

Protein-immobilized poly(2-hydroxyethyl methacrylate-co-methacrylic acid) Hydrogels for Cardiac Tissue Engineering

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Abstract: Cardiac tissue engineering requires a scaffold material that provides structural support and is conducive to cell growth. A hydrogel scaffold of hydroxyethyl methacrylate (HEMA) copolymerized with methacrylic acid (MAA) was chosen for its biocompatibility and mechanical properties. To promote cell adhesion, the surface of the hydrogel was activated with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). The activated hydrogels were then reacted with collagen and seeded with C₂C₁₂ mouse myoblasts. Mechanical testing and ESCA analysis confirms the successful activation of the gel and immobilization of collagen. Cell studies confirm the bioactivity of the immobilized collagen as a significant number of cells attached and survived on the activated p(HEMA-co-MAA) hydrogels.

1. INTRODUCTION

According to the American Heart Association [7], cardiovascular disease claims the lives of more Americans each year than cancer, chronic lower respiratory diseases, and diabetes mellitus combined. In addition, 7.9 million Americans suffer from myocardial infarctions, resulting in damaged heart tissue that cannot spontaneously regenerate [7]. Clearly, there is an overwhelming need for an effective treatment option to supplement the current shortage in organ donation [5]. Cardiac tissue engineering, specifically the replacement of cardiac scar tissue with functioning cardiomyocyte grafts, presents a treatment option that holds great promise.

Cardiac tissue engineering requires a scaffold material that provides structural support and orientation for growing cells. Hydrogels are an attractive scaffold material due to their tissue-like elasticity, high diffusion capabilities, and high water content [3]. To satisfy these requirements, poly(2-hydroxyethyl methacrylate) (pHEMA) was chosen for this project. Because the 2-hydroxyethyl pendant chains on pHEMA contain relatively non-reactive hydroxyl terminating groups, copolymerization with methacrylic acid (MAA) is necessary in order to introduce more reactive carboxylic acid pendant chains. This reactivity can be used to immobilize proteins with only minor changes to the mechanical properties of the hydrogel.

Another requirement for a viable scaffold material is the ability to promote cell attachment and survival. This can be accomplished by

adsorption of adhesion proteins onto the hydrogel, creating a surface of ligands recognized by the cell's adhesion receptors. However, hydrogels inherently resist protein adsorption due to the weak driving force associated with their hydrophilic surface [2]. The hydrogel surface must be modified to support protein immobilization before it can be used to promote cell adhesion.

The surface of pHEMA can be activated by utilizing a carbamate linkage between free hydroxyl groups and a carbonyldiimidazole (CDI) molecule [4,6]. The remaining imidazole ring is then subjected to nucleophilic attack from the primary amine groups present on collagen. Activated hydroxyls can react with non-activated hydroxyls, leading to uncontrolled crosslinking and severely altered mechanical properties. Another approach is to limit the amount of undesired crosslinking by introducing only a specified percentage of groups designated for activation. This approach is demonstrated in the attachment of a cysteine-containing oligopeptide to a copolymer of HEMA and aminoethyl methacrylate by taking advantage of the reactivity between a primary amine in the polymer and a sulfhydryl group in the peptide residue [8]. However, this immobilization scheme requires a relatively expensive custom-synthesized oligopeptide, when the majority of cell-binding ligands already contain primary amines.

Surface activation can also be carried out by way of N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide

hydrochloride (EDC) [1]. This method of coupling a primary amine and carboxylic acid was chosen for several reasons. First, the reaction does not elicit uncontrolled crosslinking as an activated carboxylic acid will not crosslink with another carboxylic acid. Second, this coupling method can take place in aqueous conditions at a mild pH and can be carried out without significantly altering the desired properties of the original polymer.

2. MATERIALS AND METHODS

2.1 Polymerization of *p*(HEMA-co-MAA)

HEMA and MAA were copolymerized from an aqueous solution of 79 mol % HEMA, 10 mol % ethylene glycol, 10 mol % water, and 1 mol % of the crosslinking agent tetraethylene glycol dimethacrylate (TEGDMA), relative to HEMA. 1, 5, and 10 mol % MAA monomer was substituted relative to HEMA to achieve various MAA percentages. A solution of ammonium persulfate and sodium metabisulfite was used to initiate polymerization. HEMA and MAA monomers were copolymerized between two glass plates for 24 h before the resulting copolymer sheets were rinsed 4 times for 1 h in deionized water to remove unreacted monomer.

2.2 Polymer activation and collagen immobilization

Before activation, gels were equilibrated for 30 min in a pH 5.6 MES buffer. The gels were then activated in a solution of 0.1M N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and 0.2 N-hydroxysuccinimide (NHS) in MES for 15 min to 2 h on a shaker (Figure 1). The gels were then washed once for 10 min in pH 7.4 phosphate buffered saline (PBS) and reacted in a 200 µg/ml solution of rat tail collagen I in phosphate buffered saline (PBS) overnight at 4°C. Finally, the disks were rinsed in PBS three times with sonication to remove unreacted reagents. Gels prepared for ESCA analysis were rinsed an additional three times for 10 min in deionized water to remove salts.

2.3 ESCA analysis of *p*(HEMA-co-MAA)

Because the unmodified polymer contains no nitrogen, ESCA can be used to detect the presence of collagen based on the nitrogen signal [4]. Analyses were conducted using a Surface Science Instruments (SSI) X-Probe ESCA using an aluminum $K_{\alpha 1,2}$ monochromatized X-ray source to stimulate photoemission. The energy

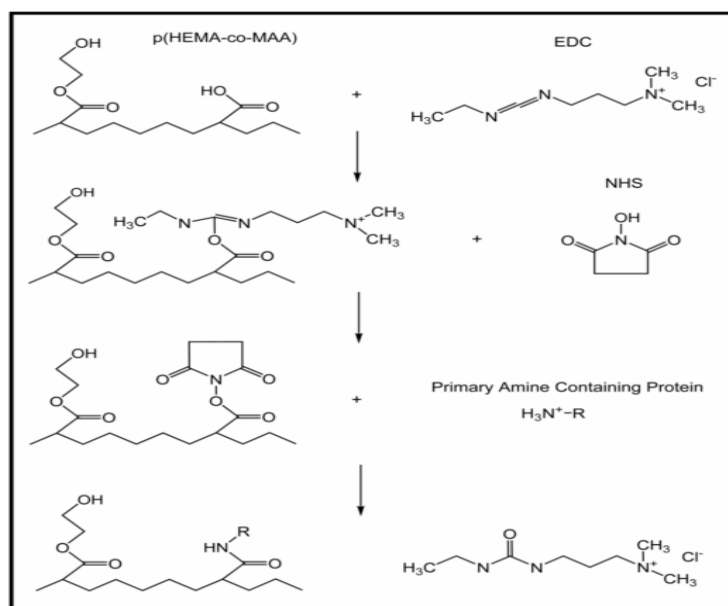


Figure 1 Reaction scheme for covalent immobilization of an amine containing protein with poly(HEMA-co-MAA) using EDC/NHS as the activating agent [1].

of the emitted electrons was measured with a hemispherical energy analyzer, where higher pass energies allow quantification of the N1s signal. SSI data analysis software was used to calculate the elemental compositions from the peak areas.

2.4 Mechanical testing

An INSTRON universal testing device was used to determine mechanical properties using a strain rate of 10 mm/min until failure. A plot of stress (force per unit area in kPa, σ) versus strain (% elongation, ϵ) was generated for six specimens of each sample. The Young's modulus (E) was calculated from the linear portion of the stress-strain curve according to Hooke's law: $\sigma = E\epsilon$.

2.5 Cell studies

Prior to activation, gels used in cell studies were cut into 6 mm diameter circles and sterilized overnight in 70% ethanol. All activation and wash solutions were sterile-filtered and all reactions were performed in a laminar flow hood. C₂C₁₂ mouse myoblasts were suspended in Advanced DMEM/F12 media (Invitrogen) supplemented with 5% FBS (Hyclone), 1% L-glutamine (Invitrogen), and 1% antibiotic-antimycotic (Invitrogen). Samples were placed in a 24-well plate and plated with 0.5 ml of media containing 25,000 cells. Images were taken at 2 and 24 h of incubation at 37°C.

3. RESULTS AND DISCUSSION

3.1 ESCA Surface Analysis

ESCA analysis shows an increase in N1s signal with an increase in EDC/NHS activation time and MAA percentage (Figure 2). Unmodified pHEMA and MAA do not contain nitrogen, therefore an increase in N1s signal can be correlated with an increase in the presence of collagen's primary amine group. ESCA confirms the immobilization of collagen onto the activated gels. Because the N1s signal increases in a predictable manner, the amount of bound collagen can easily be controlled with a careful choice of MAA percentage and activation time. Unmodified pHEMA gels show an increase in

N1s signal after 60 min of activation which is likely due to incomplete rinsing. Activation of the 10% MAA gels for 15 and 60 min and activation of the 5% MAA gels for 60 min result in similar N1s signals, suggesting that the 5% MAA gels can be used to minimize the effect of MAA on the mechanical properties of pHEMA.

3.2 Mechanical Testing

Figure 3 shows the relatively small effect of EDC/NHS activation and collagen immobilization on the Young's modulus of the p(HEMA-co-MAA) gels when compared to unmodified pHEMA. Little variability is observed within each EDC/NHS category suggesting a predictable elasticity for a given EDC/NHS immobilization scheme. In contrast, CDI activation increases the Young's modulus significantly and large variation exists between samples. This indicates the unreliability and brittleness of the CDI activated gels. The breaking strain of the EDC/NHS activated gels decreases slightly from that of unmodified pHEMA (Figure 4). However, this decrease is relatively small when compared to the nearly four-fold decrease in breaking strain exhibited by the CDI activated gels. The smallest variations in breaking strains are displayed by the EDC/NHS activated gels even when compared to unmodified pHEMA.

Mechanical testing shows only small changes in polymer characteristics with EDC/NHS immobilization schemes. Analysis of stress-strain data from the INSTRON universal tester reveals that p(HEMA-co-MAA) modified with EDC/NHS and collagen retains the favorable elasticity and strength of unmodified pHEMA gels. Slight increases in the Young's Modulus and a decrease in the breaking strain are likely a result of changes in hydrophilicity from the collagen attachment. Additionally, since collagen is ~300 kDa, the protein itself may be influencing the mechanical properties. Regardless, the elasticity, strength, and consistency of the EDC/NHS activated gels are favorable over the brittle gels that result from CDI-activation.

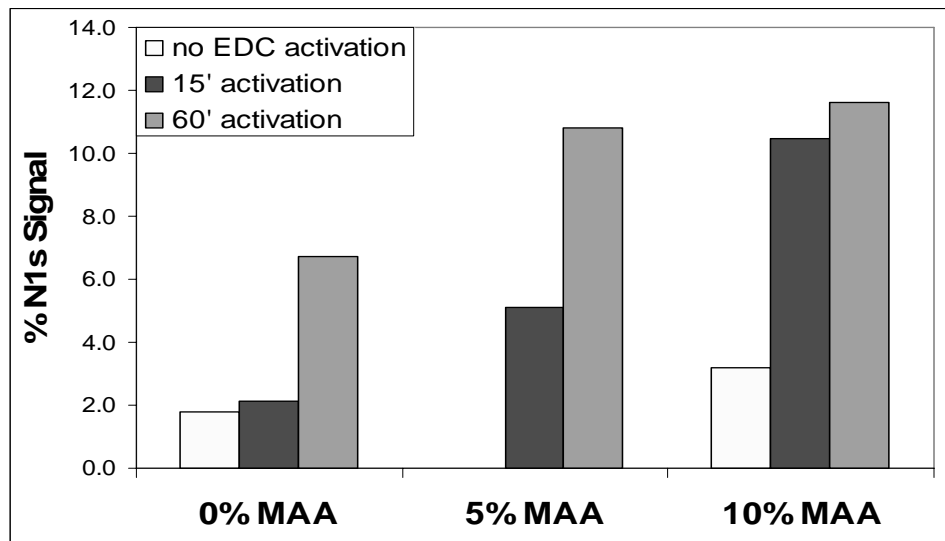


Figure 2 N1s signal significantly increases with an increase in activation time and MAA percentage. The increase in N1s signal for 0% MAA gels is likely due to incomplete rinsing since collagen immobilization is unlikely without activation of MAA's carboxylic acid group.

3.3 Cell Studies

Modification of pHEMA with 5% MAA and subsequent activation with EDC and NHS results in a gel that supports significant cell attachment and survival (Figure 5, column 2). This confirms that the bioactivity of collagen is maintained after immobilization and demonstrates the lack of toxicity associated with the EDC/NHS reaction scheme. In addition, the

necessity of the MAA carboxylic acid group for sufficient gel activation is confirmed since cell attachment only occurred with activated p(HEMA-co-MAA) gels. Without EDC/NHS activation, pHEMA and p(HEMA-co-MAA) gels do not promote cell attachment (Figure 5, column 1). Similarly, un-modified pHEMA gels also do not promote cell attachment even after 60 min of activation.

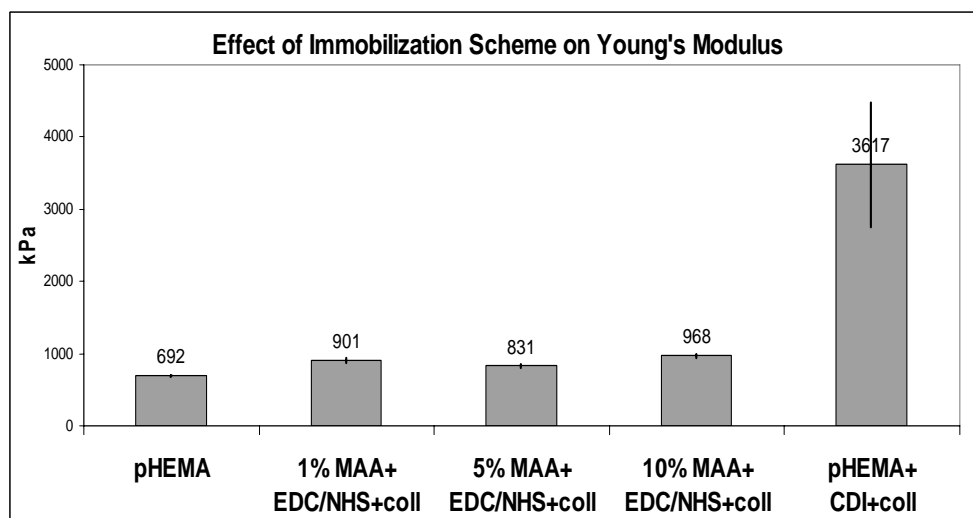


Figure 3 Young's modulus of p(HEMA-co-MAA) gels does not significantly increase with EDC/NHS activation and collagen immobilization. CDI activation and collagen immobilization increases the gel's stiffness by about four fold. Error bars represent one standard deviation from the mean.

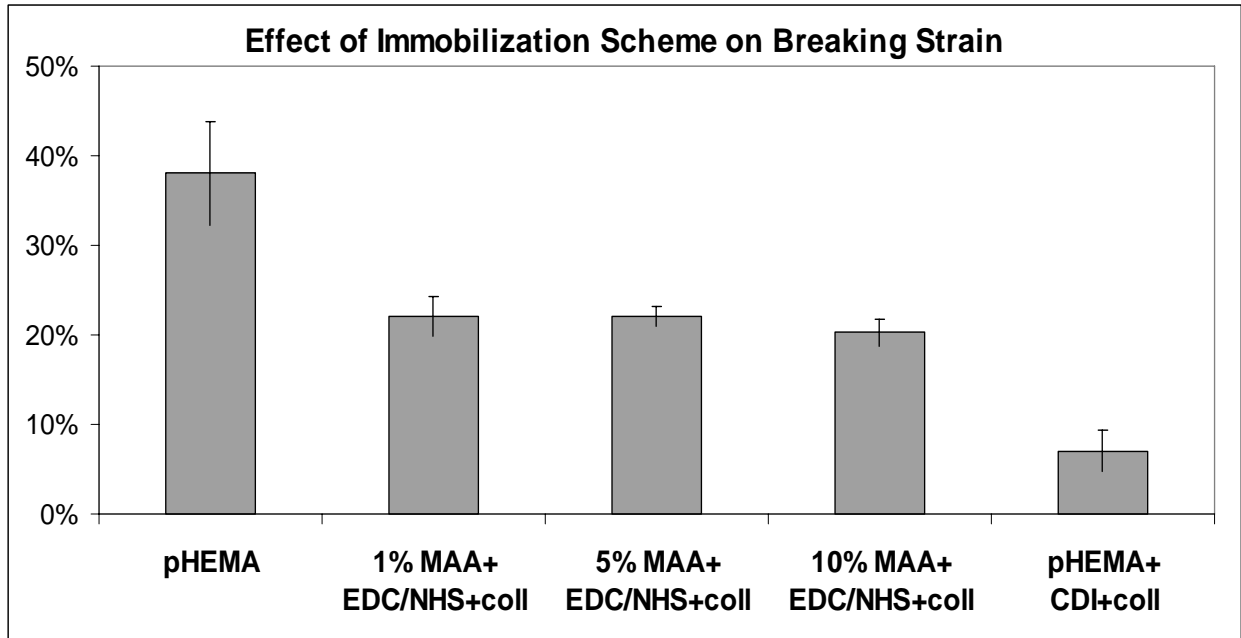


Figure 4 Breaking strain decreases slightly with EDC/NHS activation when compared to unmodified pHEMA. However, the breaking strain of EDC/NHS activated gels is still about three times larger than the breaking strain of the CDI activated gels. Error bars represent one standard deviation from the mean.

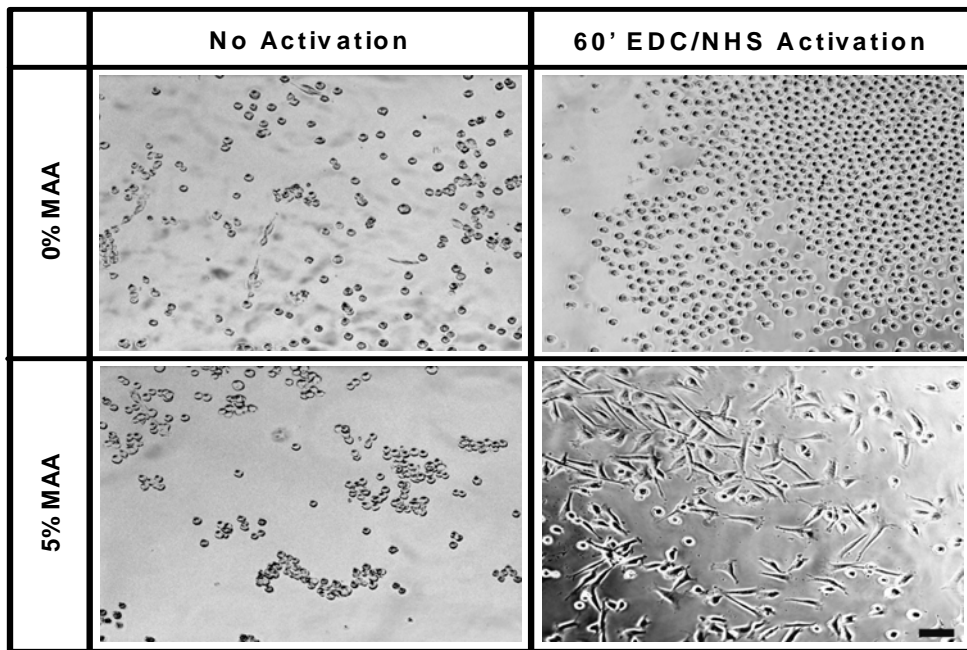


Figure 5 Gels do not promote cell attachment without EDC/NHS activation of the MAA carboxylic acid groups. 5% MAA gels activated for 60 min show significant cell attachment and survival. Images were taken 2 h after plating.

4. CONCLUSIONS

Successful EDC/NHS activation of p(HEMA-co-MAA) and subsequent collagen immobilization results in scaffolds that promote cell attachment and survival while retaining the desirable characteristics of unmodified pHEMA. The activation time and MAA percentage were preliminarily optimized with the greatest protein coverage resulting from a 60 min activation of the 5% MAA gels. This reaction is easily controlled and the percentage of MAA and EDC/NHS activation time can be selected to produce a desired amount of bound collagen. Consistency among the stress-strain mechanical testing data and its performance over the CDI activated gels indicates the reliability of p(HEMA-co-MAA) as a tissue engineering scaffold material.

ACKNOWLEDGEMENTS

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