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Elastin based cell-laden injectable hydrogels with tunable gelation, mechanical and biodegradation properties

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ABSTRACT

Injectable hydrogels made from extracellular matrix proteins such as elastin show great promise for various biomedical applications. Use of cytotoxic reagents, fixed gelling behavior, and lack of mechanical strength in these hydrogels are the main associated drawbacks. The aim of this study was to develop highly cytocompatible and injectable elastin-based hydrogels with alterable gelation characteristics, favorable mechanical properties and structural stability for load bearing applications. A thermoresponsive copolymer, poly(N-isopropylacrylamide-*co*-polylactide-2-hydroxyethyl methacrylate-*co*-oligo(ethylene glycol)monomethyl ether methacrylate, was functionalized with succinimide ester groups by incorporating N-acryloxysuccinimide monomer. These ester groups were exploited to covalently bond this polymer, denoted as PNPHO, to different proteins with primary amine groups such as α-elastin in aqueous media. The incorporation of elastin through covalent bond formation with PNPHO promotes the structural stability, mechanical properties and live cell proliferation within the structure of hydrogels. Our results demonstrated that elastin-*co*-PNPHO solutions were injectable through fine gauge needles and converted to hydrogels *in situ* at 37 °C in the absence of any crosslinking reagent. By altering PNPHO content, the gelling time of these hydrogels can be finely tuned within the range of 2–15 min to ensure compatibility with surgical requirements. In addition, these hydrogels exhibited compression moduli in the range of 40–145 kPa, which are substantially higher than those of previously developed elastin-based hydrogels. These hydrogels were highly stable in the physiological environment with the evidence of 10 wt% mass loss in 30 days of incubation in a simulated environment. This class of hydrogels is *in vivo* bioabsorbable due to the gradual increase of the lower critical solution temperature of the copolymer to above 37 °C due to the cleavage of polylactide from the PNPHO copolymer. Moreover, our results demonstrated that more than 80% of cells encapsulated in these hydrogels remained viable, and the number of encapsulated cells increased for at least 5 days. These unique properties mark elastin-*co*-PNHPO hydrogels as favorable candidates for a broad range of tissue engineering applications.

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1. Introduction

Injectable biopolymer hydrogels display great promise for *in vivo* tissue engineering due to their high water uptake capacity and mass transfer capabilities [1,2], host tissue adhesive properties [3,4], biological similarity to natural extracellular matrix [5], tunable physicochemical characteristics [6,7], potential for encapsulation of cells, drugs, or growth factors [8,9], and minimally invasive method of delivery [10]. The elastin based hydrogels exhibit great potential for *in vitro* regeneration of dermal [11,12], cartilage [13] and cardiovascular tissues [14,15]. Low mechanical strength and lack of control on the gelation behavior and the use of cytotoxic crosslinking reagents are the main associated drawbacks to most of the current elastin based injectable formulations [9].

Different thermoresponsive monomers and polypeptides were chemically copolymerized to develop injectable hydrogels with

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tunable mechanical strength and gelation properties. The characteristics of thermoresponsive copolymer based injectable systems are modulated by changing the chemical composition of the copolymer and thus can be finely tuned to address specific clinical requirements [6,7]. In addition, the gelation of these thermoresponsive systems is triggered by increasing the temperature above the lower critical solution temperature (LCST) of the copolymers. This thermosetting behavior eliminates the need for addition of crosslinking reagents.

Poly(N-isopropylacrylamide) (PNIPAAm) is a water soluble, FDA approved thermoresponsive monomer with the LCST (~32 °C) close to physiological temperature, which makes it a favorable material for biomedical applications. Bioresorbable PNIPAAm-based copolymers have been developed via the copolymerization of PNIPAAm with synthetic degradable macromonomers and peptide sequences [7,16–18]. Due to the simplicity of its synthetic process and high mechanical strength, 2-hydroxyethyl methacrylate (HEMA) based macromonomers such as polylactide/HEMA (PLA/HEMA) have been widely used as hydrophobic backbones in PNIPAAm based copolymers [19]. Fixed physicochemical properties, lack of cell motif sites [18], and a fast degradation rate, such as 100% mass loss within 7 days [7] are the main limiting factors in clinical applications of these thermoresponsive hydrogels. Injectable biomaterials with tunable and favorable characteristics, therefore, are of vital need to address different clinical requirements.

The aim of this study was to develop a new class of thermoresponsive and bioresorbable material for a broad range of biomedical applications. To achieve this, a hydrophilic segment (oligo (ethylene glycol)) monomethyl and a protein reactive site (NAS) were incorporated to the molecular structure of PNIPAAm-*co*-PLA/HEMA to form a water soluble copolymer. We anticipated that the formation of covalent bonds between the copolymer and proteins would promote the structural stability and mechanical properties of this class of hydrogels. Elastin was selected as a model protein with a relatively high number of lysine groups to investigate this effect on the characteristics of the hydrogels. We also hypothesized that the physicochemical and gelation properties of the resulting hydrogel could be finely tuned by changing the composition of the copolymer. In this study, the effect of copolymer formulation on the characteristics of hydrogels was investigated to produce a panel of materials with a wide range of properties. To the best of our knowledge this is the first injectable hydrogels with tunable gelation, mechanical and bioresorption properties that can be finely modified for a specific clinical application.

2. Materials and method

2.1. Materials

D,L-lactide (LA), stannous 2-ethylhexanoate ($Sn(OCt)_2$), N-isopropylacrylamide (NIPAAm), 2-hydroxyethyl methacrylate (HEMA), 4, 4′-azobis (4-cyanovaleric Acid) (ACVA), N-acryloxysuccinimide (NAS), Bradford reagent, Dulbecco's Modified Eagle's Medium (DMEM) and Fetal Bovine Serum (FBS) were from Sigma and were used as received. PrestoBlue® cell viability reagent was purchased from Life Technologies. Oligo(ethylene glycol) monomethyl ether methacrylate (OEGMA, Mn = 475, Sigma) was purified by passing a solution in dichloromethane (1:1 volume ratio) through a basic alumina column to remove inhibitors prior to use. LA monomer was dried under vacuum at 40 °C for 24 h prior to use. α-elastin, extracted from bovine ligament, was obtained from Elastin Products Company.

2.2. Synthesis of PLA/HEMA

PLA/HEMA macromonomer was synthesized by ring-opening polymerization of lactide with HEMA using $Sn(OCt)_2$ as catalyst [19]. Known amounts of LA and HEMA were mixed in a 250 ml three-neck flask at 110 °C under nitrogen atmosphere for 15 min. A mixture of 1 mol% of $Sn(OCt)_2$ (with respect to HEMA feed) in 1 ml anhydrous toluene was then added to the LA/HEMA solution. The resulting mixture was stirred at 300 rpm at 110 °C for 1 h under nitrogen blanket. The mixture was then dissolved in 20 ml tetrahydrofuran and precipitated in 200 ml cold (~1 °C) distilled water. The unpurified precipitated PLA/HEMA was separated by centrifugation at 3000 rpm for 5 min and the supernatant discarded. Cold distilled water was then added to the precipitated PLA/HEMA and the centrifugation process repeated twice to remove unreacted monomers and byproducts. The precipitated PLA/HEMA was then dissolved in ethyl acetate. PLA/HEMA macromonomer was further purified by centrifugation at 6000 rpm for 5 min. The supernatant was then dried by absorbing the residues of water by adding $MgSO_4$ particles and incubating this suspension for 18 h at 25 °C. The particles of $MgSO_4$ were then removed by vacuum filtration. The purified polymer solution in ethyl acetate was dried at 60 °C under reduced pressure using a rotary evaporator. The solvent residue (mainly ethyl acetate) was further removed under vacuum at 40 °C for 24 h. The resulting PLA/HEMA formed a viscous liquid and thereafter was stored at 4 °C. The feed ratio of LA:HEMA was varied from 1.5:1 and 2.5:1 to obtain PLA/HEMA macromonomers with different lactate numbers. The formation of PLA/HEMA macromonomer and the lactate number were verified using ^1H NMR (Varian, 400 MR).

2.3. Synthesis of poly(NIPAAm-co-(PLA/HEMA)-co-OEGMA-co-NAS)

Poly(NIPAAm-*co*-(PLA/HEMA)-*co*-OEGMA-*co*-NAS) denoted as PNPHO was synthesized by free radical polymerization, using ACVA as an initiator. Known amounts of NIPAAm, NAS, PLA/HEMA, OEGMA, and ACVA (7.0×10^{-5} mol as an initiator) were dissolved in 13 ml anhydrous N,N′-dimethylformamide in a 25 ml round bottom, one-neck flask. The system was then deoxygenated by at least three freeze-pump-thaw cycles, using liquid nitrogen as the coolant. The reactor was then sealed and immersed in an oil bath. The polymerization was conducted at 70 °C for 24 h under gentle stirring (300 rpm). The resulting polymeric solution was then cooled at room temperature for 1 h and precipitated in 250 ml diethyl ether. The precipitate was collected by filtering the suspension, and was subsequently dried under vacuum for 6 h. The product was further purified by dissolving the dried powder in tetrahydrofuran and precipitating it in diethyl ether. The final product, PNPHO copolymer, was dried under vacuum for at least 48 h. The PNPHO was characterized with Gel Permeation Chromatography (GPC), ^1H NMR, and gas-chromatography techniques. Subscripts in PNPHO correspond to PLA/HEMA mol %(lactate number)OEGMA mol%.

2.4. Chemical bonding of PNPHO with proteins

The presence of succinimide ester groups in the molecular structure of PNPHO polymer provided active sites to bond with lysine-containing proteins such as elastin. In each run, a solution of 30 mg/ml of elastin and 150 mg/ml of PNPHO in phosphate buffered saline (PBS; 10 mM Na phosphate, 150 mM NaCl, pH 7) was prepared and stirred for 6 h at 4 °C to chemically bond elastin and PNPHO. The solution was then moved to 37 °C to form an elastin-*co*-PNPHO hydrogel. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy was used to confirm the chemical bonding between elastin and PNPHO. ATR-FTIR spectra were collected at a resolution of 2 cm^{-1} and signal average of 32 scans in each interferogram over the range of 1800–1500 cm^{-1} using a Varian 660 IR FTIR spectrometer.

2.5. Rheological and mechanical properties of elastin-co-PNPHO hydrogels

The rheological behavior of elastin-*co*-PNPHO hydrogels was investigated at different temperatures. The temperature was increased from 10 °C to 37 °C at a rate of 0.3 °C/min using a Physica rheometer. Parallel plates with a diameter of 25 mm were used and a sample with the thickness of 0.5 mm was placed between these plates. The gelling temperatures of elastin-*co*-PNPHO hydrogels fabricated with different compositions of PNPHO were recorded at the crossover point of the dynamic storage (G') and loss (G'') moduli. In addition, the rheological behavior of elastin-*co*-PNPHO solutions was studied at 37 °C to determine the gelling time of hydrogels at physiological conditions.

An Instron (Model 5543) was used for conducting Uniaxial compression tests in an unconfined state with a 100 N load cell according to a previously described procedure [20]. Prior to the test, the samples were equilibrated in PBS for 2 h at 37 °C. The compression (mm) and load (N) were collected using Bluehill-3 software at a cross speed of 50 μm/s. The compressive modulus was obtained as the tangent slope of stress–strain curve in the linear region, between 10% and 20% strain level.

2.6. Elastin and PNPHO chemical bonding efficiency – Bradford assay

The conjugation capacity of different compositions of copolymers was assessed by evaluating the retention of elastin within the copolymer structure after 24 h of incubation in Milli-Q water (MQW). In each analysis 200 mg elastin-*co*-PNPHO hydrogel was soaked in 5 ml washing media (MQW) and left at 37 °C for 24 h. The elastin concentration in the washing media was determined by the Bradford dye-binding assay [21,22]. In each test, 5 μl of washing media was mixed with 195 μl Bradford reagent in a 96 well-plate and left at room temperature for 25 min. The solution's absorbance was then measured at 595 nm using a microplate reader (Biorad 680). A standard curve of elastin concentration-absorbance was generated from at least 6 elastin standard solutions with absorbance in the range of 0.4–0.5. The samples were either diluted or concentrated to achieve the absorbance reading in the linear region (0.4–0.5).

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