Cross-linked poly(acrylic acids) microgels and agarose as semi-interpenetrating networks for resveratrol release

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Received: 19 February 2014 / Accepted: 20 July 2014 / Published online: 11 January 2015

1 Introduction

Hydrogels are the elective materials in clinical practice for the development of innovative strategies to deliver drug candidates to a target site at a therapeutic concentration [1–3]. In spite of the large number of hydrogels for drug delivery, several challenges are still unmet; for example, drug loading is a key point especially for thermolabile and hydrophobic drugs.

Resveratrol (3,5,4′-trihydroxy-stilbene; RSV, Online Resource 1) is a natural polyphenol found in a wide variety of plants, including peanuts, grapes and berries. In vitro it shows a broad range of biological benefits, including cardioprotection [4], cancer prevention [5] and neuroprotection [6], but their confirmation in humans through randomized clinical trials is very limited [7]. In fact, RSV is subject to a presystemic metabolism through first-pass glucuronidation and sulfate conjugation [8–11], that strongly reduces its in vivo bioavailability. To overcome this limitation, the development of suitable delivery systems may thus stabilize and protect RSV from degradation and increase its blood plasma concentration to an effective level.

Particulate systems, such as nano- and microparticles, lipid-core nanocapsules, liposomes, halloysites clay nanotubes and cyclodextrin-based nanosponges, were proposed for RSV delivery in several applications [12–17], while

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only few examples related to the loading of RSV in a simple bulk hydrogel, a macrogel, are reported. Mixtures of RSV and ammonium acryloyldimethyltaurate/vinylporrolidone copolymer, sodium carboxylmethyl cellulose, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, Carbopol® 940 or Carbopol® Ultrez20 were proposed as soft gels for the topical/transdermal treatment of skin disorders [18, 19], while Sheu et al. [20] exploited RSV to cross-link oxidized hyaluronic acid for future applications in cartilage tissue engineering, rather than release. Thus, the loading of RSV in an injectable hydrogel to be easily placed into target tissues or intranasal administered (as explored for several bioactive molecules poorly available in the brain after systemic administration [21–23]) may represent an innovative strategy to enhance RSV therapeutic potential against neurodegeneration by a localized in situ administration. Furthermore, a bulk hydrogel offers the advantage to be exploitable to embed particulate systems as a reinforcement or a tool to slow the release, thus preventing their dispersion [24].

In this work, we have proposed poly(acrylic) acid/agarose hydrogels for the delivery of RSV against neurodegeneration. Starting from two well-known and characterized polymer components and aiming at tightly controlling the production steps, we have optimized a simple preparation method that allows to obtain, in one shot, a sterile and a solid-like gel, without the need for further chemical modifications or expensive purifications.

Cross-linked poly(acrylic acid) polymers, also known as carbomers or Carbopols®, have been extensively used as thickening agents in pharmaceutical formulations and hydrogels in a semi-diluted concentration [25–30]. Among them, Carbopol® 974P is a poly(acrylic acid) cross-linked with allyl ethers of pentaerythritol in ethyl acetate. In the form of powder (2–7 μm) it hydrates in water and at neutral pH it creates a dispersion of swollen microgels (20–70 μm). The rheological properties of the dispersion may be modulated by acting on pH, co-solvents and electrolyte concentration; in particular, at neutral pH, in the absence of electrolytes and in the presence of molecules able to promote hydrogen bonding (e.g. alcohols as hydroxyl donors), Carbopol® 974P behaves as a highly viscous liquid rather than a solid-like gel [31].

Among hydrogels, semi-interpenetrating polymer networks (semi-IPNs) offer the advantage to join two independent and physically interlocked networks, combining the features of both polymer components in a tunable way [3, 32]. Thanks to their abundance, low cost of production and variety of composition and properties, polysaccharides are good candidates as building blocks of semi-IPNs [33]. Agarose is particularly interesting to develop drug release systems due to its gelling ability, thermoreversibility and tunable viscoelastic properties. Depending on the degree of hydroxyethyl substitutions on the side chains, it gels in the temperature range 17–40 °C and the gels are thermally reversible [34]. These properties may be exploited for drug loading in a sol form, prior to solidification. Thus, the sol–gel transition of the proposed poly(acrylic) acid/agarose hydrogels offers the advantage to easily load RSV (or other thermolabile drugs) after their preparation and sterilization, without degrading the drug.

Carbomer/agarose-based hydrogels were previously tested for electrically-modulated drug delivery [35] and as potential cell carriers [36]. Starting from the literature available, in this work we have optimized the formulation of a semi-IPN composed of Carbopol® 974P microgels and agarose networks with a view to pave the way for the development of a simple and easily tunable drug delivery device and evaluated its potentiality for the release of RSV.

2 Materials and methods

2.1 Materials

Carbopol® 974P NF was kindly provided by Lubrizol Corporation (Wickliffe, OH, USA), while Ultra-Pure™ agarose and reagents for culturing SH-SY5Y human neuroblastoma cell line (American Type Culture Collection® code CRL-2266™) were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Dulbecco's phosphate buffered saline (PBS), tris(2-hydroxyethyl)amine (triethanolamine), *N*,*N*-di-ethylethanamine (triethylamine), sodium hydroxide, glycerol, 1,2-propanediol, ethanol and reagents for culturing L929 immortalized mouse fibroblasts (European Collection of Cell Cultures no. 85011425) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sterile polystyrene plasticware was purchased from Corning Incorporated (Corning, NY, USA), while 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-etrazolium (MTS, CellTiter 96® AQueous One Solution Cell Proliferation Assay) was from Promega Corporation (Madison, WI, USA).

2.2 Preparation of carbomer/agarose hydrogels (CarHy)

Two sets of CarHy were prepared using two different methods for the heating process: heating by microwave (P.Ethyl-m) or autoclave (P.Ethyl-a).

P.Ethyl-m hydrogels were prepared by dispersing 0.73 % (w/v) of Carbopol® 974P in PBS (pH 7.1) with moderate agitation for 1 h, then triethylamine was added (7.2 mmol/g carbomer) and the dispersion was mixed for 20 min. Preweighted amounts of 1,2-propanediol (817 mmol/g carbomer) and glycerol (27.2 mmol/g carbomer) were added

Table 1 Formulations and pH values of P.Ethyl-a, P.Ethanol, P.NaOH, P.NaOH.15, P.NaOH.0 and P.Ethanol.0 dispersions

CarHy	Neutralizing agent (7.2 mmol/g carbomer)	Hydroxyl donors (mmol/g carbomer)		pH
		1,2-Propanediol	Glycerol	
P.Ethyl-a	Triethylamine	817	27.2	5.8
P.Ethanol	Triethanolamine	817	27.2	5.1
P.NaOH	Sodium hydroxide	817	27.2	5.9
P.NaOH.15	Sodium hydroxide	408.5	27.2	5.8
P.NaOH.0	Sodium hydroxide	–	–	5.7
P.Ethanol.0	Triethanolamine	–	–	4.6

under stirring, then the dispersion was stored at 4 °C for at least 24 h. Finally, agarose (0.5 g/g carbomer) was manually mixed to the dispersion. The mixture was heated by microwave at 750 W for 1 min (for 10 mL of dispersion) until boiling and subsequently cooled to room temperature.

To prepare P.Ethyl-a hydrogels, after the addition of agarose, the dispersion was autoclaved (760 autoclave, Asal s.r.l., Italy) at 121 °C for 20 min.

The dispersions were then modified as described in Table 1 and heated by autoclave. Equimolar quantities of triethanolamine or sodium hydroxide were introduced as substitutes of triethylamine and the amount of OH donors was reduced and replaced with an equal volume of PBS.

2.3 Rheological characterization

Rheological characterization was performed with an AR 1500ex rheometer (TA Instruments, New Castle, DE, USA) equipped with parallel-plate geometry (20 mm diameter, 1000 μm working gap). The linear viscoelastic region was determined at 25 °C with an amplitude sweep test and the stress was varied from 0.1 to 100 Pa.

Frequency sweep test was performed at 25 °C in the linear viscoelastic range at a constant strain ($\gamma = 1$ %) with the angular frequency ranging from 1 to 100 rad/s. Storage (G'), loss (G'') moduli and complex viscosity were recorded as a function of frequency.

Creep test was performed in the linear viscoelastic range at a constant stress ($\sigma = 5$ Pa) and the percentage of strain was recorded as a function of time.

Storage and loss moduli were investigated with a temperature ramp test in the range of 20–100 °C, with steps of 5 °C/min.

All the experiments were performed in triplicate.

2.4 Preparation of CarHy samples

CarHy samples were prepared by pipetting 500 μL of the autoclaved dispersions within cylindrical moulds (inner diameter 11.05 mm) placed in 12-well plates. The moulds were removed after gel formation and the samples were used to evaluate the water content, weight loss and in vitro cytocompatibility with two different cell models, L929 mouse fibroblasts [37] and SH-SY5Y neuroblastoma cell line.

2.5 Water content

CarHy samples were weighted (m_{wet}) with an analytic balance (Crystal 200, Gibertini Elettronica s.r.l., Italy). To measure the variation of water content (ΔWC), each sample was immersed in 2.5 mL of PBS and distilled water (dH_2O) up to 7 days. At each time point (1, 4, 24, 48 h and 7 days) it was extracted from the solvent and the excess of fluid was gently removed with filter paper. The wet-swollen gels were weighted ($m_{wet\_swell}\ (t)$) and ΔWC was calculated as follows (1):

$$\Delta WC\,(\%) = \frac{m_{wet\_swell} - m_{wet}}{m_{wet}} \times 100 \quad (1)$$

The test was performed in triplicate and the results were expressed as mean ± standard deviation.

2.6 Swelling behavior

To quantify the swelling ratio (SR), CarHy samples were dried at 40 °C until constant weight (m_{dry}), then they were incubated in dH_2O at 37 °C up to 7 days. At each time point (1, 4, 24, 48 and 7 days) the excess of dH_2O was removed and the samples were weighted ($m_{swell}(t)$). SR was calculated using equation (2):

$$SR\,(\%) = \frac{m_{swell}(t) - m_{dry}}{m_{dry}} \times 100 \quad (2)$$

The test was performed in triplicate and the results were expressed as mean ± standard deviation.

2.7 In vitro cytocompatibility evaluation

2.7.1 Cell culture

L929 mouse fibroblasts and SH-SY5Y human neuroblastoma cell line were cultured at 37 °C in 5 % CO_2 in Dulbecco's Modified Eagle Medium supplemented with 10 % (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/mL of penicillin and 100 μg/mL streptomycin. Cells were split twice a week.

2.7.2 Elution assay with L929 cells

The biological effect of the hydrogel modifications was initially investigated on L929 cells with the elution test.

Cells were seeded in a 96-well plate at a density of 31,250 cells/cm^2. After 24 h, the medium was replaced with 100 μL of hydrogel supernatants, obtained by incubating the hydrogels in 2.5 mL of culture medium for 1, 3.5, 24 h and 7 days at 37 °C. At each time point the supernatants were collected and added to the cells. After 24 h of incubation, cell viability was assessed by MTS assay, according to the instructions provided by the manufacturer. After 3 h of incubation at 37 °C in 5 % CO_2, the absorbance was measured at 485 nm (reference wavelength 630 nm) by a spectrophotometric plate reader (Tecan GeNios Plus; Tecan Group Ltd., Switzerland). Cell viability (%) was calculated with respect to controls grown in standard conditions using eq. (3), where A is the absorbance measured:

$$\text{Viability}\,(\%) = \frac{A\_sample}{A\_control} \times 100 \qquad (3)$$

The test was performed in triplicate and the results were expressed as mean ± standard deviation.

2.7.3 Elution assay with SH-SY5Y cells

The biological effect of P.Ethanol.0 and P.NaOH.0 was also evaluated on SH-SY5Y cells with the elution test, as described. To assess the effect of hydrogel supernatants on a different number of cells, SH-SY5Y cells were seeded at different densities (31,250; 62,500 and 93,750 cells/cm^2). After 24 h, the medium was replaced with 100 μL of hydrogel supernatants, obtained by incubating the hydrogels in 2.5 mL of culture medium for 1 h, 2 and 7 days at 37 °C. After 48, 67 and 24 h of incubation, respectively, cell viability was assessed by MTS assay.

2.8 In vitro drug release

2.8.1 Resveratrol loading

RSV was dissolved in ethanol to obtain a concentration of 1.9 mM and loaded in P.NaOH.0 (Table 1). After the heating process and prior to solidification of the gel (at about 40 °C), the RSV solution was mixed to the polymeric dispersion to ensure a homogeneous dispersion and obtain a final concentration of 131.4 μM, that represents the aqueous solubility limit of RSV.

2.8.2 Resveratrol release

RSV-loaded samples were prepared in 12-well plates, then 2.5 mL of PBS or dH_2O was added and the plates were incubated at 37 °C. After 1, 4, 24 h and 7 days, the supernatants were collected and replaced with fresh release medium. The RSV content in the supernatants was measured at room temperature with a UV–visible spectrophotometer (6705 UV/vis spectrophotometer, Jenway, Bibby Scientific, UK) at 304 nm in the calibration range 2.1–131.4 μM. As a control, at each time point the absorbance of the supernatants collected from P.NaOH.0 hydrogels was subtracted from the absorbance of the release media collected from the RSV-loaded samples.

The test was performed in triplicate and the results were expressed as mean ± standard deviation.

2.9 Statistical analysis

Statistical analysis was performed using Origin-Pro 8 software (OriginLab, Northampton, MA, USA). One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used for comparisons among the groups, while the student's *t* test was used to compare two different groups of samples.The significance level was set at $P < 0.05$.

3 Results

3.1 Preparation and characterization of P.Ethyl-m

P.Ethyl-m was prepared by dispersing Carbopol® 974P in PBS. As described [36], triethylamine was added to increase the pH, while alcohols acted as hydroxyl donors and induced hydrogen-bonding, causing the polymer mol-ecule to uncoil. The gel formation was produced by ther-mal treatment through microwave radiation, that presents advantages in terms of efficiency, low energy consumption and homogeneity if compared to traditional heating methods [38].

The pH value of P.Ethyl-m hydrogels obtained from three independent experiments was 5.8 ± 0.1 (mean ± standard deviation), suggesting the reproducibility of the process. At all the frequencies investigated, the storage modulus G′ was greater than the loss modulus G″ and showed a frequency-independent response, indicating a solid-like behavior (Fig. 1). The gel showed a non-Newtonian shear thinning behavior, as the complex viscosity decreased while increasing the frequency, suggesting its injectability (data not shown). The temperature ramp test (Fig. 2) showed a transition from a solid-like to a liquid behavior by increasing the temperature, with an onset point at about 76.0 °C. However, in the range of physiological temperatures G′ did not change significantly.

3.2 Effect of the heating method

P.Ethyl-m and P.Ethyl-a share the same formulation, but the dispersion for P.Ethyl-a hydrogels was autoclaved

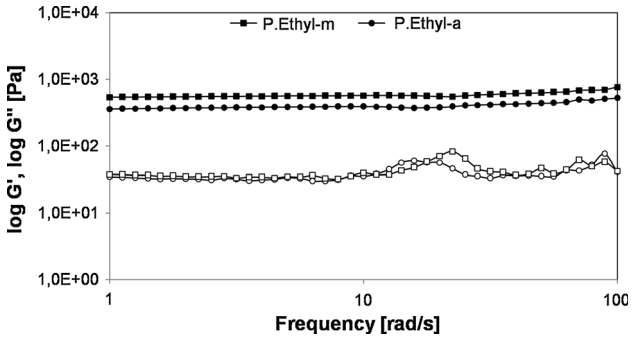

Fig. 1 Frequency sweep test: storage modulus (G′, filled symbols) and loss modulus (G″, void symbols) as a function of frequency for P.Ethyl heated by microwave (P.Ethyl-m) or autoclave (P.Ethyl-a)

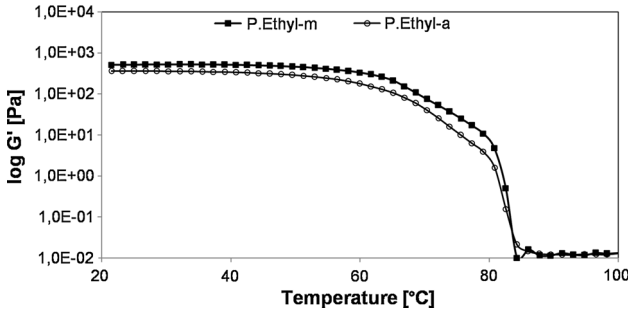

Fig. 2 Temperature ramp test: storage modulus (G′) as a function of temperature for P.Ethyl heated by microwave (P.Ethyl-m) or autoclave (P.Ethyl-a)

instead of heated by microwave. Since autoclaving represents the easiest sterilization procedure approved by the US Food and Drug Administration [39], it was introduced with the double function to sterilize the materials and produce the gel, increasing, in addition, the control over the heating process.

The effects of this modification were evaluated, observing comparable pH values (5.8 ± 0.1) and rheological properties (Figs. 1, 2) for P.Ethyl-m and P.Ethyl-a. In particular, the onset points recorded in the temperature ramp test (Fig. 2) were 73.0 ± 7.0 °C and 76.0 ± 7.0 °C for P.Ethyl-a and P.Ethyl-m, respectively.

From a biological point of view, the elution assay with L929 cells indicated that after 24 h of incubation with the supernatants collected from P.Ethyl-m and P.Ethyl-a, cell viability was reduced in comparison with controls grown in standard conditions and the change in the heating method did not lead to significant differences between the hydrogels. In particular, the average values remained lower than 50 % (data not shown).

According to these results, P.Ethyl-a was selected as a reference and subject to further optimizations to develop a RSV delivery device for future applications against neurodegeneration.

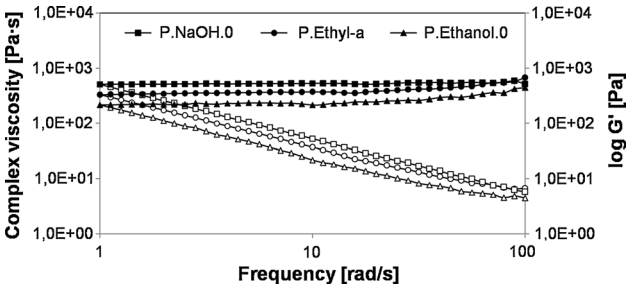

Fig. 3 Frequency sweep test: complex viscosity (*opened symbols*) and storage modulus (G′, *filled symbols*) as a function of frequency for P.Ethyl-a, P.NaOH.0 and P.Ethanol.0

3.3 Effect of the neutralizing agent and OH donors

Different hydrogels were prepared from P.Ethyl-a by varying the neutralizing agent and the concentration of OH donors (Table 1).

These modifications impacted on pH values. The hydrogels produced with triethanolamine (P.Ethanol and P.Ethanol.0) presented a lower pH (5.1 and 4.6, respectively) than those prepared with triethylamine (P.Ethyl-a) and sodium hydroxide (P.NaOH, P.NaOH.15 and NaOH.0), that exhibited a similar pH (5.8 ± 0.1).

3.3.1 Rheological characterization

Amplitude sweep tests indicated that all the hydrogels presented a linear viscoelastic region in the range from 0.1 to 10 Pa (data not shown). Above this value, a breakdown of the structure occurred and the elastic modulus decreased while increasing the stress. Therefore, all the subsequent characterization was performed with stress and strain values in the linear viscoelastic region.

The storage modulus G′ and complex viscosity of P.Ethyl-a, P.Ethanol.0 and P.NaOH.0 as a function of frequency are shown in Fig. 3. All the hydrogels presented a solid-like behaviour with a frequency-independent response. G' depended on the pH and the presence of co-solvents like glycerol and 1,2-propanediol. Greater values were measured for P.Ethyl-a and P.NaOH.0, that presented the higher pH values (5.8 vs. 4.6 for P.Ethanol.0). In fact, the increase in the pH determined the uncoiling and swelling of carbomer microparticles and consequently the formation of elastic networks and entangled structures. The comparison between P.Ethyl-a and P.NaOH.0 indicated that the hydrogels prepared without the addition of OH donors (P.NaOH.0) showed a thicker behavior.

The results from the temperature ramp test (Fig. 4) showed that in the range of physiological temperatures, all the gels presented a constant G′ profile. The onset point (mean ± standard deviation) occurred at 73.0 ± 7.0 °C for

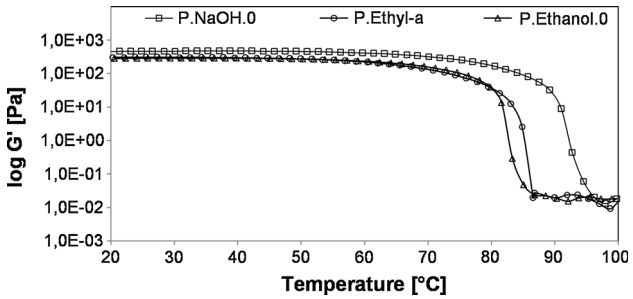

Fig. 4 Temperature ramp test: storage modulus (G′) as a function of temperature for P.Ethyl-a, P.NaOH.0 and P.Ethanol.0

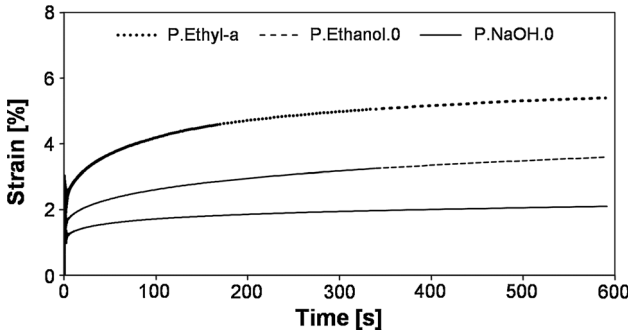

Fig. 5 Creep recovery curves as a function of time for P.Ethyl-a, P.NaOH.0 and P.Ethanol.0 with an applied stress of 5 Pa

P.Ethyl-a, 76.7 ± 2.3 ° C for P.Ethanol.0 and 89.0 ± 0.1 °C for P.NaOH.0.

On the overall, these results suggested that P.NaOH.0 behaved as a more cross-linked structure than the other investigated.

The results from the creep test are shown in Fig. 5 for P.Ethyl-a, P.Ethanol.0 and P.NaOH.0 hydrogels. The values of the strain increased with time and tended to level off after the initial elastic oscillating response. The lowest percent strain obtained for P.NaOH was in agreement with the greatest G′ values observed for this hydrogel in the frequency sweep test (Fig. 3).

3.3.2 Water content

The initial water content was lower for P.Ethyl-a than P.NaOH.0 and P.Ethanol.0 (89.3 ± 4.7, 97.8 ± 1.5 and 97.2 ± 4.3 %, respectively). The variation of water content at 1, 4, 24, 48 h and 7 day after the immersion in PBS and dH_2O is presented in Fig. 6. After the immersion in dH_2O, the weight of the hydrogels showed an increase of about 20 % with respect to the initial wet mass and after the first 4 h it was stable. After dipping in PBS, the solvent used to prepare the carbomer dispersions, the weight of the hydrogels was stable up to 7 days (with a variation lower than 10 %).

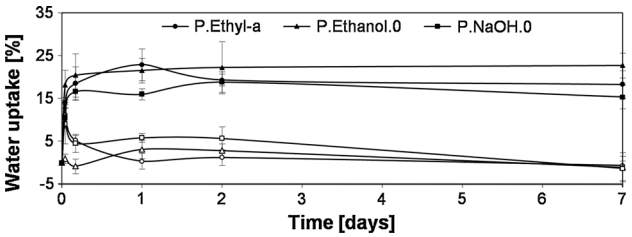

Fig. 6 Variation of water content as a function of time in water (*filled symbols*) and PBS (*opened symbols*) for P.Ethyl-a, P.NaOH.0 and P.Ethanol.0

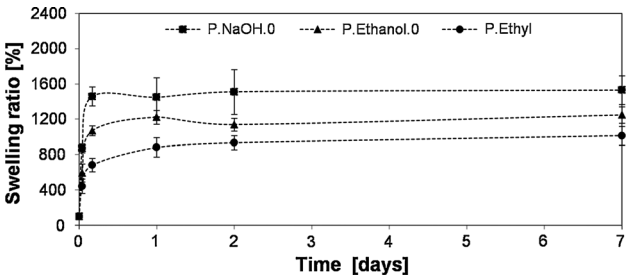

Fig. 7 Swelling ratio as a function of time in water for P.Ethyl-a, P.Ethanol.0 and P.NaOH.0

3.3.3 Swelling behavior

For all CarHy hydrogels, the swelling curves showed the same trend and reached equilibrium within 4 h. For P.Ethyl-a, P.NaOH.0 and P.Ethanol.0 (Fig. 7) the values varied as a function of composition. P.Ethyl-a presented a lower swelling capacity than P.Ethanol.0 and P.NaOH.0, that were prepared without OH donors. Between them, the gel neutralized with sodium hydroxide, exhibited the greater SR.

3.3.4 Elution assay with L929 cells

The results of the elution test with L929 mouse fibroblasts for the different CarHy hydrogels are reported in Fig. 8. Even though the viability of L929 cells was lower in comparison with controls, P.Ethanol and P.NaOH showed less cytotoxic effects than P.Ethyl-a ($P < 0.05$), suggesting that the replacement of triethylamine with triethanolamine or sodium hydroxide improved the biological performance. This result was supported by bright-field images of the cells (Online Resource 2), where the loss of the expected spindle-shaped morphology was more apparent for the cells cultured in the supernatants from P.Ethyl-a hydrogels.

An increase in cell viability in relation to a decrease of glycerol and 1,2-propanediol was observed by comparing P.NaOH with P.NaOH.15 and P.NaOH.0 and P.Ethanol with P.Ethanol.0 (Fig. 8), suggesting that the amount of

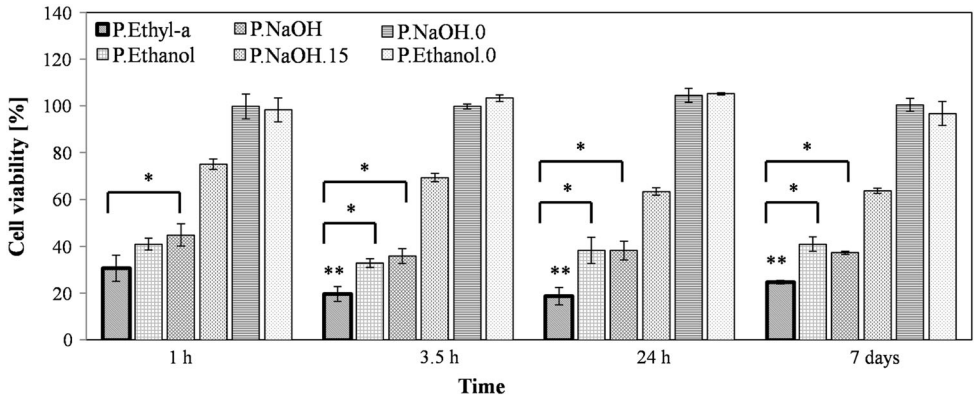

Fig. 8 Elution assay: L929 fibroblast viability after 24 h of incubation with the supernatants collected from CarHy hydrogels at 1, 3.5, 24 h and 7 days. Cell viability was expressed as a percentage with respect to controls grown in standard conditions. $*P < 0.05$ for the difference between P.Ethyl-a and P.Ethanol and P.NaOH.0 at each time point, $**P < 0.01$ for the difference in P.Ethyl-a with time

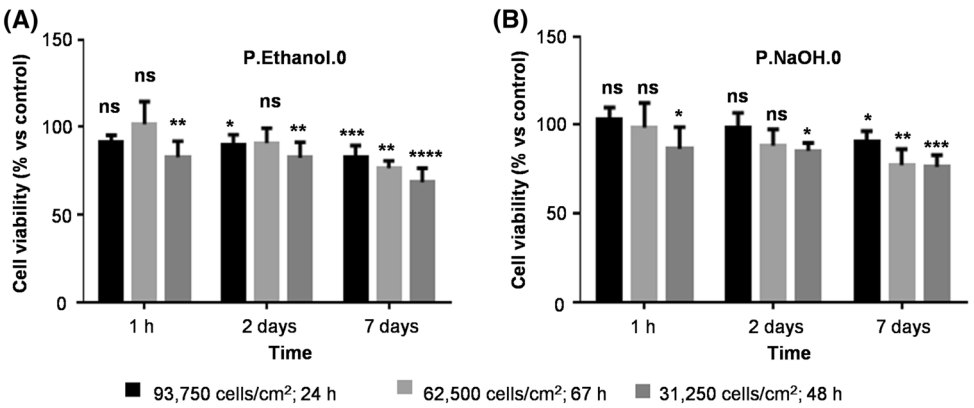

Fig. 9 Elution assay: SH-SY5Y cell viability after incubation with the supernatants collected at 1 h, 2 and 7 days from: **a** P.Ethanol.0, **b** P.NaOH.0. Cell viability was reported as a percentage with respect to controls grown in standard conditions. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, while ns indicates that cell viability was comparable with controls

OH donors has a significant impact on cell viability. In particular, for the hydrogels prepared without alcohols (P.NaOH.0 and P.Ethanol.0), cell viability was comparable with controls grown in standard medium at all the time points selected. These results were confirmed by the bright-field images of the cells (an example of which is reported in Online Resource 3), showing a recovery of the expected fibroblastic-like morphology while decreasing the amount of OH donors.

3.3.5 Elution assay with SH-SY5Y cells

Considering our future application, the gels showing the best performances with L929 cells, that is P.Ethanol.0 and P.NaOH.0, were also tested with SH-SY5Y cells (Fig. 9). To study the effect of the same volume of hydrogel supernatants on a different number of cells, three conditions were examined. In the first one (93,750 cells/cm^2), the viability of SH-SY5Y cells cultured for 24 h in the supernatants from P.NaOH.0 was lower than controls only for the supernatants collected on day 7 ($P < 0.05$), while for those grown in the supernatants from P.Ethanol.0 a decrease was also observed with the supernatants collected on day 2 ($P < 0.05$ on day 2, $P < 0.001$ on day 7). In the second condition (62,500 cells/cm^2), the results from P.Ethanol.0 were comparable to those from P.NaOH.0: the viability of SH-SY5Y cells cultured for 67 h in the hydrogel supernatants was lower than controls only for the supernatants collected on day 7 ($P < 0.01$). In the first condition (31,250 cells/cm^2, 48 h of culture in the supernatants), a significant difference was found at all the sampling times between treated and control cells, but the average viability was always greater for SH-SY5Y cells grown in the supernatants from P.NaOH.0.

Globally, these results suggested that P.NaOH.0 has a better performances than P.Ethanol.0 with neuronal-like cells. For this reason, P.NaOH.0 was selected for RSV release studies.

3.3.6 Resveratrol release

To obtain a homogeneous and reproducible system, RSV was loaded in the viscous dispersion for P.NaOH.0 hydrogels during the cooling process. The RSV release

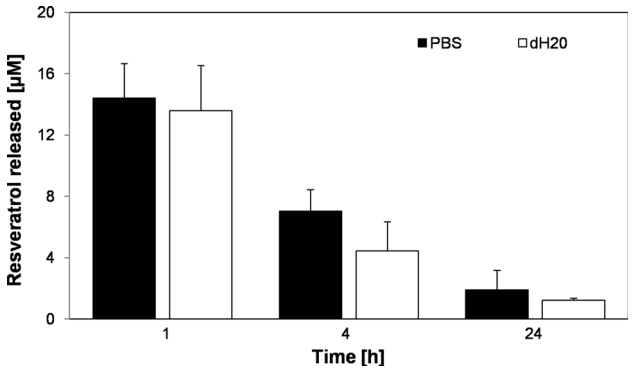

Fig. 10 RSV release profile from P.NaOH.0 hydrogels in distilled water and PBS

profile in dH_20 and PBS is shown in Fig. 10. It was independent on the incubation medium, as no significant differences were recorded between the values obtained in dH_2O and PBS at each time point ($P > 0.05$). Although the greater amount of the released drug was detected within the first hour, a moderate release was also visible at 4 and 24 h of incubation, while at 48 h and 7 days no release was observed in any of the conditions considered.

4 Discussion

The development of drug delivery systems is fundamental to overcome the limitations imposed by molecules with poor oral bioavailability but high therapeutic potential. To develop a device for the delivery of RSV due to its potential therapeutic relevance in several disorders including neurodegenerative diseases [6], we focused on the optimization of the formulation of carbomer/agarose-based hydrogels from both a rheological and in vitro biological point of view.

As a first step we selected the solvent where dispersing Carbopol® 974P by comparing water and PBS. Both prior and after the thermal treatment, the dispersion prepared in PBS was less viscous than that obtained in water. In fact, when carbomer is dispersed in water or alkaline solution, the ionization of carboxylic groups and the electrostatic repulsion between the anionic groups determine the increase in viscosity [40]. On the opposite, in the presence of electrolytes the viscosity is reduced, because the charges are partially shielded by the cations [41]. Since this characteristic appears more favorable to achieve a homogeneous dispersion of the drug in the carrier, all the hydrogels investigated were prepared by dispersing Carbopol® 974P in PBS.

Sterilization issues are a critical step in the development and clinical translation of biomaterials. Aiming at producing, in one shot, a sterile and a solid-like gel, we focused on the heating method and investigated the possibility to move from microwave heating to autoclaving. Agarose is commonly molten in a microwave oven or autoclave in the basic protocols for the resolution of DNA fragments [42], while it is reported that in liquid formulations carbomers react with oxygen, leading to a permanent reduction or loss of viscosity. This process is catalyzed by sunlight, UV light and some metals in the water; in particular, trace levels of iron and other transition metals may catalytically degrade Carbopol® polymers. Heat may accelerate this process, but when viscosity loss is experienced upon sterilization at 121 °C, the problem may be stopped by deaerating the formulation under vacuum and breaking the vacuum with inert gas [43].

The results showed that P.Ethyl-m and P-Ethyl-a were comparable in terms of rheological properties and in vitro biological performance with immortalized fibroblasts, thus autoclaving was selected as a heating method, since this preparation method offers the advantage to both produce and sterilize the gel. As a further step, we varied the neutralizing agent and the amount of OH donors and evaluated the effects of these modifications with respect to the just optimized formulation. The addition of the neutralizing agent increases the number of carboxylate groups that lead to the swelling of carbomer microgels and may facilitate the network formation. In fact, at higher pH the repulsion between the negative charges causes maximum uncoiling and the dispersion becomes more closely packed and entangled [44]. The use of alcohols to promote hydrogen bonding is a common strategy to produce carbomer-based hydrogels [45]. Polyols and sugars may be added as hydroxyl donors with the effect of thickening the gel, but their presence may also aid the dissolution of poorly water-soluble drugs, such as RSV. As described [36], in this work this approach was followed by adding 1,2-propanediol and glycerol. The pH values of P.Ethyl-a and P.NaOH.0 (5.8) are comparable, while the value decreases for P.Ethanol.0 (4.6). P.NaOH.0 is thicker and presents a stronger network with respect to the other gels, as indicated by the greatest storage modulus and complex viscosity at all the frequencies investigated (Fig. 3). The stability of this network is evidenced by the fact that it is necessary to increase the temperature over 80 °C to observe a transition from gel-like to liquid behavior (Fig. 4) and its deformation with respect to an imposed strain is lower than that measured for P.Ethyl-a and P.Ethanol.0 (Fig. 5). The greater complex viscosity of P.NaOH.0 with respect to P.Ethyl-a (Fig. 3) might be attributed to a different interaction of the alcohols with the carbomer microgels after the thermal treatment. A solid study [46], examining the effects of polymer–solvent interactions on the rheological properties of poly(acrylic acid) gels, reported that the increase in water content with respect to the amount of alcohols favors polymer–solvent

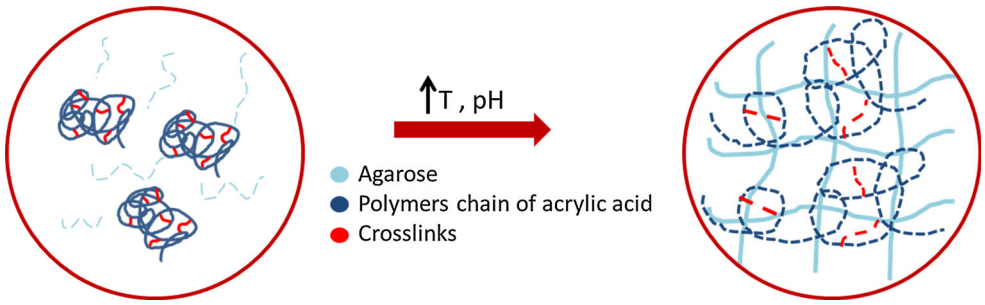

Fig. 11 A schematic representing the formation of carbomer/agarose semi-IPNs (CarHy). Before adding the neutralizing agent and heating, the mixture (*left circle*) is composed of agarose in powdered form (*light blue lines*) and carbomer in a tightly coiled structure (polymer chains, *blue lines*, interconnected by *crosslinks*, *red lines*). Increasing the pH and the temperature (*right circle*), the carbomer microgels swell and interpenetrate in the network created by the association of the agarose *double helix* (Color figure online)

interactions over polymer–polymer interactions, causing the extension of the polymer chains and increasing the viscosity of the polymer suspension. Finally, the rheological differences between P.Ethanol.0 and P.NaOH.0 (both not containing OH donors, Fig. 3, 4, 5) appear not to be related to the different neutralizing agent, but rather to the different pH value. These results are in agreement with a previous study, reporting that triethanolamine and sodium hydroxide do not result in marked rheological changes for gels with the same pH values [47].

Cytocompatibility evaluation was performed with two different cell models. L929 mouse fibroblasts, a reference cell line recommended by many standard institutions [37], were selected as a model of soft tissue response, while SH-SY5Y neuroblastoma cell line were chosen as a model of dopaminergic neurons for Parkinson's disease research.

The elution assay with L929 cells was fundamental to highlight that triethylamine shows a greater cytotoxic effect than triethanolamine and sodium hydroxide and that the removal of OH donors enhances cell viability. These results indicated that the toxicity of glycerol and 1,2-pro-panediol is concentration-dependent, but they also sug-gested that the OH donors do not strongly interact with the carbomer/agarose matrix. In fact the reduction in cell via-bility was apparent from the first time point, with no sig-nificant differences in comparison to the last time point examined. The further elution test with SH-SY5Y allowed comparing the performance of P.Ethanol.0 and P.NaOH.0 with a popular cell model of mammalian neurons, indicating that P.NaOH.0 is a better candidate for future application as a drug delivery system against neurodegeneration.

The different formulations investigated allowed us to observe that even though its concentration is relatively low (0.5 g/g carbomer), the addition of agarose is essential to obtain a gel with a solid-like behavior. In fact, a gel (P.Agar.0) prepared from agarose but without carbomer, with the same pH (5.8) and subject to the same thermal treatment as P.Ethyl-a, led to the formation of a weaker gel ($G' = 528$ Pa at 10 rad/s for P.NaOH.0 vs $G' = 300$ Pa for P.Agar.0, Online Resource 4). On the other hand, the viscosity of the dispersion prepared from carbomer but without agarose, with the same pH (5.8) and subject to autoclaving as P.Ethyl-a, was not modified by the thermal treatment. For all formulations, the viscosities of the dis-persions were unmodified when the pH was below 4. These results indicated that the contemporary presence of agarose and carbomer at a pH above 4 is required to obtain the solid-like characteristics of P.Ethyl-a, P.NaOH.0 and P.Ethanol.0. In particular, they suggested that in the pre-sence of agarose and with a pH-dependent process, car-bomer forms a semi-IPN, where agarose networks create a three-dimensional structure with the interpenetration of the cross-linked carbomer microgels (Fig. 11). This structure is stabilized by secondary bonds, that may be disrupted at about 80 °C for P.Ethyl-a and P.Ethanol.0 and about 90 °C for P.NaOH.0, when a structure-dependent transition from solid-like to liquid-like behavior occurs. As explained, an increase in pH determines the uncoiling of carbomer chains and may promote the availability of binding sites for the interactions, stabilizing the semi-IPN. Heating might also affect the mobility of polymer chains, allowing the for-mation of the network and promoting the change to an uncoiled conformation. A similar mechanism was proposed for the formation of agarose-chitosan gels and cyclodex-trin/carbopol micro-scale IPNs [48, 49].

Considering the overall results, P.NaOH.0 was selected for RSV release studies. To our knowledge, bulk hydrogels as injectable release systems for the localized delivery of RSV against neurodegeneration are not reported in literature. However, some data about the therapeutic requirements are available for several applications, suggesting that the development of a sustained delivery system may be an appealing strategy to achieve the desired efficacy while maintaining low and sustained plasma concentrations. For example, RSV shows cardioprotection when orally

delivered at 20–50 mg/kg daily (for an adult, 20 mg/kg represents approximately 140 times the maximum amount of RSV in a litre of red wine [50]), but alternative routes increase drug bioavailability; in particular, daily intravenous or intraperitoneal administration are effective at 1 mg/kg [51]. In vitro RSV inhibits the proliferation of human keratinocytes even at submicromolar concentrations, while at 40–100 μM it is cytotoxic to these cells. Furthermore, at a concentration of 50 mg/mL it inhibits *P. acnes* replication, while at a concentration of 200 mg/mL it is bactericidal [19]. With regards to neurodegeneration, it is reported that RSV is slightly toxic to SK-N-BE cells from a concentration of 10 μM, while at a concentration of 7.5 μM it prevents toxicity triggered by H_2O_2 or 6-hydroxydopamine in an in vitro model of oxidative stress [6].

For our release studies, we have mixed the polymer dispersion with a solution of RSV in ethanol at a dose of saturated solubility. The drug loading may be a critical step, in the sense that if loaded after gel formation, the dispersion of the drug could be non homogeneous within the hydrogel. On the other side, the drug could be degraded if loaded before gel formation. The loading of RSV during the cooling process (at about 40 °C) has allowed to obtain a homogeneous dispersion of the drug within the hydrogel, preventing its thermal degradation. After a burst release within the first hour, a slow release was still detected up to the end of the experiment. According to the spectrophotometric analysis of the supernatants, at the end of the test (7 days) the hydrogels released about 80 % of the drug loaded. Even though preliminary, these results suggest that RSV loading in P.NaOH.0 semi-IPNs may prolong its availability and thus enhance its therapeutic potential. In fact, previous studies have shown that after intravenous administration to rabbits (20 mg/kg), the greatest RSV concentration in plasma (42.8 ± 4.4 μM, 5 min after treatment) decreases to 0.9 ± 0.2 μM at 1 h. If the same concentration is administered orally, the greatest concen-tration in plasma (2–3 μM in mice and approximately 1 μM in rabbits or rats, measured at 5 min) decreases to less than 0.1 μM at 1 h [50].

5 Conclusions

In this study, hydrogels based on the combination of agarose networks and carbomer microgels in a semi-IPN structure were developed, optimized and tested as RSV release systems. They behaved as viscous liquids after the heating process and as solid-like gels after cooling to physiological temperatures, thus representing an advantage for drug loading. The set-up process allowed obtaining sterile hydrogels in one single step. The cytocompatibility of the formulations proposed was proved with standardized in vitro models. These results represent a starting point for future studies aimed at developing RSV release systems.

Acknowledgments The authors thank Lubrizol Corporation for providing Carbopol® 974P NF and technical bulletins about this product.

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