Direct Condensation Reaction for Grafting of Polyethylene Glycol Monomethyl Ether on Poly(Methacrylic Acid-co-Methyl Methacrylate) for Application in Biomedical Engineering

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Abstract  Synthesis, characterization and cytotoxicity evaluation of copolymers based on polyethylene glycol monomethyl ether-g-poly(methacrylic acid-co-methyl methacrylate) are reported via a polymeric precursor method. Grafting was accomplished based on direct condensation reaction in the presence of dicyclohexylcarbodiimide as an esterification-promoting agent catalyzed by dimethylamino pyridine. Polyethylene glycol grafted copolymers were characterized using various spectroscopic techniques; in addition, their biocompatibility was studied. Manifestation of bands assigned to the ester functional groups in Fourier transform infrared spectra and nuclear magnetic resonance was employed for structural characterization of the grafted copolymers. Performance of grafting reaction was guaranteed by determination of grafting efficacy. Cytotoxicity evaluations of the grafted copolymer using L929 fibroblast cell line elucidated acceptable biocompatibility profile; consequently, the applicability of the copolymers is confirmed for biomedical applications.

Keywords  Graft Copolymers, Esterification, Biocompatibility, Biological Applications of Polymers, Polycondensation

1. Introduction

Development of new, engineered materials with desired properties (e.g. mechanical[1], thermal[2] or biological[3-6]) for specific applications have been recognized as a very promising field in material sciences. More specifically, the polymeric materials are very attractive due to their multifarious possible variations in the nature (or ratio) of the initial monomer(s), synthesis method, catalysts, final processing etc. to provide new materials with specific properties[1-9].

In this regard, two major categories of physical and/or chemical approaches are being adopted in modification of polymers, which are planned to be employed in biomedical applications. For instance, plasma surface modification of polyamide 6 is frequently reported to increase its wettability[10-14] or to improve cell adhesion and proliferation on poly(L-lactide) and poly(D,L-lactide-co-glycolide)[15]. Chemical treatment of biomaterials consists of various methods for both grafting[16-18] and crosslinking[19-21], together with the structural modifications such as hydrogenation[22], halogenation[23], and hydrohalogenation[24, 25] which are widely described in the literature.

According to its desirable and intrigue properties including biocompatibility, biodegradability (up to 20 kDa nominal molecular weight), ionic conductivity, wettability and resistance to protein adsorption, polyethylene glycol (PEG) [26] has been found as an outstanding modificatory potency for biological entities, polymers and surfaces[26-30]. For instance, modification of acrylic resins using PEG has attracted much attention[29-42]. Generally, these amphiphilic PEG-grafted copolymers were prepared by either (co)polymerization of vinyl-derivatives of PEG[32-36] or synthesis of acrylic polymeric precursors followed by PEG conjugation via polymeric analogous reactions[37-42]. A procedure using polymeric precursors was adopted by Chiu et al.[37] in order to prepare a polymeric drug carrier via synthesis of a linear acrylic resin from methyl acrylate, stearyl methacrylate, acrylic acid and PEG acrylate and subsequent reaction with monomethoxy polyethylene glycol (mPEG) in different molar ratios of the reactants and mPEG nominal molecular weights. Synthesis and characterization of a variety of PEG-grafted copolymers upon polymeric precursors method was reported consecutively in the last decade of ex-century[38-42], however, there are several uncovered aspects in the mentioned researches including direct esterification of carboxyl and hydroxyl functional...
groups of polyacids and mPEG that should be defined. In addition, the biocompatibility and cell supporting capacity of these copolymers should be enhanced.

The aim of the present work is grafting of mPEG on poly(methacrylic acid-co-methyl methacrylate) (MA-co-MMA) via a polymeric precursor method. This method is based on the adoption of a direct condensation reaction in the presence of dicyclohexylcarbodiimide (DCC), which was used as an esterification-promoting agent catalyzed by dimethylamino pyridine (DMAP). PEG-grafted copolymers were characterized by Fourier transform infrared spectroscopy (FTIR) and proton nuclear magnetic resonance spectroscopy (1HNMR) techniques. Grafting efficiency and their biocompatibility was also evaluated. To our knowledge, synthesis of this specific material has not been reported yet.

2. Materials and Methods

2.1. Materials

Poly(methacrylic acid-co-methyl methacrylate) (MA-co-MMA, Mw=135000 g.mol⁻¹) was supplied by Röhm Pharma GmbH (Darmstadt, Germany) and dried by leaving in a forced-air convection oven at 110°C for 24 hrs before use. Monomethoxy polyethylene glycol (mPEG, of 350 & 750 g.mole⁻¹ nominal molecular weights) were supplied by Fluka (Ronkonkoma, USA) and dried using azeotropic distillation in toluene. N,N’-Dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino) pyridine (DMAP) were purchased from Merck Chemicals (Darmstadt, Germany) and used without further purification. Acetone (Merck Chemicals Co., Darmstadt, Germany) was purified by distillation in ambient pressure before heating under reflux condition with successive quantities of potassium permanganate. It was then dried using anhydrous potassium carbonate, filtered from the desiccant and stored over type 4Å molecular sieves. Toluene crystals, the reaction media was passed from Whatman filter paper grade 2 (Whatman, New Jersey, USA). Water was added as a non-solvent to the filtrate and the product was removed by decantation. The precipitate was dissolved again in acetone and the same purification process was repeated for 2-3 times. The resulting polymer was dried in a forced-air convection oven at 70°C for 12 hrs. The product appeared as a white, brittle and acetone-soluble powder, which was collected from filter paper and stored at -5°C in a desiccator for further use.

2.2. Grafting Reaction

In a typical procedure, 0.0163 mole of pre-purified and dried MA-co-MMA copolymer was dissolved in 100 mL of neutralized acetone and charged into a 250 mL three-necked reaction flask equipped with a reflux condenser, dropping funnel and thermometer. DMAP (0.1% mol/mol to mPEG) was added to the mixture and heated to 45°C under reflux condition. A respective amount of mPEG was dissolved in 50 mL of acetone to yield 10, 20 or 30% molar ratio to methacrylic acid units. DCC (1% mol/mol to mPEG) was dissolved in the mPEG solution at room temperature. This solution was added dropwise to the reaction flask in the period of 15 minutes. The precise compositions used in the synthesis of grafted polymers are tabulated in Table 1. In the samples' nomenclature the molar ratio of mPEG to methacrylic acid units and nominal molecular weights of mPEG were described by the first and second numbers, respectively.

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>mPEG (mole)</th>
<th>DCC (mole)</th>
<th>DMAP (mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10P350</td>
<td>0.0016</td>
<td>0.0016</td>
<td>0.0001</td>
</tr>
<tr>
<td>20P350</td>
<td>0.0033</td>
<td>0.0033</td>
<td>0.0003</td>
</tr>
<tr>
<td>30P350</td>
<td>0.0049</td>
<td>0.0049</td>
<td>0.0005</td>
</tr>
<tr>
<td>10P750</td>
<td>0.0016</td>
<td>0.0016</td>
<td>0.0001</td>
</tr>
<tr>
<td>20P750</td>
<td>0.0033</td>
<td>0.0033</td>
<td>0.0003</td>
</tr>
<tr>
<td>30P750</td>
<td>0.0049</td>
<td>0.0049</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

The reaction mixture was then left overnight under reflux condition afterwards transferred to a refrigerator at 5°C for 24 hrs. In order to remove the needle-like dicyclohexylurea crystals, the reaction media was passed through a Whatman filter paper grade 2 (Whatman, New Jersey, USA). Water was added as a non-solvent to the filtrate and the product was removed by decantation. The precipitate was dissolved again in acetone and the same purification process was repeated for 2-3 times. The resulting polymer was dried in a forced-air convection oven at 70°C for 12 hrs. The product appeared as a white, brittle and acetone-soluble powder, which was collected from filter paper and stored at -5°C in a desiccator for further use.

2.3. Fourier Transform Infrared (FTIR) Spectroscopy

Fourier transform infrared (FTIR) spectra (4000-400 cm⁻¹) were acquired using an Equinox 55 spectrophotometer (Bruker, Germany) at 4 cm⁻¹ resolution and 32 scans at room temperature. FTIR spectra of mPEGs were collected by applying the materials on KBr disks while they were in liquid or molten state. Grafted copolymers were mixed thoroughly in 1:70 ratios with KBr in a mortar and pestle and equal weights (±212 mg) were used to prepare compressed disks. All measurements were made in transmittance mode.

2.4. Proton Nuclear Magnetic Resonance Spectroscopy (1HNMR)

Proton nuclear magnetic resonance spectra (1HNMR) of the purified copolymers were recorded using a Bruker UltraShield 400 system (Bruker, Germany) at 25°C to characterize the copolymer structure and confirm the inclusion of...
mPEG chains on MA-co-MMA backbone. Samples were dissolved in deuterated acetone and chemical shifts were recorded in ppm from the signal of tetramethylsilane.

2.5. Grafting Efficiency

Grafting efficiency (GE%) was evaluated by determination of the unreacted carboxylic acid content of MA-co-MMA-g-mPEG by a titration technique as previously described in the section under determination of methacrylic acid content. Titrations were performed kinetically on 0.5, 1, 2 and 4 hrs after initiation of the grafting reaction *i.e.* complete addition of mPEG to the reaction flask. GE (%) was calculated according to the following equation:

\[
GE(\%) = \frac{MA_I - MA_G}{MA_I} \times 100
\]

where, MA<sub>I</sub> and MA<sub>G</sub> represent to the methacrylic acid content of the initial and grafted copolymers, respectively.

2.6. In vitro Cell Culture

The mouse fibroblast connective tissue (L929) cell line (NCBI C-161, National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran) were cultured in RPMI-1640 (GIBCO, Scotland) supplemented with 10% fetal calf serum (FCS) (SeraMed, Germany), 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma, Milwaukee, USA). L929 cells were then harvested with 0.25% trypsin-EDTA solution (Sigma, Milwaukee, USA) in phosphate-buffered saline (PBS, pH 7.4) and seeded onto the 96-well microtiter plates (NUNC, Denmark) at a density of 1×10⁴ cells/well for direct contact tests. Tissue culture polystyrene (TCPS) was used as a reference to perfect cell culturing. The cells were incubated at 37°C in humified air with 5% CO2 for one week.

2.7. Statistical Analysis

Statistical analysis was performed using MiniTab software (Release 11.12, Minitab Inc., State College, PA, USA). Data were reported as mean ± standard deviation at significant level of *p* < 0.05. Outliers were rejected from processing using the *T* procedure. Differences between groups were analyzed using one-way analysis of variance (ANOVA) and considered statistically significant when the *p* value was less than 0.05.

3. Results and Discussion

Using the previously described method in determination of acid value for MA-co-MMA copolymer, 28.02% methacrylic acid units (equivalent to 1.625 mmol/g based on dry polymer weight) was present in the polymer composition. Three levels of mPEG concentration, in feed, were considered for grafting *i.e.* 10, 20 and 30% (molar ratio to methacrylic acid units) in order to provide various copolymers of different hydrophilic characteristics. The effect of varying molecular weight of the hydrophilic units (mPEG) on the hydrophilic nature of copolymers was previously shown by Hashemi Doulabi *et al.* [44]. Grafted copolymers were synthesized by adopting a direct condensation procedure based on the employment of an esterification promoting agent *i.e.* DCC. According to the supposed reaction mechanism for Steglich esterification *i.e.* the production of the ester bonds in the presence of DCC and DMAP [45], carboxylic acid functional groups present in MA-co-MMA copolymer were converted to an O-acylisourea intermediate upon reaction with DCC, which offers reactivity similar to the corresponding carboxylic acid anhydride functional groups. This reactive intermediate then forms an acyl pyridinium species with DMAP (I), followed by equilibration of (I) with the mPEG to produce ion pair (II). Upon recovery of the DMAP, grafted copolymer and stable dicyclohexylurea (DHU) by-product were generated via nucleophilic attack by R’O- on the acyl group of (II) to provide a terpolymer of methyl methacrylate, methacrylic acid and monomethyl polyethylene glycol methacrylate. The reaction mechanism is illustrated in Figure 1.

![Figure 1. The Proposed Scheme for Grafting Reaction Mechanism.](image-url)
FTIR spectra of the initial MA-co-MMA copolymer along with polyethylene glycol mono methyl ether-g-poly (methacrylic acid-co-methyl methacrylate) in different molar ratio and mPEG nominal molecular weights are shown in Figure 2.

FTIR spectra of the initial MA-co-MMA copolymer revealed the specific signals related to hydroxyl (3443 cm⁻¹), methylene (2999 and 2953 cm⁻¹), carbonyl (1732 cm⁻¹) and alkyl-substituted ether (1155 cm⁻¹) functional groups [46] which were declined in the finally grafted material’s spectra, whereas performance of the esterification reaction was guaranteed by the appearance of new methoxy and methyl ether band signals at 2853 and 1020 cm⁻¹, respectively.

Proton nuclear magnetic resonance (¹HNMR) spectroscopy was used to confirm FTIR results. Appearance of mPEG ethoxy protons signal at 1.2 ppm chemical shift clearly indicated the promotion of esterification reaction between MA-co-MMA and mPEG as shown in Figure 3a. Assignment of signals appeared in ¹HNMR spectra of starting materials and mPEG-grafted copolymer are as follows:

¹HNMR: δ 3.58 (methoxy protons of MMA), δ 2.80 (methylene functional group of mPEG adjacent to the formed carboxyl ester), δ 2.25 (methyl functional group of MMA repeating units), δ 2.09 (CH₃ of deuterated acetone solvent signal), δ 1.87 (methylene protons in mPEG backbone), δ 1.28 (methylene protons on MA-co-MMA backbone), δ 1.2 (ethoxy protons of mPEG), δ 0.88 (methyl protons of MA-co-MMA side chains). The ratio of integrals for signal appeared at 2.8 ppm to any signal originated by copolymer backbone can be correlated to the degree of grafting. The ¹HNMR spectra of MA-co-MMA and mPEG-grafted copolymers are depicted in Figure 3b.

Grafting efficiency (GE) was measured to assure the performance of the grafting reaction. GE was enhanced upon increasing in the mPEG percentage in the feed for a constant molecular weight (see Figure 4). This can be attributed to the well-known mass effect law; however, GE was reduced upon increasing in the molecular weight of mPEG in the feed, which can be attributed to the lower reactivity ratios of hydroxyl functional groups for higher molecular weight analogues of mPEG. The observed reduction in the GE upon increasing in the molecular weights of mPEG may also be assigned to the increasing potential of intra-molecular hydrogen bond formation between the polyacid and polyol i.e. mPEG chains. The reaction time usually plays an important role in a condensation reaction but in this specific case the grafting was nearly completed after first hour hence, no statistically significant differences (p>0.5) was observed after that.

Cytotoxicity evaluation of the grafted copolymers using L929 fibroblast cell line elucidated acceptable biocompatibility profile also applicability of the copolymers in biomedical field. Direct observation of cells in the close proximity of the PEG-grafted samples revealed that a considerable amount of cells on the copolymers who were started spreading and obtaining their fibroblastic morphology. Morphology of L929 cells for 20P750 sample after one-week incubation in the presence of polymeric sample is illustrated in Figure 5 in comparison to control group (TCPS).
analysis using Image-pro plus software. Data were collected for at least 6000 cells in each sample and the test was run in quadruplicate. As it is evident from data represented in Table 2, cells were got more flattened upon introduction of mPEG into the structure of copolymer ($p<0.05$), however, there were no statistically significant difference between 10, 20 and 30\% mPEG grafted samples ($p>0.5$). According to the results significant changes will be occurred by incorporation a limited amount of mPEG in the structure of the grafted copolymer (10\% mPEG) but incorporation of these higher ratios of hydrophilic moieties will not end to better results.

Table 2. Surface Area of L929 Fibroblasts Cultured on PEG Grafted Copolymer Samples After Two Weeks Exposure

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell Area (pixel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCPS</td>
<td>15.10±5.06</td>
</tr>
<tr>
<td>10P750</td>
<td>42.99±19.26</td>
</tr>
<tr>
<td>20P750</td>
<td>56.57±11.52</td>
</tr>
<tr>
<td>30P750</td>
<td>69.84±48.88</td>
</tr>
</tbody>
</table>

4. Conclusions

The terpolymer of methyl methacrylate, methacrylic acid and monomethoxy polyethylene glycol was synthesized by direct condensation reaction of mPEG and MA-co-MMA via Steglich mechanism using DCC as an esterification-promoting agent in the presence of DMAP. Chemical structure of the resulting materials were characterized spectroscopically by FTIR and $^1$HNMR techniques which clearly confirmed the significant changes upon grafting reaction including the appearance of carbonyl ester signal band absorption in FTIR also chemical shift at 2.8 ppm in $^1$HNMR spectroscopy which can be assigned to the methylene groups of mPEG just adjacent to carbonyl functional groups of MA upon the reaction. GE was dependent on the initial mPEG molecular weights and its concentration in the feed ratio but no significant changes were observed after one hour due to the rapid progression of the reaction at first times. Moreover, seven days in vitro cytotoxicity examination to evaluate cellular proliferation in the neighborhood of the grafted copolymer was in good agreement with the control groups showing higher biocompatibility profile for samples grafted with 10\% of mPEG in comparison to neat ones. Increasing mPEG content to higher values did not improve the results further on.

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