Macromolecules
ARTICLE

Macromolecules **2010**, *43*, 1371–1378 **1371**
DOI: 10.1021/ma902469f

Alginate−Poly(ethylene glycol) Hybrid Microspheres with Adjustable Physical Properties

Redouan Mahou and Christine Wandrey*

Institut de Ingénierie Biologique et Institut des Sciences et Ingénierie Chimiques, Ecole Polytechnique Fédérale de Lausanne, EPFL-SV-IBI-LMRP, Station 15, CH-1015 Lausanne, Switzerland

Received November 8, 2009; Revised Manuscript Received December 30, 2009

ABSTRACT: A one-step extrusion process under physiological conditions yielded calcium alginate-poly(ethylene glycol) hybrid microspheres (Alg-PEG-M), for which the physical properties were adjustable by the macromolecular characteristics of the components, their concentration as well as the process conditions. A solution containing a mixture of sodium alginate (Naalg) and multiarm vinyl sulfone-terminated PEG (PEG-VS) was extruded into a receiving bath providing calcium ions and a thiol cross-linker. Covalent cross-linking of PEG-VS occurred in the rapidly gelled spherical calcium alginate (Caalg) matrix. After liquefaction of the Caalg, the cross-linked PEG remained spherical. The stoichiometric ratio thiol/VS was decisive for the PEG gel stability. The permeability of the hydrogels could be tuned by adequate choice of the arm length of PEG-VS, while the swelling behavior was influenced by its concentration, the quality of the storage solvent, and the presence or absence of the Caalg matrix. Only slight differences of the mechanical resistance were observed after the dissolution of Caalg.

Introduction

Microencapsulation has advanced to a multidisciplinary topic with impact on many fields including agriculture, food, cosmetics, construction, pharmacology, analytics, biotechnology, and medicine. Thereby, the dimensions cover the range from millimeters to the submicrometer scale, and the functionality ranges from simple storage devices to intelligent systems responding reproducibly to the environment. Among the most ambitious challenges are the encapsulation/immobilization of bioactives, cells, and tissue, while maintaining the functionality and/or viability. For such applications, also the term bioencapsulation is frequently used. Because of their high water content, hydrogels composed of either covalently or electrostatically cross-linked macromolecular networks were found to meet the requirements for bioencapsulation. Despite considerable progress during the past decade in better understanding the interactions between "inert" encapsulation material and "living" actives, materials without negative side effects need to be discovered/developed considering recent debates whether such material should be inert or active.[1]

A variety of hydrogels prepared from different macromolecular origin, synthetic, natural, and modified natural, are under study.[2,3] In particular, sodium alginate (Naalg) and poly(ethylene glycol) (PEG), as well as derivatives of these, have been reported as promising.

Naalg forms hydrogels with a number of divalent cations and polycations. Alginate-based hydrogels are being intensely studied as materials for cell encapsulation/immobilization intended for subsequent transplantation therapy.[4−7] In spite of the nontoxicity, nonimmunogenicity, and advantageous gelling behavior of alginate (alg) allowing for encapsulation of various biologically active substances, these hydrogels are suffering from some drawbacks such as limited mechanical stability, durability, and permeability problems. Reinforcing initially formed alg hydrogel microbeads with one or more coatings of polyelectrolyte complexes seems to induce serious biocompatibility problems. Polycations such as, for example, the frequently used poly(L-lysine) hydrohalogenids have been identified as the main component responsible for the fibrotic overgrowth often observed after implantation of microspheres.[8] Moreover, any additional coating step complicates the technology and can compromise cell survival.

It was demonstrated that surface modification with PEG derivatives can significantly reduce the unwanted host response to any implant by masking the underlying charged surfaces and sterically hindering the approach of protein molecules present in the implant environment,[9] thus avoiding cell attachment and spreading. PEG hydrogels itself are also attractive for cell immobilization and scaffolds for tissue regeneration because of their hydrophilic character and their high water content which resembles, to some extent, the environment of native tissues.[10] Several types of PEG macromers have been extensively investigated as precursors to form cross-linked PEG scaffolds.[11−17] However, formation of covalently cross-linked PEG hydrogels of spherical shape and suitable surface/volume ratio, which is essential for cell nutrition and active delivery, is a challenge. Very recently, production of PEG beads was reported using microfludics.[18]

Considering the advantages of both alg- and PEG-based hydrogels, novel calcium alginate−PEG hybrid microspheres have been produced combining the fast ionotropic gelation of Naalg with divalent calcium ions and the more time-consuming covalent cross-linking of PEG derivatives. Under physiological conditions, a one-step process yielded interpenetrating macromolecular hydrogel networks of spherical shape for which the size, the mechanical resistance, the permeability, and the swelling behavior are adjustable and tunable. Moreover, after liquefaction of the Caalg, PEG microspheres are obtainable. The mechanical stability, permeability, and swelling of the hybrid microspheres and the PEG microspheres were studied with the purpose to identify correlations between their preparation conditions and

*Corresponding author: Tel +41 21 693 96 61; Fax +41 21 693 96 85; e-mail christine.wandrey@epfl.ch.

Published on Web 01/13/2010

physical properties. This paper reports and discusses the results of these studies. The conclusions thereof create the basis for the selective production of such microspheres for future application studies.

Experimental Section

Materials. Naalg (LV Kelton, lot no. 46198 A) was obtained from Kelco, San Diego, CA. The intrinsic viscosity [η] in 0.1 M NaCl at 20 °C and the molar guluronic acid fraction F_G have been analyzed as $[\eta]_{0.1\,M\,NaCl}$ = 477 mL/g and F_G = 0.41, as described previously.[19] Multiarm PEG (8-arm, 10 and 40 kg/mol; 4-arm, 20 kg/mol) were purchased from Shearwater Polymers (Huntsville, AL). These polymers consist of a poly(glycerol) backbone with multiple PEG arms attached to it through an ether bond (PEG-OH). These three types of multiarm PEG will subsequently be designated as PEG-8-10, PEG-8-40, and PEG-4-20. Threo-1,4-dimercapto-2,3-butanediol (DTT) and fluorescein isothiocyanate−dextran (FITC-dextran; 70 and 150 kg/mol) were purchased from Sigma (Sigma-Aldrich, Switzerland). Unless otherwise mentioned, all reagents were analytical grade and were used without further purification.

The chemicals for the PEG functionalization and their suppliers were toluene (VWR, Nyon, Switzerland), dichloromethane (Fisher Scientific, Wohlen, Switzerland), sodium hydride, divinyl sulfone (Sigma-Aldrich, Switzerland), acetic acid (Fluka, Switzerland), filter cell cake (Macherey-Nagel, Switzerland), and diethyl ether (Fisher Scientific, UK).

Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), sodium chloride (NaCl), sodium citrate, and 3-(*N*-morpholino)propanesulfonic acid (MOPS) were purchased from Sigma (Sigma-Aldrich, Switzerland) and used to prepare the stock solutions, storage, and washing media.

Analytical and Characterization Methods. ^{1}H NMR spectra were recorded on a Bruker AV-400 NMR spectrometer at room temperature. Chemical shift was referred to the solvent peak (δ = 7.26 for $CDCl_3$).

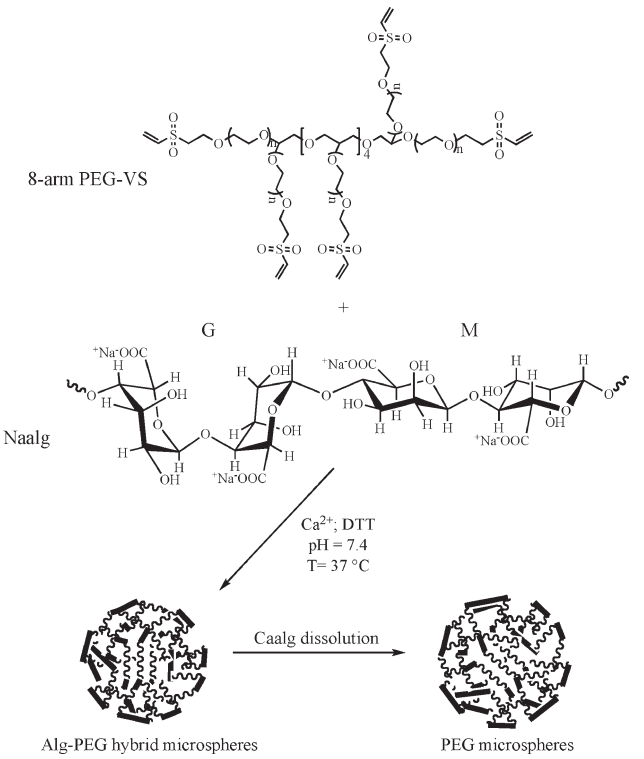
Gel permeation chromatography (GPC) was carried out using a Waters system (Waters AG, Rapperswil, Switzerland) with a column heater, manual injector, and both refractive index and UV/vis detectors. Separation of PEG-OH and PEG-VS was achieved combining three columns (Waters Styragel THF HR 2, HR 3, and HR 4) at a flow rate of 1.0 mL/min with THF as the mobile phase at 40 °C. PEG standards were used for calibration.

A texture analyzer (model TA-XT2i, Stable Micro Systems, Godalming, UK) equipped with a force transducer of a resolution of 1 mN and the software Texture Expert v.1.16 were used to study mechanical resistance of the microspheres at room temperature.

Absorbance and fluorescence spectra were measured at room temperature using a Multiplate Reader (Safire-II Tecan, Maennedorf, Switzerland). Microphotographs were recorded on an Olympus AX70 microscope connected to an Olympus DP70 color digital camera. Olympus DP Manager software was used for image analysis (Olympus, UK).

Functionalization of Poly(ethylene glycol) with Vinyl Sulfone End Groups. Differently than the known and previously published four-step synthesis,[20] PEG-VS was synthesized in one step by coupling PEG-OH with an excess of divinyl sulfone. Before starting the reaction, 5 g of PEG-OH was dried for 4 h by azeotropic distillation in toluene using a Dean−Stark trap. After cooling to room temperature, PEG-OH was dissolved in 300 mL of dichloromethane previously dried by molecular sieves. Sodium hydride was added at 25-fold excess over OH groups under an argon atmosphere. After hydrogen evolution (~10 min), divinyl sulfone was quickly added at 50-fold molar excess over OH groups. The reaction mixture was left under argon at room temperature with constant stirring for 2 days. The sodium hydride excess was neutralized with concentrated acetic acid, and the reaction mixture was filtered over a filter cell cake, reduced to a small volume by rotary evaporation, and finally added dropwise into ice-cold diethyl ether. The polymer was collected by filtration and washed with diethyl ether to give a white solid. The precipitation step was repeated twice in order to remove the no reacted divinyl sulfone. The three types of functionalized PEG will subsequently be designated as PEG-VS-8-10, PEG-VS-8-40, and PEG-VS-4-20.

Scheme 1. Formation of Calcium Alginate−PEG Hybrid Microspheres and PEG Microspheres under Physiological Conditions[a]



[a] G and M denote the guluronic and mannuronic monomer units in the alginate backbone.

Preparation of Stock Solutions. All components used for the formation of the hybrid microspheres and PEG microspheres were dissolved in MOPS stock solution (10 mM MOPS + 0.45 wt % NaCl, pH 7.4). Considering the humidity of the Naalg powder, the Naalg solution was prepared with a final concentration of 1.5 wt %. The gelation bath was prepared with 2.2 wt % $CaCl_2$. To liquefy the Caalg gel, sodium citrate, 50 mM in MOPS stock solution, was used. All solutions were sterile filtered (0.2 μm) and stored at 4 °C or at room temperature.

Caalg−PEG Hybrid Microsphere Formation. Microspheres were prepared employing a coaxial air-flow droplet generator.[21] The procedure is illustrated in Scheme 1.

PEG-VS was dissolved at defined concentrations in Naalg stock solution and shaken at 4 °C until complete dissolution, usually overnight. The solution was then sterile filtered (0.2 μm) and extruded into the gelation bath containing $CaCl_2$ and the cross-linker DTT. For example, 100−200 mg of PEG-VS-8-40 (98% functionalized) were added to Naalg stock solution to obtain a final volume of 1 mL with 10, 15, or 20% (w/v) concentration. The solution containing a homogeneous mixture of Naalg and PEG-VS was then extruded into 10 mL of the gelation bath containing the corresponding amount of cross-linker. For a PEG-VS concentration of 100 mg/mL, 1.52 mg of DTT correspond to 1 equiv of VS. Gelation occurs within a few minutes; however, the receiving bath was incubated in a shaker (80 rpm) at 37 °C for up to 3 h. The hybrid microspheres were collected by filtration, washed twice with MOPS stock solution, and finally stored in this solution at 4 °C. PEG microspheres were produced by subsequent liquefaction of the Caalg gel embedding the cross-linked PEG using sodium citrate as

calcium chelating agent. In practice, the hybrid microspheres obtained after extrusion of 1 mL of polymer solution was incubated at room temperature in 10 mL of sodium citrate solution for 15 min, washed twice with MOPS solution, and stored at 4 °C in the washing solution. Calcium alginate−PEG hybrid microspheres and PEG microspheres will subsequently be designated as Alg-PEG-M and PEG-M, respectively.

Optimization of the Stoichiometric Ratio. In order to optimize the stoichiometric ratio r, the molar ratio of thiol (SH) to vinyl sulfone (VS) groups, six series of PEG-M were prepared by varying r. Both the water uptake W accompanied by swelling of the microspheres as well as the mechanical resistance were determined as indicators. The water uptake, g of water/g of cross-linked polymer, of PEG-M was defined as

$$W = \frac{w_{sw} - w_d}{w_d} \quad (1)$$

where w_{sw} and w_d represent the mass of the swollen and freeze-dried PEG-M, respectively.

Water Uptake. PEG-M microspheres were prepared as described above, collected by filtration, separated from the storage medium, and then allowed to swell at room temperature in distilled water for 48 h. PEG-M batches obtained from 1 mL polymer solution were divided in three portions, weighed with an accuracy of 0.1 mg, and subsequently freeze-dried until constant weight. The value of W was calculated as the average of the three portions. For the determination of W it was assumed that the main quantity of the buffer components diffuse into the excess of water during the 48 h equilibrium swelling.

Mechanical Resistance. The gel strength of Alg-PEG-M and PEG-M was studied by measuring the mechanical resistances of the hydrogels under compression. The technique was previously successfully used for microencapsules.[22−24] Single microspheres were placed below the probe, which traveled toward the lower plane with a constant speed set as 0.5 mm/s. During probe displacement, the resistance of the samples to the compression was recorded up to a compression of 98% of the microsphere diameter. Thirty spheres of each batch were measured separately at room temperature.

Permeability. Studying the ingress diffusion of labeled dextran standards of different molar masses over a given time assessed the permeability of Alg-PEG-M and PEG-M. 0.5 g of Alg-PEG-M or PEG-M was stored in MOPS solution for 2 days to equilibrate. The microspheres were then collected by filtration and incubated at room temperature in 1 mL MOPS solution containing 1 mg of FITC−dextran. An aliquot of the supernatant was withdrawn immediately after mixing, corresponding to zero time concentration, and at distinct time intervals for 6 h. The concentration decrease in the supernatant was analyzed by measuring the fluorescence (excitation wavelength: 490 nm; emission wavelength: 525 nm). All samples were analyzed in triplicate.

Swelling. Swelling behavior of Alg-PEG-M was studied by measuring their swelling degree S_V in water and in PBS. S_V is defined as[25]

$$S_V\,(\%) = 100\left[\frac{V_t - V_0}{V_0}\right] \quad (2)$$

V_0 and V_t represent the volume of the microspheres before and after swelling. Because of the good sphericity, the volume was replaced by the diameter:

$$S_D\,(\%) = 100\left[\left(\frac{D_t}{D_0}\right)^3 - 1\right] \quad (3)$$

D_0 and D_t represent the diameter of microspheres before and after swelling. Diameters of 30 Alg-PEG-M isolated directly from the gelation bath and after 2 days incubation either in water or in PBS were randomly measured using an optical microscope. The impact of the presence of Caalg on swelling was also studied. For this purpose, hybrid microspheres were incubated in sodium citrate solution (10 mL solution/g of Alg-PEG-M) for different time intervals (15 and 30 min) and subsequently left to swell in water or PBS for 2 days. PEG-M were collected by filtration, and the diameters of 30 PEG-M randomly chosen from each batch were measured. Samples were analyzed in triplicate.

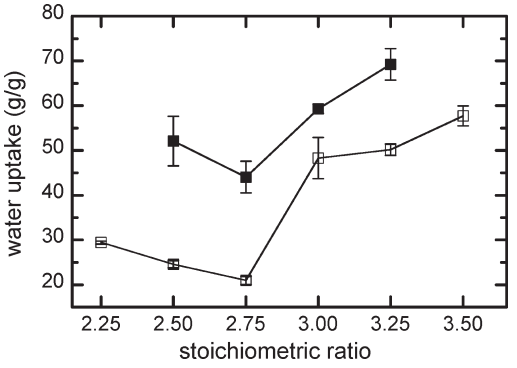


Figure 1. Water uptake of PEG-M calculated according to eq 1 as a function of the stoichiometric ratio r = SH/VS. PEG-M prepared with 100 mg/mL (■) PEG-VS-4-20 and (□) PEG-VS-8-40.

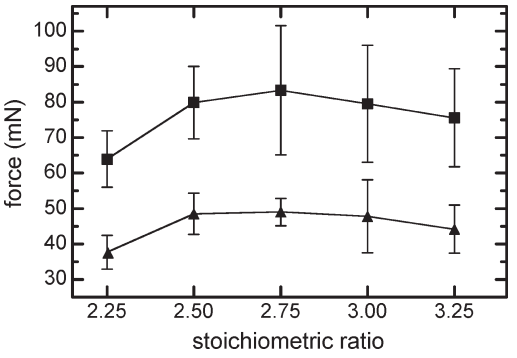


Figure 2. Mechanical resistance of Alg-PEG-M as a function of the stoichiometric ratio r for microspheres prepared with 100 mg/mL PEG-VS-8-40: ▲, 60% compression; ■, 70% compression.

Results

Functionalization of Poly(ethylene glycol). The degree of functionalization determined by ^{1}H NMR was in the range of 91−98% ($CDCl_3$, δ): 3.63 ppm (s, PEG backbone), 6.05−6.08 (d, 1H, $=CH_2$, $j \approx 10$ Hz), 6.35−6.39 ppm (d, 1H, $=CH_2$; $j \approx 16$ Hz), 6.77−6.84 ppm (dd, 1H, $-SO_2-CH=$). The excess of divinyl sulfone over −OH groups avoided cross-linking. GPC elution curves revealed a single peak confirming the absence of side products. (Spectra, chromatograms, and analytical details are provided as Supporting Information.)

Optimization of the Stoichiometric Ratio. To achieve optimal cross-linking via Michael-type addition,[26] the stoichiometric ratio of the reacting substituents should equal 1. However, insufficient cross-linking occurred using such amount here. Only PEG gels formed with $r > 2$ kept their spherical shape after Caalg liquefaction. Comparing the water uptake of PEG-M and the mechanical resistance of Alg-PEG-M prepared with r ranging from 2.25 to 3.5, minimal values of W and maximum mechanical resistance were obtained for $r = 2.75$, as shown in Figures 1 and 2.

Size and Shape. Alg-PEG-M were formed in one step under physiological conditions, pH = 7.4, T = 37 °C. The

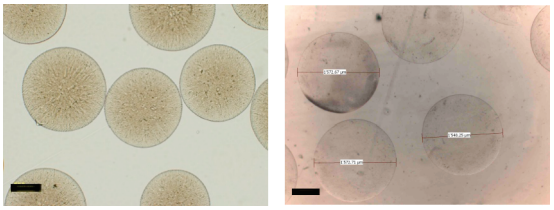


Figure 3. Alg-PEG-M (left) and PEG-M (right). Scale bar: 500 μm.

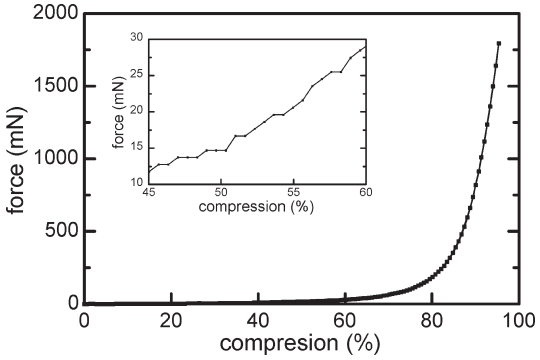


Figure 4. Typical force−compression curve obtained by uniaxial compression of Alg-PEG-M and PEG-M.

diameter of the hybrid microspheres was tunable modifying process conditions such as air flow, syringe diameter, or the extrusion rate. Focus was on diameters ranging from 0.4 to 1.5 mm. The majority of experiments was performed with 1 mm diameter. After removal of the Caalg, the covalently cross-linked PEG remained spherical. Good uniformity was obtained with $< 5\%$ relative standard deviation of diameter. Figure 3 shows examples of microspheres before and after Caalg liquefaction.

Mechanical Resistance. Alg-PEG-M prepared from the same PEG-VS derivative exhibited significantly different mechanical properties when the initial PEG-VS concentration was varied from 100 to 200 mg/mL, but the stoichiometric ratio SH/VS was maintained at its optimum $r = 2.75$. Figure 4 shows a typical force−compression curve as recorded for all microspheres. Low resistance was observed until about 30−45% compression. Subsequently, the resistance increased slightly until about 60−70% compression, before increasing exponentially and approaching values of > 1 N at 90% compression.

Generally, the mechanical resistance increased with the PEG-VS concentration. This is demonstrated in Figure 5 for 100, 150, and 200 mg/mL PEG-VS concentration in the extrusion solution. Furthermore, as also shown in Figure 5, the mechanical resistance slightly increased after Caalg liquefaction.

Elasticity. Besides the adjustable mechanical resistance, Alg-PEG-M demonstrated good elasticity. Microspheres were subject to 20 successive compression steps. A decrease of the mechanical resistance after the first compression step was observed. However, after this initial reduction the resistance remained almost constant as can be seen in Figure 6. Optical microscopy confirmed complete recovery of the original shape and dimension after putting the Alg-PEG-M back into the storage medium.

Swelling. The presence/absence of Caalg gel strongly affected the swelling behavior of the microspheres in water and PBS. As is visible in Figure 7, for the initially formed Alg-PEG-M, only slight swelling occurred in pure water, less than in PBS.

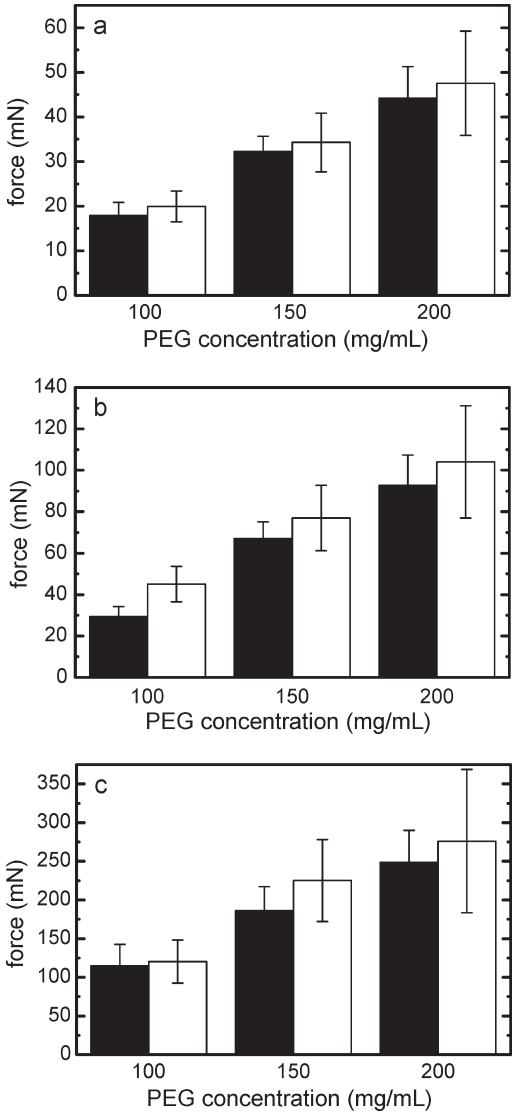


Figure 5. Mechanical resistance to compression for different initial PEG-VS-8-40 concentrations (■) before and (□) after Caalg liquefaction: (a) 60%, (b) 70%, and (c) 80% compression. Note the different scale of the y-axes.

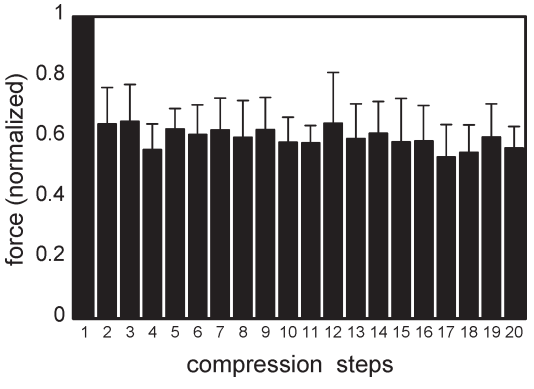


Figure 6. Mechanical resistance of Alg-PEG-M prepared with 100 mg/mL PEG-VS-8-40; 20 successive compression steps until 70% compression at room temperature.

The microsphere diameters increased in both media upon Caalg liquefaction. Differently than PBS, the swelling degree S_D in water was higher after longer incubation of the Alg-PEG-M in sodium citrate. Contrary to Alg-PEG-M, after

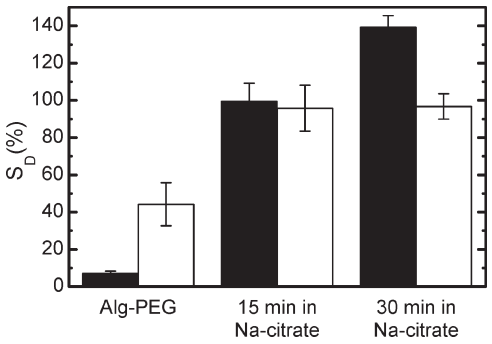


Figure 7. Swelling degree S_D of Alg-PEG-M prepared with 100 mg/mL PEG-VS-8-40 (■) in water and (□) in PBS, before and after different periods of Caalg liquefaction. S_D calculation according to eq 3.

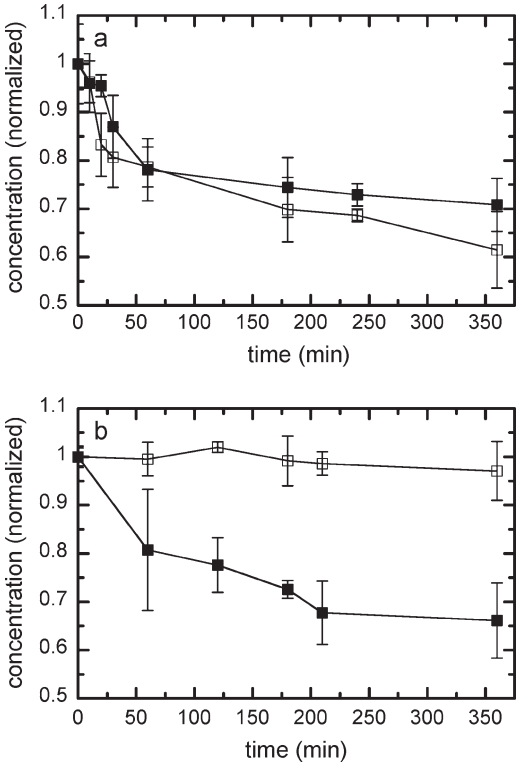


Figure 8. Diffusion of FITC-labeled dextran into Alg-PEG-M prepared with 100 mg/mL (a) PEG-VS-8-40 and (b) PEG-VS-8-10: (■) dextran 70 kg/mol; (□) dextran 150 kg/mol.

Caalg liquefaction, PEG-M exhibited higher ability to swell in water than in PBS. Comparing PEG-M prepared from different initial PEG-VS concentrations, less water was taken up for higher PEG content (data not shown), confirming results of other authors who prepared pure PEG gels.[26]

Permeability. The permeability of Alg-PEG-M and PEG-M prepared from PEG-VS-8-40 and PEG-VS-8-10 and assessed by the ingress diffusion of labeled dextran standards revealed significant differences for the two molar masses. Figure 8 shows the results of the diffusion studies.

70 kg/mol dextran diffused into both microsphere preparations. Alg-PEG-M produced from the higher molar mass PEG-VS-8-40 allowed for the diffusion of FITC−dextran 150 kg/mol, while the latter was completely excluded when using the lower molar mass PEG-VS-8-10.

Interestingly, the MWCO was found to be independent of the presence/absence of Caalg. Comparing the diffusion of

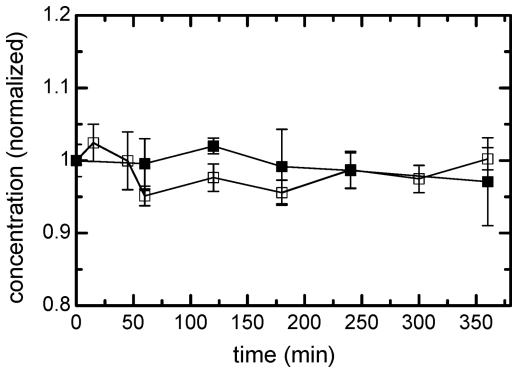


Figure 9. Exclusion of FITC-labeled dextran 150 kg/mol from Alg-PEG-M prepared with 100 mg/mL PEG-VS-8-10 (■) before and (□) after liquefaction of Caalg.

FITC−dextran into Alg-PEG-M and PEG-M, as shown in Figure 9, no significant difference was observed.

Discussion

Gelation and Cross-Linking. The hybrid microspheres were formed by simultaneous ionotropic gelation of Naalg with divalent calcium ions and covalent cross-linking of multiarm PEG-VS with DTT. The receiving bath, into which the polymer solution was extruded, contained a molar excess of $CaCl_2$ and an optimized amount of DTT. The small calcium ions immediately diffuse into the extruded polymer solution drops and gel the alginate backbone by a mechanism extensively described previously.[27−29] The Caalg gel properties such as mechanical stability, durability, and permeability depend on the type of the alginate, its molar mass, concentration, and the medium.[30] Here, Naalg of not too high molar mass was used to achieve sufficient PEG-VS solubility at not too low Naalg concentration. In the case of too low Naalg concentration, no sphericity was maintained, and PEG-VS could diffuse out of the drops before covalent cross-linking completed. 1.5 wt % of the Naalg type used here allowed for a PEG-VS concentration up to 200 mg/mL.

Optimally cross-linked PEG hydrogel is a prerequisite for efficient protection of encapsulated bioactives. As visible in Figures 1 and 2, the stoichiometric ratio r = SH/VS provided in the gelation bath affects the final properties of the hydrogel. Minimum water uptake and maximum mechanical resistance were achieved performing the cross-linking reaction with $r = 2.75$. Interestingly, the optimum r appeared to be independent of the PEG arm length. For both 4- and 8-arm PEG-VS, the same r was identified when the PEG-VS concentration was 100 mg/mL. However, the gel of the 8-arm PEG-VS took up less water. To recall, $r = 2.75$ referred to the amount of DTT provided in 10 mL gelation bath for 1 mL extruded Naalg/PEG-VS solution droplets. This result needs experimental confirmation for other PEG-VS concentrations in case other PEG-VS concentrations become relevant during application studies.

To study the DTT consumption more in detail, the decrease of the DTT concentration in the gelation bath was analyzed for 3 h using Ellman's essay.[31] The thiol consumption curve in Figure 10 shows a rapid concentration decrease to about 50% of the initial concentration during the first 50 min; i.e., an equivalent of $r \approx 1.35$ diffused into the droplets.

By separate analyses, Ellman's essay revealed that the thiol concentration in the gelation bath decreased by 20% after 3 h even in the absence of multiarm PEG-VS due to oxidation yielding nonreactive disulfide. Noteworthy, the DTT

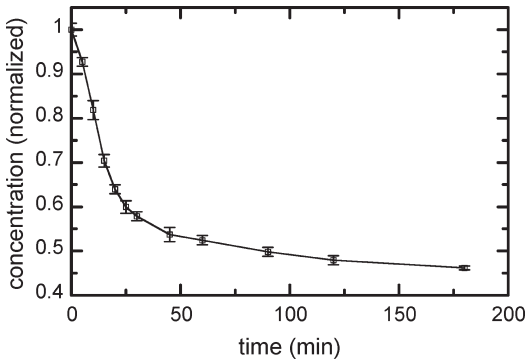


Figure 10. Consumption of thiol in the gelation bath during the formation of Alg-PEG-M, 100 mg/mL PEG-VS-8-40, $r = 2.75$, $T = 37$ °C.

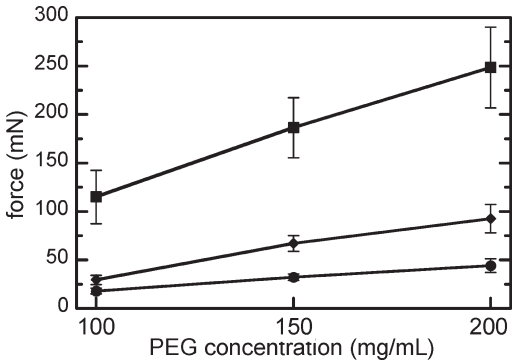


Figure 11. Mechanical resistance to compression of Alg-PEG-M for different initial PEG-VS-8-40 concentrations at (●) 60%, (◆) 70%, and (■) 80% compression.

solutions were freshly prepared only few minutes before the hydrogel formation. Considering such side reaction also inside the gelling droplets, the DTT available for cross-linking further reduces to $r \approx 1.1$. In addition, a 10% dilution of the DTT occurs when extruding the 1 mL polymer solution into the 10 mL gelation bath. Therefore, it can be assumed that almost the stoichiometric amount of DTT was consumed to cross-link PEG-VS in the droplets.

Concluding, it is probably crucial to provide an optimum amount of DTT at the beginning of the gel forming reaction, which is high enough to ensure fast diffusion of the stoichiometric amount into the gelling drops before the initially formed Caalg and PEG gels hinder the diffusion, but less than an amount that exceeds the stoichiometric amount. Such excess would result in reacting more than 50% of the VS units with one DTT molecule leaving not sufficient VS groups for optimal cross-linking.

No stable hydrogel remained after the water uptake test if the PEG-M was prepared from 4-arm PEG-VS with $r = 2.25$ and $r = 3.5$ (missing data points in Figure 1). If the water uptake was much higher than 60 g/g, the gel beads became so weak that they could not be collected by filtration. This also proves that both deficiency and high excess of DTT are unfavorable. Furthermore, a PEG-VS concentration > 50 mg/mL was necessary to obtain stable spherical beads, but no more than 200 mg/mL could be homogeneously dissolved in the 1.5 wt % Naalg solution.

Mechanical Properties, Shape, and Size. There are several methods to test the mechanical properties of hydrogel microspheres.[32−35] Here, a method was selected which provides data necessary for spherical hydrogels preferably intended for implantation by injection. Such properties are, for example, good mechanical resistance, deformability, elasticity, size constancy, and size/shape recovery after deformation.

It was found that the initial PEG concentration, more precisely, the concentration of the functional SH groups, strongly affects the mechanical resistance (Figure 5). This finding is in agreement with the theoretically expected correlation between mechanical characteristics and the number of cross-links. Increasing the number of cross-links creates a denser network being more rigid.[36] The continuity of the force−compression curves of Alg-PEG-M (Figure 4) suggests a homogeneous hydrogel. In contrast, for microcapsules having an external layer or membrane a discontinuity of mechanical resistance upon compression and/or bursting at high compression was observed.[37] Figure 11 quantitatively confirms the exponential increase of the resistance to compression at higher percentage of compression for all three PEG-VS concentrations studied here.

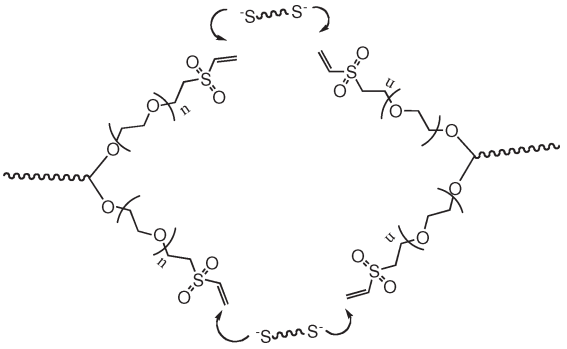
Moreover, the mechanical resistance slightly increased after Caalg dissolution. This behavior might be explained by the higher water absorption upon Caalg liquefaction compensating for the mechanical resistance of the dissolved Caalg gel.

Beside the tunable mechanical resistance, Alg-PEG-M possess good elasticity. The decrease of mechanical resistance after the first compression shown in Figure 6, most likely due to the release of a quantity of water, should not be relevant in any biological environment, for example after transplantation, because of the direct contact of the microspheres with body fluids, mainly constituted of water.

Increasing the number of covalent cross-links creates networks exhibiting restricted water uptake due to stronger retraction forces caused by the higher number of covalent bridges within the gel.[36] In contrast to the mechanical resistance, for which a slight change/increase was observed after Caalg dissolution, the swelling behavior strongly depends on the presence/absence of Caalg. The results suggest that the Caalg gel plays the role of a template avoiding swelling of the embedded PEG gel and thus limiting the water uptake. Upon gradual dissolution of Caalg, PEG hydrogel acquires the ability to take up more water than the Alg-PEG-M. These results are in agreement with the previous hypothesis that the water uptake compensates the mechanical resistance of the obtained PEG-M after liquefaction of Caalg.

It is further known that the swelling behavior of hydrogels depends on the molar mass of the gel components, their concentration, and the quality of the swelling solvent.[26] Here, the swelling of the Alg-PEG-M was affected by the quality of the solvent as well as by the presence or the absence of Caalg gel. The higher swellability of Alg-PEG-M in PBS than in water might be explained by partial disintegration of Caalg through the exchange of calcium ions by sodium ions present with higher concentration in PBS than in MOPS.[38] In contrast, only a limited quantity of calcium ions seems to be leached from Caalg upon incubation in water (Figure 7). In Figure 7, the S_D calculated according to eq 3 and ranging from 10 to 140% refers to diameter increases of 3 to 34%. The study of the swelling behavior of Alg-PEG-M upon time-dependent dissolution of Caalg by incubation in sodium citrate solution supports the hypothesis that the Caalg plays the role of a "template" avoiding swelling of the cross-linked PEG chains. These results are also in agreement with the hypothesis claiming that the mechanical resistance of the PEG-M after liquefaction of Caalg relies on the absorption of water and that the decrease of mechanical resistance observed after the first compression during the elasticity test (Figure 6) is due to water loss. Using stronger

Scheme 2. Influence of the Molar Mass on the Covalent Network Density of Cross-Linked PEG-VS-8, $n = 28$ for PEG-VS-8-10, $n = 112$ for PEG-VS-8-40



binding divalent cations such as barium or strontium could avoid leaching by PBS or other physiological sodium containing media.[39]

Permeability. Defined and controllable permeability is one of the most important characteristics of hydrogels used for cell immobilization. For Caalg gels, the MWCO was reported as >1000 kg/mol.[40] As to the authors' knowledge, the adjustability of the permeability of PEG hydrogels has not yet been reported in detail. To ensure immunoprotection and survival of cells, the network density/pore size of hydrogels intended for cell microencapsulation must be carefully designed and controlled to allow for bidirectional diffusion of factors and molecules essential for cell survival, e.g., oxygen and glucose, while staying impervious to immunoglobulins, leukocyte antigens, or antibodies, all of them having molar masses >150 kg/mol. As shown in Figures 8 and 9, the extent of ingress diffusion is determined by the molar mass of the PEG derivatives in the case of the same chain architecture, 8-arm PEG. The use of PEG-VS-8-10 yielded smaller pores than formed when using PEG-VS-8-40. This difference can be attributed to the arm length directly influencing the hydrogel network density. As schematically demonstrated in Scheme 2, pores formed from PEG-VS-8-10 will be smaller than those formed from PEG-VS-8-40 because of the lower number of ethylene glycol repeating units along the arms.

Interestingly, this MWCO seems to be independent of the presence of Caalg. Comparing the diffusion of FITC-dextran into Alg-PEG-M and PEG-M prepared from PEG-VS-8-10, no significant difference was observed (Figure 9), suggesting that the hydrogel remains impervious to molecules having molar masses >150 kg/mol, even with the higher swelling potential of PEG-M after Caalg liquefaction. This appears obvious since Caalg networks are known to be quite porous. Nevertheless, with the ingress diffusion method used here, permeability changes occurring in the range of 70 kg/mol < MWCO < 150 kg/mol, which cannot a priori be excluded, could not be detected.

Conclusions

A new type of hydrogel microspheres was synthesized. The combination of electrostatic interaction of calcium ions with Naalg and the chemical reaction of vinyl sulfone-terminated PEG with DTT yielded interpenetrating networks with well-controllable physical properties. The sensitivity of these hydrogels to their preparation conditions including stoichiometry, precursor concentration, and to the presence/absence of Caalg was investigated. It was demonstrated that the permeability of the hydrogel can be tailored by adequate choice of the arm length of PEG-VS, while the swelling degree can be tuned by varying the PEG-VS concentration and/or by liquefaction of Caalg. It was also demonstrated that dissolution of Caalg has no significant impact on the mechanical resistance of the obtained PEG-M. Overall, important physical properties of the microspheres are obtainable in the range requested for biotechnological, biomedical, and pharmaceutical applications.

Acknowledgment. The authors thank the Swiss National Science Foundation for financial support, Grant 205321-116397/1, the COST 865 for granting a STSM, and Dr. I. Lacik, Polymer Institute Bratislava, for experimental support and helpful discussions.

Supporting Information Available: Figure S1 showing the ^{1}H NMR spectrum of PEG-VS, Figure S2 showing the GPC elution curves of PEG-OH and PEG-VS, and Table S1 related to the functionalization of different PEG-OH types. This material is available free of charge via the Internet at http://pubs.acs.org.

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