarpine released from the hydrogel was determined using a calibration curve. Three replicates showed reliable results.

2.7. Cytotoxicity

2.7.1. Cell cultures

Mouse immortalized fibroblasts NIH3T3 cells were utilized for cytotoxicity experiments. NIH3T3 were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was supplemented with 10% foetal calf serum, 1% L-glutamine–penicillin–streptomycin solution, and 1% MEM non-essential amino acid solution. Once at confluence, the cells were washed with PBS 0.1 M, taken up with trypsin–EDTA solution and then centrifuged at 1000 rpm for 5 min. The pellet was re-suspended in complete medium solution (dilution 1:15).

2.7.2. Samples preparation

The hydrogels (HVa1, HVa2, and HVa5) were tested as native ones and loaded with pilocarpine (Pcp). Ten milligrams of each swollen hydrogel was sterilised with 70% EtOH for one hour at room temperature. The EtOH was removed, and the samples were washed three times by culture medium or loaded with pilocarpine (Pcp) and then tested by cell cultures. To load Pcp, ten milligrams of each swollen hydrogel was dipped in a 2 wt% pilocarpine solution in PBS (w/v) for 24 h at 37 °C. Then, the hydrogels were gently washed by culture medium and put in contact with seeded fibroblasts. High-density polyethylene (HDPE) was used as negative control; organo-tin stabilized poly(vinylchloride) (PVC) was used as positive material. All samples were set up in triplicate.

2.7.3. Cytotoxicity assay: NIH3T3 viability

To evaluate the in vitro cytotoxicity of the hydrogels, the direct contact test, proposed by "ISO 10993-5 Biological evaluation of medical devices – Part 5: Tests for cytotoxicity: in vitro methods", was utilised [31]. This test is suitable for samples with various shapes, sizes or physical states (i.e. liquid or solid). The same procedure was utilised in order to test the cytocompatibility of the hydrogels. 1.0×10^3 NIH3T3 cells suspended in 1 mL of complete medium were seeded in each well of a 24-well round multidish and incubated at 37 °C in an atmosphere of 5% CO_2 for 24 h. Then, the hydrogel samples were carefully placed on the cell layer ensuring that each specimen covered approximately one-tenth of the cell-layer surface. In order to prevent unnecessary movement of the specimens, as this could cause physical trauma to the cells, each sample was covered and fixed by a co-culture insert having a PET membrane with pores of Ø = 0.4 μm (Falcon, USA).

2.7.4. Evaluation of cell viability

Cell viability, after 24 h of incubation with the native and loaded hydrogel with pilocarpine, was evaluated by Neutral Red Uptake (Sigma–Aldrich, Switzerland) by the following procedure. First, the following solutions were prepared in order to determine

the percentage of viable cells: (a) neutral red (NR) stock solution: 0.33 g NR dye powder in 100 mL sterile H_2O; (b) NR medium: 1.0 mL NR stock solution +99.0 routine culture medium pre-warmed to 37 °C; (c) NR desorb solution: 1% glacial acetic acid solution +50% ethanol +49% H_2O. At the end of the incubation, the co-culture inserts and the routine culture medium were removed from each well, and cells were carefully rinsed with 1 mL of pre-warmed D-PBS. One mL of NR medium was added to each well and further incubated at 37 °C, 95% humidity, 5.0% CO_2 for 3 h. The cells were checked during the NR incubation for NR crystal formation. After incubation, the NR medium was removed, cells were carefully rinsed with 1 mL of pre-warmed D-PBS. Then, the PBS was decanted and blotted from the dishes and covers, and exactly 1 mL of NR desorb solution was added to each sample. Samples were shacked for 20–45 min to extract NR from the cells and form an homogeneous solution, keeping them covered in order to protect the cells from light. Five minutes later, absorbance was measured at 540 nm on the UV–visible spectrophotometer.

2.7.5. Effect of pilocarpine release on cell proliferation

As the HVa2 hydrogel was demonstrated to be the most compatible towards NIH3T3 cells both in the native form and loaded with Pcp, the effect of Pcp release from the hydrogels on cell viability as a function of time was also evaluated. Ten milligrams of medium swollen HVa2 hydrogel was sterilised with 70% EtOH for one hour, at room temperature. Then, the EtOH was removed and the hydrogel washed three times by culture medium or loaded with Pcp following the procedure reported above (see Section 2.7.2). All samples were set up in triplicate. The experimental protocol was the same reported above (see Section 2.7.3). Every 24 h the HDPE, HVa2, and HVa2-Pcp samples were removed from the wells where they have been seeded and were placed on new cell layer in different wells with fresh medium. This procedure was performed for 24, 48, 72, and 96 h. At each time of incubation, the NIH3T3 viability was evaluated by NRU following the procedure reported above (see Section 2.7.3).

2.7.6. Statistical analysis

Multiple comparisons were performed by one-way ANOVA and individual differences tested by Fisher's test after the demonstration of significant intergroup differences by ANOVA. Differences with $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Properties of the hydrogels

Most of the hydrogel properties considered in this study were already reported in previous papers [17,22,23]. Contrary to the fast swelling of the two hydrogels based on L-histidine residues (H5 and CH1), the three hydrogels carrying the L-valine moiety (HVa) showed slower swelling kinetic. The low solubility of the polymer in the neutral form and the different morphology of the gels were responsible for the different behavior of the three cross-linked compounds. In the carboxyl acid form, the degree of swelling (DS), in physiological buffer (PBS, pH 7.40), of the hydrogel samples, revealed different swelling kinetics. Fig. 1 shows the comparative characteristic DS/time plot for the swelling of the three hydrogels in the same conditions of temperature, pH and solvent. Unlike the hydrogel HVa5, that reached its equilibrium degree of swelling in about 3 days, the other two less cross-linked materials HVa1 and HVa2 reached a plateau in 8 and 12 days, respectively. The different compactness of the three different cross-linked polymers plays a role in the swelling process. Contrary to the solid state, that was friable and porous, the less cross-linked gel HVa1 becomes almost

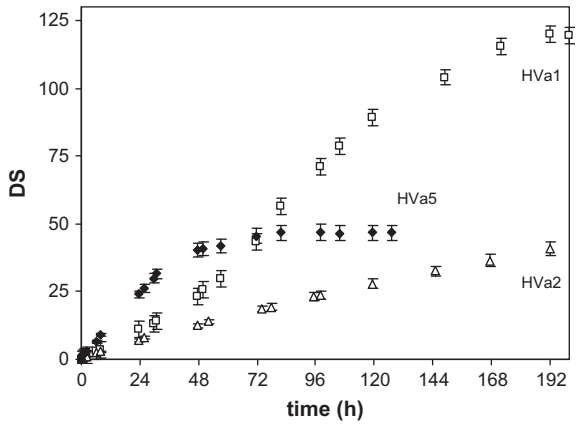


Fig. 1. Swelling degree (DS) in relation to time (h) of the hydrogels HVa1 (open square), HVa2 (open triangle), and HVa5 (dark square) in PBS buffer pH 7.40 at 25.0 ± 0.1 °C. Data are means ± standard error of three replicates.

of liquid consistency and showed some difficulty in its management; the water up-take was very high, reaching an EDS of about 1600 in pure water. On the other hand, the HVa2 showed a more compact and rigid structure in both dry and swollen states. The different morphology of the materials caused a different up-take and front of water inside the hydrogel. The proton splitting-off from the COOH group and its departure far with the front of solvent caused the longer equilibration to be reached for the swelling in PBS. In different solvent conditions, like in the alkaline medium and organic solvents (ethanol/DMF), the equilibrium degree of swelling was reached faster. The swelling profile of the three HVa samples was evaluated following the Fickian diffusional treatment usually applied for pH-sensitive hydrogels [32]. The simple power law expression proposed by Peppas [33] ($M_t/M_\infty = k t^n$) is useful in many experimental situations (M_t and M_∞ are the mass of water absorbed by the hydrogel at the time t and at the equilibrium, respectively). The structural/geometric constant k and particularly the parameter n may comparatively indicate the diffusion-controlled mechanism. Table 2 summarizes the values obtained in our system.

Since a slab-shaped hydrogel was used in this study, the value of the n parameter implies the following conceptual meaning; while the less cross-linked hydrogel HVa1 shows a case-II transport, both HVa2 and HVa5 follow a non-Fickian (anomalous) transport [33,34].

The optical transmission (OT) of the hydrogels in the swollen state linearly depends on the cross-link density (Fig. 2). Among the three hydrogels, the HVa1 showed the best performance with 97% OT (in PBS buffer, pH 7.40), while the other two hydrogels HVa2 and HVa5 revealed, respectively 95% and 90% of OT.

3.2. Effect of pH and ionic strength

Besides the zwitterionic nature of the gels H5 and CH1, previously reported, showing greater EDS values before and after the

Table 2
Values of parameters k and n of the empirical Peppas's model ($M_t/M_\infty = kt^n$) for the swelling of the HVa hydrogels in PBS buffer (pH 7.40) at 25.0 ± 0.1 °C.

Hydrogel	n	k	R^2 [a]
HVa1	1.05	0.0039	0.99
HVa2	0.76	0.0115	0.99
HVa5	0.76	0.0447	0.99

[a] Correlation coefficient.

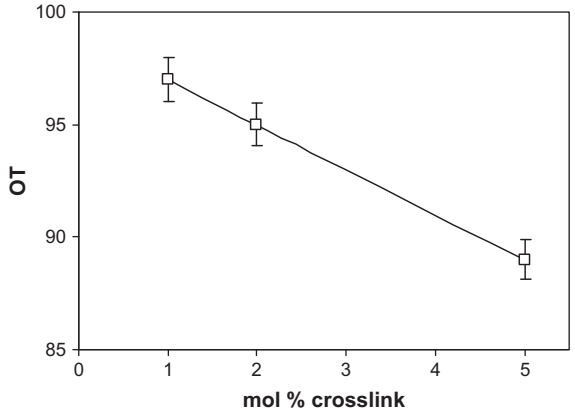


Fig. 2. Optical transmission (OT) in relation to the cross-link density (mol%) of the pilocarpine-loaded HVa hydrogels in PBS solution (pH 7.40) and at room temperature. Data and standard errors are from two replicates.

isoelectric point (pH 5), the acidic hydrogels HVa shows a sharp EDS decrease in correspondence of a pH value close to the critical degree of protonation (α) of the carboxylate anion [22]. This α, corresponding to 0.66, was related to the collapse of the macromolecular coil that forces the isopropyl groups in a close contact, outweighing the repulsive electrostatic interactions of the partially ionized polymer in a more extended and hydrated conformation. Fig. 3 shows the subtle collapse of the three hydrogels from the swollen to a shrunk state around pH 4, in acetate buffer. At pH > 4, the EDS value regularly increases with the increasing charge density of the network. As evaluated by the pK_a of the free polymer analogue [25], the charge density of the COO^- group reaches about 100% in physiological conditions (pH 7.4), while only a value of 34% may be considered at pH 4.0. It is evident that at pH > 4, the EDS increase is greater for the hydrogel HVa1, due to a lower amount of cross-links in the network. Unlike the HVa2 and HVa5, the hydrogel HVa1 allows a greater water content, becoming almost fluid and suitable for injectability purposes.

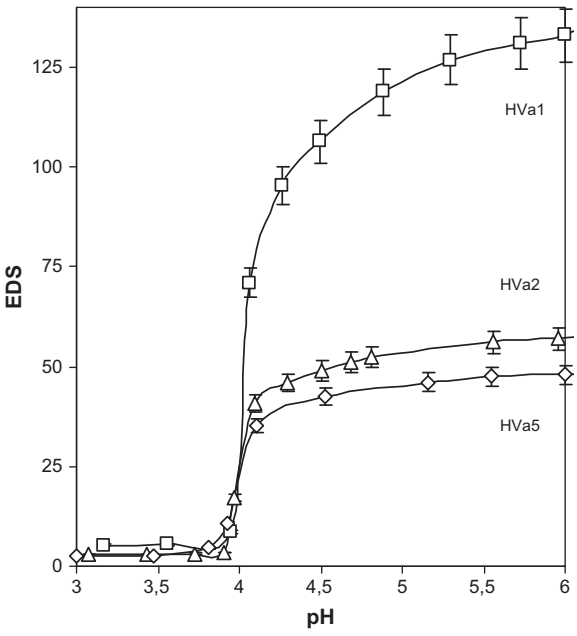


Fig. 3. Equilibrium degree of swelling (EDS) in relation to pH for the hydrogels HVa1, HVa2, and HVa5 at 25.0 ± 0.1 °C and 0.15 M ionic strength (NaCl). Data and standard errors of three replicates.

The pH of the phase transition becomes slightly higher at low ionic strength. This is in line with the fact that low ionic strength increases the basicity constant of the carboxylate group [35]. In Fig. 4 is reported for comparison the EDS/pH plot of the hydrogel HVa2 in two different buffer conditions: acetate 0.05 M in water and acetate 0.01 M in 0.15 M NaCl. The ionic strength plays a sensitive role in the swelling process. In all cases, the hydrogels HVa in the fully ionized state revealed a sharp EDS decrease till a concentration of 0.15 M NaCl; after this value, the swelling remained almost flat till a concentration of about 3 M NaCl [17]. The higher concentration of the simple salt does not lead to a further collapse for lower hydration states, as occurred instead for uncharged hydrogels based on NIPAAm [36] and charged hydrogels containing different α-aminoacid (L-phenylalanine) residues [30].

3.3. Effect of temperature

The hydration state of the three HVa hydrogels under study seems to behave quite differently for the effect of temperature and at different pHs. In all cases, the increase of temperature led to a decrease of EDS. As the pH decreased, approaching the critical pH 4.0, the lower hydration state led to a subtle EDS decrease till the hydrogel collapsed, triggered by the temperature. In Fig. 5 is reported a characteristic plot of EDS in relation to the temperature (°C) for the HVa2 at different pHs, in acetate buffer solutions, and at two different ionic strengths. At greater pHs, in acetate as well as in PBS buffers, a linear decrease of EDS over a wide range of temperature is observed either at low or high ionic strengths. As the pH approaches the critical range, when the hydrophilic–hydrophobic forces become competitive, the EDS/T plot interplays significant phase transitions. These are sensitively dependent on the charge density of the polymer, and thus the pH. As the latter decreases, the hydrophobic forces outweigh the electrostatic one, and the gel shows collapse at lower temperature. This is the normal behavior of temperature-sensitive polymers that show lower LCST for the incorporation of hydrophobic moieties; the contrary is true for the incorporation of hydrophilic substituents [37,38], thus leading to a greater phase transition temperature that vanishes at high charge density. In the case of HVa2, we can regulate the pH, the temperature, and the ionic strength to tune the collapsing process of the hydrogel in suitable conditions to trigger the drug release. The presence of a shrunk region may suggest to tailor-made and perform tunable dual-stimuli-responsive materials of

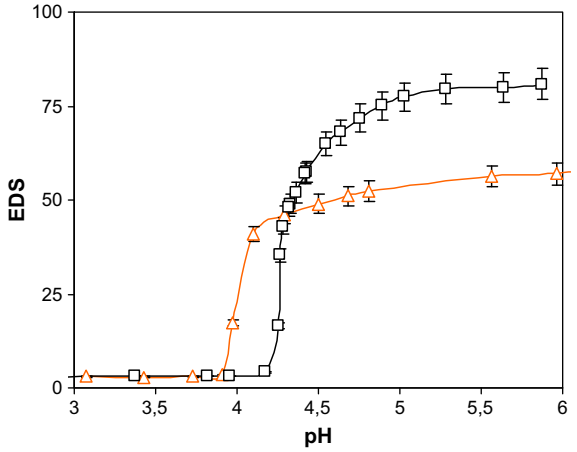


Fig. 4. Equilibrium degree of swelling (EDS) in relation to pH for the hydrogel HVa2 at 25.0 ± 0.1 °C and different ionic strengths (square, 0.05 M acetate buffer; red triangle, 0.01 M acetate buffer in 0.15 M NaCl). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

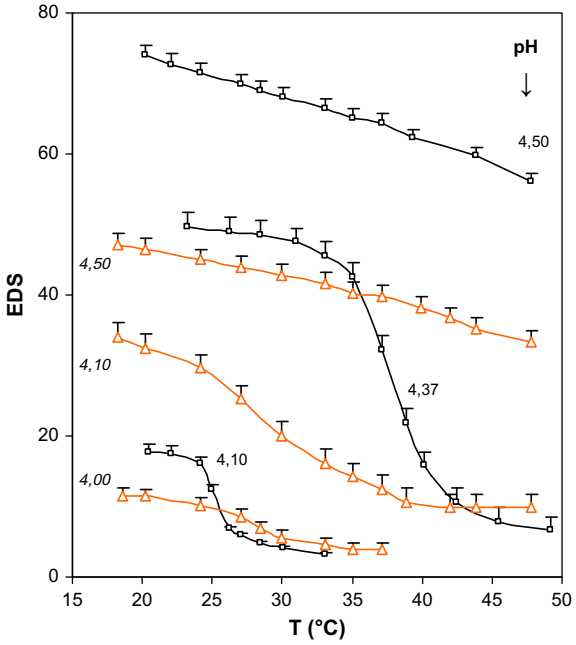


Fig. 5. Equilibrium degree of swelling (EDS, in different acetate buffer solutions) in relation to temperature (°C) for HVa2 at different pH values. Square, acetate buffer 0.05 M; red triangle, acetate buffer 0.01 M in 0.15 M NaCl).). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

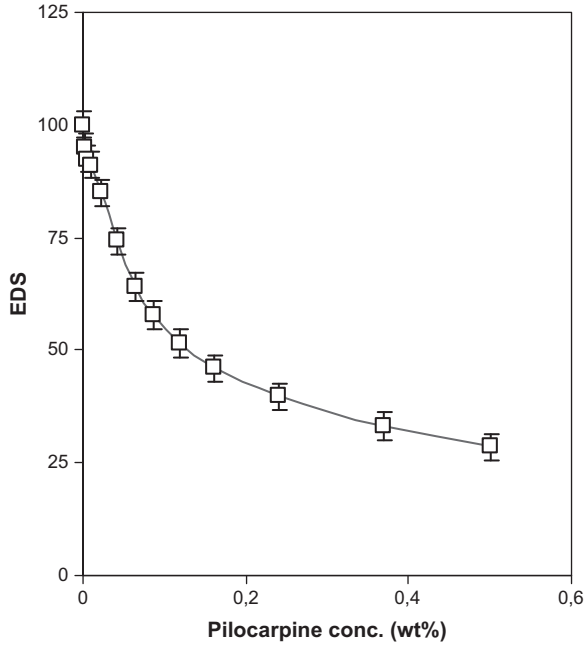


Fig. 6. Equilibrium degree of swelling (EDS, in deionized water) of the hydrogel HVa2 (10.2 mg) in relation to the pilocarpine concentration (wt%) at 25.0 ± 0.1 °C.

great potential applications [3,4]. In the present investigation, we limited our approach to suitable platforms that are useful in the controlled release of pilocarpine for the glaucoma therapy [9], as in the previous paper, the same hydrogels were considered as a platform for the cisplatin chemotherapy [21,22].

3.4. Loading and release of pilocarpine

Pilocarpine is usually administered in the eye by drops of 3 wt% solution. It comes also in eye gel, and in a controlled release system (Ocusert Pilo) [19]. The loading of the ocular drug into the network of the considered solid material was obtained by keeping for at least one week the swollen hydrogels in a 3 wt% of pilocarpine hydrochloride dissolved in distilled water, with occasional stirring. The hydrogels considered in this investigation included anionic and zwitterionic compounds, for electrostatic purposes. Besides the three novel carboxyl acid materials (based on L-valine), the more two compounds of different zwitterionic charge density (containing the L-histidine residues) were taken into consideration [17,23]. While the hydrogel H5 was based on a polymeric network of acrylic structure and containing only the L-histidine residues, the hydrogel CH1 was a copolymer of the *N*-acryloyl-L-histidine and *N*-isopropylacrylamide cross-linked with EBA at 5 and 1 mol%, respectively. During the loading process, a reduced size of the swollen HVa hydrogels was revealed. This was attributed to the partial charge neutralization due to electrostatic interactions between the negative ionized groups of the hydrogel and the positively ionized nitrogen of the pilocarpine, being in the hydrochloride form. Fig. 6 shows the deswelling effect of the HVa2, previously swollen in deionized water, upon the addition of pilocarpine in the form of a stock solution and as a solid dissolved into the medium. The initial sharper decrease of the EDS in the titration curve EDS/wt% may be simply ascribed to the ionic interaction of the pilocarpine hydrochloride and the negatively ionized gel. This interaction is not so strong because of the conceptual low stability of the forming complex species between the COO^- of the gel and the protonated pilocarpine molecule. An higher amount of pilocarpine is necessary to reach a stoichiometric break-point, that is close to a pilocarpine concentration of 0.02 wt%; the fact that for the end-point a 5–10-fold excess of pilocarpine is required, is consistent with a low equilibrium constant, that is small enough and products are not quantitatively formed as titrant is added. This hypothesis was also supported by FT-IR spectra of the gel HVa2 in the ionized form and its complex with protonated pilocarpine. The small shift of characteristic bands belonging to pilocarpine and C=O stretching of the gel was indicative of the low interaction. Overall, the pilocarpine is loaded into the gel, and its release becomes easier for the low electrostatic interaction. Once loaded, the hydrogels were filtered, washed with distilled water, and finally dried to a constant weight. The release of pilocarpine was monitored in PBS buffer (pH 7.40) by measuring the absorbance at 216 nm of sample solutions taken at intervals. A calibration procedure was previously performed to compare and to evaluate the amount of pilocarpine. Fig. 7 summarizes the amount of pilocarpine/gel (in mg/g) released from all the hydrogels investigated for one week. The results clearly show two basic points. First, a burst effect is present in all cases; second, the amount of released pilocarpine is different as different is the nature of the hydrogel. The zwitterionic hydrogel H5 reached almost a plateau after 24 h with less of 200 mg of drug released per gram of gel. On the other hand, the less ionized hydrogel CH1 showed, after the initial burst effect, a sustained release pattern around 400 mg Pcp/g of gel. A more releasing pattern around 600 mg/g and more was shown by the hydrogel of the HVa series. The different releasing pattern may be ascribed to the different electrostatic interaction between the pilocarpine and the ionized groups into the network. Pilocarpine is an etherocyclic imidazole derivative having a pK_a of 7.2 [13,14]; at physiological pH (PBS, pH 7.40), the molecules are mostly in the neutral and positively ionized form. As the pilocarpine comes inside the zwitterionic hydrogel H5, its positively ionized molecules display electrostatic repulsions with the positively ionized imidazole residues of the gel H5, despite the presence of negative carboxylate charges. The latter, having a low pK_a [23], weakly interacts with the protonated drug. Moreover, the low swelling degree of the gel H5 at pH 7.40 leads to a small pilocarpine

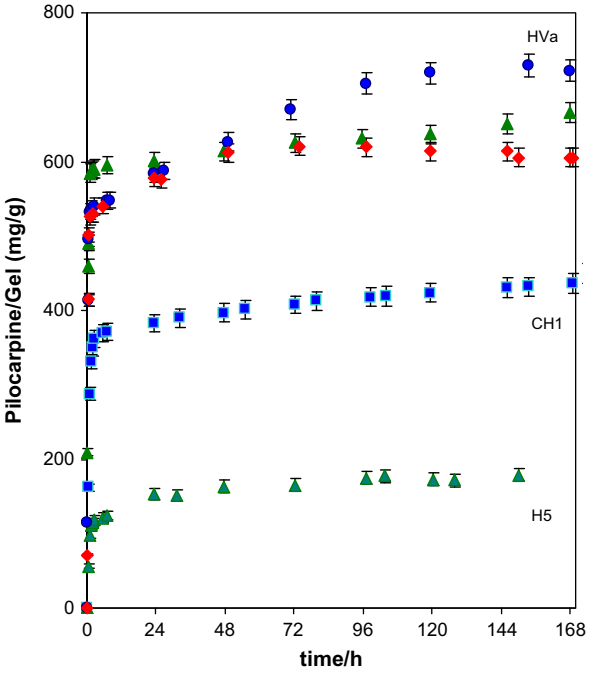


Fig. 7. Release pattern of pilocarpine from the hydrogels H5 (green, bottom), CH1 (blue, middle), and HVa series (top: HVa1, blue circle; HVa2, green triangle; HVa5, red square) in PBS buffer pH 7.40 at 25.0 ± 0.1 °C. Data and standard errors of three replicates.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

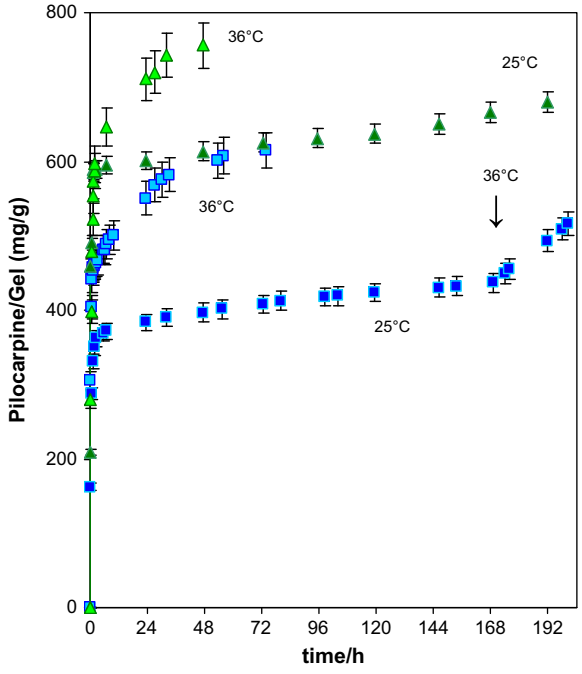


Fig. 8. Release pattern of pilocarpine at different temperatures from the hydrogels CH1 (blue square, 25.0 ± 0.1 °C and 36.0 ± 0.1 °C) and HVa2 (green triangle, 25.0 ± 0.1 °C and 36.0 ± 0.1 °C) in PBS buffer pH 7.40. Data and standard errors of three replicates.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

loading. On the other hand, as the number of charges decreases and the swelling of the hydrogel CH1 increases, because of the lower cross-linking density, the loading of the pilocarpine becomes two times higher. Furthermore, in this case, a slow sustained release was improved for a week. This behavior was already found for the release of the ferulic acid from the zwitterionic hydrogels [17]. As a matter of fact, the presence of only negative charges, due to carboxylate anions of greater pK_a values, in the HVa hydrogels, clearly improves the effect of the electrostatic interactions [24,25]. In all cases, the amount of incorporated pilocarpine is almost the same and becomes three times greater with respect to the comparable gel H5. The less cross-linked HVa1 gel shows, after the initial burst effect, a greater slope for the sustained release of the pilocarpine within six days. As the cross-link density increased, the releasing profile was flatter. Unlike the gel HVa2, that showed a longer sustained release, the gel HVa5 released pilocarpine only for three days. It is evident that the cross-link density plays a role in the controlled release of the drug: any increase of the EBA content leads to a lower concentration of pilocarpine able to be therapeutically available. This may be tailor-made for applications to design polymeric systems of improved efficacy, avoiding thus possible toxic side effects.

The effect of temperature was also evaluated for two hydrogels: CH1 and HVa2. In Fig. 8 is compared the releasing profile of pilocarpine at 25.0 ± 0.1 °C and 36.0 ± 0.1 °C in the same PBS solution. It is noteworthy that the increased temperature displays an increased amount of released pilocarpine. For the hydrogel CH1, the sustained release of pilocarpine at 25.0 ± 0.1 °C, monitored for a week, notably increased for more than one day as the temperature increased to 36.0 ± 0.1 °C. At the same temperature of 36.0 ± 0.1 °C, the amount of pilocarpine released from the gel CH1 was higher, within the three experimental days, reaching almost the quantity released from the gel HVa2. The effect of temperature improved the same behavior for the gel HVa2, because the releasing profile showed either a greater slope and a greater amount of pilocarpine released within two days at 36.0 ± 0.1 °C. This behavior was correlated to the shrinking phenomenon occurring in temperature-sensitive hydrogels [39–41]. In the gel CH1, the presence of the greater NIPAAm content displayed a lower solvation at increased temperature. This caused the gel to shrink and the pilocarpine molecules gradually squeezed out from the polymeric network. Likewise, the gel HVa2 showed the same behavior. This is in line with the fact that this gel showed a temperature-responsiveness, as described above.

3.5. Biological evaluation

3.5.1. Cytotoxicity assay: NIH3T3 viability

The evaluation of the in vitro acute toxicity does not depend on the final use the material is intended for and the document ISO 10993-5 recommends many cell lines from American Type Culture Collection [31]. Among them, for the evaluation of the HVa hydrogels cytotoxicity, immortalized mouse fibroblasts NIH3T3 cells were chosen. Not confluent adhered cells were incubated with 10 mg of each swollen sample, both in the native form and loaded with Pcp. Samples were analyzed after 24 h of incubation, and the results are reported in Fig. 9. All the tested samples were not cytotoxic for mouse fibroblast 3T3. In particular, the percentage of viable cells in contact with the native samples (i.e. HVa1, HVa2, and HVa5) and with the HVa5-Pcp loaded was not statistically different in comparison to the negative control (HDPE). On the contrary, viable cells in contact with Pcp, HVa1-Pcp and HVa2-Pcp were significantly more numerous than both in contact with HDPE and Pcp. Looking at graph reported in Fig. 9, it is evident that the number of cells in contact with the hydrogel reached the highest value for HVa2-Pcp, decreased for HVa1-Pcp and reached the lowest value for the HVa5-Pcp. This trend may be due to the different cross-linking degree of the hydrogels. In fact, the HVa2, with an intermediate cross-linking degree, showed a structure which promotes an optimal drug release.

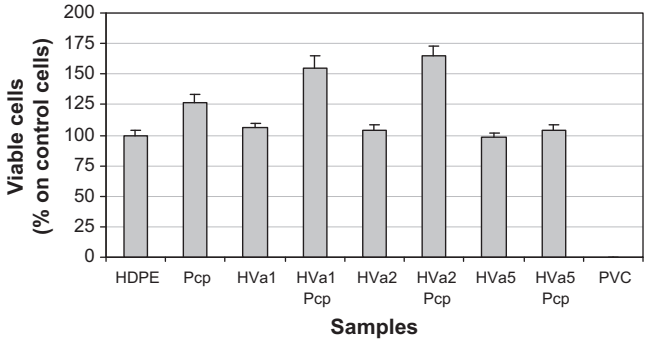


Fig. 9. Cytotoxicity assay: percentage of viable NIH3T3 cells after 24 h of contact with the different hydrogels (HVa1, HVa2, and HVa5) loaded and not with pilocarpine (2 wt%). Data are means ± standard error of three experiments run in triplicate. HDPE: high-density polyethylene (control cells); Pcp: pilocarpine; PVC: organo-tin stabilized poly(vinylchloride) [Pcp, HVa1-Pcp, and HVa2-Pcp are statistically different from HDPE ($p < 0.05$). HVa1-Pcp and HVa2-Pcp are statistically different from Pcp].

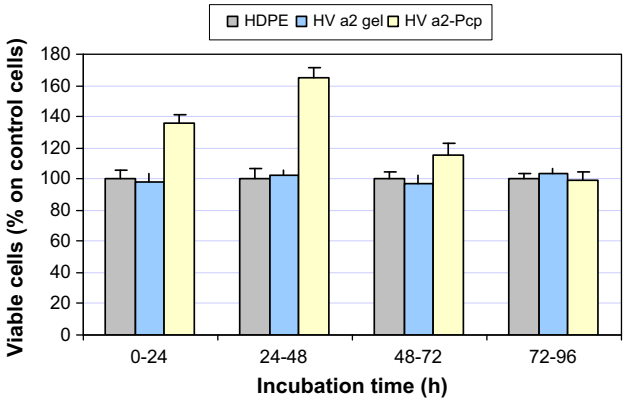


Fig. 10. Effect of pilocarpine release from HVa2 on NIH3T3 cells proliferation from 0 to 24 h, from 24 to 48 h, from 48 to 72 h, and from 72 to 96 h. [HVa2-Pcp 24 h of incubation, HVa2-Pcp 48 h of incubation, and HVa2-Pcp 72 h of incubation are statistically different from both HDPE and HVa2 gel ($p < 0.05$)]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5.2. Effect of pilocarpine release from HVa2 on cell proliferation

The effect of Pcp released from the HVa2 sample was evaluated at intervals of 24 h for a total incubation time of 96 h. Every 24 h the samples were removed and placed on new cell layer in the presence of fresh medium. The number of viable cells is reported in Fig. 10. The released Pcp showed to increase the percentage of viable cells from 0 to 24 h, from 24 to 48 h and from 48 to 72 h. The maximum of viable cells was obtained with the drug released from 24 to 48 h. From 72 to 96 h, the amount of released drug was not able to significantly influence the number of cells, which resulted to be the same of both HDPE and native HVa2. This is in line with the above reported results on the release of Pcp from the gel HVa2 at 36.0 ± 0.1 °C (see Fig. 8).

From the obtained results, we can suppose that the pilocarpine is able to influence the rate of cell proliferation, even if its mechanism of action towards fibroblasts NIH3T3 is not known.

4. Conclusions

The proposed platforms based on stimuli-responsive hydrogels may have a wide interest in many biomedical fields to release drug to the target site [1–4,9,21,22]. In the glaucoma therapy, they provide a sustained pilocarpine release, show resistance and good optical transmission in the swollen state. The hydrogels, being non-toxic towards the cell-line 3T3, may be inserted in purposely designed Ocusert-pilocarpine devices [19].

The pilocarpine molecule remains loaded into the hydrogel by ionic interaction and also entrapped into the network. After the initial burst effect, a sustained release may be improved for long time depending on the cross-link density and on the nature of the material. Moreover, the release of the drug may be tuned by changing the external conditions, like temperature.

The preliminary biological evaluation shows a good biocompatibility that allows them to be promising candidates as soft materials, at low cross-link density.

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