International Journal of Pharmaceutics 385 (2010) 163–169

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

"Smart" alginate–hydroxypropylcellulose microbeads for controlled release of heparin

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ARTICLE INFO

Article history:
Received 14 July 2009
Received in revised form 5 October 2009
Accepted 7 October 2009
Available online 17 October 2009

Keywords:
Alginate
Hydroxypropylcellulose
Heparin
Microbeads
Thermo-responsive

ABSTRACT

"Smart" (thermosensitive) alginate–hydroxypropylcellulose (Alg/HPC) microbeads for controlled release of heparin were synthesized and the release profiles at various temperatures and for various alginate/HPC compositions were measured. Microbeads of regular spherical shape (ca. 3 μm in diameter) containing efficiently encapsulated heparin were obtained using an emulsification method. The internal structure of the bead was estimated by fluorescence microscopy using dansyl alginate as a labelled component. The microbeads surface structure and morphology were imaged in a dry state using scanning electron microscopy (SEM) and in water using atomic force microscopy (AFM). The microbead surface was shown to be covered by the regular network of pores with a mesh of ca. 30–60 nm. Lower critical solution temperature (LCST) of the Alg/HPC systems was measured spectrophotometrically (cloud point measurements). Heparin release profiles were obtained based on spectrophotometric detection of heparin complex with Azure A. Three-stage sustained release for at least 16 days was observed at 37 °C. This was correlated with the size of the pores present at the surface of microbeads. The release profile can be controlled by the temperature and composition of the Alg/HPC microbeads.

1. Introduction

Calcium alginate microbeads are widely studied as possible drug carriers (Morgan et al., 1995; González Ferreiro et al., 2002; Zahoor et al., 2005). Although they are very easy to obtain, their practical applications are hindered by the limited control over the kinetics of drug release. There has been a number of attempts to eliminate this problem (Leonard et al., 2004; Halder et al., 2005; Martins et al., 2007). Various methods and compositions of the systems for alginate-based microbeads preparation have been suggested. One of the possible solutions is based on making blends of alginate with other polymeric components (Dong et al., 2006; Şanli et al., 2007). Application of natural polymers for this purpose seems to be the most attractive solution. Chan et al. (1997) have demonstrated the effect of addition of cellulose derivatives to sodium alginate prior to microbead formation on the efficiency of encapsulation of model drug (sulphaguanidine) and its release profiles. They have observed that these effects are strongly dependent on the hydrophilicity of the cellulose derivatives used and the viscosity of the formulations, most likely due to the induced changes in the morphology of the microspheres formed. Interestingly, however, only hydroxypropylcellulose (HPC) added to the alginate formulation retarded the release of drug from the microspheres. The authors have explained this considering the markedly higher viscosity of the alginate–HPC solution than that of the other solutions containing cellulose derivatives and alginate at the same concentration and composition.

In this paper we would like to follow these studies and try to explain this observation considering the morphology of the microbeads and structure of their surface. We have used heparin as a model drug in our studies. Our choice has served two purposes. Methodologically, heparin is a large molecule, thus the size dependent mass transport effects can be monitored. Moreover, heparin is a very important anticoagulant drug and there has been a growing interest in the development of the effective methods of its administration (Ebert and Kim, 1982; Innes et al., 1997; Oliveira et al., 2003). The microbeads, which would be able to encapsulate heparin efficiently and release it in a controlled manner under physiological conditions could serve this purpose. Optimally, they should be fabricated from the biodegradable and non-toxic materials. The alginate–hydroxypropylcellulose systems are very promising in this respect. Considering the fact that HPC is a thermo-responsive polysaccharide, which displays the lower critical solution

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doi:10.1016/j.ijpharm.2009.10.021

Table 1
Composition of the emulsion for various Alg/HPC ratios (per 5 ml of solution).

	Alginate/HPC ratio (w/w)			
	2:1	3:1	4:1	5:1
Alginate [mg]	100.38	100.46	100.01	100.17
HPC [mg]	50.19	33.18	25.01	20.52
Heparin [mg]	50.12	50.28	50.23	50.10

temperature (LCST) (Uraki et al., 2004), one can consider additional way to control the behaviour of these materials.

2. Materials and methods

2.1. Materials

Alginic acid sodium salt (Alg) from brown algae (viscosity ≥ 2000 cP, 2% at 25 °C, lit.; Sigma), dansyl alginate (Sigma), unfractionated heparin sodium salt from bovine intestinal mucosa (Sigma), hydroxypropylcellulose (HPC, Brookfield viscosity 150,000–700,000 cps for 10% in water, Spindle #2, 60 rpm, 25 °C; Aldrich), Azure A chloride (standard Fluka, for microscopy; Fluka), TWEEN® 85 (polyoxyethylenesorbitan trioleate; Sigma), calcium chloride min 93%, anhydrous (Sigma–Aldrich), cyclohexane p.a. (Idalia, Poland) and 2-propanol p.a. (Idalia, Poland) were used as received.

2.2. Preparation of the alginate/HPC microbeads

The aqueous solution of sodium alginate (2%, w/v) was mixed with the appropriate amount of hydroxypropylcellulose (HPC) to yield a viscous, homogeneous, clear solution. To 5 ml of this solution heparin was added and the solution was then stirred with a magnetic stirrer for 24 h. The exact amounts of the polymers used to form each blend and the amount of heparin added are summarized in Table 1.

The round-bottom flask (100 ml) was charged with 45 ml of cyclohexane to which 0.702 g of TWEEN® 85, an emulsion stabilizing agent, was added and the mixture was stirred for 10 min at 600 rpm to assure complete dissolution of the stabilizer. Then 5 ml of the Alg/HPC/heparin mixture was added dropwise to that solution and stirred at the same speed for another 10 min to obtain the milk-white emulsion. Ten milliliters of 0.2 M calcium chloride was subsequently added to achieve the physical crosslinking of the polymeric microbeads formed. The resulting mixture was stirred for another 30 min to allow for microbeads hardening. Finally, the microbeads were filtered off and washed with water and isopropanol.

For fluorescence measurements 4:1 (w/w) Alg/HPC microbeads were prepared according to the above procedure (see Table 1 for details), but 33 wt% of alginate was replaced with dansyl alginate.

2.3. Optical microscopy study

Optical microscope imaging was performed using Nikon Eclipse TE2000 Inverted Research Microscope System. All the images were obtained in the air using Plan Achromat 50× objective. A small amount of the sample studied (ca. 2 mg) was dispersed in water using ultrasonic bath and spread over the glass slide directly before the measurement. The size of the microbeads was measured for the central, fixed area of the image, as a mean value for the 250 microbeads. Histograms were made on the basis of these measurements to show the size distribution profiles of the microbeads.

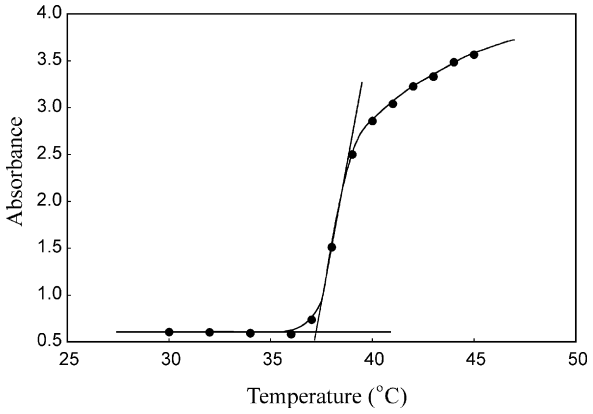

Fig. 1. Dependence of the turbidity of 3:1 Alg/HPC gel on temperature and linear fit for LCST determination.

2.4. Fluorescence microscopy

Nikon Eclipse TE2000, fitted with fluorescence and Hoffman Modulation Contrast microscope techniques was used. Fluorescence images were measured using BV-2E/C filter (excitation wavelengths: 465–495 nm; emission wavelengths: 515–555 nm).

2.5. SEM measurements

Field-emission scanning electron microscopy (FE-SEM, Hitachi S-4700) equipped with energy dispersive X-ray spectroscope (EDS) was used to study the morphology and microstructure of microbeads (magnifications from 2500× to 100,000×).

2.6. AFM measurements

AFM measurements were performed in water using Nanoscope IV microscope (Digital Instruments, Veeco, USA) equipped with liquid cell and working in tapping mode. Standard V-shaped silicon nitride cantilevers (Veeco) with nominal spring constant $k = 0.12$ N/m were used. This technique was used to study the microstructure of the Alg/HPC bead surface in water. The measurements were very difficult due to the spherical shape of the beads and their significant softness.

2.7. DLS measurements

The light scattering measurements were performed using Malvern Instruments ZETASIZER NANO-ZS. The samples were prepared in aqueous solution, filtered through a Chromafil filter (0.45 μm) and measured at room temperature. Data were analyzed using the Malvern software and the average of six measurements was calculated. All the experiments were performed in triplicate.

2.8. LCST measurements

LCST measurements were performed using a Hewlett–Packard 8452A diode array spectrophotometer equipped with a Hewlett–Packard 89090A Peltier temperature control accessory allowing for the precise digital temperature control (±0.1 °C). Alg/HPC solutions of the same composition and concentration as those used to obtain microspheres were placed in a 1-cm cuvette and appropriate amount of calcium chloride was added (same as used for microbead crosslinking). Temperature was measured with a Hewlett–Packard 89102A temperature sensor immersed in the solution. LCST values were determined from changes in the sample transmittance at $\lambda = 400$ nm (see Fig. 1).

2.9. Release studies

The weighed amount (ca. 3 mg) of the microbeads was placed in a 7 ml centrifuge tube, 1 ml of distilled water was added, and the sample was placed on the magnetic stirrer with temperature control (±1 °C) in the oil bath and stirred continuously at the fixed temperature. After defined time intervals the sample was centrifuged at 6000 rpm for 4 min, and then the solution from above the microbeads was decanted. The new portion of distilled water was added to the microbeads and the system was placed again in the oil bath. To the collected solution 100 μl of 0.2 M calcium chloride was added to remove any trace of dissolved alginate, which could interfere with heparin detection. The alginate gel crosslinked with calcium ions was removed from the solution by subsequent centrifugation.

The spectrophotometric method of heparin detection was based on colour changes of the solution occurring during interactions of heparin anion with cationic Azure A dye, which aggregates on the surface of the heparin macromolecular anion (Němcová et al., 1999). To determine the amount of heparin in the collected solution 0.8 ml of the solution was mixed with 0.8 ml of Azure A (8×10^{-5} M) aqueous solution and the absorption was measured at 512 nm. The concentration of heparin was calculated using the calibration curve for heparin. In the procedure of calibration curve preparation the 100 μl of 0.2 M calcium chloride solution was also added to account for the fact, that increased salt concentration caused a decrease in the sensitivity of the Azure A–heparin assay (Jiao and Liu, 1998).

2.10. Encapsulation efficiency determination

To determine the efficiency of heparin encapsulation, ca. 3 mg samples of the microbeads were placed in a small glass bottle and 1 ml of 5 mM EDTA solution was added. EDTA is a strong chelator of divalent cations and was used to remove the Ca^{2+} crosslinks in the alginate gels in order to solubilize the alginate. The sample was stirred with the magnetic stirrer for 24 h to ensure complete dissolution of the microbeads and then the amount of heparin was determined using the same method as in the release studies. The calculations were done based on the calibration curve obtained for the standard solutions prepared in 5 mM EDTA. Each experiment was done in triplicate.

3. Results and discussion

3.1. Thermosensitivity of sodium alginate–hydroxypropylcellulose aqueous solutions and hydrogels

Sodium alginate and hydroxypropylcellulose have been shown to be miscible in aqueous solution as well as in the hydrogel for all the compositions used. Hydroxypropylcellulose exhibits LCST at 43 °C. Its thermosensitivity is retained in Alg/HPC solutions and gels and can be easily observed as a growing turbidity of these systems upon heating. The LCST for the Alg/HPC blends has been determined at the same concentrations of polymers and calcium chloride as those further used to prepare microbeads. Importantly, the LCST values for all samples were found to be in the range of physiological temperatures (36–38 °C), i.e. 5–7 °C lower than those of the native HPC. The observed decrease in the LCST can be explained considering the high ionic strength values of the solution (about 0.4) resulting from the addition of calcium chloride. Such a considerable decrease of the LCST value is in agreement with our previous studies on the effect of ionic strength on the LCST of HPC (Szczubiałka et al., 2006; Rosół et al., 2008). The effect of the Alg/HPC ratio on the LCST of the gel was also established (see Table 2). LCST of the gel decreases with increasing HPC content. To explain that tendency, the dependence of the LCST values of HPC aqueous solutions on HPC concentration was analyzed (experimental data not shown). It was established that LCST of the solution decreases with HPC concentration. During microbeads formation alginate concentration was kept constant while the amount of HPC was lowered. The results obtained for the gels suggest that HPC concentration in the microparticles' sample is a main factor influencing the LCST changes of the gel. Dependence of LCST on the gel composition allows for accurate (to 0.1 °C) tailoring of the LCST of the system to match the requirements of various biomedical applications.

Table 2
LCST values for various Alg/HPC gels.

Microbeads composition	LCST [°C]
Alginate/HPC 2:1	36.0
Alginate/HPC 5:2	36.7
Alginate/HPC 3:1	36.9
Alginate/HPC 4:1	37.1
Alginate/HPC 5:1	37.5

3.2. Size, microstructure and morphology of Alg/HPC beads

The shape, size, and size distribution of microbeads were analyzed using an optical microscope. Fig. 2 shows optical microscope images of the microbeads of various compositions and radius distribution obtained for each composition from the measurements of the representative sample of 250 particles.

When the content of HPC in the Alg/HPC mixture exceeds 30% (w/w), only unsymmetrical objects of various, irregular shapes are produced in small amount. That may be explained considering the fact that due to the high content of HPC in the blend the effective physical crosslinking is difficult to achieve. Part of the material is washed out from the beads and the collapse of the structure occurs. Therefore, those microparticles were not used in further studies. Average diameter of the obtained particles was estimated to be ca. 3.0 ± 1.6 μm for all the samples and decreased slightly with decreasing content of HPC in the blend.

In the fluorescence microscopy studies fluorescently labelled microbeads were used. They were prepared from the formulation in which 30% of sodium alginate was replaced with dansyl alginate. Fig. 5 presents the images of such microbeads. As expected, alginate is almost homogeneously distributed in the beads, although in some particles the higher density of the alginate was observed close to the bead surface.

Scanning electron microscopy (SEM) was used to study further the morphology and microstructure of the microbeads. The most regular beads were obtained for 4:1 Alg/HPC composition. SEM images show mostly regular spherical shaped beads, although some of them show slight deformations (Fig. 3). The microbead surface is rough with pores visible at highest magnifications achieved (100,000×). The pore sizes can be estimated to be in the range of tens of nanometers. SEM measurements, although very useful, can be performed only using the dry samples. It can be expected, however, that when hydrogel microparticles swell, the bead microstructure and pore sizes change compared to the dry sample.

Despite the difficulties with the measurements (see Section 2) we have obtained a high-quality AFM image of the microbead surface with a regular network of pores with mesh of ca. 30–60 nm (Fig. 4). To the best of our knowledge this is the first time when the pore structure of such a small microsphere has been visualized by AFM in the aqueous environment.

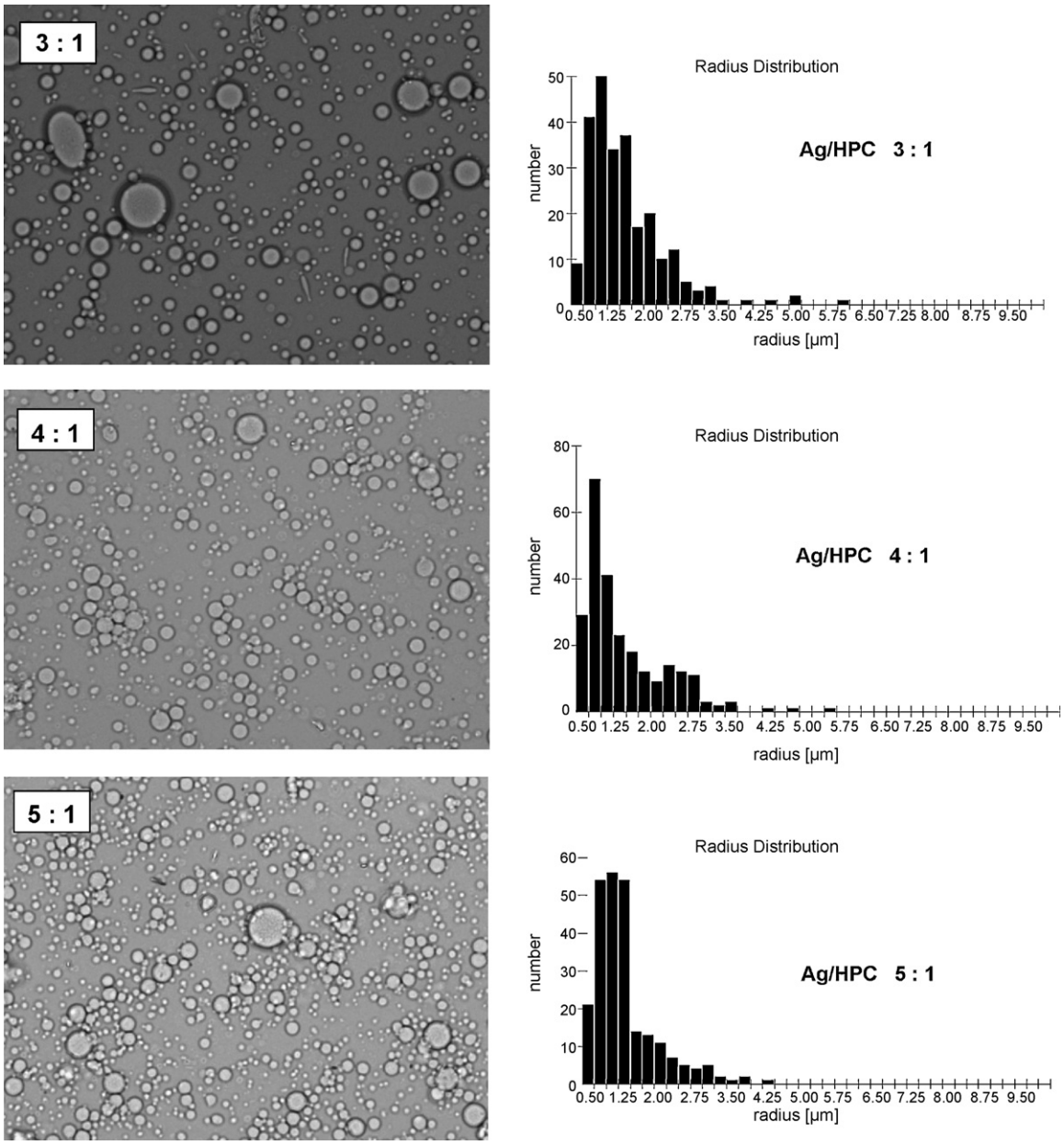

Fig. 2. Optical microscope images for the Alg/HPC microbeads of the various compositions and radius histograms of the microbeads of spherical shape.

3.3. Encapsulation and release of heparin

Heparin encapsulation efficiency (Table 3) was comparable for all blends of Alg/HPC studied with a small tendency to decrease with the increase in the HPC content in the sample. This tendency is similar to that reported earlier for sulphaguanidine (Chan et al., 1997).

The studies on heparin release were performed for all the samples in water. The concentration of released drug was determined spectrophotometrically using Azure A as a heparin-sensitive dye. In the aqueous solution Azure A shows the absorption band with the maximum at 634 nm (blue solution), whereas in the presence of heparin the absorption maximum shifts to 512 nm (violet solution), characteristic of the dye aggregated at the surface of the heparin macromolecular chain. The calibration was done using seven standard solutions and excellent fit was obtained ($R^2 = 0.9931$).

Table 3
Heparin encapsulation efficiency for various microbeads compositions.

Microbeads type	Encapsulation efficiency [%]
Alginate/HPC 3:1	57.0 ± 3.0
Alginate/HPC 4:1	61.3 ± 3.1
Alginate/HPC 5:1	63.3 ± 6.2

The release profiles for heparin-loaded microbeads of various compositions have a similar shape; however, they differ in the amounts of the heparin released in the same time intervals (Fig. 6). These differences may be explained considering the differences in encapsulation efficiency and in the microbead morphology.

As mentioned above, the pore sizes in the wet (hydrated) Alg/HPC microbead surface was determined using AFM to be in the range of 30–60 nm. That sets the upper limit for the size of the entrapped drug molecule which can be released. The size of heparin macromolecules was determined using the dynamic light scattering technique (data not shown). As expected, the measurements have shown that the unfractionated heparin sample

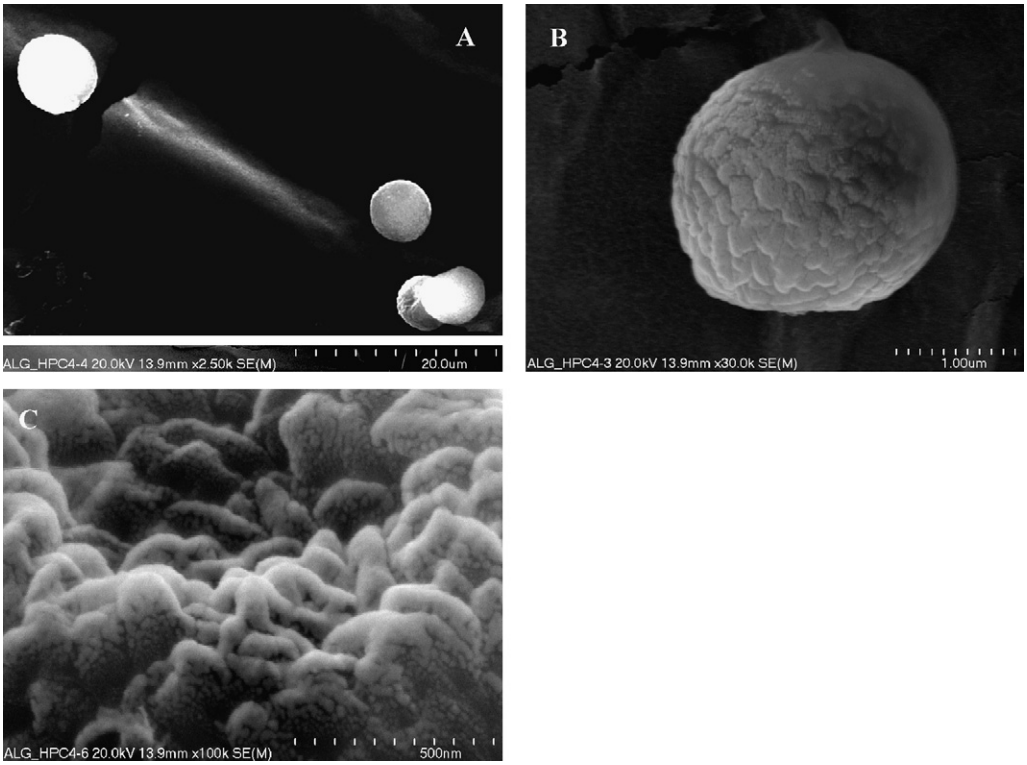

Fig. 3. SEM images of Alg/HPC 4:1 microparticles: (A) 2500× magnification—several non-aggregated microparticles; (B) 30,000× magnification—single particle of spherical shape and rough surface; (C) 100,000× magnification—microstructure of the particle surface.

used is not homogenous; there are two populations of macromolecules; one characterized by hydrodynamic diameter in the range of 20–100 nm and the second one in the range of 200–500 nm. Thus, one can expect that the smallest molecules up to 30 nm will be released fast while the bigger ones (up to 60 nm) will diffuse much slower. The largest heparin molecules will be trapped in the bulk of the beads and stay there until the particle is gradually degraded and the drug molecule finally released.

The experiments confirmed this expectation—the heparin release is a three-stage process. Initially, during the first 4 h fast release is observed which is followed by the slower one occurring during the next 5 days. Then the third stage begins, characterized by even slower, linear release. After as long as 16 days slow heparin release is still observed.

To gain a better insight into the release process, DLS analysis of the released heparin at various release times was performed. Fig. 7 shows the particle size distribution for the samples collected after 2 h, 55 h and 4 days of release. Initially (after 2 h) the size of 80% of the released heparin molecules does not exceed 60 nm

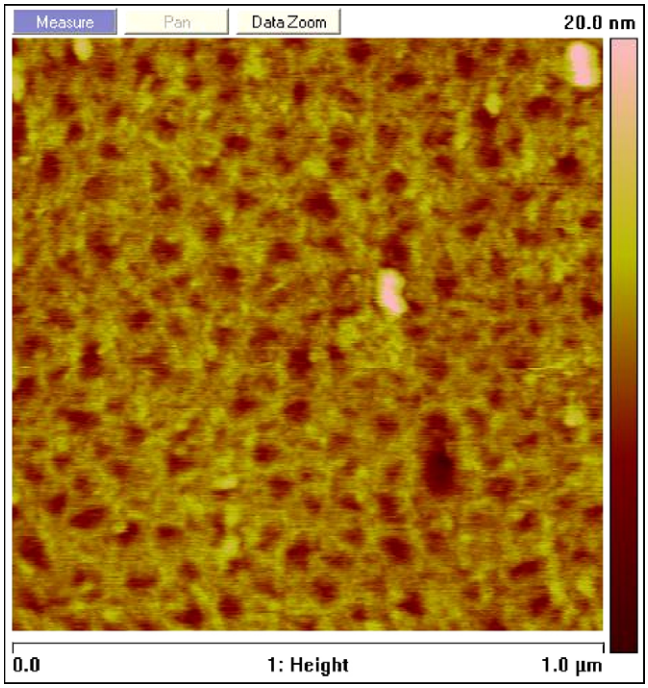

Fig. 4. AFM image of 1 μm × 1 μm fragment of the Alg/HPC 4:1 microparticle surface in 2D representation.

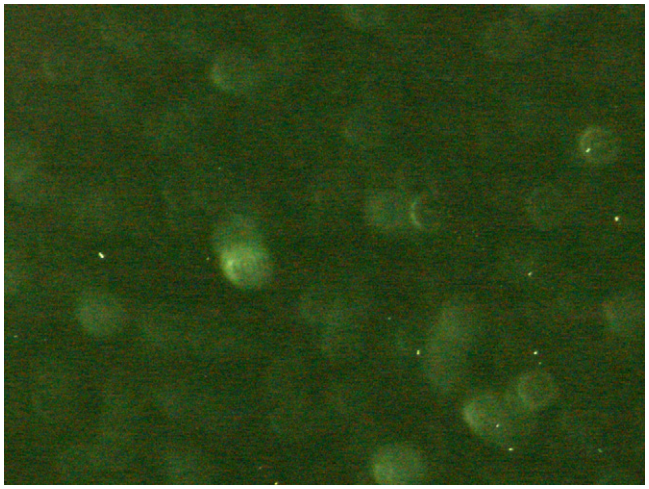

Fig. 5. Fluorescence microscope image of the 4:1 Alg/HPC microbeads, B-2E/C filter (excitation wavelengths: 465–495 nm; emission wavelengths: 515–555 nm).

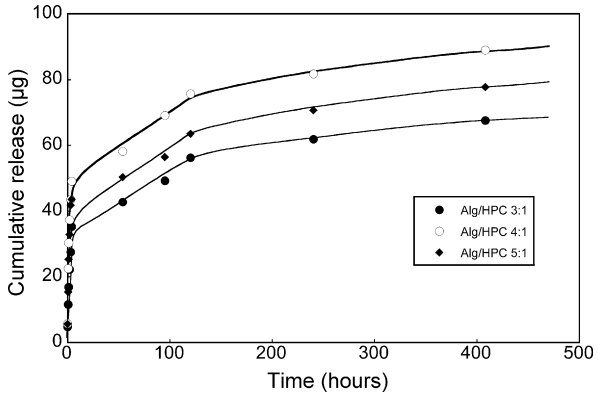

Fig. 6. Release profiles for the microbeads of various Alg/HPC compositions at 25 °C: 3:1 (●), 4:1 (○), and 5:1 (♦).

and 50% of them are smaller than 30 nm. In the second stage (after 55 h) the heparin molecules smaller than 60 nm constitute only 36% and smaller than 30 nm only 14% of the released material. In the last stage (4 days) the percentage of the particles below 60 nm decreases down to 11%. These observations are in the excellent agreement with our explanation of the release profile.

Such a release profile is desirable in many applications. After initial "shot" of a drug, it is continuously released for a few days to maintain the high level of the active substance and after that its release is sustained for at least 2 weeks, which can assure the long-lasting therapeutic effect. The most effective release was obtained for 4:1 Alg/HPC gel and these microbeads were used in further tests.

3.4. Temperature effect on the heparin release profile

In the next step the temperature effect on the heparin release profile from the 4:1 Alg/HPC thermosensitive microbeads was studied. All the profiles have been shown to have similar shape, with the initial fast stage, followed by the slow release. It was observed, however, that the efficiency of release is lower at elevated temperatures than that at room temperature (Fig. 8). Considering the effect of temperature on the drug diffusion the opposite effect could be expected. The observed effect reflects the morphological changes of the thermosensitive bead on heating. Similar finding was previously reported by Higginbotham et al. for another type of a thermosensitive drug-loaded hydrogel (Geever et al., 2008). Such

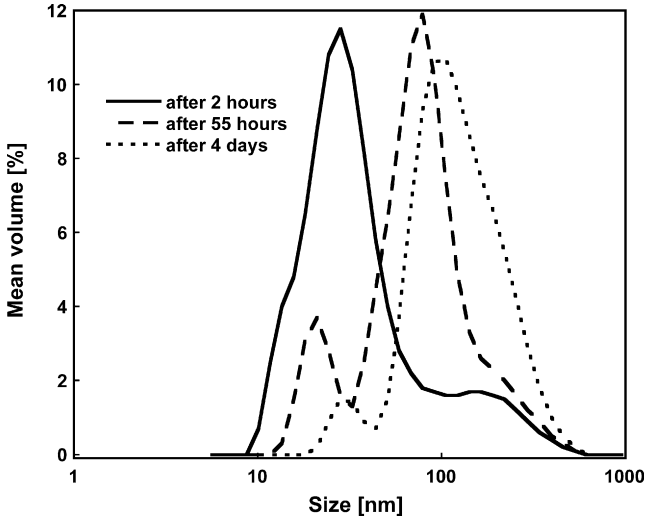

Fig. 7. DLS analysis for the heparin samples measured after different release times from the 4:1 Alg/HPC microbeads at 25 °C.

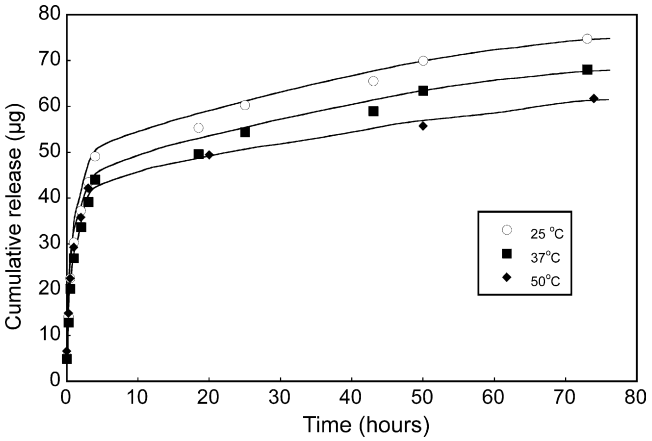

Fig. 8. Release profiles for 4:1 Alg/HPC microbeads at various temperatures: 25 °C (○), 37 °C (■), and 50 °C (♦).

behaviour of hydrogel can be explained considering the conformational changes of its thermosensitive component in response to the temperature increase above the LCST. Due to the more compact conformation of HPC chains above LCST, heparin macromolecules have less rotational freedom and, as a result, the heparin release is slower.

Slow heparin release from the gel at the physiological temperature constitutes the real advantage of the system. Fast initial stage followed by the long-term slow steady release allows for attaining quickly the necessary concentration of the delivered drug and maintaining this level for the time sufficient to assure its therapeutic effect.

The experimentally determined profiles of the heparin release at various temperatures were fitted to the known theoretical models using the equations presented in Table 4. Both Higuchi (1961) and Ritger and Peppas (1987) models are widely used to characterize the release mechanisms. In Higuchi approach the fraction of drug released is proportional to the square root of time, a is a constant characteristic of the formulation, M_t and M_∞ are cumulative amounts of compound released at time t and at infinite time, respectively. For Peppas semi-empirical equation, known also as "power law" (see Table 4), a is the kinetic constant and b is an exponent characterizing the diffusion mechanism. For $b = 0.5$ the Peppas model becomes identical with Higuchi model. Both models are short time approximations and their use is limited to the first 60% of the release. The Weibull model is an alternative empirical equation that can be used to describe the entire set of experimental data. Although its parameters do not have direct physical interpretation, Papadopoulou et al. (2006) in their work provided a link between the values of b and the diffusional mechanism of the release. They have found that for $b \leq 0.75$ the Fickian diffusion in either fractal of Euclidian spaces is dominant while for b values in the range $0.75 < b < 1$ a combined mechanism (Fickian diffusion and Case II transport) operates. For b values higher than 1 the drug release occurs according to a complex transport mechanism (Papadopoulou et al., 2006). As can be seen in Table 5 the best fits (all R^2 above 0.99) were obtained using Peppas equation applied to first 60% of heparin released. Fitted curves were shown in Fig. 9.

Table 4
Release models considered.

Higuchi	$\frac{M_t}{M_\infty} = a\sqrt{t}$
Peppas	$\frac{M_t}{M_\infty} = at^b$
Weibull	$\frac{M_t}{M_\infty} = a(1 - \exp(-(kt)^b))$

Table 5
Parameters and coefficients obtained for different release kinetic models fitted to the experimental heparin release profiles from Alg/HPC 4:1 hydrogel beads at various temperatures.

Release model	25 °C	37 °C	50 °C
Higuchi			
a	36.0 ± 1.43	31.3 ± 1.06	43.7 ± 2.57
R^2	0.9625	0.9666	0.9199
Peppas			
a	38.0 ± 0.97	34.1 ± 0.69	44.7 ± 0.87
b	0.400 ± 0.028	0.397 ± 0.018	0.364 ± 0.021
R^2	0.9906	0.9949	0.9931
Weibull			
a	103 ± 17.4	92 ± 13.2	85 ± 6.7
b	0.37 ± 0.160	0.40 ± 0.138	0.42 ± 0.147
K	0.19 ± 0.138	0.17 ± 0.105	0.34 ± 0.143
R^2	0.9733	0.9781	0.9813

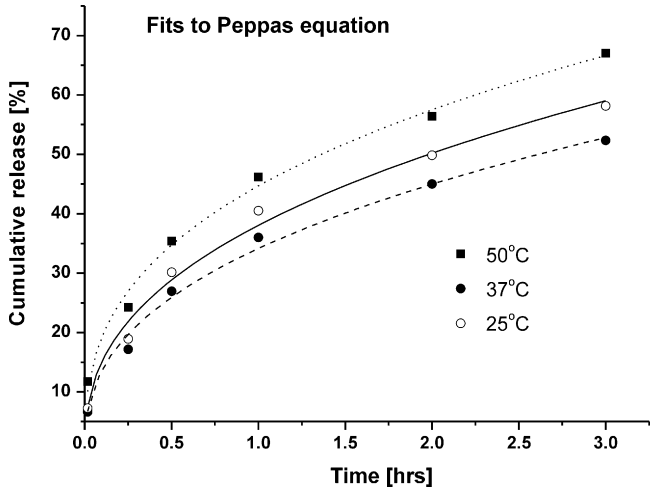

Fig. 9. Release data fits to Peppas model for various temperatures.

At all temperatures studied the *b* values were below 0.43, which, according to Ritger and Peppas (1987), suggests the spherical matrix domination of a Fickian diffusion. For longer release times, however, release kinetics could be successfully described using Weibull equation. At longer release times the release model is much more complicated and can be associated with drug diffusion from the fractal spaces, as suggested by Papadopoulou et al. (2006).

4. Conclusions

A series of alginate/hydroxypropylcellulose microbeads of various compositions was prepared by emulsification method. Spherical hydrogel microparticles of, ca. 3 μm in diameter were obtained and no significant aggregation was observed. All Alg/HPC gels were thermosensitive, showing LCST in the physiological range. Heparin was successfully encapsulated in the microbeads with high encapsulation efficiency. The AFM images of the surface of microsphere under water were successfully obtained (to the best of our knowledge this is the first time when this kind of measurements was performed for such a small particle surface of soft microobject in the aqueous medium). The morphology of the wet surface shows the network of the pores with sizes estimated to be in the range of 30–60 nm. That explains well the three-stage heparin release profiles. By fitting the experimental data to the theoretical models the dominant release mechanism for up to the 60% of heparin released can be described as Fickian diffusion from the porous matrix of the microsphere. At longer times the kinetics of release become more complicated. Thus, in the first step, characterized by fast release, the smallest heparin macromolecules (less than 30 nm) are involved, while in the second step the larger ones (up to 60 nm, which diffuse slower) participate. The release of the macromolecules larger than 60 nm is possible when the slow degradation of the microbead structure occurs.

The heparin release profile can be easily controlled by the temperature and composition of the beads with decreased release rate upon exceeding LCST. The 4:1 Alg/HPC gel proved to be the best material for microbeads formation and for heparin release.

Acknowledgements

Project operated within the Foundation for Polish Science Team Programme co-financed by the EU European Regional Development Fund, PolyMed, TEAM/2008-2/6 and partly supported by Polish Ministry of Science and Education in the frame of SURUZ network.

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