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Citation	Yuan, S., Xiong, G., Roguin, A., & Choong, C. (2012). Immobilization of Gelatin onto Poly(Glycidyl Methacrylate) -Grafted Polycaprolactone Substrates for Improved Cell–Material Interactions. Biointerphases, 7(1-4), 1-12.
Date	2012
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Biointerphases (2012) 7:30
DOI 10.1007/s13758-012-0030-1

ARTICLE

Immobilization of Gelatin onto Poly(Glycidyl Methacrylate)-Grafted Polycaprolactone Substrates for Improved Cell–Material Interactions

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Received: 10 February 2012 / Accepted: 5 April 2012 / Published online: 24 April 2012
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Abstract To enhance the cytocompatibility of polycaprolactone (PCL), cell-adhesive gelatin is covalently immobilized onto the PCL film surface via two surface-modified approaches: a conventional chemical immobilization process and a surface-initiated atom transfer radical polymerization (ATRP) process. Kinetics studies reveal that the polymer chain growth from the PCL film using the ATRP process is formed in a controlled manner, and that the amount of immobilized gelatin increases with an increasing concentration of epoxide groups on the grafted P(GMA) brushes. In vitro cell adhesion and proliferation studies demonstrate that cell affinity and growth are significantly improved by the immobilization of gelatin on PCL film surfaces, and that this improvement is positively correlated to the amount of covalently immobilized gelatin. With the versatility of the ATRP process and tunable grafting efficacy of gelatin, this study offers a suitable methodology for the functionalization of biodegradable polyesters scaffolds to improve cell–material interactions.

Electronic supplementary material The online version of this article (doi:10.1007/s13758-012-0030-1) contains supplementary material, which is available to authorized users.

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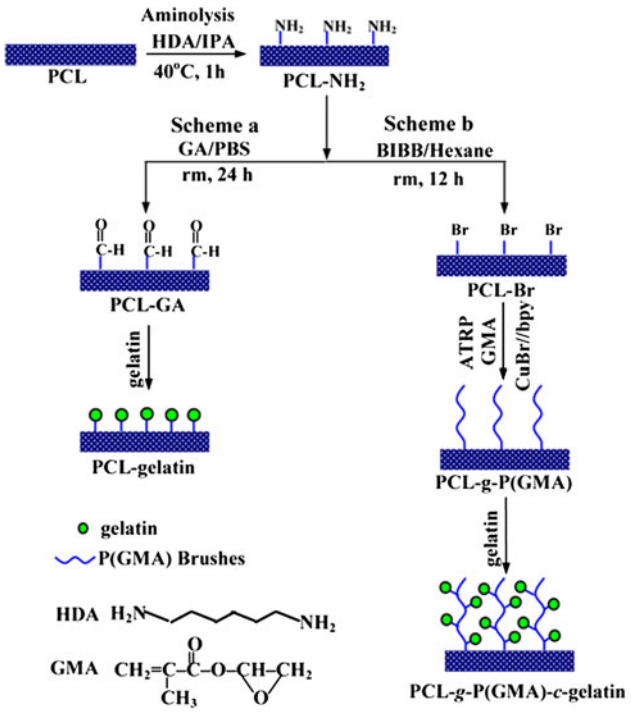
1 Introduction

Due to its slow degradation rate in vivo, good processability, and appropriate mechanical properties, polycaprolactone (PCL) is currently being extensively investigated as a scaffold material for tissue engineering applications [1–8]. However, the intrinsic hydrophobicity of PCL substrates results in poor cell attachment properties, thereby restricting their applications as biomaterials [8–17]. Modification of PCL substrate surfaces with physiological or biological activities has proven to be an effective strategy to promote cell adhesion and growth. Various methods, such as hydrolysis [13, 18, 19], aminolysis [5, 10, 20–23], plasma treatment [8, 11–16, 24, 25], UV-induced copolymerization [9, 26], ion-beam irradiation [27], and ozone treatment [28], have been employed in immobilizing extracellular matrix (ECM) molecules (e.g., collagen, gelatin and chitosan) and small active peptide sequences (e.g. Arg-Gly-Asp (RGD)) onto the PCL substrates to induce cell-specific interactions. Alternatively, functional polymer brushes containing reactive hydroxyl, carboxyl or amine groups have been grafted onto the PCL surfaces using γ-ray irradiated, ozone or photo-induced grafting to introduce hydrophilicity [14, 29–31]. These flexible reactive groups on the polymer brushes are well-suited to conjugate bioactive macromolecules for improved cytocompatibility. However, γ-ray irradiated, ozone or photo-induced polymerization grafting of polymer brushes has several limitations, including low density of grafting due to steric hindrance, uncontrollable graft yield of polymer brushes, and undesired formation of a covalent bond between reactive groups on the polymer brushes and the surface [32]. Hence, alternative methods that allow control over brush density, polydispersity and composition are desired.

One such alternative is the use of surface-initiated atom transfer radical polymerization (ATRP) method to covalently attach polymer brushes in a tunable and controllable manner [33–35]. This approach allows the preparation of polymer brushes bearing reactive pendant groups, such as hydroxyl, carboxylic acid, or epoxide groups, which provide highly reactive binding sites for bioactive macromolecules at the brush interfaces [36]. Hence, surface-initiated ATRP is a promising approach for the functionalization of the PCL surface, as it allows for the control of the length and density of the polymer brushes, which leads to tunable grafting efficiency for the desired biologically active molecules. However, to the best of our knowledge, few studies have been devoted to modifying biodegradable polyester polymers using surface-initiated ATRP for the improvement of their cytocompatibility [37]. Moreover, no systematic study has been performed to investigate the relationship between the surface density of the grafted bioactive molecules and cellular functions in order to demonstrate the tunable grafting efficiency of this approach.

In the current study, gelatin was used as the model protein, as its cell-binding properties have been previously demonstrated [38]. Using both a conventional chemical immobilization process and surface-initiated ATRP, gelatin was covalently immobilized onto the PCL substrates with different surface-grafting densities. As schematically illustrated in Scheme 1, the aminolysis reaction with 1,6-hexanediamine resulted in an activated PCL surface with free amino and hydroxyl groups [10, 20, 28]. A monolayer of gelatin was covalently conjugated to the aminolyzed PCL film surface with glutaraldehyde (GA) as bifunctional cross-linking agent, using the conventional chemical immobilization process in Scheme a. To further increase the binding capacity of gelatin on the PCL substrates, well-defined polymer brushes of glycidyl methacrylate (GMA) were prepared via surface-initiated ATRP from the alkyl halide initiator-immobilized PCL film surface in Scheme b. Gelatin was subsequently coupled to the pendant active epoxide groups of the grafted P(GMA) brushes. The efficacy of each functionalization step was ascertained by ATR-FTIR, XPS, AFM, and static water contact angle measurements. Subsequently, in vitro cell adhesion, spreading and proliferation studies were carried out to evaluate the cytocompatibility of the modified PCL surface.



Scheme 1 Schematic diagram illustrating the aminolysis process used to introduce free amino groups to PCL (i.e. PCL-NH_2 surface) and the two subsequent surface-modification schemes used to create gelatinized substrates. In scheme a, gelatin is covalently immobilized onto the aminolyzed PCL surface with glutaradehyde as a cross-linking agent, resulting in the PCL-gelatin surface. In Scheme b, immobilization of an alkyl bromine-containing initiator via condensation reaction leads to the formation of the PCL-Br surface. Surface-initiated ATRP of GMA from the PCL-Br surface results in the formation of the PCL-*g*-P(GMA) surface, and subsequently covalent conjugation of gelatin will result in the PCL-*g*-P(GMA)-*c*-gelatin surface

2 Experimental

2.1 Materials

Polycaprolactone pellets (PCL, average M_n 45,000), 1,6-hexanediamine (98 %), glycidyl methacrylate (GMA, >97 %), 2-bromoisobutyrl bromide (BIBB, 98 %), 2,2′-bipyridine (Bpy, 98 %), dichloromethane (anhydrous, >99.8 %), triethylamine (TEA, 98 %), isopropyl alcohol, hexane (anhydrous, >95 %), glutaraldehyde (25 %, Grade I), ninhydrin (>95 %), copper (I) bromide (CuBr, 99 %), copper (II) bromide ($CuBr_2$, 98 %), and gelatin (Porcine skin, Type A) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO), and were used without further purification. GMA was passed through a silica gel column to remove the inhibitor, and stored under a nitrogen atmosphere at −4 °C. All other chemical reagents and solvents were used as received. Human Umbilical Vein Endothelial cells (HUVECs, ATCC CRL-1730^{TM}) were purchased from American Type Culture Collection (Manassas, VA, USA). Cell culture medium (MCDB131), heparin and paraformaldehyde (4 %, v/v) were obtained from Sigma-Aldrich Chemical Co. Medium supplements, such as Foetal Bovine Serum (FBS), bovine brain extract, amphotericin, penicillin, streptomycin, and Trypsin–EDTA (0.25 %), were obtained from Life Technologies (Carlsbad, CA,

USA). LIVE/DEAD® Cell Viability assays and Alamar-Blue™ assay reagents were also purchased from Life Technologies. Dulbecco's phosphate buffered saline (PBS, pH 7.4) solution was freshly prepared.

2.2 Preparation of Polycaprolactone Film and Aminolysis Process

Polycaprolactone (PCL) films were prepared by solution casting method. Five gram of the PCL pellets were dissolved in 40 ml of dichloromethane to form the PCL solution. The polymer solution was then cast onto the glass substrate with predetermined thickness using the automatic film applicator (PA-2105, BYK). The solvent was removed at room temperature by slow evaporation over a 24-h period, and was further dried in a vacuum oven for another 24 h at 35 °C to obtain translucent PCL films with a thickness of about 150 μm. The resultant pristine PCL films were cut into round-shaped specimens with a diameter of 2 cm, followed by washing with copious amount of deionized water and isopropanol. The films were dried in a vacuum oven at room temperature prior to use. The cleaned PCL films were subsequently aminolyzed to introduce amino groups onto the surface of PCL films using a procedure described previously [10, 20, 23]. Briefly, the PCL films were immersed in a 10 % (w/w) isopropanol solution of 1,6-hexanediamine at 40 °C for 10 min, 30 min, 1 h, 1.5 h, 2 h, and 3 h. After aminolysis treatment, the PCL films were thoroughly rinsed with copious amount of deionized water to remove free 1,6-hexanediamine, and dried in a vacuum oven at 30 °C for 24 h.

2.3 Immobilization of Gelatin onto the Aminolyzed PCL Film Surface

The aminolyzed PCL films from 1 h of aminolysis were immobilized with gelatin using glutaraldehyde (GA) as cross-linking agent in a two-step method, as shown schematically in scheme a of Scheme 1. In the first step, the PCL-NH_2 films were immersed in a 2.5 wt % glutaraldehyde (GA)/PBS solution at room temperature for 12 h to produce the PCL-GA surface. The reaction was stopped by rinsing the samples rigorously with copious amount of deionized water to remove free GA. Subsequently, the resultant PCL-GA surface was incubated in 3 mg/ml gelatin/PBS solution at room temperature for 24 h under continuous stirring to form the PCL-gelatin surface. After the reaction, the gelatin-immobilized PCL films were washed thoroughly with copious amounts of deionized water, followed by immersion in a large volume of PBS solution for 24 h to ensure the complete removal of any physically adsorbed gelatin.

2.4 Surface-initiated ATRP of GMA and Conjugation of Gelatin

As shown schematically in scheme b of Scheme 1, the introduction of alkyl halide ATRP initiator on the PCL-NH_2 surface was accomplished through the reaction of the amine groups with 2-bromoisobutyrate bromide (BIBB) [33]. Briefly, the PCL-NH_2 films were immersed in 30 ml of anhydrous hexane solution containing 1.0 ml (7.2 mmol) of triethylamine (TEA). After 30 min of degassing with nitrogen, the reaction mixture was cooled in an ice bath, and 0.89 ml (1.65 g, 7.2 mmol) of BIBB was added dropwise via a syringe. The reaction was allowed to proceed with gentle stirring at 0 °C for 2 h and then at room temperature for 12 h to give rise to the 2-bromoisobuty-immobilized PCL surface (the PCL-Br surface). The PCL-Br surface was washed thoroughly with copious amounts of hexane, ethanol, and finally deionized water, in that order, and was subsequently dried in a vacuum oven at room temperature overnight.

For the grafting of P(GMA) brushes from the PCL-Br film surfaces, surface-initiated ATRP of GMA was carried out using a [GMA (3 ml)]:[CuBr]:[$CuBr_2$]:[Bpy] molar feed ratio of 100:1.0:0.2:2.0 in 5 ml of methanol/water mixture (5/1, v/v) at room temperature in a Pyrex® tube. The reaction was allowed to proceed for 0.5–3 h to generate the PCL-*g*-P(GMA) films. At the end of predetermined reaction time, the films were removed and washed thoroughly with methanol and deionized water to ensure the complete removal of the physical adsorbed reactants prior to being dried under vacuum. To directly couple gelatin onto the pendant epoxide groups, the PCL-*g*-P(GMA) films were incubated in 10 ml of the phosphate buffered saline (PBS, pH 7.4) containing 3 mg/ml gelatin. The coupling reaction was allowed to proceed at room temperature for 24 h under continuous stirring to produce the corresponding PCL-*g*-P(GMA)-*c*-gelatin surface. The physically adsorbed (reversibly bound) gelatin was desorbed in a large volume of PBS over 24 h at room temperature with gentle stirring, followed by rinsing with copious amounts of PBS and deionized water, respectively.

2.5 Determination of Grafting Density of Polymer Brushes and Conjugated Gelatin

The grafting density of the gelatin and the P(GMA) brushes on the PCL films were determined by the grafting yield using the following equation [2, 27]:

$$GY = \frac{W_a - W_b}{A} \quad (1)$$

where W_a and W_b are the weights of the dry film after and before graft polymerization (or conjugation of gelatin)

respectively, and A is the film area (3.2 cm^2). For each GY measurement, at least three PCL films were investigated and the resulting values were averaged. Subsequently, XPS measurement was carried out to quantitatively determine the relative amount of gelatin immobilized on the PCL film surfaces. The N 1 s core-level signal was used as an indicator of the conjugation of gelatin on the film surface. The [N]/[C] ratio, determined from the sensitivity-factor-corrected N 1 s and C 1 s core-level XPS spectral area, indicated the relative abundance of gelatin on the PCL film surface.

2.6 Characterization of Surface-Functionalized PCL films

Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS), atom force microscopy (AFM), and water contact angle (WCA) were used to characterize the surface properties of the functionalized PCL films. Detailed procedures for all measurements are described in the Supporting Information section.

2.7 Human Umbilical Vein Endothelial Cell (HUVEC) Culture

Human Umbilical Vein Endothelial Cells (HUVECs, ATCC CRL-1730TM) were cultured in gelatin-coated T25 flasks containing MCDB131 cell culture medium, supplemented with Foetal Bovine Serum, 0.2 % Bovine Brain Extract, 0.25 μg/ml amphotericin, 0.1 mg/ml heparin, 100 U/ml penicillin, and 100 μg/ml streptomycin, in a CO_2 environment at 37 °C. The MCDB131 medium was changed every other day. Upon 90 % confluency, cells were harvested by trypsinization by 0.25 % Trypsin–EDTA. HUVECs between passages 4–6 were used for subsequent experiments.

2.8 Cell Adhesion and Proliferation

To assess HUVECs adhesion and proliferation on the surface-functionalized PCL films, gelatin-coated coverslips (0.1 %) were used as positive controls. The pristine and functionalized PCL films were sterilized by immersing into 75 % (v/v) ethanol solution for 60 min, and then rinsed three times with sterile PBS, followed by MCDB131 medium incubation overnight. For cell adhesion assay, PCL films were first placed at the bottom of each well of a 24-well plate. 0.5 ml aliquots of HUVECs cell suspension (2×10^4 cells/ml) were seeded onto the surface of pre-wetted PCL films in each well. Cell-seeded films were incubated in a 5 % CO_2 atmosphere at 37 °C for 24 h to allow for cell attachment. After this initial incubation period, the films were washed twice with PBS solution to remove any unattached cells. The number of adhesive cells was counted using a haemocytometer (n = 3). The adhesion ratio was defined by the following equation:

$$Adhesion\ ratio\ (\%) = \frac{N_1}{N_0} \times 100\% \quad (2)$$

where N_1 is the number cells on the surfaces of PCL films after incubation for 24 h, and N_0 is the initial number of cells seeded into each well.

Cell viability and proliferation were determined using the AlamarBlue™ (AB) assay. 0.5 ml of HUVECs cell suspension (2×10^4 cells/ml) were seeded into each well of a 24-well plate containing the pristine and functionalized PCL films, and incubated in a 5 % CO_2 environment at 37 °C for 1, 3, 5 and 7 days. The cell culture medium was changed every other day. At the end of each incubation period, culture medium was removed from the wells, and 0.5 ml of the AB solution (10 % AB solution in culture media without FBS) was added to the wells. The plates were incubated in a 5 % CO_2 atmosphere at 37 °C for 4 h and the fluorescence density was measured using a microplate reader (Model 680, Bio-Rad Laboratories, Inc. Hercules, CA, USA) at an excitation wavelength of 570 nm and an emission wavelength of 580 nm. Cell numbers were calculated by seeding known quantities of cells and correlation with fluorescence emission.

2.9 Cell Imaging

In vitro qualitative analysis of cell coverage and viability was performed using the LIVE/DEAD® viability/cytotoxicity assay to assess the extent of endothelialisation on the functionalised PCL surfaces. For this procedure, calcein AM (4 mM in anhydrous dimethyl sulfoxide, DMSO) and EthD-1 (2 mM in DMSO/H_2O, 1:4 v:v) were added to PBS (1:1,000 ratio) to produce a LIVE/DEAD® staining solution. The cell-seeded PCL samples, obtained after 7 days of cell culture, were first washed thrice with PBS to eliminate the nonadherent cells, followed by staining using 0.1 ml of LIVE/DEAD staining solution. After incubation in a 5 % CO_2 atmosphere at 37 °C for 30 min, the samples were visualised with a Nikon Image Ti fluorescence microscope (emission at 515 nm and 635 nm (Nikon Instruments, Tokyo, Japan) to acquire fluorescent images using NIS-Elements Br software.

2.10 Statistical Analysis

All the quantitative results were expressed as mean ± standard deviation (SD). Statistical analysis was carried out by means of one-way analysis of variance (ANOVA) with Tukey's post hoc test. A p value less than 0.05 was considered statistically significant.

3 Results and Discussion

3.1 Aminolysis of PCL Film Surface

Aminolysis modification represents an easy-to-perform chemical technique to engraft amino groups along polyester chains [5, 20–23]. In this study, one terminal amine group of 1,6-hexanediamine is able to react with the ester groups ($-COO^-$) of the PCL films to produce the covalent bond, $-CONH^-$, and the other end amine group remains free. The free amine density on the aminolyzed PCL surface (referred to as the PCL-NH_2) was quantitatively determined using the ninhydrin assay. The density of amino groups on the PCL surface increased with aminolysis time and reached a maximal value at 1 h, estimated to be approximately 0.265 ± 0.02 μmol/cm^2 (Fig. 1). This result is consistent with the data reported previously [10, 20, 23]. In addition, the aminolysis reaction was found to proceed preferentially at the amorphous regions of polymer in the initial period, as the result of bulk degradation in the diamine solution [23]. Upon prolonging reaction time, the decrease in the number of bound amine groups may be caused by chain scission, formation of oligomers and the removal of other low mass fragments during the reaction and the rinsing process [39]. Since aminolysis takes place by nucleophilic attack of the carbonyl groups in PCL by diamine, the density of amine groups on the PCL substrates was reported to be dependent on the concentration of diamine, temperature and reaction time [23]. For this study, the optimal aminolysis time for the PCL film was found to be 1 h, and this reaction time was used for subsequent cellular and surface functionalization studies.

Figures 2a and b show the ATR-FTIR spectrum of the pristine PCL and PCL-NH_2 films, respectively. The characteristic peaks of carbonyl (C=O) and aliphatic groups at 1,725 cm^{-1} (ν(C=O)) and 2,947, 2,865 cm^{-1} (ν_{as}(C–H) and ν_s(C–H)) [23], were observed on the pristine PCL film (Fig. 2a). After 1 h of aminolysis treatment, three additional peaks at wavelengths of 3,321, 1,650 and 1,550 cm^{-1}, attributable to ν_{N-H}, $\nu_{C=O}$ (amide I) and ν_{N-H} (amide II) [40], respectively, were clearly observed (Fig. 2b). An additonal N 1 s signal at a binding energy (BE) of about 400 eV in the wide scan XPS spectrum of the PCL-NH_2 film was also observed (Fig. 3), which was absent in the pristine PCL film. Hence, these results confirm the successful introduction of amine groups onto PCL substrates. The [N]/[C] ratio, as determined from sensitivity factor-corrected N 1 s and C 1 s XPS spectral area, of the PCL-NH_2, was found to be about 0.043 (Table 1).

3.2 Immobilization of Gelatin onto the Aminolyzed PCL Surfaces

In this study, a monolayer of gelatin was immobilized onto the PCL-NH_2 surface with glutaraldehyde (GA) as the cross-linking agent. The resulting surface is referred to as the PCL-gelatin surface. Figure 2d shows the FTIR

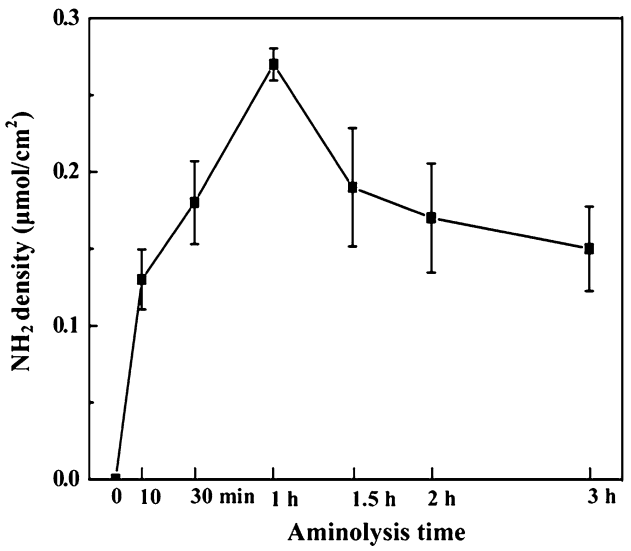


Fig. 1 The density of amine groups of the aminolyzed PCL film surface as a function of aminolysis time as determined by the ninhydrin assay. The analysis reaction of PCL films proceeded at 40 °C in 10 wt% 1,6-hexanediamine/2-propanol solution. Error bars represent the standard deviation over three separate PCL films. The optimized aminolysis time was observed at 1 h with NH_2 density at about 0.265 μmol/cm^2

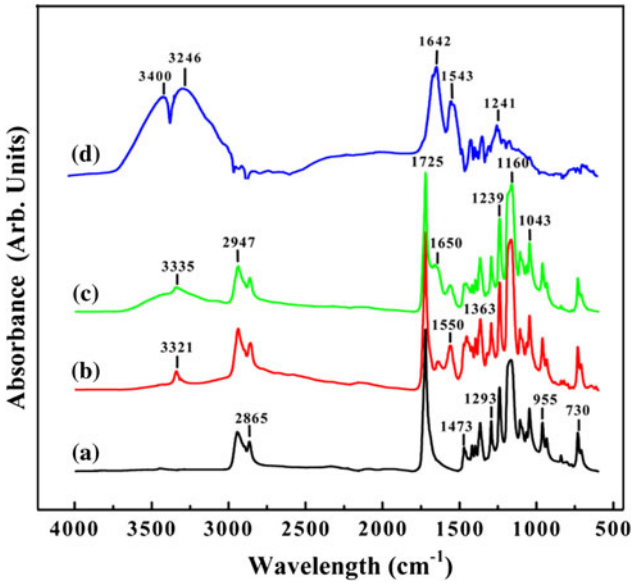


Fig. 2 ATR-FTIR spectra of the **a** pristine PCL, **b** PCL-NH_2 from 1 h of aminolysis, **c** PCL-gelatin, **d** pure gelatin. The successful immobilization of gelatin was deduced from the presence of a broad band of free O–H and N–H groups (at 3,295 cm^{-1}), amide I band (at 1,650 cm^{-1}) and amide II band (at 1,550 cm^{-1}) as compared to the FTIR spectrum of pure gelatin

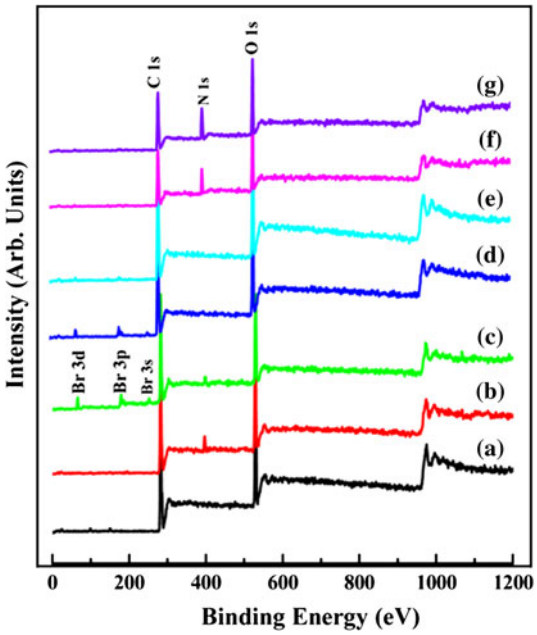


Fig. 3 Wide scan XPS spectra of the **a** pristine PCL, **b** PCL-NH_2 from 1 h of aminolysis, **c** PCL-Br, **d** PCL-*g*-P(GMA)1 from 1 h of ATRP, **e** PCL-*g*-P(GMA)2 from 3 h of ATRP, **f** PCL-*g*-P(GMA)1-*c*-gelatin, and **g** PCL-*g*-P(GMA)2-*c*-gelatin surfaces

spectrum of pure gelatin with characteristic peaks for *v*(O–H) (3,400 cm^{-1}), *v*(N–H) (3,246 cm^{-1}), amide I (1,642 cm^{-1}) and amide II (1,543 cm^{-1}) [23]. Consequently, the successful immobilization of gelatin onto the PCL-NH_2 surface could be deduced by the presence of a broad band at 3,295 cm^{-1}, which is due to the overlap of free O–H and N–H stretching vibrations, and by the increase in relative intensity of the amide I band (1,650 cm^{-1}) (Fig. 2c). The immobilized amount of gelatin for the PCL-gelatin surface, 0.42 ± 0.11 μg/cm^2 (Table 1), was found to be similar to results obtained by other researchers [20, 23].

3.3 Surface-Initiated ATRP of GMA and Immobilization of Gelatin

For the grafting of polymer brushes via surface-initiated ATRP, a uniform layer of initiators immobilized on the PCL film surface is indispensible [33]. The introduction of an alkyl bromide ATRP initiator was achieved via a TEA-catalyzed condensation reaction between the amine groups of the PCL-NH_2 surface and 2-bromoisobutyryl bromide (BIBB). Successful immobilization of an alkyl bromide-containing ATRP initiator onto the PCL-NH_2 surface could be deduced from the appearance of three additional signals of Br 3d (BE, 70 eV), Br 3p (BE, 189 eV), and Br 3 s (BE, 256 eV) in the wide scan spectrum of the PCL-Br surface (Fig. 3c) [41], as compared to that of the PCL-NH_2 film (Fig. 3b). The [Br]/[C] ratio, as determined from the Br 3d and C 1 s core-level spectral area ratio, was found to be about 3.17×10^{-2} (Table 1). Thus, the alkyl bromine groups were successfully immobilized onto the PCL-NH_2 surface in preparation for the subsequent ATRP process.

In this study, the molar ratio of [GMA (monomer)]:[$CuBr_2$ (deactivator)]:[CuBr (catalyst)]: [Bpy (ligands)] for the surface-initiated ATRP process was controlled at 100:0.2:1.0:2.0 to produce the P(GMA) polymer brushes. The resulting surfaces from 0.5, 1 and 3 h of ATRP are referred to as the PCL-*g*-P(GMA)0, PCL-*g*-P(GMA)1 and PCL-*g*-P(GMA)2 surfaces, respectively. The presence of the P(GMA) brushes on the PCL film surfaces was confirmed by XPS and ATR-FTIR analyses. Figures 4a and b show the FTIR spectra of the PCL-*g*-P(GMA)1 and PCL-*g*-P(GMA)2 surfaces, respectively. Three obvious characteristic bands of epoxide groups at 903, 846, and 756 cm^{-1} were clearly visible in the FTIR spectrum [42]. The strong peak at 1,726 cm^{-1} is associated with the ester *v*(C=O) in COO^- of the P(GMA) chains [43]. The grafting yield (GY) was used to evaluate the kinetics of polymer chain growth. As shown in Fig. 5, an approximately linear increase in GY of the grafted P(GMA) chains with polymerization time could be observed for the PCL-Br surface. GY values of the PCL-*g*-P(GMA)0, PCL-*g*-P(GMA)1 and PCL-*g*-P(GMA)2 surfaces were found to be 2.72 ± 0.68, 6.31 ± 1.32 and 14.76 ± 2.63 μg/cm^2 (Table 1) respectively, indicating that the amount of P(GMA) grafted onto the PCL surfaces could be modulated by varying the reaction time. However, the molecular weight and molecular weight distribution of the surface-graft polymer could not be determined with sufficient accuracy without precise cleavage of the grafted P(GMA) chains from the PCL film surfaces [44].

Poly(glycidyl methacrylate) (P(GMA) is an effective spacer for biomolecules, such as the immobilization of proteins, antibodies and enzymes, for tissue engineering applications [44]. As such, the nucleophilic reaction between $-NH_2$ moieties of biomolecules and epoxide groups has been widely reported [45, 46]. Therefore, PCL-*g*-P(GMA) surfaces with terminal halide groups and a high density of epoxide groups are well-suited for the immobilization of gelatin. In this work, the gelatin immobilized P(GMA)-grafted PCL substrates are defined as the PCL-*g*-P(GMA)0-*c*-gelatin, PCL-*g*-P(GMA)1-*c*-gelatin and PCL-*g*-P(GMA)2-*c*-gelatin surfaces, respectively. Figure 4c and d show the ATR-FTIR spectra of the PCL-*g*-P(GMA)1-*c*-gelatin and PCL-*g*-P(GMA)2-*c*-gelatin surfaces, respectively. The appearance of three additional bands at 3,295, 1,642, and 1,543 cm^{-1}, attributable to the overlap of free O–H and N–H stretching, amide I and amide II, is associated with the coupling of gelatin on the film surfaces, as compared to the spectra of pure gelatin and the PCL-*g*-P(GMA) surfaces.

Table 1 Reaction time, grafting yield, and surface composition of the pristine PCL and surface-functionalized PCL surfaces

Sample	Reaction time (h)	GY (μg/cm^2) (mean ± SD)	[Br]/[C][j]	[N]/[C][j]
Pristine PCL[a]	–	–	–	–
PCL-NH_2[b]	1	–	–	0.043
PCL-GA[c]	12	–	–	–
PCL-gelatin[d]	24	0.42 ± 0.11	–	0.097
PCL-Br[e]	12	–	3.17×10^{-2}	–
PCL-*g*-P(GMA)0[f]	0.5	2.72 ± 0.68	2.34×10^{-2}	–
PCL-*g*-P(GMA)1[g]	1	6.31 ± 1.32	9.29×10^{-3}	–
PCL-*g*-P(GMA)2[h]	3	14.76 ± 2.63	4.72×10^{-3}	–
PCL-g-P(GMA)0-c-gelatin[i]	24	0.93 ± 0.25	–	
PCL-*g*-P(GMA)1-*c*-gelatin[i]	24	2.63 ± 0.52	–	0.169
PCL-*g*-P(GMA)2-*c*-gelatin[i]	24	3.79 ± 0.73	–	0.202

SD standard deviation

[a] Pristine PCL refers to the cleaned PCL film after rigorous washing with alcohol and deionized water

[b] PCL-NH_2 was obtained after 1 h of aminolysis in a 10 % (w/w) 1,6-hexanediamine/isopropanol mixture at 40 °C

[c] PCL-GA was obtained after the PCL-NH_2 surface immersed in a 2.5 wt% PBS solution of glutaradehyde (GA) at room temperature for 12 h

[d] PCL-gelatin refers to the PCL-GA surface conjugated with gelatin after incubated in the PBS (pH 7.4) solution containing the gelatin at a concentration of 3 mg/ml at room temperature for 24 h

[e] PCL-Br was obtained after the PCL-NH_2 surface reacted with 2-bromoisobutyryl bromide (BIBB) in dried hexane containing 1:1 (molar ratio) BIBB and triehtylamine (TEA)

[f,g] Reaction conditions: [GMA]:[CuBr]:[$CuBr_2$]:[bpy] = 100:1:0.2:2 in methanol/water solution (1:1, v:v) at room temperature for 1 and 3 h to produce the PCL-*g*-P(GMA)1 and PCL-*g*-P(GMA)2 surfaces, respectively

[h] Reaction conditions: the PCL-*g*-P(GMA)1 and PCL-*g*-P(GMA)2 surfaces incubated in the PBS (pH 7.4) solution containing the gelatin at a concentration of 3 mg/ml at room temperature for 24 h

[i] GY denotes grafting yield, and is defined as GY = $(W_a - W_b)/A$, where W_a and W_b corresponds to the weight of the dry films before and after grafting, respectively, and A is the film area (about 3.2 cm^2)

[j] Determined from their corresponding sensitivity factor-corrected element core-level spectral area ratios

The [N]/[C] ratio, determined from the sensitivity factor-corrected N 1 s and C 1 s core-level spectral area, is used to assess the relative amount of immobilized gelatin. The [N]/[C] ratios of the PCL-*g*-P(GMA)0-*c*-gelatin, PCL-*g*-P(GMA)1-*c*-gelatin and PCL-*g*-P(GMA)2-*c*-gelatin surfaces were found to be about 0.134, 0.169 and 0.202 (Table 1) respectively, indicating that the P(GMA) brushes from 3 h of ATRP possess higher binding capability to gelatin. Earlier studies have reported that the concentration of epoxide groups of the grafted P(GMA) brushes played a dominant role in the immobilization of biomolecules [44]. The immobilized gelatin amounts of 0.93 ± 0.25, 2.63 ± 0.52 and 3.79 ± 0.73 μg/cm^2 (Table 1) for the PCL-*g*-P(GMA)0-*c*-gelatin, PCL-*g*-P(GMA)1-*c*-gelatin and the PCL-*g*-P(GMA)2-*c*-gelatin films respectively, showed that the amount of the immobilized gelatin is tunable by varying the concentration of epoxide groups on the P(GMA) brushes.

3.4 Surface Wettability and Topography

Surface wettability is a prerequisite for proper material recognition by cells and thus effective adhesion to the substrates [17]. Static water contact angles (WCA) and the representative WCA images of the pristine PCL and functionalized PCL film surfaces are summarized in Table 2. The pristine PCL film was found to be a highly hydrophobic surface with a WCA of 93 ± 4°. The aminolysis treatment led to a more hydrophilic PCL film surface as the WCA decreased to 66 ± 3° owing to the protonation of the primary amine group in water. The average WCAs of the PCL-*g*-P(GMA)1 and PCL-*g*-P(GMA)2 surfaces were found to be 62 ± 3° and 61 ± 2°, respectively, due to the presence of hydrophilic epoxide groups [45]. Overall, a significant improvement in surface hydrophilicity was observed after the immobilization of gelatin. The WCA of the PCL-gelatin surface decreased to 49 ± 3°, while the immobilization of higher surface density of gelatin onto the grafted P(GMA) polymer chains resulted in an even more hydrophilic surface, with the WCAs as low as 37 ± 2° (for the PCL-*g*-P(GMA)1-*c*-gelatin surface) and 35 ± 3° (for the PCL-*g*-P(GMA)2-*c*-gelatin surface). Gelatin has been reported to contain large amounts of glycine (Gly) and proline (Pro), which are hydrophilic amino acids [17]. The hydroxyl groups (−OH)

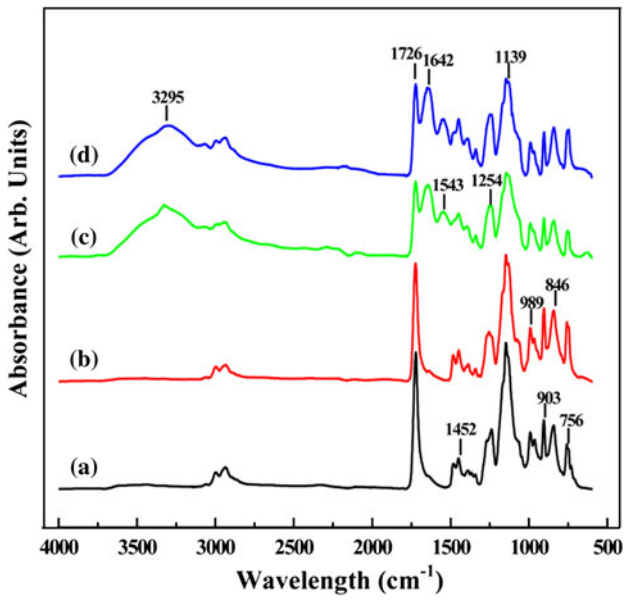


Fig. 4 ATR-FTIR spectra of the **a** PCL-*g*-P(GMA)1 from 1 h of ATRP, **b** PCL-*g*-P(GMA)2 from 3 h of ATRP, **c** PCL-*g*-P(GMA)1-*c*-gelatin, and **d** PCL-*g*-P(GMA)2-*c*-gelatin. The successful immobilization of gelatin on the P(GMA) brushes can be deduced from the presence of a broad band of free O–H and N–H groups (at 3,295 cm^{-1}), amide I band (at 1,642 cm^{-1}) and amide II band (at 1,543 cm^{-1}) as compared to the pure gelatin FTIR spectrum

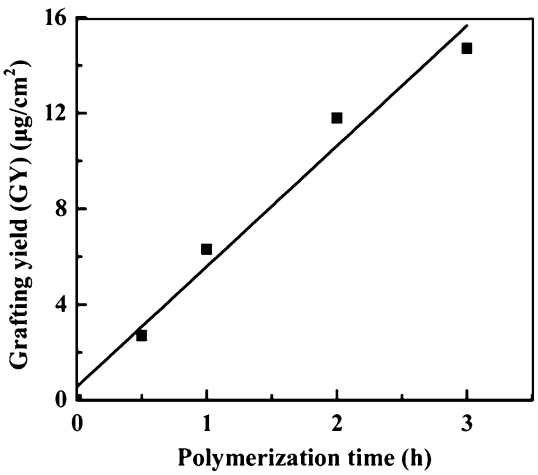


Fig. 5 A linear relationship between the grafting yield (GY) of the P(GMA) brushes with the surface-initiated ATRP time was observed. The polymer chain growth from the PCL-Br surface could be controlled by varying reaction time

generated in the ring-opening reaction of epoxide groups by the coupling of gelatin also contributed to the decrease in WCA for the PCL-*g*-P(GMA)-*c*-gelatin surfaces.

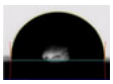
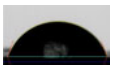
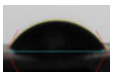
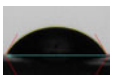
The changes in topography of the PCL film surfaces after each functionalization step were investigated by AFM. The surface roughness and the corresponding three-dimensional (3D) AFM images of the pristine and functionalized PCL films are shown in Table 2 and Fig. S3 (Supporting Information). The pristine PCL film surface was found to be relatively uniform and smooth with a root-mean-square surface roughness value (R_a) of about 19 ± 2 nm. A significant increase in the R_a value of 31 ± 5 nm was observed after the aminolysis treatment. This result is in agreement with the findings by other groups [31]. After the immobilization of gelatin monolayer, no further significant difference in the R_a value was observed. After graft polymerization of GMA, the R_a values increased significantly to about 57 ± 4 nm (for the PCL-g-P(GMA)1) and 68 ± 3 nm (for the PCL-g-P(GMA)2 surfaces), respectively. The subsequent coupling of gelatin to P(GMA)-grafted films resulted in a further increase in the R_a values to 59 ± 2 nm (for the PCL-*g*-P(GMA)1-*c*-gelatin surface) and 71.5 ± 3 nm (for the PCL-*g*-P(GMA)2-*c*-gelatin surface).

3.5 Cell Adhesion

The interaction of cells with different PCL substrates was investigated by seeding endothelial cells (ECs) onto the films for 24 h to determine the initial adhesion ratios. Gelatin-coated coverslips were used as a positive control. The results (Table 2) showed that the ECs had the least affinity for the pristine PCL film, since less than 50 % of the total number of cells initially seeded onto the film remained on the film after 24 h. Despite obvious changes in surface hydrophilicity and roughness achieved after aminolysis treatment and the grafting of P(GMA) brushes, no significant improvement in cell adhesion ratio was observed on the PCL-NH_2 and the P(GMA)-grafted surfaces, as compared to the pristine PCL surfaces, this suggests that other factors (e.g. biological cues) may be required for positive cell interaction. This hypothesis is confirmed by the fact that the gelatinized films (PCL-gelatin film) had a higher affinity for cells, as compared to the films that did not contain the bioactive components of gelatin. More evident improvement in cell adhesion was observed in the PCL-*g*-P(GMA)1-*c*-gelatin and PCL-*g*-P(GMA)2-*c*-gelatin surfaces (Table 2). In fact, the number of attached cells increased with increasing concentration of exposed gelatin on the PCL surface. As a result, the PCL-*g*-P(GMA)2-*c*-gelatin surface, which had the highest density of immobilized gelatin, exhibited the most significant increase in cell adhesion ratio, comparable to that of the gelatin-coated coverslips (positive controls).

As compared to the PCL-gelatin films, the PCL-*g*-P(GMA)-*c*-gelatin films had a higher amount of immobilized gelatin, since gelatin was directly coupled to the pendant epoxide group of the repeat unit of P(GMA) brushes, resulting in the immobilized gelatin appearing in a dispersed form among the grafted P(GMA) chains rather

Table 2 Surface roughness, surface wettability and cell adhesion on the pristine PCL and surface-functionalized PCL surfaces

Samples	Surface roughness (R_a^a, mean ± SD nm)	Cell adhesion ratio[b] (%)	Surface wettability (°)	Representative images of WCA[c]
Pristine PCL	19.3 ± 1.7	49.1 ± 7.2	93 ± 4	
PCL-NH_2	30.2 ± 4.5	63.0 ± 4.4	66 ± 3	
PCL-gelatin	34.5 ± 1.8	116.1 ± 3.5	49 ± 3	
PCL-*g*-P(GMA)1	56.9 ± 4.4	50.1 ± 3.3	62 ± 3	
PCL-*g*-P(GMA)2	59.4 ± 2.9	53.2 ± 4.0	61 ± 2	
PCL-*g*-P(GMA)1-*c*-gelatin	67.8 ± 6.2	127.2 ± 3.8	37 ± 2	
PCL-*g*-P(GMA)2-*c*-gelatin	72.3 ± 3.6	134.4 ± 4.4	35 ± 3	

[a] R_a denotes a root-mean-square surface roughness, *SD* denotes standard deviation

[b] Cell adhesion ratio was calculated by ($N_1/N_0 \times 100$ %), where N_1 is the number cells on the PCL surfaces after 24 h, and N_0 is the initial number of cells seeded into each well. The cell adhesion ratio of gelatin-coated coverslips is about 131.1 ± 2.6

[c] WCA denotes static water contact angles

than in the formation of a continuous surface layer. The increase in cell adhesion ratio of the PCL-*g*-P(GMA)2-*c*-gelatin surface (3.79 ± 0.73 μg/cm^2) with respect to the PCL-*g*-P(GMA)1-*c*-gelatin surface (2.63 ± 0.52 μg/cm^2) suggests that the cell adhesion is positively correlated to the amount of immobilized gelatin.

3.6 Cell Proliferation

Figure 6 shows the proliferation profiles of the attached EC cells over time on the different PCL film surfaces. The pristine PCL film surface is the least conducive for supporting cellular growth, as the number of cells remains almost unchanged between the first and the seventh day of culture. Generally, anchorage-dependent cells such as HUVECs need to strongly adhere to substrates, in order to survive, spread, and transfer signals into the cytosol to maintain cell homeostasis [46]. Hence, the extent of cell spreading on substrates plays a crucial role in cell proliferation of ECs. As observed from the SEM images (Supporting Information, Fig. S6a), only a few ECs were firmly attached to the pristine PCL films after 3 days of incubation, and these attached cells showed poor spreading behavior. The cells attached to the PCL-NH_2 film surfaces showed a slight improvement in terms of proliferation and spreading behaviors (Fig. S6b), as compared to the pristine PCL surface. This result is consistent with previous findings that the presence of amine groups on the PCL surfaces leads to a positive effect on cell proliferation, albeit to a limited extent [7, 10]. However, no improvement in EC proliferation was observed on the P(GMA)-grafted PCL surfaces, and SEM observations also demonstrated that the ECs presented a poor spreading morphology (Fig. 6Sd). This phenomenon could be due to the cytotoxic and mutagenic effects of the epoxide groups on cells [47].

In the case of the gelatin-immobilized film surfaces, ECs were observed to adopt a flat and spreading morphology (Figs. S6c and 6e), which resulted in the formation of a confluent cell layer (Fig. S6f). Cell proliferation on the gelatin-immobilized surfaces was not only significantly enhanced, but was also found to be positively correlated to the amount of covalently immobilized gelatin. Despite the low surface density of immobilized gelatin for the PCL-gelatin films (about 0.42 ± 0.11 μg cm^{-1}), a 13- and fivefold improvement in cell proliferation, as compared to the pristine PCL and the PCL-NH_2 surfaces respectively, was observed. With the increase in surface density of immobilized gelatin, more pronounced enhancements in cell proliferation were observed for the PCL-*g*-P(GMA)1-*c*-gelatin and PCL-*g*-P(GMA)2-*c*-gelatin surfaces as compared to the PCL-gelatin surface. Moreover, the proliferation rate of the ECs on the PCL-*g*-P(GMA)2-*c*-gelatin

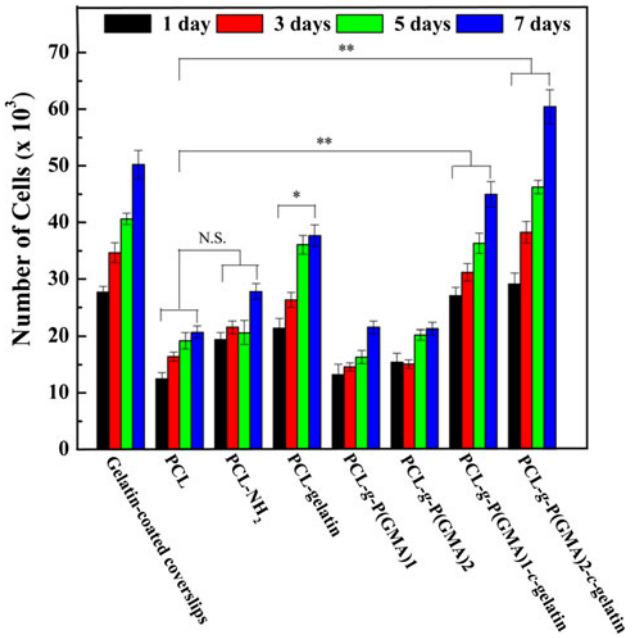


Fig. 6 Cell proliferation profile of the gelatin-coated coverslips, pristine PCL and functionalized PCL surfaces after 1, 3, 5 and 7 days of incubation at 37 °C in a 5 % CO_2 atmosphere as determined by the AlamarBlue™ (AB) assay. Data is presented as means ± SD. $*p < 0.05$, $**p < 0.01$ compared to the pristine PCL substrates. The proliferation rate of HUVECs seeded on the PCL-*g*-P(GMA)-*c*-gelatin surfaces was significantly improved as compared to the pristine PCL and PCL-*g*-P(GMA) film surfaces

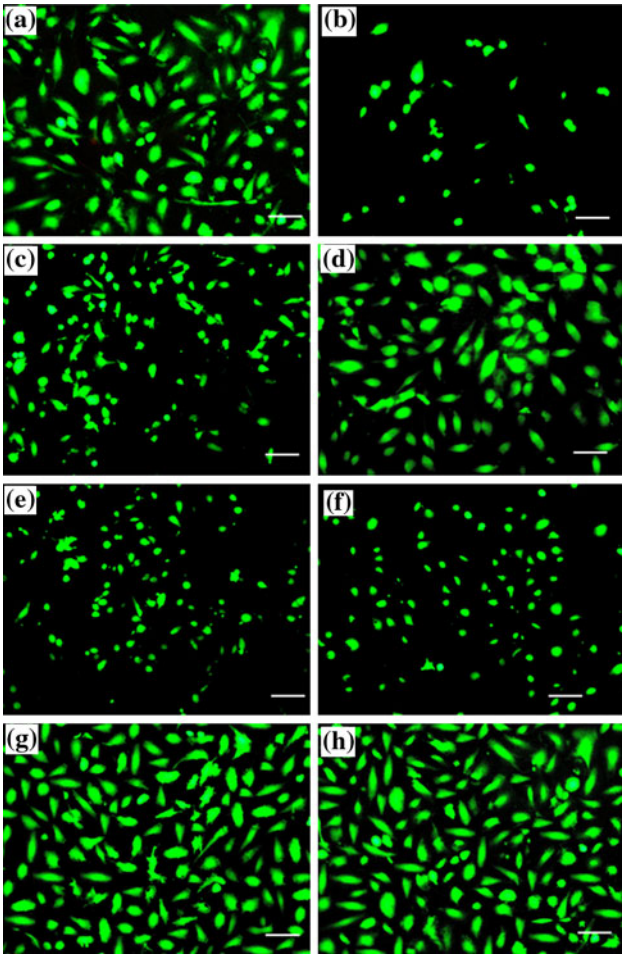


Fig. 7 Representative fluorescence images of LIVE/DEAD®-stained HUVECs on the **a** gelatin-coated coverslips, **b** pristine PCL, **c** PCL-NH_2, **d** PCL-gelatin, **e** PCL-*g*-P(GMA)1 from 1 h of ATRP, **f** PCL-*g*-P(GMA)2 from 3 h of ATRP, **g** PCL-*g*-P(GMA)1-*c*-gelatin, and **h** PCL-*g*-P(GMA)2-*c*-gelatin surfaces. Rapid endothelialization was observed on all the gelatinized PCL surfaces. *Scale bar* 20 μm

surface was higher than that of the PCL-*g*-P(GMA)1-*c*-gelatin surface. In fact, the results obtained for the PCL-*g*-P(GMA)1-*c*-gelatin and PCL-*g*-P(GMA)2-*c*-gelatin surfaces were comparable to those of the gelatin-coated coverslips (positive controls), which showed that in general, the presence of immobilized gelatin led to an overall positive effect on cell proliferation.

3.7 Cell Imaging

Fluorescence images of LIVE/DEAD®-stained ECs were captured on the functionalized PCL film surfaces after 7 days of incubation to evaluate the extent of endothelialization (Fig. 7). The sparsely distributed cells on the pristine PCL substrate further confirmed the unfavorable surface properties of pristine PCL for cell adhesion and proliferation (Fig. 7b). Similarly, ECs were found to be sparsely distributed over the PCL-NH_2 and the P(GMA)-grafted surfaces (Figs. 7c, e and f), indicative of poor endothelialization. However, the endothelialization on the gelatin-immobilized PCL surfaces was substantially improved (Figs. 7d, g and h). The extent of endothelialization appeared to be positively correlated to the amount of immobilized gelatin on the PCL film surface, since the PCL-*g*-P(GMA)1-*c*-gelatin and PCL-*g*-P(GMA)2-*c*-gelatin surfaces had a higher amount of attached cells, as compared to the PCL-gelatin surface. In general, the extent of endothelialization within a given period of time was determined by the surface concentration of immobilized gelatin.

3.8 Stability of Gelatin-Immobilized Surface

The stability of the grafted gelatin-coupled P(GMA) layers on the PCL substrate surface was investigated, as this had direct influence over the long-term viability of the functionlized substrates. In previous studies, surface-initiated ATRP-grafted polymer brushes on various substrates, including glass, fiber, paper, silicon wafer and titanium, were shown to be stable under harsh environments [33–37]. For this study, the gelatin molecules were immobilized

directly onto the side chains of P(GMA) brushes via robust covalent bonding (O=CNH), which led to the formation of highly stable gelatinized surfaces.

However, to further investigate the possible release of gelatin from the side chains of the P(GMA) brushes, the gelatin-immobilized PCL substrates were immersed in 50 ml of the PBS solution at 25 °C for 10 days under slight agitation. After 10 days, the gelatin-immobilized substrates were washed vigorously with deionized water and dried under reduced pressure prior to XPS characterization. The XPS results (Supporting Information, Fig. S7) showed that the composition of the PCL-*g*-P(GMA)-*c*-gelatin surface remained relatively unchanged even after immersion in the PBS solution, as seen from the [N]/[C] ratios for the PCL-*g*-P(GMA)1-*c*-gelatin and PCL-*g*-P(GMA)2-*c*-gelatin substrates before and after immersion. The results therefore confirm the high stability of the gelatin on the P(GMA) brushes.

4 Conclusion

PCL substrates were successfully modified via the conventional chemical immobilization process and surface-initiated ATRP of GMA. Kinetics studies revealed an approximately linear increase in grafting yield of the functional P(GMA) brushes using the ATRP process with polymerization time, and that the amount of immobilized gelatin on the P(GMA) chains increased with the pendant epoxide concentration of the grafted P(GMA) brushes. Subsequent in vitro cell adhesion and proliferation studies using HUVECs revealed better cell affinity and growth on the gelatin-immobilized PCL film surface, as compared to the poor performance of the pristine PCL and aminolyzed PCL film surfaces. Cell proliferation was also found to be positively correlated with the surface density of the immobilized gelatin, which was why the PCL-*g*-P(GMA)2-*c*-gelatin substrates showed significantly improved cell attachment properties as compared to the other gelatinized substrates. With the inherent versatility of surface-initiated ATRP, and the good cell-adhesive nature of gelatin, biodegradable polyesters can be readily tailored with high surface concentrations of gelatin to facilitate rapid endothelialization for cardiovascular applications. The approach described in this study can potentially be used for other bioactive molecules to improve cell–material interactions of biodegradable polyester polymers currently used for biomedical applications.

Acknowledgments This research is supported by the Singapore National Research Foundation under CREATE programme: The Regenerative Medicine Initiative in Cardiac Restoration Therapy (NRF-Technion).

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