Design, synthesis, and degradation studies of new enzymatically erodible poly(hydroxyethyl methacrylate)/poly(ethylene oxide) hydrogels

Nawel S. Khelfallah, Gero Decher, and Philippe J. Mésini[a)]
Institut Charles Sadron CNRS UPR 022, 6 rue Boussingault, 67083 Strasbourg, France

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This work describes the synthesis and the study of poly(hydroxyethyl methacrylate) PHEMA hydrogels, cross-linked by poly(ethylene oxide)(PEO) chains containing the Gly-Gly-Leu tripeptide. This sequence was selected for its ability to be cleaved by subtilisin, a bacterial protease. The cross-linker was synthesized by coupling the peptide with two amino-terminated PEO chains of M_w=3400 g/mol. The resulting polymer was characterized by size exclusion chromatography, nuclear magnetic resonance, and mass spectroscopy, and was shown to be readily cleaved by subtilisin. Its esterification of the hydroxyl end groups into methacrylate afforded a macromonomer that was used as a degradable cross-linker and copolymerized with hydroxyethylmethacrylate to form hydrogels. The swelling ratio of the gels increases when the PEO cross-linker/polyHEMA ratio increases. Incubation of these gels with the enzyme led to the total degradation of the gels. These assays show that these gels can be used as drug-delivery systems where the release is triggered by the presence of proteases. © *2007 American Vacuum Society.* [DOI: 10.1116/1.2799034]

I. INTRODUCTION

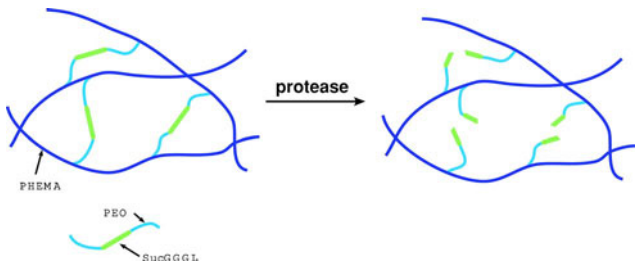
Hydrogels have been of great interest to biomaterial scientists[1–3] because they offer interesting properties: high water content and a good permeability toward oxygen and metabolites. Their viscoelastic properties can be tuned to approach the mechanical properties of common human tissues. For biomedical applications of hydrogels, the polymer network has to be composed of polymers with low toxicity and antigenicity such as poly(hydroxethyl methacrylate) (PHEMA), poly(ethylene oxide) (PEO), or chitosan. However, these polymers are not degradable under physiological conditions. Degradability is essential to design, for example, temporary prosthesis, tissue scaffolds, or devices to release pharmaceutical molecules. It is often challenging to gather in one single polymeric network all required functions: mechanical properties, biocompatibility, and degradability. A successful strategy[4–7] consists of introducing degradable blocks such as poly(lactic acid) or poly(glycolic acid) in block copolymers that are then cross-linked for obtaining a gel. Such gels can then undergo degradation under physiological conditions with a rate that can be adjusted. For some specific application, it is more advantageous when the degradation starts only when the gels are solicited by a given stimulus.[8] Considerable work has been carried out on gels whose degradation is triggered by *p*H changes[9,10] or enzymatic action. In this last case, gels were synthesized that can be degraded by azoreductase[11–13] for drug release in the colon, chymotrypsine,[14,15] or by collagenases[16–18] for tissue repair.

The aim of the present study is to develop gels that can be degraded by the action of proteases in this case using the well-known bacterial enzyme subtilisin as a model. Here, we report on the design and the synthesis of a new class of PHEMA/PEO-based hydrogel (Scheme 1). The cross-linker is a PEO polymer chain and contains a short peptide spacer whose sequence has been selected to be easily hydrolyzed by subtilisin. When such a gel is incubated with the protein, the enzymatic hydrolysis will cleave the cross-linkers and release the short PHEMA segments. In support of this concept, we report on both the enzymatic assay performed on the cross-linker and on the whole gels.

II. MATERIALS AND METHODS

A. Materials

Amino-terminated PEO was purchased from Shearwater, triethylamine from Aldrich, *N*′-(3-dimethylamino propyl-*N*-ethyl hydrochloride (EDC) from Fluka, subtilisin A type VIII from Sigma; all substances were used without further purification. PEO (M_w=8000 g/mol) was purified by precipitation from diethyl ether. Succinyle-Glycine-Glycine-Leucine (SucGGL) was obtained by succinylation[19] of the commercial tripeptide Gly-Gly-Leu (Bachem). An aqueous solution of 2-hydroxyethyl methacrylate (25 vol %) was washed with hexane (4×200 mL) to remove the dimethacrylate. NaCl was then added to the aqueous phase



SCHEME 1. Principle of this study: the cross-linkers incorporate a peptide sequence that is cleaved by the protease.

a)Electronic mail: mesini@ics.u-strasbg.fr

and the organic phase was dried over Na_2SO_4. The crude product was distilled in vacuum to yield the pure monomer.

B. Characterization of the polymers

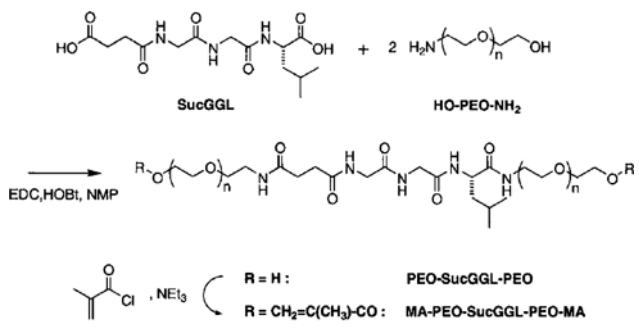
The average molecular weight of the polymers was estimated by size exclusion chromatography (SEC) in THF against PEO standards using a refractive index detector. In addition, the molar masses were corroborated using a multiangle light scattering detector. Nuclear magnetic resonance (NMR) measurements were performed with a 400 MHz Bruker.

The mass spectra were recorded with an autoflex apparatus from Bruker Daltonics using the matrix assisted laser desorption ionization (MALDI) technique and time-of-flight analysis. The compounds were dissolved in a water/MeOH mixture with α-cyano-4-hydroxycinnamic acid as the matrix and NaCl as the cationization medium, and an aliquot of the solution was dried on the target-plate. The spectrometer was calibrated with a sample of PEO with known mass (M_w =6400 g/mol). The mass distributions observed for this PEO sample were shown to correspond to the composition of $H_2O+n(C_2H_4O)$ with an accuracy of 0.1 g/mol. The spectra of the samples to be analyzed were recorded alone and compared to this reference spectrum for the mass calculations. The spectra of the same samples mixed with the standard were also recorded and the same mass values were found.

C. Synthesis of the cross-linker

The macromonomers were synthesized according to Scheme 2. Coupling of Suc-Gly-Gly-Leu with heterobifunctional hydroxyl-amino-PEO was achieved with *N*-(3-dimethylaminopropyle)- *N′*-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxy-7-azabenzotriazole (HOBt).

O-(2-aminoethyl)-poly(ethylene oxide) or $H_2N(EO)_n$-NHBOC (2 eq., 0.58 mmol) was mixed with Suc-Gly-Gly-Leu (1 eq., 0.29 mmol), EDC (2.5 eq, 0.735 mmol), and HOBt (0.1 eq, 0.029 mmol) in *N*-methyl pyrrolidone (25 mL) at room temperature. After 1 week, the reaction medium was diluted with water (20 mL). The polymer was recovered by extraction with dichloromethane and precipitation in diethyl ether. The material obtained was dialyzed against deionized water using a cellulose ester membrane (Spectra/Por) with a cutoff molecular mass of 5000 g/mol and freeze dried.



Scheme 2

SCHEME 2. Synthesis of the macromonomer used as cross-linking agent.

The macromonomers were prepared from the reaction of HO-PEO-Suc-Gly-Gly-Leu-PEO-OH for the case of degradable gels, and with PEO (M_w=8000) for the gels to be used as nondegradable reference. In a typical procedure 0.24 mmol of the respective polymer and triethyl amine (0.53 mmol, 2.2 equiv.) were dissolved in THF (20 mL) and methacroyl chloride was added dropwise at 0 °C. The mixture was allowed to reach 25 °C and stirred for a further 3 h. The reaction was stopped by the addition of water (100 mL), and the mixture was extracted with CH_2Cl_2. The organic phase was washed with a phosphate buffer (*p*H 7), dried over Na_2SO_4, and finally evaporated. The residue was precipitated in Et_2O and washed repetitively with Et_2O. The yield was typically 85%.

D. Synthesis of the gels

The hydrogels were prepared by copolymerization of hydroxyethyl methacrylate (HEMA) with the above-described cross-linker in a mixture of water-ethanol at 60 °C. A stock solution of the initiator $K_2S_2O_8$ was prepared at a concentration of 0.021 mol/l in water. All experiments were performed in pyrex glass tubes. The polymerization was carried out until the complete conversion of HEMA and the macromonomer, which was typically reached after 72 h, and always resulted in transparent gels. The hydrogels were removed from the cups and the conical bottom part was cut off to obtain cylindrical hydrogels of 1.3 cm in diameter and 3 cm in length. To leach out the unreacted PEG as well as residual HEMA monomers presumably entrapped in the network, the hydrogels were immersed in both water and ethanol for 3 days each at room temperature.

E. Extractible and swelling rates

Extractible: gels were prepared as described before, but before their extraction they were lyophilized for at least 24 h and their dry weight W_0 was measured. The gels were then immersed in water and ethanol for 3 days each in order to remove unreacted HEMA and PEO cross-linker, and lyophilized again. The dry extracted weight W_e was measured. The amount of extractible material was calculated according to Eq. (1),

$$e\% = \frac{W_0 - W_{\text{ex}}}{W_0} \times 100. \quad (1)$$

Swelling rates: Dry cylinders of the gels (1 cm diameter and 0.9 cm height) were incubated in deionized water at 25 °C and the swollen weight of each sample was recorded at regular time intervals after excess surface water was carefully removed with Kimwipes paper. The degradation experiments were continued until a constant weight was reached. The swelling ratio was calculated by Eq. (2), where W_1 and W_0 are the weight of the gel in wet and dry state, respectively,

$$SR = \frac{W_1 - W_0}{W_0} \times 100. \quad (2)$$

F. Degradation of the gels

Hydrogel cylinders with diameters of 1 cm and height of 0.9 cm were incubated at 37 °C in 10 mL of phosphate buffer solution (Na_2HPO_4 30 mM, KH_2PO_4 8.7 mM, *p*H 7.4) that contained subtilisin (50 μg/l) and 0.2 mg/mL sodium azide to prevent any bacterial contamination and additional protease sources.

III. RESULTS AND DISCUSSION

A. Selection of the peptide sequence

The choice of the peptide moiety was guided by results from the literature: Subtilisin cleaves preferentially after Leucine and, as many proteases, is not sensitive to the residue in the $P1'$ position. Moreover, it is known that short peptides such as Cbz-Gly-Gly-Leu-NH_2,[20] Cbz-Gly-Gly-Leu *p*-nitrophenylester,[21] and methylcoumarin esters[22] are substrates for subtilisin. The fact that the $P1'$ position can tolerate a wide variety of groups suggests that Gly-Gly-Leu-PEO would also be substrate. The choice of a short sequence offers two advantages: the synthesis is less effort consuming, and second, short peptides have a low immugenicity. In a preliminary study, we tested the compound BOCNH-$(EO)_3$-SucGlyGlyGlyLeu-$(EO)_3$-NHBOC, where $(EO)_3$ represents triethyleneoxide and NHBOC a BOC-protected aminogroup. It turned out that this compound is a substrate for subtilisin, demonstrating that when the *C* terminus is linked to amino-terminated oligo(ethylenene oxide) it is readily cleaved by the protease.

B. Synthesis of the cross-linkers

The coupling of the peptide sequence with oligomers or with aminoethyl-PEO was achieved with standard coupling agents (EDCI 2.5 equiv., HOBt) (Scheme 2). The yields were quantitative for short oligomers. For the amino-ethyl-PEO, the product was analyzed by SEC and showed a major peak at M_w=8000 g/mol, which corresponds to twice the mass of the starting polymer. This peak can unambiguously be attributed to the target compound HO-PEO-SucGlyGly-PEO-OH. On the elugram, a second peak with a mass of M_w =3500 g/mol can be superimposed with the signal of the starting material, and therefore be attributed to either the unreacted polymer or to the mono-adduct of PEO. Integration of both peaks leads to a ratio of 5 to 1 between them and, in our hands, could not be improved. The mixture was then purified by dialysis using cellulose ester membranes. The best results were obtained with a membrane possessing a cutoff of 5000. Figure 1 shows elution diagrams of the mixture after different times of dialysis leading to a complete separation only after 4 weeks. Despite the long duration of this purification step, the whole synthesis can be processed easily to yield the pure polymer at a scale of 10 g. Its analysis by MALDI-TOF revealed only one series of peaks, thus

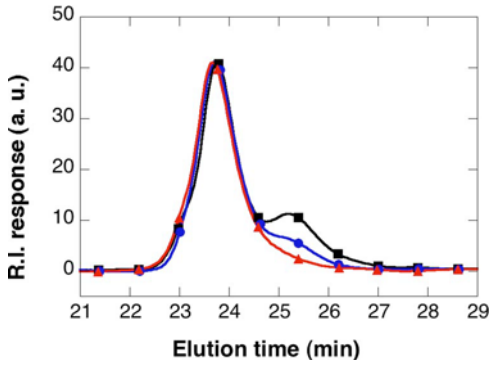


FIG. 1. SEC of HO-PEO-SucGGL-PEO-OH. ■: crude after synthesis; ●: after 7 days dialysis with a cellulose ester membrane (5000 Da cut-off); ▲: after 4 weeks dialysis.

suggesting a good purity of the sample (Fig. 2). The masses have a distribution in an arithmetic progression with an increment of 44 g mol^{-1}, which corresponds to the mass of the monomer unit. The peak distribution fits with the composition $nC_2H_4O+Na+C_{14}H_{25}N_5O_5$. These fragments correspond to the monomer, the sodium (cationization medium), and the diamide of SucGGL respectively, which proves that the synthesized cross-linker has the expected structure. The mass distribution is centered around 7150 g/mol, which is in agreement with the value obtained from size exclusion chromatography.

C. Synthesis and characterization of the gels

The hydroxyl end groups of the polymer described above were esterified with methacroyl chloride to yield the bifunctional macromonomer MA-PEO-SucGlyGlyLeu-PEO-MA.

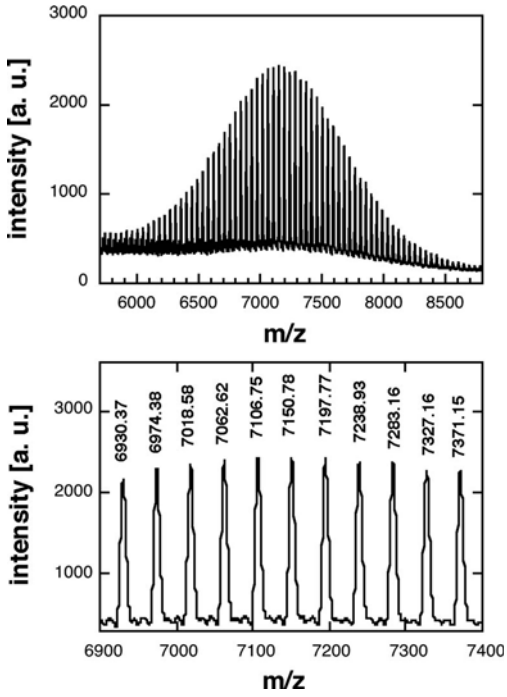


FIG. 2. MALDI-TOF spectrum of HO-PEO-SucGlyGlyLeu-PEO-OH.

TABLE I. Feed composition of the synthesized hydrogels.

Gel	HEMA (mmol)	PEO cross-linker (μmol)	EO unit (mmol)	EO/HEMA
*G*1	7.63	107.9	14.3	1.87
*G*2	7.63	74.5	9.9	1.29
*G*3	7.63	27.9	3.7	0.54
*G*4	7.63	16.7	2.2	0.30
*G*5	7.63	8.4	2.1	0.15
*GM*1	7.63	75.0	13.6	1.77
*GM*3	7.63	2.5	4.5	0.59
*GM*4	7.63	0.8	2.3	0.30

The degree of end-group functionalization was analyzed by ^1H nuclear magnetic resonance spectroscopy (NMR) and estimated to be 80%. The polymerization of the macromonomer was subsequently carried out by the free radical mechanism, in a 1:1 mixture of water and ethanol.

The feed content of the synthesized gels *G*1 to *G*5 is given in Table I. In addition to the gels synthesized with the peptide containing cross-linkers, we also synthesized gels with a PEO cross-linker lacking the central peptide sequence but with a molar mass (M_w=8000 g/mol), very close to the one of MA-PEO-SucGlyGlyLeu-PEO-MA. This undegradable cross-linker was synthesized by acylation of amino-terminated PEO(NH_2-PEO-NH_2), in the same way as the degradable cross-linker above. These gels are denoted as *GM*1, *GM*3, and *GM*4 in Table I. The content of extractable species (unreacted monomer and the polymer chains not covalently linked to the network) was determined to be between 3 and 5%, which shows that the polymerization reaction proceeds to high degrees of conversion.

The dried gels swell in water to form soft, rubbery, and transparent hydrogels. The swelling rate of dried hydrogels was studied by a general gravimetric method as described in Sec. II. The swelling behavior of the different gels is depicted in Fig. 3. The hydrogels absorb water until their weight reaches a plateau that represents the swelling ratio at equilibrium. This ratio is very sensitive to the feed composition of the gels and increases with the EO content. The gels from *G*1 to *G*5 have swelling ratios of 120, 100, 80, 66, and

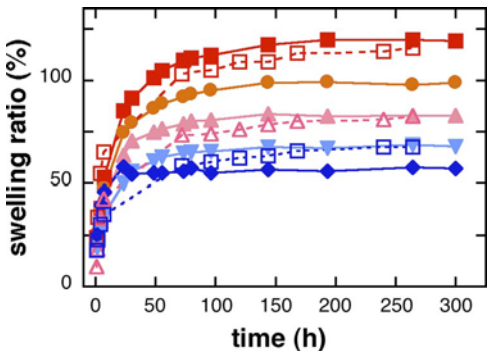


FIG. 3. Swelling ratio of the PHEMA/PEO hydrogel. Peptide containing gels: ■: *G*1; ●: *G*2; ▲: *G*3; ▼: *G*4; ◆: *G*5. Model gels, without peptides: □: *GM*1; △: *GM*3; ▽: *GM*4.

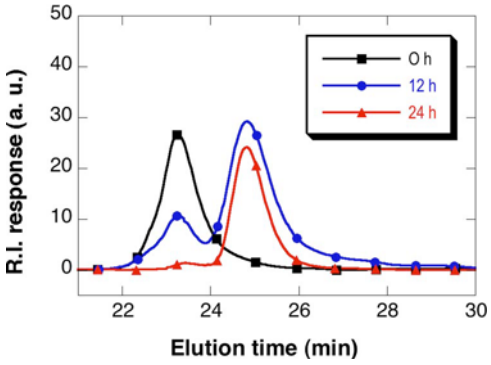


FIG. 4. SEC of HO-PEO-SucGlyGlyLeu-PEO-OH incubated with the protease at physiological conditions (37 °C, *p*H 7.4).

56%, respectively, whereas their EO/HEMA feed contents drop from 2.09 to 0.16 for *G*5. A very likely explanation for this phenomenon is that PEO is more hydrophilic than PHEMA, and therefore a higher PEO content should increase the swelling ratio. It has also been reported that for macronets, the swelling ratio in poor solvents increases when the cross-linking rate increases.[23–25]

The kinetics of the swelling process also depend on the composition of the gel. The time necessary to reach the plateau is less than 48 h for *G*5, and increases up to 200 h for *G*1; as expected, the diffusion of water is slower for higher degrees of cross-inking.

A comparison of gels with and without a degradable peptide sequence shows that the peptide sequence itself has little influence on the swelling process. For the gel *GM*1, which has a feed composition close to that of *G*1, the swelling ratio is approximately 110%. Similar results were obtained for the gels *GM*3/*G*3 and *GM*4/*G*4.

D. Degradability assays

In order to verify that the cleavable cross-linker is effectively degraded by the enzyme, it was incubated with the enzyme in physiological buffer and the resulting products were monitored by SEC (Fig. 4). The elution diagrams showed that the peak at 7000 g/mol corresponding to the intact cross-linker gradually disappeared, and that a peak with a lower mass (3500 g/mol) appeared and increased with time. This shows indeed that the degradable cross-linker is a substrate for the protease and that it is readily cleaved in its center to yield two identical fragments with half the mass of the starting material.

The gels were also subjected to enzymatic degradation assays. They were incubated with a solution of the protease (50 μg/mL) in phosphate buffer (*p*H 7) at 35 °C and their residual weights were measured at different times (Fig. 5) as described in Sec. II F. In order to prevent any bacterial contamination, NaN_3 was added to the buffer. The gels obtained with the degradable cross-linker underwent a slow degradation, which most likely takes place at the surface of the gel and eventually results in their complete dissolution and the formation of a clear solution. The reference gels without peptide sequences were subjected to the same degradation con-

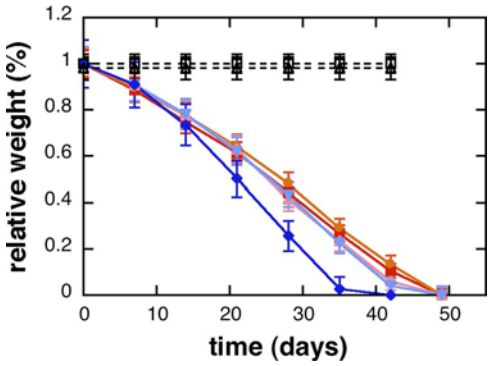


FIG. 5. Rate of degradation of the hydrogels by subtilisin followed by gravimetry. Peptide containing gels incubated with subtilisin: ■: *G*1; ●: *G*2; ▲: *G*3; ▼: *G*4; ♦: *G*5. Model gel GM1, without peptide, incubated with subtilisin: □; peptide containing gels, incubated in the same conditions, without subtilisin: △.

ditions but remained unaltered. The fact that the degradation of the gels is triggered specifically by the presence of the enzyme was demonstrated by incubating degradable gels under the same conditions but in the absence of a protease: the gels remained intact under these circumstances.

The time required for the complete dissolution of the samples increases slightly with the amount of cross-linker used to form the gels. Cylindrical hydrogel samples of 1.3 cm in diameter and 3 cm in length were completely degraded within 35 days in the case of *G*5, within 42 days in the case of *G*3, and within 50 days in the case of *G*1. The degradation took place by a depletion of material from the surface of the gel without swelling of the gel prior to degradation. The profile of continuous mass observed on Fig. 5 is also indicative of a surface degradation, as opposed to a bulk degradation process, where the mass of the gels would increase and then decrease. The process of bulk degradation occurs when the diffusion of the hydrolytic species is faster than the rate of the hydrolysis. To the contrary, the surface degradation occurs when the diffusion of the hydrolytic species is slow compared to the rate of hydrolysis. In the present study, the observed profile shows that for all the gels, the network is dense enough to slow down the diffusion of the enzyme ($M=27$ kDa) into the gel. The decrease of the degradation times from *G*1 to *G*5 can be attributed to the decrease of the amount of cleavable peptides.

IV. CONCLUSION

This study shows that it is possible to design and to synthesize well-defined PEO macromonomers containing an enzymatically cleavable peptide sequence, in this case Suc-Gly-Gly-Leu. We have shown that this polymer is a substrate for subtilisin and is actually cleaved in its center where the peptide sequence is located. We have copolymerized this macromonomer with HEMA to form new types of degradable gels. The capacity of the gels to absorb water increases with increasing macromonomer content, which is likely explained by the highly hydrophilic character of the PEO. Comparison with reference gels based on cross-linkers without degradable units and on long-term stability of gels in the absence of protease demonstrates that the only mechanism of gel degradation is enzymatic action. The enzymes attack the gels from their surface, thus reducing the gel volume with time until complete dissolution of the gels. The next steps of this work will be the inclusion of other peptide sequences to control the degradation process with proteases from different bacterial strains. The loading of gels with therapeutically active molecules and their release from our degradable gels will be also addressed.

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