Photo-crosslinkable, thermo-sensitive and biodegradable Pluronic hydrogels for sustained release of protein

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Received 17 December 2003; accepted 13 April 2004

Abstract—Thermo-sensitive and biodegradable hydrogels based on Pluronic tri-block copolymers were prepared by a photo-polymerization method. Two terminal hydroxyl groups in Pluronic F-127 were acrylated to form a Pluronic macromer. Photo-cross-linked Pluronic hydrogels prepared by UV radiation showed a gradually decreased swelling ratio with increasing temperature and exhibited a thermally-responsive change in the swelling ratio when the temperature was cycled between 10°C and 37°C. These hydrogels degraded slowly due to the cleavage of ester linkage in the acrylated Pluronic terminal end. When lysozyme, a model protein drug, was loaded in the hydrogels, bi-phasic protein release profiles were attained: a burst-free and rapid controlled release profile was initially observed for a one week period and a much slower sustained release was followed thereafter. The release rates could be controlled by varying the amount of Pluronic macromer for photo-polymerization.

Key words: Block copolymers; hydrogels; photopolymerization; drug-delivery systems; biodegradable.

INTRODUCTION

Many kinds of natural and synthetic polymeric hydrogels have been widely used for sustained delivery of bioactive macromolecules [1–7]. Water-soluble polymer chains were cross-linked by chemical and physical means to form a network structure that swells in water [8, 9]. Synthetic polymeric hydrogels have been prepared by free radical polymerization of water-soluble vinyl monomers with a small amount of divinyl cross-linker or by intra-molecular cross-linking of...
functionalized water soluble polymer chains using a bi-functional cross-linker [10].
Most of these hydrogels are chemically cross-linked by covalent linkages [11]. On
the other hand, physical hydrogels are formed by self-association of polymer chains
by non-covalent interactions such as ionic interaction [12], hydrogen bonding [13],
hydrophobic interaction [14] and stereocomplex formation [15].

Tri-block copolymers of poly(ethylene oxide)-b-poly(propylene oxide)-b-poly
(ethylene oxide) (Poloxamers and Pluronics) self-associate to form micelles in an
aqueous solution in a dilute condition [16, 17]. At high concentrations above ca. 20% (w/v), they exhibit a temperature-dependent sol–gel transition behavior [18].

The thermo-sensitive property of Pluronic hydrogels can be attributed to a lower
critical solution temperature (LCST) behavior that is mainly caused by a delicate
balance of hydrophilic and hydrophobic moieties on the polymer structure [19].
The LCST is closely related to critical micelle temperature (CMT) in Pluronic
copolymers. Highly concentrated Pluronic copolymer solution instantaneously
turns into a thermo-setting gel state upon increasing the temperature above the
LCST by the formation of closely packed spherical micelles [20]. The formation
of Pluronic hydrogels has been widely used for sustained delivery of various
macromolecular drugs [21]. However, the semi-solidified gel structure formed
above the CMT loses its structural integrity upon dilution with other aqueous fluids,
due to an immediate decrease in the copolymer concentration required for the close
packing of Pluronic micelles. When a Pluronic sol solution was injected into a body,
a thermo-setting gel structure was produced immediately at the site, but rapidly
dissolved out hereafter [22]. This rapid dissolution problem at the injection site
severely limits the use of Pluronic copolymers for sustained release of various
drugs. Recently, it was shown that biodegradable tri-block copolymers based
on poly(lactic-co-glycolic acid) (PLGA) and poly(ethylene oxide) also exhibit a
temperature-dependent sol–gel transition behavior and become a semi-solidified gel
state at high concentration similar to Pluronic copolymers. In contrast to Pluronic
hydrogels, however, they do not dissolve out upon dilution with aqueous buffer
solutions and maintain their structural gel integrity at the injection site in vivo. This
may be caused by the presence of more hydrophobic PLGA block in the copolymer
structure. Long-term sustained release of macromolecular protein drugs could be
successfully achieved by using tri-block copolymers of PLGA and PEO, while
Pluronic copolymers often failed to exhibit such sustained release in vitro and in vivo [23].

In situ-formed biodegradable hydrogels prepared by photo-irradiation were pre-
viously reported for sustained delivery of proteins and cells in the body [24, 25].
Poly(ethylene glycol) (PEG) terminated with oligo-lactic acid at both ends was di-
acrylated to synthesize a biodegradable PEG macromer [26, 27]. In the presence
of a small amount of photo-initiator, biodegradable hydrogels could be produced
by UV radiation. These in situ-formed biodegradable hydrogels have some advan-
tages in delivering various therapeutic proteins and cells at the desired site. Af-
ter injecting a mixture solution of macromer, photo-initiator and proteins or cells
through a needle in the body tissue, a hydrogel depot can be produced with subsequent photo-polymerization via a UV-transmissible optical fiber. A schematic illustration of injectable Pluronic hydrogel delivery system is shown in Fig. 1. In a previous report, we prepared photo-cross-linked hyaluronic acid (HA)/Pluronic composite biodegradable hydrogels for sustained release of human growth hormone [28]. Vinyl monomer conjugated HA and di-acrylated Pluronic F-127 were photo-polymerized in the presence of human growth hormone, which resulted in sustained release of human growth hormone over 30 days. In this paper, chemically cross-linked Pluronic F-127 hydrogels were prepared by photo-polymerization of di-acrylated Pluronic F-127 macromer without using other monomers or macromers. It was hypothesized that by simply conjugating an acrylate group to the terminal ends of Pluronic copolymer, a photo-cross-linkable hydrogel could be obtained without using any other vinyl monomers as polymerization aiding agents. The photo-polymerization was expected to occur due to the presence of enriched acrylate groups segregated within peripheral interstitial space between self-assembled spherical Pluronic micelles. This photo-polymerization strategy is different from that of the previous study which used vinyl group conjugated hyaluronic acid [28]. The main objective was to form a non-dissolving and semi-solid Pluronic hydrogel depot for sustained release of proteins.

MATERIALS AND METHODS

Materials

Pluronic® F-127 ((PEG)_{99}(PPG)_{69}(PEG)_{99}) was purchased from BASF (Ludwigshafen, Germany). (4-Benzoylbenzyl)trimethylammonium chloride, acryloyl chloride and triethylamine were purchased from Aldrich (Milwaukee, WI, USA). Lysozyme was obtained from Amore Pacific (Seoul, South Korea). Methylene chloride, diethylether and other reagents were of analytical quality.
Synthesis of di-acryloyl Pluronic F-127

Pluronic copolymers were dried at 110°C under vacuum for 1 h. Dried Pluronic F-127 (40 g, 3.18 mmol) and triethylamine (1.00 ml, 7.24 mmol), dissolved in 100 ml methylene chloride, were placed in a 500-ml round-bottomed flask. Acryloyl chloride (0.50 ml, 12.72 mmol) was added drop-wise. The mixture was stirred at 4°C for 24 h and then at room temperature for 24 h. After stirring, the product was purified by precipitation in an excess amount of diethylether and dried under vacuum overnight. The extent of acrylation was determined by \(^1\)H-NMR (Brucker DRX 400 spectrometer operating at 400 MHz) as determined previously [28]. Chemical shift (δ) was measured in ppm using CDCl\(_3\) as an internal reference. The substitution degree was about 72%. It was determined at the relative peak ratio of acryloyl protons at each terminal of di-acryloyl Pluronic F-127 (\(=\text{CH}_2\), 5.8–6.4 ppm) and three protons of the methyl group of the propylene oxide unit (\(=\text{CH}_3\), 1.1 ppm).

Synthesis of Pluronic hydrogels

Three different amounts of di-acryloyl Pluronic F-127 were dissolved in 16 ml of degassed deionized water to make 15, 20 and 25% (w/v) solutions. (4-Benzyolbenzyl)trimethylammonium chloride was added as a photo-initiator to the mixture at a final concentration of 2% (w/w) relative to the polymer. For lysozyme loading, 100 mg of lysozyme was dissolved in the Pluronic macromer solution. The sol-state solution was placed at 4°C overnight for homogeneous mixing and then poured into a glass dish at room temperature. After equilibrating at room temperature, the gel-state mixture was exposed to long-wavelength UV by a 100-W UV source (UVP model B 100AP, Upland, CA, USA) for 5 min under a nitrogen atmosphere. The UV source was located 10 cm far from the sample. After UV induced polymerization, gel-disks with a diameter of 1 cm were excised with a cork borer and washed two times in deionized water to remove unreacted macromers and other molecules. The above gel-disks were dried in air for 1 day, and then in a vacuum oven for 1 day.

Swelling behavior experiments

To demonstrate the temperature-sensitive characteristics of Pluronic hydrogels, swelling ratios were investigated at different temperatures. The swelling ratios were gravimetrically determined in duplicate by dividing the wet hydrogel weight by the dry hydrogel weight at each temperature point. For estimation of the swelling ratio, hydrogels with known dry weights were incubated in phosphate-buffered saline (10 mM, pH 7.4) containing 0.01% (w/v) sodium azide at the desired temperature. The hydrogels were equilibrated for 12 h before measuring the wet weight. In determining oscillatory swelling ratios, hydrogels were incubated in the above PBS and the temperature was changed between 10°C and 37°C in a step-wise fashion.
Degradation experiments

Various Pluronic hydrogel disks were weighed and swollen in PBS solution at 37°C for a desired period. At pre-determined time intervals, the hydrogel disks were dried under vacuum for 12 h and then weighed. Mass erosion percent was determined by calculating the difference of dry weight before and after the incubation. The measurements were carried out in duplicate.

Analysis of protein release from hydrogels

For protein release experiments, lysozyme was used as a model protein. Three different Pluronic macromer solutions containing lysozyme was photo-polymerized as described above. The amount of lysozyme released from the hydrogels in the incubation medium was determined by using a Micro-BCA™ protein assay kit (Pierce, Rockford, IL, USA).

Circular dichroism (CD) study

Conformational change of released lysozyme from hydrogels was examined by a far-UV CD spectroscopy. The concentration of native or released lysozyme from 25% (w/v) Pluronic hydrogel was adjusted to 300 µg/ml. The CD spectra were recorded on a Jasco J-720A spectropolarimeter over the 200–250 nm range using a 1-mm path-length cell. Scan speed was set at 50 nm/min and five scans of each sample were averaged.

RESULTS AND DISCUSSION

Synthesis of hydrogels

Three different concentrations of di-acrylated Pluronic F-127 (15, 20 and 25% (w/v)) were prepared in a PBS buffer solution. The macromer solutions were in a clear viscous fluid state at 4°C, but they became a semi-solidified gel when incubated at room temperature, although they showed different degrees of gel strength depending on the concentration. The gelation was attributed to the micellization of Pluronic tri-block copolymers upon increasing temperature. When the temperature was raised above a certain critical micelle temperature range, hydrophobic PPO blocks were dehydrated and self-associated, while hydrophilic PEO blocks were hydrated, resulting in the formation of a core-shell type polymeric micellar structure [29]. The temperature-induced sol–gel transition was due to the formation of closely packed Pluronic micelles in a confined volume [30]. By UV irradiation in the presence of (4-benzoylbenzyl) trimethylammonium chloride as a photo-initiator, vinyl groups present on the periphery of Pluronic micelles could be polymerized to covalently cross-link the closely packed micelles, thereby producing non-dissolving and chemically cross-linked Pluronic hydrogel networks. Figure 2
Figure 2. Sol–gel transition and UV-induced photo-cross-linking of di-acrylated Pluronic macromers. Pluronic macromers were assumed to be in a closely packed state prior to photo-polymerization.

shows a schematic illustration for the sol–gel transition and UV-induced photo-cross-linking of Pluronic macromers. Pluronic hydrogels prepared from different concentrations maintained their shape and mechanical integrity upon incubation in a large volume of PBS buffer solution. It should be noted that the resultant hydrogels had some mono-acrylated Pluronic chains grafted to a polymerized network backbone, because the degree of Pluronic acrylation was about 72% as estimated by $^1$H-NMR. The acrylation degree calculated from $^1$H-NMR spectra included not only di-acrylated Pluronic fraction, but also mono-acrylated Pluronic fraction. Thus, it was possible that mono-acrylated Pluronic copolymers were grafted on the polymer network while non-acrylated Pluronic fraction was removed after a washing step.

Swelling ratio of hydrogels

Since the photo-induced cross-linking between Pluronic micelles was performed in a closely packed state, self-associated Pluronic micelles were entrapped within a cross-linked polymer network. Thus, the synthesized Pluronic hydrogels were expected to demonstrate a thermo-responsive swelling property as a function of temperature. Figure 3 shows the swelling degree of three different Pluronic hydrogels at different temperatures. The swelling ratio monotonically decreases with
Figure 3. Swelling behaviors of three different Pluronic hydrogels in PBS buffer (pH 7.4) as a function of temperature: (●) 15% (w/v) Pluronic hydrogel, (○) 20% (w/v) Pluronic hydrogel, (▼) 25% (w/v) Pluronic hydrogel.

Increasing temperature, suggesting that the entrapped thermo-sensitive Pluronic micelles played a critical role in controlling the swelling ratio. The swelling ratio changes broadly over a wide range of temperature, from 6°C to 50°C. The broad transition might be caused by the effect of photo-cross-linking between Pluronic tri-block copolymers. Typically, Pluronic F-127 solution with 25% (w/v) concentration shows a sol–gel transition around 20°C [31]. At higher temperatures, hydrophobic interactions between PPO blocks in the tri-copolymer structure became stronger, and as a result, a more compact Pluronic micellar structure was entrapped in the network. Consequently, the polymerized chain network around the micelles collapsed, leading to a decrease in the swelling ratio. In contrast, at lower temperatures, a disintegrated Pluronic micelle structure, which was induced by weak hydrophobic interactions between PPO blocks in the inner core, permitted the expansion of the surrounding polymer chain network. This resulted in an increase in the swelling ratio. The hydrogels with 15% and 20% (w/v) Pluronic show higher swelling ratios than those prepared from 25% (w/v). The significantly reduced swelling extent for 25% (w/v) Pluronic was caused by the formation of more closely packed Pluronic micelles at the higher concentration. The initial packing state of di-
acrylated Pluronic macromers at room temperature prior to UV radiation determines the cross-linking density of the resultant photo-cross-linked Pluronic hydrogels, resulting in the different swelling behaviors. Pluronic hydrogels exhibited a semi-reversible swelling/deswelling behavior as temperature was cycled between 10°C and 37°C, as shown in Fig. 4. This is due to the temperature-dependent association and dissociation of hydrophobic PPO blocks within the core of micelles surrounded by the polymerized chain network. The synthesized Pluronic hydrogels were slowly degradable because of a water-hydrolyzable ester linkage in the acrylate group attached to the terminal end of Pluronic copolymer. Thus the swelling ratios shown in Figs 3 and 4 were pseudo-equilibrium values determined over a short incubation period.

Degradation of hydrogels

Figure 5 shows the remaining gel mass percentage in a long-term incubation, period up to 35 days. After 35 days, it was difficult to determine the mass erosion percent in a reliable manner because the Pluronic hydrogels were fragmented into many
small pieces. They degraded slowly as a function of time due to gradual cleavage of an ester linkage in the polymerized acrylate group. Hydrolytic cleavage of the ester linkage at the junction of Pluronic copolymer to a polymerized network of polyacrylate eventually generated negatively charged poly(acrylic acid) chains. Although the formation of negatively charged poly(acrylic acid) backbone by the erosion of Pluronic hydrogels was not directly proved in this study, it was previously demonstrated that ester linkages in the hydrogels photo-cross-linked with di-acrylated oligo-lactic PEG macromers were readily hydrolyzed to produce negatively charged backbone [25]. The three hydrogels degraded up to ca. 50% in a dry weight basis over a 35-day incubation period. The mass erosion profiles for the three samples were almost similar in an early incubation stage, but were significantly different in a later stage after 15 days, depending on the Pluronic concentration. Pluronic hydrogels with 15 and 20% (w/v) concentrations exhibit faster degradation than those with 25% (w/v) probably due to their higher swelling states. The disintegration speed of the polymer gel network depends on the cross-linking density that directly affects the swelling ratio. Thus, in the later incubation stage, the erosion rates for the less cross-linked hydrogels were accelerated to a greater extent by increasing the water content.
Lysozyme (14.4 kDa) was used as a model protein to explore the possibility of sustained protein release using biodegradable and in situ photo-polymerized hydrogels. Lysozyme was homogeneously mixed with di-acrylated Pluronic macromer solutions in the sol state at a lower temperature below critical micelle temperature (CMT). By raising the temperature at room temperature that is above the CMT, the mixture instantaneously became a semi-solidified physical gel state. Through UV photo-irradiation, cross-linked Pluronic hydrogels containing lysozyme were formed. In vitro release profiles of lysozyme from three different Pluronic hydrogels are shown in Fig. 6. Bi-phasic kinetic profiles can be seen: a faster linear release up to 7 days followed by a more sustained release over a one month. Pluronic hydrogel with 25% (w/v) concentration shows slower release rate than the other two hydrogels because of reduced water swelling ratio. It is of interest to note that there are very minimal burst releases of ca. 10% on day 1. This suggests that lysozyme was homogeneously loaded within the bulk phase, not loaded in the surface region of the hydrogels. Lysozyme in the early stages was likely to be released in a controlled diffusion mechanism through an insufficiently degraded gel network. After the initial linear release phases for 7 days, much slower but linear release phases ensued. The slower release of lysozyme at the later stage can be attributed to ionic interactions of positively charged lysozyme with negatively charged polymer backbone that was produced as a result of ester bond hydrolysis. Thus, ionic interactions of lysozyme to carboxylate groups in the hydrolyzed polymer network might impede the release of lysozyme from the degrading hydrogels, although the pore size was enlarged due to the erosion. Hence, a complicated mechanism including diffusion, erosion and ionic interaction may play a role in determining the lysozyme release profile. In our previous study of HA/Pluronic composite hydrogels for sustained release of human growth hormone, an accelerated release behavior was observed when the HA/Pluronic hydrogels eroded sufficiently [28]. Because human growth hormone was negatively charged, its release rate was governed by the diffusion through the enlarged pores by polymer degradation. Therefore, release profiles of proteins from the photo-cross-linked Pluronic hydrogels are dependent on various factors; namely, the kinds of proteins, Pluronic concentration, cross-linking extent of Pluronic macromer and the presence of other polymers. Figure 7 shows CD spectra of native lysozyme and released lysozyme from 25% (w/v) Pluronic hydrogel at day 7. Both of the CD spectra are almost identical, indicating that the UV irradiation used in this study did not significantly affect the conformation of lysozyme.

Sustained delivery of therapeutic proteins has been attempted using various biodegradable polymers. Typically, poly(lactic-co-glycolic acid) microspheres (PLGA) were widely used for encapsulating various proteins for controlled release [32]. Since PLGA is hydrophobic and water insoluble, the protein encapsulation process requires the use of an organic solvent. The direct contact of protein molecules with an organic solvent resulted in protein denaturation and aggregation during the formulation [33]. In addition, acidic micro-environment created by the
Figure 6. *In vitro* release profiles of lysozyme from three different Pluronic hydrogels at 37°C.

Figure 7. Far-UV CD spectra of native lysozyme and released lysozyme at day 7.
degradation of PLGA and protein adsorption to hydrophobic PLGA leads to protein aggregation within the microspheres and subsequent incomplete release [34]. Thus, high water containing hydrogel materials relative to PLGA polymers would be more benign for protein delivery in terms of their physical stability. The release of proteins from these hydrogels could be modulated by controlling the mesh size and their degradability. Photo-cross-linked Pluronic hydrogels are promising candidates for protein delivery because Pluronic copolymers were accepted as a safe polymer for human use.

CONCLUSIONS

New thermo-sensitive and biodegradable hydrogels based on Pluronic F-127 triblock copolymers were prepared by UV-induced photo-polymerization. Di-acrylated Pluronic macromer was synthesized and cross-linked in a micellar gel state. These hydrogels exhibited a temperature-dependent swelling behavior in an aqueous solution over a wide range of temperature. This was because the inner core structure of Pluronic micelles entrapped within a photo-cross-linked polymer network sensitively responded to temperature. Semi-reversible swelling/deswelling behaviors were observed during thermal cycles between 10°C and 37°C in a short incubation period. The ester linkage located at the junctions of Pluronic micelles to a cross-linked polymer network was slowly cleaved by hydrolysis, making these hydrogels biodegradable. Bi-phasic release profiles from photo-cross-linked Pluronic hydrogels were observed. At an early stage, a burst-free and rapidly controlled release pattern was observed. A much slower sustained release was observed thereafter. This is due to the ionic interactions of the positively charged lysozyme with the negatively charged polymer backbone that was produced as a result of the ester linkage hydrolysis. These in situ forming photo-cross-linked and biodegradable Pluronic hydrogels are expected to be very useful for achieving sustained release of proteins at the injection site.

Acknowledgements

This research was supported by the Korea Science and Engineering Foundation, Korea (# R01-2003-000-10362-0). We also appreciate Amore Pacific Co. (Seoul, South Korea) for providing lysozyme.

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