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Encapsulation of the peptide Ac–Glu–Thr–Lys–Thr–Tyr–Phe–Trp–Lys–NH_2 into polyvinyl alcohol biodegradable formulations—Effect of calcium alginate

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ABSTRACT

It has been recently reported that the peptide Ac–Glu–Thr–Lys–Thr–Tyr–Phe–Trp–Lys–NH_2, analogue of the Glu1811–Lys1818 region of A3 light chain of blood coagulation factor VIII, presents in vitro significant anticoagulant activity. The encapsulation of this peptide into different polyvinyl alcohol formulations is examined here. The formulations were prepared using polyvinyl alcohol cross-linked with either boric acid or glutaraldehyde, giving a series of twelve different hydrogels. In case of PVA–boric acid method, a small percentage of sodium alginate was used in order to avoid bead's agglomeration. In that case, the most efficient encapsulation of the octapeptide (74%) was achieved with 0.2% (w/w) sodium alginate. It was also observed that the increase in sodium alginate percentage leads to beads with increased peptide release time, ranging from 60 to 90 min at 0.02% and 1% (w/w) sodium alginate respectively. The water holding of the PVA gels was estimated to be 27% regardless of the cross-linking reagent used, while it was increased with increasing sodium alginate concentration and reached about 60% for 1% sodium alginate. The longer octapeptide release, at 120 min, was observed with PVA–glutaraldehyde hydrogel, with encapsulation efficiency comparable to those obtained with boric acid, indicating that this hydrogel may be further used in drug delivery systems.

1. Introduction

The most popular route for drug administration is oral, although, many protein and peptide drugs are not able to be administered through the oral route, due to their degradation by the digestive enzymes of the stomach and the small intestine (Xing, Dawei, Liping, & Rongqing, 2003). The permeability of the gastrointestinal (GI) mucosa to a drug and the residence time of the dosage form in absorptive GI tract are two major factors determining the absorption of an orally administered drug. In the case of peptide drugs there are two major problems to be overcome: proteolytic degradation in the GI track and poor mucosal permeability to large molecular and high hydrophilic drugs (Lee, 1990). The prolongation of the residence time can be achieved by protecting them from degradation (Kimura et al., 1996). Gastric digestion takes about 1 h, under acidic conditions and in the presence of proteolytic enzymes, while the intestinal digestion takes 2 h under alkaline conditions (Hornero-Méndez & Mínguez-Mosquera, 2007; Miller, Schricker, Rasmussen, & VanCampen, 1981). In order to control their release, drugs are encapsulated into systems which are expected to provide a certain site with a predetermined amount of drug over a well-defined period of time (Zalfen et al., 2008).

Hydrogels, which are cross-linked polymer networks, are insoluble in water but able to swell in its presence. Due to their close resemblance to natural tissues hydrogels have been used for tissue engineering and drug delivery systems (Brazel & Peppas, 1999; Hoffman, 2002; Lee, Kung, & Lee, 2005; Peppas, Bures, Leobandung, & Ichikawa, 2000). The release of the drug from hydrogel-controlled release systems is affected by the rate of water diffusion into the polymer, which in turn depends on the chemical structure of the polymer (polarity, glass transition temperature, flexibility of the polymer backbone) and on the cross-link density and inter-chain interactions (Brazel & Peppas, 1999; Peppas et al., 2000).

Thromboembolic diseases are a major cause of mortality worldwide. Traditional antithrombotic treatments consist mainly of low-molecular weight heparins, as well as inhibitors of vitamin K, such as coumarin. Both of these anticoagulant families have shown undisputed efficacy in thromboembolism treatment, but display a range of undesirable clinical side effects (as haemorrhaging), which lead to the need for new and improved antithrombotic agents (Spiegel, Kaiser, Simon, & Stoddard, 2004). These include inhibitors of the factor VIIa, Xa and VIIIa. Factor XIIIa is a transglutaminase that catalyzes gamma-glutamyl–lysyl bonds between fibrin and other proteins involved in haemostasis. Blood coagulation mechanism consists of two pathways (extrinsic and intrinsic) that are initiated by the exposure of tissue factor groups of activated platelet membranes to circulating protein factors. Factor VIII in the presence of thrombin is activated and with FIXa, phospholipids and calcium

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forms the intrinsic tenase complex, which proteolytically activates factor X to factor Xa. The activated factor X serine-protease then assembles with cofactor Va and II to form the prothrombinase complex, which in turn cleaves factor II to release thrombin (factor IIa) and finally results in clot formation (Spiegel et al., 2004). The inhibition of the formation of the FVIIIazFIXa complex by blocking the interaction sites of the two factors could be a possible mechanism for anticoagulant activity of novel drugs. Recent studies have identified the FVIII region Glu1811–Lys1818 of the A3 subunit of the light chain A3–C1–C2 as being involved (Lenting, van de Loo, Marie-Jose, van Mourik, & Mertens, 1996; Rodgers, Duncan, Barbulescu, Quinn, & Lloyd, 2006) in FIXa binding and in the assembly of the FX-activating FIXazFVIIIa complex. The synthesis of a series of peptides analogues of the 1811–1818 sequence and their in vitro anticoagulant activity has been recently reported (Patsialas, Koutsas, Makris, & Liakopoulou-Kyriakides, 2010).

PVA, a water-soluble (Fonseca dos Reis et al., 2006) and non-toxic synthetic polymer, has been used for cell immobilization (Hashimoto & Furukawa, 1987; Idris, Zain, & Suhaimi, 2008). Due to its mechanical strength, durability, cell viability (Lee et al., 2005), and economical feasibility PVA is also used in a wide range of industrial, commercial, food and medical applications. Cross-linked polyvinyl alcohol microspheres are also used for controlled release of oral drugs. Orally administered PVA is relatively harmless. As an industrial and commercial product, PVA is valued for its solubility and biodegradability, which contributes to its very low environmental impact (DeMerlis & Schoneker, 2003). Other materials which have been successfully used for cell entrapment include agar, agarose, kappa-carragennan, collagen, alginates, chitosan, polyacrylamide, polyurethane, and cellulose (Tampion & Tampion, 1987). However, each one of these polymers has drawbacks, such as poor mechanical strength and durability (agar, agarose, kappa-carragennan, collagen, alginates, chitosan), toxicity to microorganisms (polyacrylamide, polyurethane), or high cost (Ariga, Takagi, Nishizawa, & Sano, 1987; Kuu & Polack, 1983).

A simple and economical technique for cell immobilization includes PVA–boric acid (Idris et al., 2008). Crosslinking of PVA with the use of boric acid produces a monodiol-type PVA–boric acid gel lattice (Ochiai, Shimizu, Tadokoro, & Murakami, 1981). Hashimoto and Furukawa (1987) used such PVA beads for the entrapment of activated sludge. Chemical cross-linking is a highly versatile method to create and modify polymers, and several properties of them, such as mechanical, thermal and chemical stability can be improved (Fonseca dos Reis et al., 2006). An alternative to boric acid method for the cross-linking of PVA is the formation of acetal with glutaraldehyde (Fonseca dos Reis et al., 2006; Korsmeyer & Peppas, 1981; Lee et al., 2005). PVA crosslinked with glutaraldehyde has been used in case of theophylline as drug delivery system (Korsmeyer & Peppas, 1981).

The present study is focused on the investigation of the feasibility of PVA cross-linked either with boric acid under different experimental conditions and alginate content or with glutaraldehyde, to encapsulate the octapeptide Ac–Glu–Thr–Lys–Thr–Tyr–Phe–Trp–Lys–NH_2, analogue of the Glu1811–Lys1818 region of A3 light chain of blood coagulation factor VIII, which was found to present anticoagulant activity in vitro (Patsialas et al., 2010).

2. Materials and methods

2.1. Materials

All amino acids and derivatives used in this work were of the S-configuration. 2-Chlorotrityl-chloride resin (2-CLTR) and Rink amide MBHA resin were purchased from CBL, Patras, Greece. Methanol (MeOH) HPLC grade LiChrosolv, H_3BO_3 and H_2SO_4 (95–98%) were from Merck KGaA, Germany. Glutaraldehyde (50% aqueous solution), NaCl, KCl, $Na_2HPO_4{\cdot}2H_2O$, KH_2PO_4, Na_2SO_4 (anhydrous), $CaCl_2$ were from Sigma–Aldrich Inc. Chemicals Co., Germany, and sodium alginate from BDH Chemicals Ltd., England. PVA, Elvanol 82–85®, was obtained from DuPont, Switzerland.

Table 1
Composition of PVA–octapeptide gels.

Gel #	PVA (%, w/v)	Boric acid (%, w/v)	Sodium alginate (%, w/w)	Glutaraldehyde
1	13	7.5	–	
2	13	5	0.02	
3	13	7.5		
4	13	10		
5	13	5	0.2	–
6	13	7.5		
7	13	10		
8	13	5	1	
9	13	7.5		
10	13	10		
11	10	7.5		
12	13	–	–	50 μl

2.2. Peptide synthesis

Octapeptide synthesis, purification and biological evaluation are given elsewhere (Patsialas et al., 2010).

2.3. Octapeptide encapsulation

2.3.1. PVA–boric acid method

PVA (4 g) in 30 ml water was heated to 70 °C, until dissolution. Then it was cooled down to 35 °C and 1.5 mg of the octapeptide was added. One ml of this solution was added drop wise into 10 ml of 7.5% (w/v) boric acid and the beads produced were used as a reference (gel **1**, Table 1). The rest of the initial octapeptide solution was divided into three parts of 9 ml each and sodium alginate at 0.02, 0.2 and 1% (w/w) respectively, was added to them. Droplets of 1 ml of these solutions were then mixed with 10 ml solution containing calcium chloride (2%, w/v) and boric acid at 5, 7.5 and 10% (w/v) respectively, at room temperature. The formed beads (gels **2**–**10**) were kept under gentle stirring for 24 h, and then rinsed extensively with distilled water.

The same experiments were repeated for gel **11** (Table 1) containing 10% (w/v) PVA, 1% sodium alginate and 7.5% boric acid.

2.3.2. PVA–glutaraldehyde method

An aqueous PVA–octapeptide solution (13%, w/v) prepared as described above, was added drop wise into a mixture of 20 ml Na_2SO_4 (30%, w/v), 1 ml MeOH, 40 μl H_2SO_4, and 50 μl glutaraldehyde, at room temperature. The mixture was kept for 24 h at room temperature and the formed beads were filtered and rinsed with distilled water (gel **12**).

2.4. Peptide release study

PVA beads (180 mg) from each gel obtained as reported previously were suspended in 2 ml distilled water at room temperature and kinetics of the peptide release was performed. The release profile of the octapeptide with gel **7**, which presented the higher encapsulation efficiency, was also performed in phosphate buffer saline (PBS). Octapeptide was determined by HPLC analysis using a diode array detector, at 280 nm, with a C18 column, Separon SGX C18 7 μm, 200 mm × 4.6 mm, isocratic elution with MeCN:H_2O (68:32) and flow rate 1 ml/min.

All experiments were performed in triplicate and the values reported are mean values (SD ≤ 5%).

2.5. Diffusion coefficient determination

Molecular coefficient or molecular transport can be defined as the transfer of individual molecules through a fluid by means of the random, individual movements. It is also referred to as a random-walk process as the molecules travel in a random path. The determination of effective diffusion coefficient (D_e) of the substrate molecules within the gel matrices is the prerequisite for optimizing the preparation conditions of the immobilized octapeptide (Tanriseven & Doğan, 2001).

Diffusion of molecules in a sphere-shaped bead with radius R can be appropriately modelled by the following Eq. (1) (Grunwald, Hansen, & Gunber, 1997):

$$\frac{c_t - c_\infty}{c_0 - c_\infty} = \frac{6}{\pi^2}\sum_{n-1}^{\infty} n^{-2}\ \exp\left(-\frac{n^2\pi^2 D_e t}{R^2}\right) \quad (1)$$

At the beginning of the release experiment ($t = 0$) it is assumed that the whole entrapped compound is within the beads (c_0), and the concentration 'c' in the bulk solution is zero ($c = 0$). For a given time 't' ($c = c_t$) the compound concentration within the bead is still higher than the surrounding solution, but lower than the initial. Finally, as $t \to \infty$ ($1/t = 0$, $c = c_\infty$) the compound concentration tends to be equally distributed in both phases.

For an extended period, the expression can be simplified as:

$$\frac{c_t - c_\infty}{c_0 - c_\infty} \cong \frac{6}{\pi^2}\ \exp\left(-\frac{\pi^2 D_e t}{R^2}\right) \quad (2)$$

or

$$-\ln\left(\frac{c_t - c_\infty}{c_0 - c_\infty}\right) = \frac{\pi^2 \times D_e}{R^2} \times t - \text{const.} \quad (3)$$

By plotting $\ln[(c_t - c_\infty)/(c_0 - c_\infty)]$ versus time, the diffusion coefficient D_e could be readily determined (Grunwald et al., 1997).

2.6. Entrapment efficiency and water uptake

The entrapment efficiency of the octapeptide in the beads was determined using the following relation:

$$\text{Octapeptide entrapment efficiency} = \frac{\text{Real octapeptide loading}}{\text{Theoretical loading}} \times 100 \quad (4)$$

The water uptake (S_w) of hydrogel was calculated by Eq. (5).

$$S_w = \frac{m_w - m_d}{m_w} \times 100 \quad (5)$$

where, m_w is the weight of the swollen hydrogel, m_d its dry weight.

3. Results

A series of twelve hydrogels were produced as shown in Table 1. Gel **1** was obtained with 13% PVA cross-linked with 7.5% boric acid. Gels **2–10** contain 13% PVA, a combination of boric acid at different percentages ranging from 5 to 10% and sodium alginate at 0.02, 0.2 and 1% respectively. Gel **11** was prepared with 10% PVA cross-linked with 7.5% boric acid and 1% sodium alginate, while gel **12** is the only one where glutaraldehyde was used as cross-linking reagent.

Fig. 1 gives the entrapment efficiency of these gels for the octapeptide. The entrapment efficiency was estimated according to Eq. (4). As Fig. 1 shows, the higher encapsulation efficiency (74.5%) was achieved in case of gel **7** with 0.2% alginate and 10% boric acid and the lower one about 51%, with gel **1**. The entrapment efficiency of all the other gels ranged from 51 to 74.5%. As it is shown in this figure, upon addition of alginate up to 0.2% an increase in the entrapment efficiency occurs, whereas further increase in alginate (gels **8–10**), or a decrease in PVA percentage (gel **11**) result to beads with lower entrapment efficiency values.

Table 2
D_e values for octapeptide release from the hydrogels.

Gel #	D_e ($\times 10^{-4}$ cm^2 s^{-1})
1	4.36
2	5.08
3	5.04
4	4.90
5	5.18
6	5.04
7	5.01
8	5.33
9	5.30
10	5.22
11	5.37
12	2.46

In almost all cases the prepared beads (gels **1–11**) were almost spherical with a radius of 2 mm with the exception of gel **12** where ellipsoid beads were obtained. Fig. 2 gives the pictures of gels **1**, **2**, **9** and **12** indicatively. As it can be seen from Fig. 2a agglomerated beads were obtained in case of gel **1**.

Fig. 3 gives the release profile of the octapeptide indicatively from gels **1**, **9**, **11** and **12**. The longer release time was observed in case of gel **12** containing glutaraldeyde. Fig. 4 depicts the release time of the gels containing the same percentage of PVA and boric acid with sodium alginate at different concentrations (gels **3**, **6** and **9**). In Fig. 5 is also given the octapeptide release profile for gel **7** in distilled water and in PBS, in an attempt to examine the peptide release in a solution which is near to human body as far as the osmolarity and ions concentrations concern.

The water uptake (water remaining into the beads after their formation) of the produced PVA gels was also estimated according to Eq. (5) and the results are given in Fig. 6. Water uptake is increasing upon increasing alginate concentration with the higher values obtained in case of gels **8–10**. Gel **11** with the same alginate concentration but lower PVA content, presented a little bit lower value, indicating that not only alginate but also PVA concentration determine water holding of these gels.

After the beads were transferred into distilled water the octapeptide starts to diffuse into water, and it was possible to calculate the efficient diffusion coefficient D_e value of peptide molecules within the carrier matrix according to Eq. (3). In Fig. 7 is given indicatively the plot of $\ln[(c_t - c_\infty)/(c_0 - c_\infty)]$ versus time, for the diffusion coefficient D_e calculation from the slope for the hydrogels **1** and **6** with the lowest and higher encapsulation efficiency respectively, gel **11** with same percentage of boric acid and different PVA percentage and gel **12** where glutaraldehyde was used as cross linking reagent and showed the slower release time for the octapeptide. The D_e values are given in Table 2.

4. Discussion

Ac–Glu–Thr–Lys–Thr–Tyr–Phe–Trp–Lys–NH_2 has been found to present significant inhibition of factor VIII activation and a prolongation of activated partial thromboplastin time (aPTT) from in vitro clotting assays (Patsialas et al., 2010). In an attempt to entrap this peptide into various systems and study its release, PVA was chosen as the support material due to its nontoxicity, rapid gel beads formation, low cost and relatively simple procedure using aqueous solutions.

Initial experiments were performed at PVA concentrations in the range of 5–15%. The gels obtained from 5% PVA were not stable

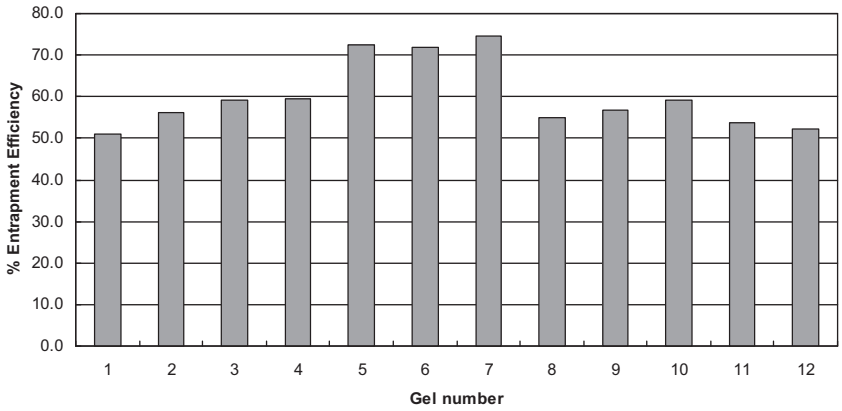

Fig. 1. Entrapment efficiency of octapeptide in the PVA gel formulations.

and 15% PVA solution was really too viscous and the beads could not be formed (data obtained but not shown). PVA 13% was then chosen in almost all cases here, since this percentage has been successfully used in other cases of cell entrapment (Hashimoto & Furukawa, 1987). As it is reported, the fabricated beads with 13% PVA remained insoluble in aqueous solution, giving stable gels that are not dissolved in subsequent release study experiments. Here, the release of the octapeptide into water was accomplished in about 60 min (Fig. 3). These beads though, have a strong tendency to agglomerate, as it was shown in Fig. 2a, but do not turn to a mass of PVA and is rather difficult to break up. This agglomeration problem has been attributed to the relatively slow cross-linking reaction kinetic of PVA by boric acid (Wu & Wisecarver, 1992). Droplets of PVA which had not been sufficiently cross-linked tended to agglomerate into a mass, even under vigorous stirring.

Trials to overcome this problem resulted in a series of the other gels (**2**–**11**) containing alginate in addition to PVA and boric acid. As it has been reported in the case of cell immobilization, PVA might contribute durability and strength to the beads, while calcium alginate might improve their surface properties reducing their tendency to agglomerate (Hashimoto & Furukawa, 1987). In addition, it is considered that calcium alginate lattice is formed nearly instantaneously and the resulting polymeric structure is sufficient to keep the beads apart during the PVA slower crosslinking process, preventing agglomeration (Wu & Wisecarver, 1992). The proposed concentration of sodium alginate added to PVA–boric acid beads is usually below 0.4% and the highest 1% (Idris et al., 2008). It has also been reported that the lowest alginate concentration that would prevent bead's agglomeration was approximately 0.02% (Kim, Eldon, & Park, 2008; Tampion & Tampion, 1987; Yujian, Xiaojuan, Hongyu, & Wei, 2006). Accordingly, we kept alginate concentrations into this range (0.02–1%) since the use of higher than 1% alginate concentrations, gave high viscous solution that was very difficult to extrude and obtain beads (data not shown). The beads with alginate were strong, highly elastic, and of nearly spherical shape (Fig. 2b and c) indicating that alginate concentration

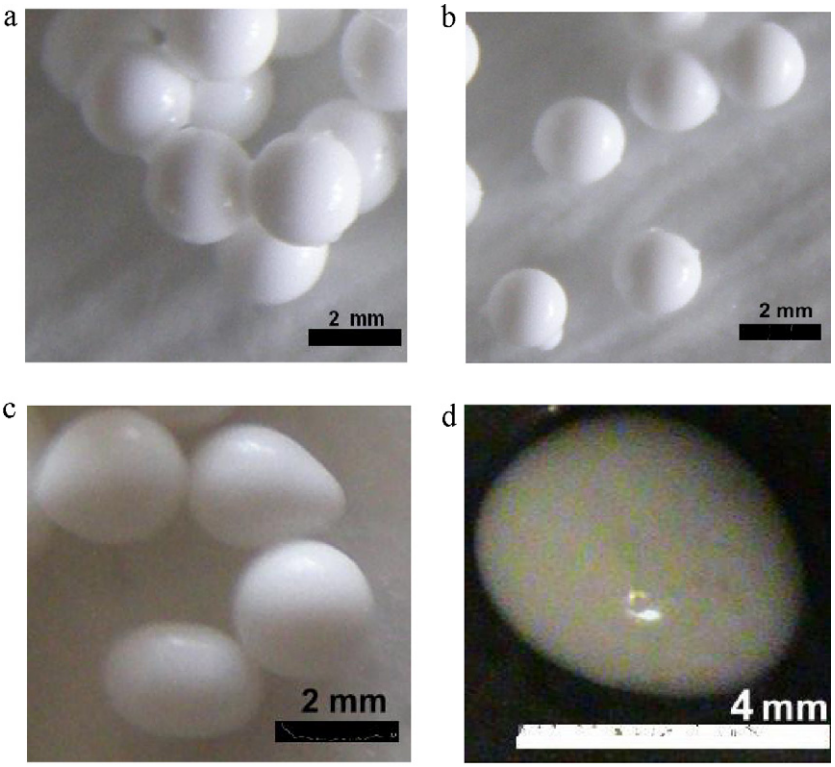

Fig. 2. Photographs of gels **1** (a), **2** (b), **9** (c) and **12** (d).

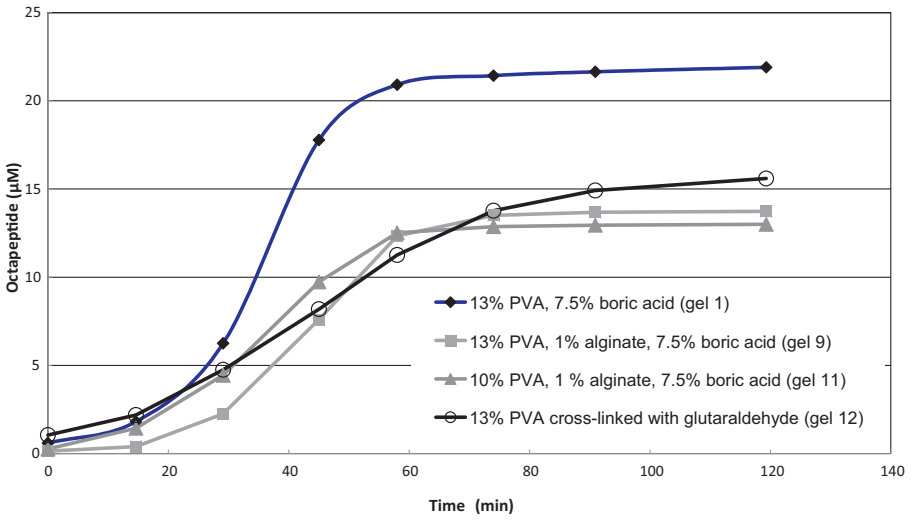

Fig. 3. Octapeptide release from gels **1**, **9**, **11** and **12**.

did not affect significantly their morphology. In case of gels with 0.02% alginate there is no significant change in the release time of the octapeptide, as shown in Fig. 4 and is almost the same, at 60 min, with it from gel **1** (Fig. 3). The release of the peptide was accomplished a little later at 70 and 90 min respectively, when higher alginate concentrations of 0.2 and 1%, were used, as it is shown in Fig. 4, whereas bead's morphology did not change (Fig. 2c). Furthermore, boric acid concentration did not seem to affect the release time of the octapeptide (gels **2**–**10**). The slight differences observed (data not shown) in their release profile can be explained by their specific D_e values (Table 2). In case of sludge immobilization, as it has been reported, the beads produced from 12% (w/v) PVA and 5% (w/v) boric acid possess at least 10% higher enzyme activity than those from the same PVA percentage and boric acid 7%. They also presented at least 28% higher mechanical stability compared to other formulations (Idris et al., 2008) thus enhancing the role of boric acid into the produced polymer beads. Fig. 3 gives the release profile of the peptide from gel **11**. This gel was prepared indicatively from lower PVA concentration (10%) and the higher alginate and boric acid concentrations used here (1 and 7.5% respectively) in an attempt to examine the effect of PVA concentration. The octapeptide release in case of gel **11** was achieved earlier than that of gel **9** (Fig. 4) and in about the same time with gel **1** and **3** (Figs. 3 and 4), meaning that PVA content is also a crucial factor for the release of the octapeptide, besides alginate content. As it is shown in Fig. 5, the release rate of the peptide for gel **7** is higher at the beginning in case of PBS solution, while equilibrium time is about the same (~75 min) in both solutions.

In all the above examined cases the produced beads were nearly spherical with a radius of 2 mm (Fig. 2a–c) with exception of gel **12** which was rather elliptic-shaped, with dimensions 2 mm × 1 mm (major and minor semi axes of the ellipse, Fig. 2d) which is another interesting finding concerning morphology of the beads and cross linking reagent. The octapeptide release from PVA–glutaraldehyde gel was accomplished at 120 min (Fig. 3), which is the maximum time obtained here and may be attributed to more intense cross-linked polymer formed.

As it is shown in Fig. 6, the water holding in case of PVA–alginate–boric acid gels was increased upon increasing the alginate content, while alteration in PVA content, slightly affected water holding. The maximum water content 60% was observed with

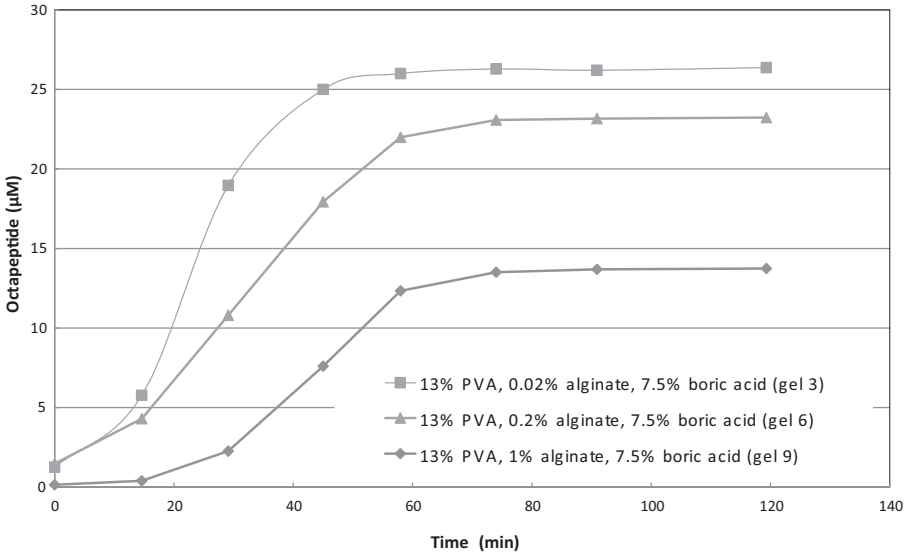

Fig. 4. Octapeptide release from the gels **3**, **6** and **9** with 13% PVA, 7.5% boric acid and different alginate concentrations.

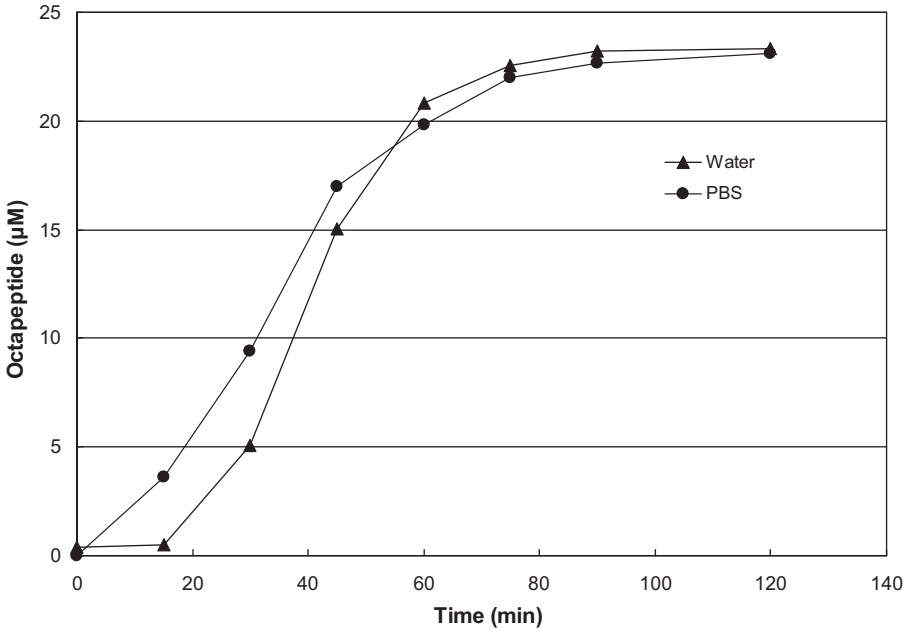

Fig. 5. Octapeptide release from gel **7** in water (-▲-) and PBS (-●-).

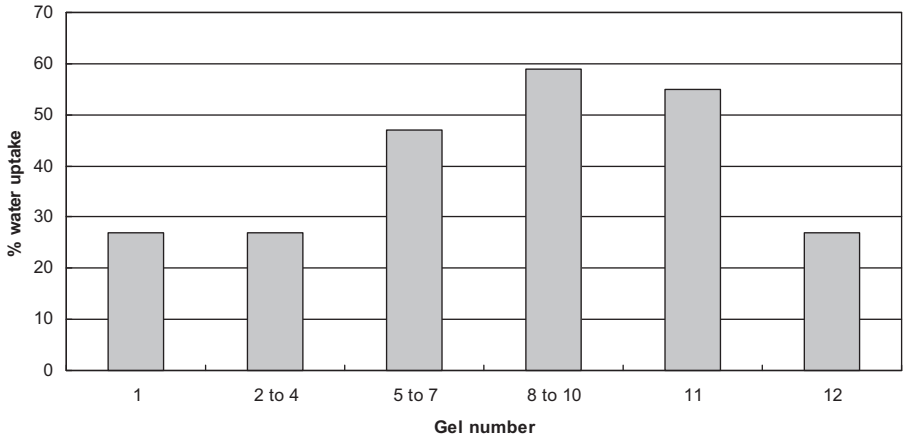

Fig. 6. Average water uptake of hydrogels.

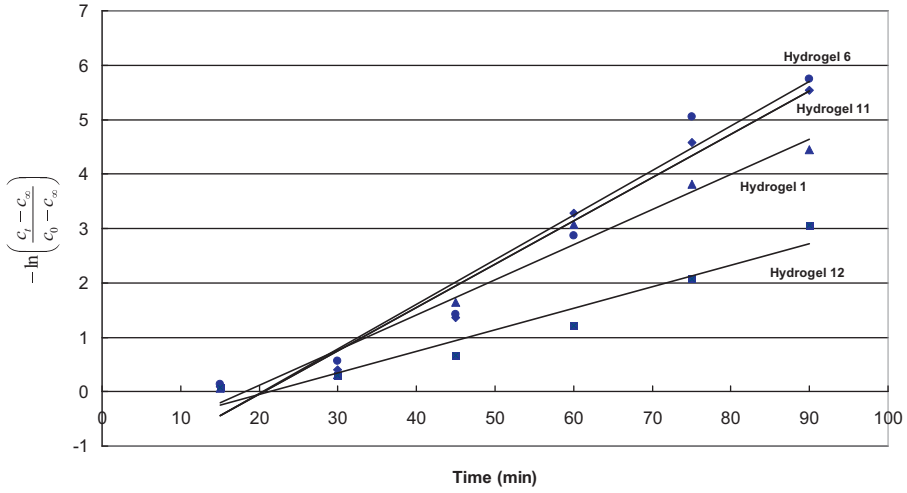

Fig. 7. Plot of $\ln[(c_t - c_\infty)/(c_0 - c_\infty)]$ versus time, where from the slope is calculated the D_e for hydrogels **1**, **6**, **11** and **12**.

PVA 13% and 1% alginate. On the other hand, no differences in water uptake were observed in case of gels **1** and **12** produced by different cross linkers as shown also in Fig. 6, indicating that the increase in the water uptake is mainly due to sodium alginate addition.

Beads composition also is responsible for the variation in D_e values. As it is shown in Table 2, all the D_e values obtained here are of the same magnitude, meaning that the produced gels differ slightly in their diffusion characteristics. Gels (**2**–**4**) with higher PVA concentration and low alginate concentrations were denser and possibly with smaller pores. The slightly higher D_e value for gel **11** is possibly a result of a weakly cross-linked polymer matrix formed around the peptide molecule due to lower PVA concentration. Another interesting observation at this point is that increase in sodium alginate percentage leads to higher D_e values, probably due to a more oriented gel matrix of the resulting gels or to wider pores. In contrary, the increase of boric acid concentration gives gels with slightly lower D_e values due to higher crosslinking. In the case of glutaraldehyde gel, the D_e value is the lower one, indicating that gel **12** is denser than all the other gels produced here. It is worth mentioning that the D_e value for the PVA–glutaraldehyde gel was calculated initially for spheres of the same volume with the rest, as they have been produced from droplets with the same PVA concentration, and the value given here is after correction, taking into account a shape factor equal to 0.917 (Gabitto & Aquerre, 1986). Thus the true coefficient calculated for these particles is lower than that calculated for the spheres of equal volume.

5. Conclusions

PVA cross-linked with either boric acid or glutaraldehyde was successfully used for the entrapment of the anticoagulant peptide Ac–Glu–Thr–Lys–Thr–Tyr–Phe–Trp–Lys–NH_2. The addition of alginate in case of PVA–boric acid gave more stable gels with longer release time and higher efficient diffusion coefficient values upon increasing of its percentage. The entrapment efficiency (higher than 50% in all examined here cases) is also acceptable for this hydrophilic octapeptide. The optimum alginate concentration for the slower octapeptide release was 1%, regardless of the boric acid used, giving a release time of about 90 min. The longer release time (120 min) of the octapeptide was achieved in case of gel with PVA cross-linked with glutaraldehyde. Also, from the effective diffusion coefficient values, it is further confirmed that the PVA cross-linked with glutaraldehyde gel exhibits the slower mass transfer indicating that it may be more appropriate for use in oral drug delivery systems.

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