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ORIGINAL PAPER

Biological buffered saline solution as solvent in agar–carbomer hydrogel synthesis

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The role of phosphate buffer saline solution (PBS) was investigated here as a solvent in the polycondensation synthesis of an injectable agar–carbomer based hydrogel, a promising new material specifically intended for regenerative medicine applications. The effects of PBS, with respect to standard distilled water (DW), were quantitatively assessed. Experiments were performed both from physico-chemical and biological points of view. Titration showed higher stability due to the presence of the buffer solution; ESEM analysis confirmed its distribution along the polymeric fibers and infrared spectroscopy showed the consequent anionic nature of the polymeric network. This electrostatic nature of the matrix was confirmed by mass equilibrium swelling data performed at different pH values of the swelling medium. A very relevant role of the solvent was observed also with respect to cell housing inside such hydrogels: living cell counts showed a high amount of cells surviving the latency period of encapsulation in hydrogel when PBS was applied while only very few survived in a deionized water based gel. Obtained data allowed a novel understanding of the cause-effect cascades of all observed phenomena which suggest the PBS fundamental role both in fine control of hydrogel preparation and in material tuning according to the specific needs of different target tissues; the latter being a feature of primary importance when applying hydrogels as cell carriers in regenerative medicine applications.

Keywords: hydrogels, phosphate buffered saline solution, cell housing, agar, carbomer

Introduction

Tissue engineering, a smart combination of cells, active substances, and smart materials replacing damaged or missing parts of living tissues, is widely accepted as the future of regenerative medicine and health care (Shoichet, 2010; Lanza et al., 2000; Hynd et al., 2007). Among all known and used materials (Slaughter et al., 2009; Little et al., 2008), emerging strategies in regenerative medicine confirm a very strong interest in hydrogels as great candidates for both cell and drug delivery purposes (Garripelli et al., 2010; Perale et al., 2008; Khan et al., 2009; Slaughter et al., 2009; Tabata, 2009; Hynd et al., 2007; Crompton et al., 2007; Shim et al., 2005; Luo & Shoichet, 2004; Dumitriu, 2002) as they allow building water-based systems with cells directly encapsulated in and drugs solubilized within the water molecule (Perale et al., 2008; Tunesi et al., 2009; Brännvall et al., 2007; Dumitriu, 2002). When synthesizing such hydrogels, most attention is focused on material aspects, i.e. on used polymers and on their properties (Khan et al., 2009; Crompton et al., 2007; Brännvall et al., 2007; Shim et al., 2005; Luo & Shoichet, 2004) while very little attention is given to solvents because deionized water (DW) is the most common choice in the vast majority of gels described in literature (Kuckling, 2009; Khan et al., 2009; Slaughter et al., 2009). It is indeed a sort of implicit and commonly accepted belief that DW is the best solvent to be used in hydrogels for biomedical applications, mainly for the self-evident reason that water is the most basic solvent in all bio-

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logical systems but also because organic solvents are unsuitable. Nevertheless, the use of different water-based solutions is also mentioned in literature; these works, however, represent minor efforts, as the investigated systems are just generally described and, above all, effects of the specific solutions are not quantitatively assessed (Gorbet et al., 2010; Choi et al., 2007). Among these, PBS, a water based phosphate buffered saline solution, deserves being cited. It is a very well known commercial product used in many biological applications and its use is also standardized in many laboratory protocols. It is a buffered salty solution containing sodium chloride, sodium phosphate, and, in some formulations such as those used here, potassium chloride and potassium phosphate. The buffer nature helps to keep the pH constant; the osmolarity and ion concentrations of the solution usually match those of the human body (i.e. isotonic) (Sambrook et al., 1989; Dulbecco & Vogt, 1954). It was applied in the synthesis of biomedical hydrogels but, as already said, barely qualitative descriptions were given (Gorbet et al., 2010; Choi et al., 2007).

Hence, the use of PBS, with respect to pure DW, in hydrogel synthesis is quantitatively investigated in this paper. Specifically, a promising recently developed nanostructured hydrogel for regenerative medicine applications in the central nervous system was chosen (Perale et al., 2008; Tunesi et al., 2009) and the research was aimed at the effects of PBS used as a solvent in the polycondensation reaction between the two components used to form this gel. Particularly, PBS effects on pH which is a critical parameter in hydrogel synthesis (Fatimi et al., 2009; Rajagopal et al., 2009) were thoroughly investigated. This study was completed by a physico-chemical characterization of the obtained hydrogel samples and by a preliminary biological investigation by counting living cells extracted after being in hydrogel samples for several days.

Experimental

Components chosen for building hydrogel interpenetrating matrix were two pharmaceutical-grade polymers: carbomer 974P (CAS 151687-96-6, Fagron, The Netherlands) and agarose (CAS 9012-36-6, Invitrogen Corp., USA), both at 0.25 mass %. Propylene glycol (CAS 504-63-2, Sigma, Germany) (30 mass %) and glycerol (CAS 56-81-5, Merck Chemicals, Germany) (0.5 mass %) were added as crosslinking agents (Perale et al., 2008; Tunesi et al., 2009). The polymers were blended together with cross-linkers either in PBS (D8537 Dulbecco's Phosphate Buffered Saline, Sigma, Germany) or in deionized water (in-house made on a Millipore Elix sterile filtering system) obtaining thus a "PBS hydrogel" and a "DW hydrogel", respectively, both at 60 mass %. Both polymeric solutions were kept under mild agitation and then electromagnetically heated up to 90 °C to start the condensation reactions. The hydrogels were finally poured into metal cylinders (0.5 mL) and left to cool down to 37 °C under thermostatic conditions.

In order to obtain a biomaterial, i.e. a material suitable for biological use with living cells, pH of the polymeric suspension needed to be neutralized before the electromagnetic heating. For this purpose, triethylamine (briefly TEA, CAS 121-44-8, Sigma, Germany) was used and the sensibility of the liquid suspension was tested against TEA concentration and studied via standard titration. Titration was done starting from the polymeric solution and the effects of each solvent (DW and PBS) were measured by monitoring pH against the TEA concentration assessing thus the stability of polycondensation.

Morphological studies of PBS hydrogel samples were performed by ESEM imaging (Evo 50 EP, Zeiss, Germany). Prepared hydrogel samples were left immersed in excess PBS for 24 h and then freeze-dried for other 24 h before undergoing the ESEM analysis.

Both PBS and DW hydrogel samples were immersed for 24 h in excess of their respective solvents. Infrared spectra were then recorded using ATR (attenuated total reflection) absorbance detection (TENSOR Series FT-IR spectrometer, Bruker, Germany). For each sample, scans were recorded between 4000 cm^{-1} and 500 cm^{-1} (resolution = 8 cm^{-1}).

PBS hydrogel samples were weighted (W_d) and then immersed in excess of the solvent at different pH values, removed after 24 h, i.e. when mass equilibrium was reached (Flory, 1953), and then weighted again (W_s) (Mettler-Toledo AL204, USA). The swelling equilibrium was evaluated as the ratio between W_d and W_s.

The N9 murine microglial cell line was generated as previously reported (Perale et al., 2008). The dissociated cells were plated onto glass coverslips at the density of 0.5×10^6 cells per mL and grown in MEM cellular medium (Invitrogen Life Technologies, Invitrogen Corp., USA) supplemented with 20 % FCS (Euroclone, Italy), 100 IU mL^{-1} penicillin, 10 mg mL^{-1} streptomycin, and 5.5 g L^{-1} glucose, and kept in cellular incubator at 37 °C and 5 % CO_2.

A solution of suspended cells (density of 0.5×10^6 cells per mL) was added to the condensating PBS and DW hydrogels at neutral pH, in 1 : 1 volume ratio, and then homogenized into 48-multiwell cell culture plates (Perale et al., 2008; Tunesi et al., 2009). Each sample of the so obtained biohybrid structures was covered by a cell culturing medium and kept in cellular incubator under 5 % CO_2 at 37 °C. At chosen time points (i.e. days 1, 4, 7, and 10), cells were mechanically extracted using a Pasteur pipette with fresh and warm (37 °C) medium. The obtained cell suspension (100 μL) was diluted at the 1 : 1 ratio using the Trypan Blue (CAS 72-57-1, Sigma, Germany) staining solution and live cells were counted by a hematocytometer (Perale et al., 2008; Tunesi et al., 2009).

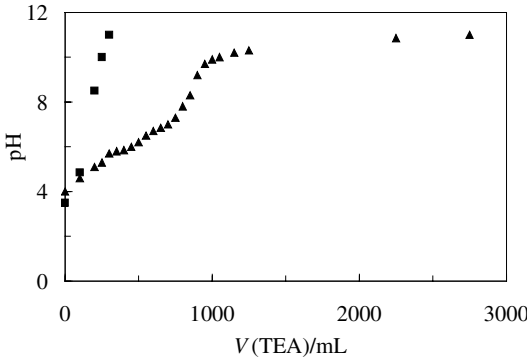

Fig. 1. Triethylamine (TEA) pH titration of the polymeric solutions (PBS and DW).

Results and discussion

Hydrogels synthesis reactions exhibit high dependence on the pH value of the ambient reaction (Fatimi et al., 2009; Shim et al., 2005). Flory (1953) defined gelation as "a sort of mistake in polymer chemistry" because if condensation starts, it precedes fast and randomly. Accordingly, its start needs to be carefully controlled and the pH value is one of the most important parameters to consider (Fatimi et al., 2009; Rajagopal et al., 2009; Chan et al., 2009; Flory, 1953). Generally, if the solution reaches a critical pH value, which depends on the chosen polymer, the reaction starts without any control making it very difficult to control the polymer synthesis and consequently the experimental reproducibility becomes very low.

To evaluate pH sensitivity of the here investigated synthesis, titration of the polymeric solutions was done comparing DW with PBS as solvents. Their trends are presented in Fig. 1. Initial pH values, i.e. determined before the titration, were approximately the same. Increasing the TEA concentration in the two systems resulted in very different behaviors: the DW hydrogel showed very high instability as a little variation in the amine concentration resulted in an extremely high pH variation. The critical pH value was reached by keeping high molar fraction of monomers so that uncontrollable reaction started at room temperature without electromagnetic heating. In the PBS hydrogel system, the pH trend showed lower sensitivity due to the buffer nature of the solvent itself. In this case, polycondensation did not start at room temperature, not even at much higher pH values due to high dilution of the monomers in the solution. Furthermore, it has to be noted that the behavior of the two hydro-

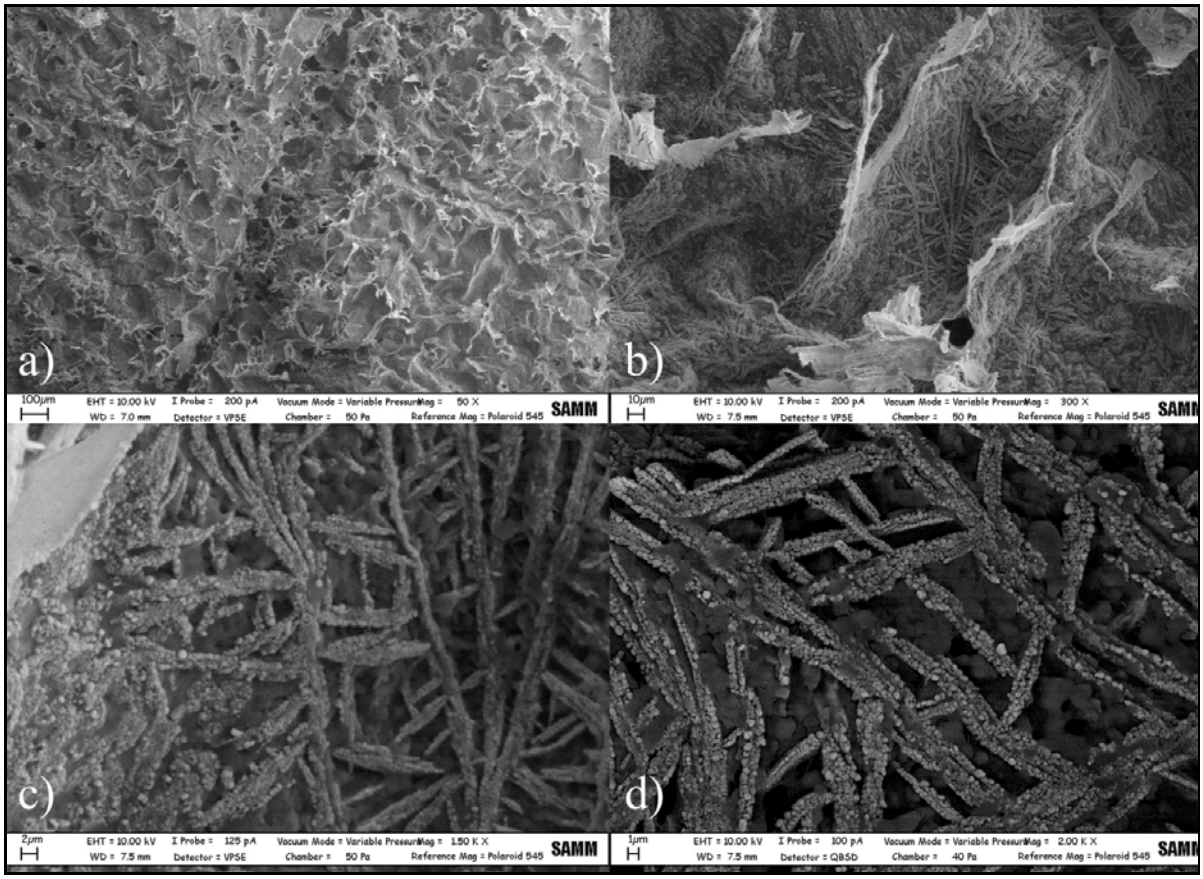

Fig. 2. ESEM images of salt depositions due to the PBS presence inside the polymeric matrix at different magnification: 50× (a), 300× (b), 1500× (c), 6000× (d).

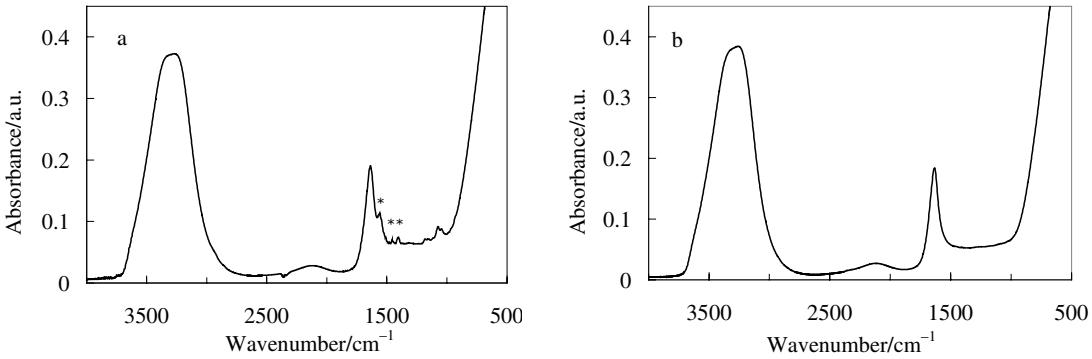

Fig. 3. FTIR spectra of hydrogel samples: a) PBS hydrogel (* asymmetric CO_2 stretch, ** symmetric CO_2 stretch; both present only in PBS hydrogel); b) DW hydrogel.

gels is critically different in the pH range of 6–8, which are the physiological lower and higher references: DW gels showed very high instability; on the other hand, PBS gels retained their stability. This last fact shows how PBS enables fine pH tuning, within the physiological range, during hydrogel synthesis and thus enables the adaptation of the material in different biological tissues, each time finally minimizing irritation to the surrounding tissues (Dumitriu, 2002).

Macroscopic results of PBS effects on pH titration were complemented, by consent, by salt arrangement analysis of a 3D hydrogel matrix. The presence of PBS salts was confirmed by the ESEM analysis as it can be observed in Fig. 2 which shows high amounts of Na^+ and K^+ ions along the polymeric fibers of the hydrogel network structure.

As shown in Fig. 2a, a very compact matrix is formed. Focusing on the deposits of PBS salts, it can be noted that they are very well detectable from panoramic (Fig. 2b) down to the fiber level (Figs. 2c and 2d). Deposits appear to be regular and well arranged in the entire polymeric network.

Further confirmation of structural effects of the PBS presence was obtained from the hydrogel analysis performed via FTIR measurements on network chemical bonds (Vidović et al., 2009). ATR spectra of the two hydrogels are presented in Fig. 3. Particularly, differences between the two spectra (Figs. 3a vs 3b) are visible in peaks corresponding to symmetric ** (1406 cm^{-1}) and asymmetric * CO_2 stretch (1560 cm^{-1}) which are due to the carboxylation products (Fig. 3a). These carboxylates are the products of the carboxylation reaction occurring between the polyacrylic acid (i.e. carbomer 974P) and PBS ions (K^+ and Na^+). Fig. 3b shows the absence of both CO_2 peak stretches as a consequence of carboxylates salts when DW was used as solvent. These results are in full agreement with literature, as all carboxylic acids dissociate in water and the presence of a carbonyl group enhances this reaction (Luo & Li, 2009; Simonetta & Carrè, 1969). In general, carboxylates cause high-delocalized negative charges between carbon and the two atoms of oxygen.

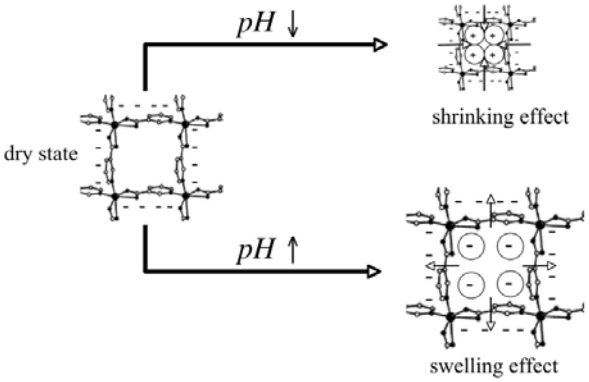

Fig. 4. Tuning of PBS hydrogel ability to retain water using different pH values.

The presence of negative charges makes the PBS hydrogel an anionic hydrogel (Slaughter et al., 2009), while the DW based is not an anionic hydrogel. Furthermore, mesh size of this 3D polymeric network can be tuned by managing the swelling solvent pH, according to the needs of the used cells which were chosen with respect to their specific regenerative medicine application. An example of such tuning is presented in Fig. 4: adding positive charges (acid pH values of swelling solvent) of the attractive electrostatic forces with the negative charges of the network hinder the swelling process favoring so the shrinking one. On the other hand, adding negative charges (basic pH values) prevailing forces are the repulsive ones which results in higher stretching degrees of the structure.

This behavior can also be used to tailor hydrogel mesh size in order to control, enhance or worsen, the release profiles and kinetics of eventually loaded drugs (Garripelli et al., 2010).

Anionic nature of the PBS hydrogel was also confirmed by swelling studies. Data are reported in Fig. 5, where mass equilibrium swelling values are plotted against pH of the used solution. As expected, mass equilibrium swelling ratio reflecting the ability of a hydrogel to swell and thus to retain its solvent increased very sensibly with the increase of the pH value

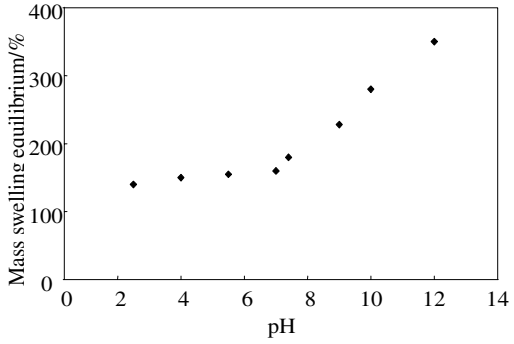

Fig. 5. Trend of PBS hydrogel equilibrium swelling ratio at different solvent pH values.

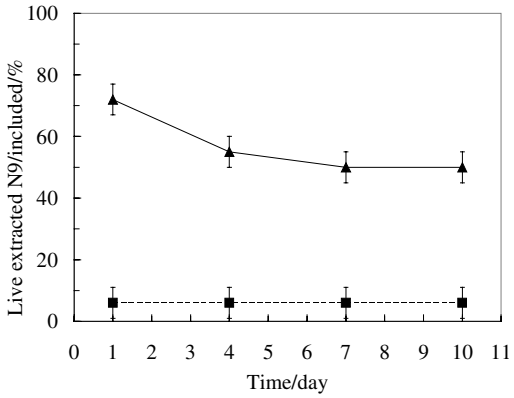

Fig. 6. Live extracted N9 line cells from hydrogel matrixes in PBS (▲) or DW (■) as solvent.

(Garripelli et al., 2010; Luo & Li, 2009). More importantly, it has to be underlined that swelling behavior presented good stability along the physiological pH range, i.e. 6–8. This allows tuning pH according to the cell needs, as previously described, but without losing their swelling properties thus maintaining all physico-chemical properties.

Being inspired by previous studies showing a relevant contribution of carboxylate moieties in cell spreading and attachments (Perale et al., 2008; Ebara et al., 2004), PBS was also investigated as a solvent in the building of bio-hybrid structures and thus as an enhancer of cell viability. DW was kept for comparison. The percentage of live cells extracted from both gel types (DW and PBS) was evaluated in different time points (days 1, 4, 7, and 10, respectively) and the data are plotted in Fig. 6.

The average number of live extracted cells from PBS hydrogels was much higher than the corresponding value measured in the DW based hydrogel. Moreover, the plot for the PBS hydrogels shows a very high value of cell viability on the first days, higher than those reported in the literature for similar systems (Crompton et al., 2007; Bränvall et al., 2007), followed by a decrease in time, which is compatible with cell viability expected in in vitro biohybrid systems (Crompton et al., 2007; Wang et al., 2009; Brannvall et al., 2007). This behavior confirms high cell survival inside PBS hydrogels. On the other hand, this effect is not visible in the cell data related to the DW hydrogels because cell viability in these gels was too low to even consider them valid as cell carriers.

Conclusions

The role of phosphate saline solution as a solvent in the synthesis of hydrogels was investigated. The study was conducted using a recently formulated gel, very promising for regenerative medical applications. Hydrogel performances were evaluated with respect to deionized water considering both traditional physico-chemical and biological aspects, the former being the first related to gelation control, swelling capability and chemical bond structures, while the latter are measured by means of assessing cell viability inside the gels.

It was observed that the presence of PBS allowed the possibility of fine tuning of pH during hydrogel synthesis thus enabling precise control of the gelation process. Furthermore, the presence of PBS induces the formation of carboxylates causing hydrogel network to be negatively charged influencing thus the network structure size affecting the overall swelling capability. All these aspects were easily controllable. Moreover, as this hydrogel was considered for biomedical applications, attention was focused on its behavior within physiological pH range, i.e. 6–8. PBS-based solutions showed high stability during gelation, which was easily kept under control, while swelling properties were slightly influenced by pH variations within this range. All this physico-chemical evidence justifies the positive and significant cell viability data observed in PBS based gels.

It has to be strongly underlined than none of these results could have been achieved using deionized water instead of PBS.

Moreover, all investigations were performed quantitatively providing reliable data for a novel understanding of the cause-effect cascades of all observed phenomena and evidence. PBS, despite being a very simple and well-known solution, showed several cascades of effects which dramatically impact processes from gelation control to cell viability of the gel matrix structure. This allowed tuning pH according to different cell needs, always remaining within the physiological range but without losing the swelling properties and maintaining all physico-chemical properties.

In the end, the presence of PBS enables adaptation of the material in different biological tissues each time minimizing the irritation to surrounding tissues, which is a feature of primary importance when agar-carbomer hydrogels are applied as cell carriers in regenerative medical applications.

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