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A cell-compatible PEO–PPO–PEO (Pluronic®)-based hydrogel stabilized through secondary structures

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ABSTRACT

Pluronic F-127 (PF127) is a thermosensitive polymer that has been widely recognized as a potential candidate for various bio-applications. However, in hydrogel form, its rapid disintegration and inhospitality toward cells have significantly limited its usage. As a means to increase the integrity and cell compatibility of a PF127 hydrogel, we propose the introduction of stabilizing secondary structures to the gel network by the addition of secondary structure-forming oligo-alanine and oligo-phenylalanine. Results indicate that increasing the oligo(peptides) attached to PF127 led to a significant decrease in the gelation concentration and temperature. A selected oligo(peptide)-modified PF127 was capable of forming a stable hydrogel network at 5% and suffered only 20% weight loss after 7 days of incubation in media. Scanning electron microscopy (SEM) revealed comparably more interconnected morphology in modified hydrogels which may be attributed to the presence of secondary structures, as verified by circular dichroism (CD) and Fourier-transformed infrared (FT-IR) spectroscopy. Nuclear magnetic resonance (NMR) provided insights into the extensive interactions at the micelle core, which is the key to altered gelation behavior. Furthermore, modified hydrogels maintained structural integrity within culturing media and supported the proliferation of encapsulated chondrocytes. In addition, *in vivo* residence time was extended to well beyond 2 weeks after oligo(peptide) modification, thereby broadening the application scope of the PF127 hydrogel to encompass long-term drug delivery and cell culturing.

1. Introduction

Hydrogels are highly swollen three dimensional polymeric networks that have been extensively studied for bio-applications including contact lenses [1–3], wound dressings [4,5], drug delivery depots [6,7], and cell delivery depots [8,9]. Their broad spectrum of uses can be attributed to their superior tunability both chemically and physically. Pluronic F-127 (PF127) is a FDA approved synthetic polymer of poly(propylene oxide) (PPO) blocks flanked by poly(ethylene oxide) (PEO) blocks that, when solubilized, undergoes thermo-reversible gelation at concentrations above 18% (*w*/*w*) [10,11]. The PF127 hydrogel has been evaluated in literature as a potential topical drug delivery system due to its ease of application and practical use as a short-term drug depot [12–14]. Thus far, the application of PF127 is limited by its weak modulus and rapid disintegration. A 30% PF127 hydrogel erodes entirely within one week in *in vitro* culture and results in comparably less tissue formation than natural materials such as alginate and collagen [15]. The half-life of a 30% PF127 solution after peritoneal injection is 21 h and the material is generally considered to possess inadequate mechanical integrity [16]. In the past, various efforts have been made to circumvent the rapid dissolution of PF-based copolymers such as the use of ethoxysilane-capped Pluronic, which hydrolyzes over time to form silanol groups that covalently crosslink to improve the mechanical properties of the hydrogel [17]. Polymers have also been added to the terminal ends of Pluronic to provide ABCBA pentablock copolymers, such as the incorporation of poly(esters) as a chain extender to improve the mechanical integrity [18,19]. Other recent strategies include the use of polymer-cyclodextrin [20] to form inclusion complexes and the introduction of graphene to provide a composite [21].

In addition to concerns involving long-term stability, studies focusing on the *in vitro* culturing of cells within PF127 are limited due to polymer toxicity at low concentrations. The growth of endothelial cells was inhibited at PF127 concentrations as low as 5%. In a separate study, 10% solution of PF127 significantly decreased HepG2 cell viability while a 15% solution resulted in complete cell death after 5 days of culture [22,23]. The toxicity of ethylene oxide and propylene oxide block copolymers has been attributed to the tendency of these polymers to adsorb

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onto cell surfaces which causes drastic changes in membrane microviscosity [24]. Although the addition of hydrocortisone to maintain membrane homeostasis has been shown to significantly increase cell viability, the long term effects of hydrocortisone on normal cell functions remain unknown [23].

Alanine is an amino acid with a high α-helix propensity due to its small size and neutral side group. Therefore, poly(peptides) with a high alanine content are known to assemble into helical structures in aqueous solution [25]. Furthermore, multiple α-helices can associate to form helix bundles that are crucial to the folding, stability, and signaling of transmembrane proteins. Similar assemblies are also present in structural proteins such as collagen [26]. Phenylalanine is an aromatic amino acid with high β-sheet propensity and is a site for π–π stacking between strands which supports a stronger network, as observed in the self-assembly of amyloid structures. [27] One approach to improve the biocompatibility of PF127 will be to introduce stabilizing secondary structures to control the disintegration rate of a hydrogel, thus eliminating the detrimental effects of toxic monomers and provide a suitable environment to accommodate cells. Previously, one group presented an interesting study discussing the assembly properties of an alanine and lysine containing poly(peptide) in the presence of PF127 and found that the addition of PF127 increased the helicity of poly(peptides) [28]. The resulting hydrogel exhibited high storage modulus as a result of fibril formation. However, its application for minimally invasive drug and cell delivery is limited by its non-thermosensitivity and Michael-type addition process which is incompatible with mammalian cells.

In this study, we proposed that by modifying PF127 with oligo(peptides), improvements in hydrogel stability and cell compatibility may be accomplished. Apart from other strategies used to stabilize Pluronic hydrogel, the addition of oligo(peptides) is unique because it allows hierarchical self-assembly of peptides, a concept borrowed from proteomics. Previously, we showed that PF127-oligo(alanine) micelles arrange in such fashion so that the hydrophobic oligo(alanine) segments are tucked within the micelle core and that this arrangement increases drug entrapment and delays the release of hydrophobic drugs partitioned inside the core [29]. However, the rearrangement of these polymers at higher concentrations and temperatures has yet to be elucidated. Specifically, we studied the presence of secondary structures and their effects on gelation, applicability of modified copolymer hydrogels for cell culture *in vitro*, and long term stability of the network *in vivo*.

2. Materials and methods

2.1. Materials

Poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) block copolymers (PEO–PPO–PEO) sold as Pluronic-F127 (PF-127), L-alanine, L-phenylalanine, triphosgene, triethylamine (TEA), dimethylformamide (DMF), 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS), trifluoroacetic acid-d (TFA-d), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reagent were obtained from Sigma (St. Louis, MO). Mesyl chloride was purchased from Aldrich (St. Louis, MO). Dichloromethane (DCM), tetrahydrofuran (THF), chloroform, and ammonia water (31–33%) were obtained from J.T. Baker (Phillipsburg, NJ). Dimethyl sulfoxide (DMSO), hexane, and ethyl ether were acquired from Echo Chemicals (Taiwan). Phosphate buffered saline (PBS) at pH 7.4, Dulbecco's Modified Eagle Medium (DMEM), and fetal bovine serum (FBS) were obtained from Gibco (Carlsbad, CA). All other materials were used as purchased and according to the manufacturer's instructions.

2.2. Synthesis and characterization of oligo(peptide)-modified PF127

PF127 was dried in a vacuum oven overnight and exposed to repeated vacuum and nitrogen purge cycles prior to processing. The terminal hydroxyls were modified into primary amines by nucleophilic substitution [30]. Briefly, 10 g of PF127 was dissolved in 150 mL of DCM and chilled prior to the addition of 3.6 mL of TEA. After drop-wise introduction of 1.96 mL of mesyl chloride, the reaction carried out for 24 h under N_2 atmosphere. The product was concentrated by rotary evaporation, precipitated in ethyl ether, and dried. Ammonia water was added to the product and the mixture was stirred vigorously for 3 days. Amine-terminated PF127 was extracted with DCM, concentrated, and lyophilized. Amination of PF127 was verified by the ninhydrin assay.

The N-carboxyanhydride form of alanine was prepared according to literature [31]. The same method was adapted for phenylalanine using 2.5 g of L-phenylalanine (15 mmol) and 1.8 g of triphosgene (6 mmol). The reaction was completed within 1 h and the product was concentrated and precipitated in ice-cold hexane.

Ring-opening polymerization was carried out using amine-terminated PF127 and either alanine-NCA or phenylalanine-NCA in a mixture of DMF and chloroform (1:3 *v*/*v*). The amount of amino acid NCA added was varied according to the oligo(peptide) chain length desired. The final product was precipitated in cold ethyl ether, solubilized in DMSO, and dialyzed (MwCO = 3000 Da). Final products were dried and stored under vacuum prior to use.

^{1}H nuclear magnetic resonance (NMR) spectra were recorded using a Varian Unity Inova 700 instrument with TFA-d as solvent at a dilute concentration (5 mg/mL) to confirm copolymerization. The number average molecular weight and polydispersity were determined using a Jasco gel permeation chromatography (GPC) system (Tokyo, Japan) equipped with a Shodex OHpak SB-803 HQ column (Tokyo, Japan) and 0.9% NaCl as the eluent at a flow rate of 1 mL/min. Copolymers solubilized in D_2O at a higher concentration (100 mg/mL) were studied using ^{1}H NMR with DSS as the internal standard.

Circular dichroism (CD) spectra were recorded using an Aviv 202 circular dichroism spectrometer (Lakewood, NJ) with a temperature control unit. A diluted copolymer solution (0.02% *w*/*v*) was prepared using deionized water, filtered through a 0.45 μm filter, and loaded into a 0.1-cm pathlength quartz cell. Results are a representation of the average of ten scans per sample. An attenuated total reflectance (ATR) module was used on a BX-FT-IR system (Perkin Elmer, USA) to analyze the secondary structure present in both powder and concentrated solution forms of native and modified PF127.

2.3. Characterization of hydrogels

The phase diagram of aqueous copolymer solutions prepared in PBS was recorded using the inverted test tube method in the temperature range of 4 °C to 80 °C at 2 °C increments. Copolymers were solubilized at various concentrations and stirred overnight to allow complete dissolution. The tubes were then placed in an equilibrated dry bath. At each temperature, the tubes were equilibrated for 5 min. The gelation point was taken as the temperature where the gel was non-flowing after agitation while inverted and the upper sol point was defined as when the hydrogel regained fluidity. This method provides a gelation window that is useful for thermosensitive hydrogel studies. The critical gel concentration (CGC) is defined as the minimum polymer concentration required before gelation can be observed.

Rheology of hydrogels was studied using a TA instrument AR-1500ex rheometer (New Castle, DE) equipped with a 40 mm parallel plate geometry and a gap of 50 mm. The working volume of each copolymer solution was mixed thoroughly and loaded onto a pre-chilled plate for measurements under a dynamic temperature sweep between 10 and 50 °C at a heating rate of 2 °C/min (2% strain, 6 rad/s). The evolution of storage modulus (G′) and loss modulus (G″) was recorded. Phase reversibility of the copolymer solution was confirmed by reversing the temperature scan.

The interior structure of lyophilized hydrogels was studied using a JEOL JSM-7001F scanning electron microscope (SEM) (Peabody, MA). Briefly, hydrogels were submerged in liquid nitrogen prior to