PEG-HYDROGELS: SYNTHESIS, CHARACTERIZATION, AND CELL ENCAPSULATION

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Introduction

Hydrogels produced by photo-polymerization have been investigated extensively as biomaterials for tissue engineering scaffolds and drug delivery carriers, in the prevention of thrombosis, for post-operative adhesion formation, and as coatings for biosensors.[1] The photo-polymerization process allows the hydrogel to be generated *in vitro* or *in vivo* from a low viscosity solution of monomer, oligomer, or low molecular mass polymer (macromer) by a free radical pathway in a minimally invasive manner. Chemically cross-linked hydrogels contain high water content yet possess mechanical properties similar to those of soft tissues. The use of photo-polymerized hydrogels as opposed to natural physical gels such as alginate also allows for the material properties to be more easily controlled.

Here, we describe a simple reaction protocol to prepare controlled molecular mass (MM) poly(ethylene glycol) dimethacrylates (PEGDMs) of high purity and low polydispersity. PEGDMs were photo-polymerized in water or growth medium (in the presence of cells) to form hydrogels. We also used a facile synthetic approach to covalently attach peptides to the hydrogels. Detailed synthesis and characterization of PEGDM oligomers were carried out. The structure and mechanical properties of corresponding hydrogels were studied using small angle neutron scattering (SANS) and compression tests, respectively. Structure and mechanical studies provide the foundation for subsequent cell response investigation. Finally, we describe the use of PEGDM hydrogels as model scaffolds for cartilage regeneration (via chondrocyte encapsulation) and bone regeneration (via osteoblast encapsulation). Factors such as gel modulus and application of external stimuli will be discussed.

Experimental[¥]

Synthesis of PEGDM oligomers and bioactive moities. PEGDMs (Mn ≈ 2000 g/mol (2k), 4000 g/mol (4k), and 8000 g/mol (8k)) were prepared by reacting PEG with methacrylic anhydride. The molecular mass and molecular mass distributions were determined using a combination of ^{1}H nuclear magnetic resonance spectroscopy (NMR, 270 MHz proton NMR spectra, 6.35 T JEOL GX270 spectrometer, JEOL, Ltd.) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker REFLEX II).[2]

The protein integrin binding domain of fibronectin (GRGDS) was synthesized by standard solid-phase synthesis using FMOC (N-9-Fluorenylmethoxycarbonyl) chemistry. The purity was confirmed by MALDI-TOF MS. MS (MALDI): 491.36 u $[M+H]^+$ (calculated molecular mass: 489.45 u). The GRGDS peptide sequence was reacted with glycidyl methacrylate in dimethylformamide (DMF) at room temperature for 2 d to form GRGDS-dimethacrylate.

Synthesis and characterization of PEGDM hydrogels and bioactive hydrogels. Photo-polymerized hydrogels were prepared according to a previously described procedure. PEGDM and aqueous I2959 solution (0.05 % by mass fraction) were mixed in distilled deionized water for mechanical testing, in D_2O for the SANS measurements, and in PBS or growth media for cell encapsulation. Photo-polymerization was achieved using a long wavelength UV source (365 nm, 300 μW/cm^2) for 10 min to obtain hydrogels.[3]

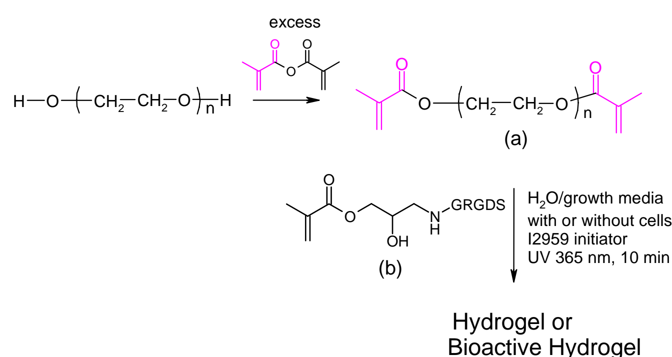

Figure 1. Synthesis of PEGDM (a) and photopolymerization with or without GRGDS-methacrylate (b) to form hydrogels.

SANS measurements were performed on the NG-7 30 m SANS instrument at the NIST Center for Neutron Research (NCNR).[4] Sample-to-detector distances were 15 m, 2 m, and in some measurements 1 m, and an incident wavelength (λ) of 8.44 Å for the 15 m and 2 m configuration and 5 Å for the 1 m configuration were used. Data were reduced by established methods with the software provided by the Center for Neutron Research at NIST.

Shear modulus was determined using uniaxial compression measurements performed using a TA.XT2I HR Texture Analyser (Stable Micro Systems, UK).

Cell culture and quantification. Bovine chondrocytes were added for a seeding density of 2×10^6 cells/mL of PEGDM solution[5] and MC3T3-E1 pre-osteoblast cells were suspended at 2.5×10^6 cells/mL of the PEGDM solution. PEGDM solutions containing cells were photo-polymerized using the same protocol described above. Gels were transferred to culture media (alpha-MEM with 10% by volume of fetal bovine serum) immediately after curing. Cell viability was determined from the DNA content assayed using the Picogreen DNA kit (Invitrogen). Differentiation was determined by measuring alkaline phosphatase activity (Stanbio lab) and alizarin red S staining.

Results and Discussion

High purity PEGDMs of controlled molecular mass and molecular mass distribution were synthesized by reacting PEG hydroxyl endgroups with methacrylic anhydride via a solution reaction at room temperature or via a microwave assisted route. Combined ^{1}H NMR and MALDI-TOF MS analyses were necessary for a complete characterization of the product purity and the degree of methacrylate conversion. For all PEGDMs, the number average molecular masses (M_n) obtained by ^{1}H NMR closely matched those calculated by MALDI-TOF MS. Complementary techniques demonstrated high reaction conversion and low impurity in the reaction product.

SANS measurements were performed on semidilute PEGDM solutions and corresponding hydrogels of different molecular masses and varied oligomer mass fractions. A marked difference in the scattering pattern was evident as the solutions photo-cross-linked to form hydrogels. At high PEGDM mass fractions, a ring developed in the scattering pattern indicating the presence of a well-defined structural length scale (correlation length ξ). Results suggested that the cross-linking of PEGDM favors the formation of clusters. Although the PEGDM only has a functionality of 4 (2 on each oligomer chain end), the way in which the cross-linking process occurs allows the cross-linking sites to be more aggregated relative to the flexible PEG moieties, thereby creating clusters of methacrylate rich regions reminiscent of the gel structures formed by star-shaped oligomers.

The shear moduli of hydrogels were measured using a uniaxial compression test and calculated using equations derived from the strain-energy function. The shear moduli of PEGDM hydrogels prepared from different molecular mass oligomers and as a function of PEGDM mass fraction. As expected, the shear modulus monotonically increased as the

[¥] Identification of materials and equipment in this paper does not imply recommendation by NIST, nor does it imply that the materials are the best available for the purpose.

oligomer mass fraction increased for PEGDMs of all molecular masses. Shear modulus also increased as the oligomer molecular mass decreased.

PEGDM (4k) was used as a model hydrogel scaffold for all cell studies, including chondrocyte, for cartilage formation, and MC3T3-E1pre-osteoblast, for bone formation. The inclusion of integrin binding peptide RGD motif on cell morphology was also investigated. Chondrocytes were encapsulated in PEGDM hydrogels as a model system to mimic the 3-D conformational environment of natural cartilage tissue. A perfusion flow bioreactor was built to apply fluid flows to chondrocytes embedded in photopolymerized hydrogels. We observed variations in proteoglycan synthesis and matrix deposition as a result of different mechanical stimulus environments. The mechanotransduction of chondrocytes is a key variable to regulate the gene expression and ECM production by applying fluid stress to chondrocytes encapsulated in PEGDM gels.

Murine osteoblasts (MC3T3-E1) were encapsulated by the same polymerization process, and cell responses, including viability and differentiation, were investigated by multiple biological assays. Using a simple fabrication technique, hydrogels with a gradient in mechanical stiffness spanning nearly a 25-fold change in compressive modulus was fabricated by varying the PEGDM concentration in solution. The survival of encapsulated cells was higher in the softer end, but an increase in the matrix stiffness induced spontaneous differentiation of the cells. Prolonged cell culture resulted in visible mineral deposits in the stiffer regions of the gel (Figure 2).

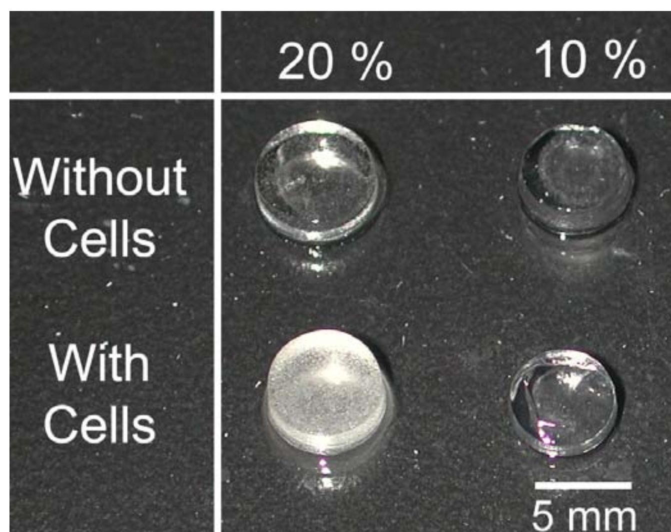

Figure 2: Experiments confirmed that hydrogel mineralization was cell-mediated. Hydrogels of uniform composition (0.05 mL volume, 5 mm diameter, 2.5 mm thick) were fabricated from 10 % or 20 % by mass PEGDM with or without cells. Gels were cultured 56 d and then photographed to image mineralization (representative images are shown). Both 10 % and 20 % PEGDM gels cultured without cells in complete cell medium were transparent and did not mineralize. The 10 % gels containing cells also did not mineralize while the 20 % gels did mineralize.

PEGDM hydrogels with and without RGD were prepared to investigate the effects of peptide modification on the cell response. Since the amount of RGD-MA is small relative to the PEGDM, we expect the mechanical properties of the PEGDM hydrogels and RGD-modified hydrogels to be similar. Cells, seeded onto both types of hydrogels, show the incorporation of RGD has a clear effect on the cell attachment to hydrogels.

Conclusions

PEGDM oligomers of high purity and high degree of endgroup conversion were achieved. Their hydrogel structure and mechanical properties depended on the oligomer molecular mass and the mass fraction. The shear modulus increased as ξ_{gel} decreased, consistent with the theory of rubber elasticity. PEGDM hydrogels serve as suitable model systems to study various biological responses, including understanding the effect of external mechanical stimuli on chondrocyte response and the effect of scaffold modulus on osteoblast differentiation, and as a model system to study the effect of modification by bioactive molecules.

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