Addition of Biological Functionality to Poly(ϵ-caprolactone) Films

Emma L. Prime, Zuratul Ain Abdul Hamid, Justin J. Cooper-White,† and Greg G. Qiao*

Polymer Science Group, Department of Chemical and Biomolecular Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

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Biodegradable polyesters such as poly(ϵ-caprolactone) (PCL) have a number of biomedical applications; however, their usage is often limited by a lack of biological functionality. In this paper, a PCL-based polymer containing pendent groups activated by 4-nitrophenyl chloroformate (NPC) and reactive toward primary amines has been cast into thin films. The reactivity of the films toward poly(L-lysine) and the cell adhesion peptide, GRGDS, was assessed, and their cell adhesive capabilities were characterized. ATR-FTIR analysis found that NPC functional groups were present on the surface of the cast film, and the synthesis, conjugation, and visualization of a fluorescent molecule on these films further demonstrated the success of this functionalization methodology. The immersion of these films into a solution of either poly(L-lysine) (PLL) or GRGDS in PBS (pH 7.4) and subsequent 3T3 fibroblast adhesion studies demonstrated significant improvement in cell adhesion and spreading over films cast from unmodified PCL. This investigation has shown that this novel NPC-containing polymer can be utilized in many applications where increased cellular adhesion is required, or the coupling of specific molecules to polymer surfaces is of interest.

Introduction

The introduction of biologically active groups that elicit positive cellular responses to biomaterial surfaces is an increasingly investigated area. The Arg-Gly-Asp (RGD) motif has been found to be the minimum peptide sequence needed for recognition by the adhesive integrins on cell surfaces or other extracellular matrix proteins.[1,2] As such, the immobilization of oligopeptides containing this RGD sequence has been a much investigated approach aiming to improve the cell adhesiveness of biomaterial surfaces.[3−6]

Aliphatic polyesters, such as poly(lactic-*co*-glycolic acid) (PLGA) and poly(ϵ-caprolactone) (PCL), have been extensively investigated as the base polymer substrate in three-dimensional scaffolds for tissue engineering and microparticles for drug delivery. One disadvantage of this class of polymers is their lack of functional groups, apart from chain end groups, to which biologically active molecules can be covalently attached. Several, postprocessing, surface modification methods have been investigated in an attempt to overcome this limitation. The introduction of functional groups on biodegradable polyester surfaces has previously been achieved by, for example, hydrolysis,[7,8] aminolysis,[7] plasma treatment,[9,10] or simple adsorption.[11,12] In the case of poly(ϵ-caprolactone), previous work has introduced amine groups onto film surfaces through treatment with a diamine, before attaching peptide sequences such as RGD by using either carbodiimide,[13] gluteraldehyde, or epoxy-amine chemistry.[6] Subsequent cell adhesion studies have demonstrated an increase in adhesion and spreading of cells on these modified surfaces.[13] Recent work has also modified PCL with poly(ethylene oxide) grafts before coupling with RGD-containing peptides, again resulting in enhanced cellular responses.[14] However, the majority of these modification methods have several drawbacks including difficulty in controlling the level and distribution of functional groups and the possibility of unknown degradation products. Further, some methods leave the bioactive groups merely adsorbed onto the surface (i.e., they are not covalently attached), meaning that there is a danger of being exchanged or removed upon introduction into existing *in vitro* culture or *in vivo* implantation.

Previous work by this group has reported the synthesis of an amine-specific reactive poly(ϵ-caprolactone) (PCL) and the subsequent attachment of poly(L-lysine) (PLL) to this polymer in solution.[15] Basically, a ketone-containing PCL-based polymer was synthesized as has previously been described by Jerome et al.[16] Chemoselective hydrazine chemistry allowed for the introduction of reactive hydroxyl groups now distanced from the polymer backbone. These hydroxyl groups were activated by 4-nitrophenyl chloroformate, resulting in a polymer now specifically reactive toward amines which could be coupled to desired bioactive molecules. One of the challenges encountered with this approach was the difficulty in finding a solvent system that would adequately dissolve the hydrophobic functional polymer and the hydrophilic bioactive molecule, such as poly(L-lysine) or cell adhesion peptides. In addition, it was unclear as to whether the PCL-PLL comb copolymer would survive subsequent processing steps to prepare the polymer for end-use applications, or further, as to whether the bioactive groups would end up on the surface of the structure where they are desired. To overcome these limitations, we here present a new method for producing polyester constructs with surface-active biological groups, by casting this novel amine-reactive PCL-based polymer into thin films. Compared to the solution coupling technique, coupling the bioactive molecules directly onto solid surfaces also avoids unnecessary loss of expensive biomolecules through being embedded inside the solid substrate and hence out of reach of their intended target. Several techniques have

* Corresponding author. E-mail: gregghq@unimelb.edu.au. Fax: (+61) 383444153.

† Present address: Tissue Engineering and Microfluidics Laboratory, Australian Institute for Bioengineering and Nanotechnology, University of Queensland, St Lucia QLD 4072, Australia.

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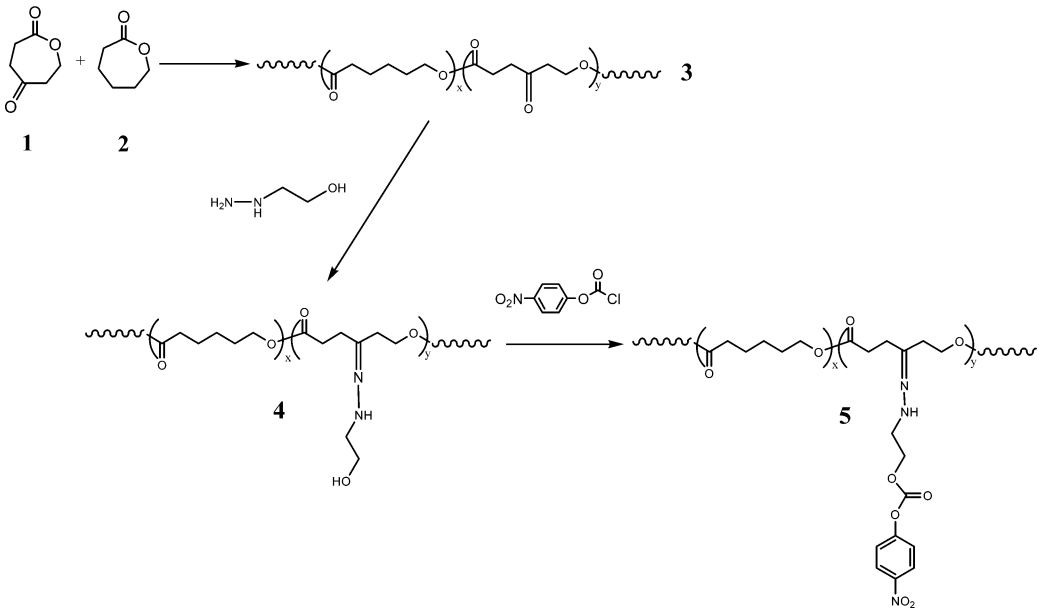

Figure 1. Synthetic scheme showing the copolymerization of the synthesized monomer 2-oxepane-1,5-dione (**1**) and ϵ-caprolactone (**2**) to produce a random copolymer with pendent ketone groups **3**. Hydroxyethyl hydrazine is then conjugated to the ketone groups to produce polymer **4** with pendent hydroxyl groups. These are then activated with 4-nitrophenyl chloroformate to produce NPC-activated polymer **5**.

previously been used to produce poly(ϵ-caprolactone) thin films including solvent casting[17,18] or spin coating[19] onto the appropriate substrate. In this work, films were produced by a variety of methods depending on the film properties required for particular analysis techniques. A wet chemistry method was subsequently used to react the functional groups on the film surface with the hydrophilic bioactive molecules poly(L-lysine) and GRGDS, and cell adhesion to these films was then examined and compared to control films cast from commercially available PCL.

Experimental Section

Materials. 9-Anthracenecarboxylic acid, thionyl chloride, tris(2-aminoethyl)amine (TAEA), poly(ϵ-caprolactone) (MW = 80 000), and poly(L-lysine) HBr (MW = 4000) were all purchased from Sigma Aldrich and used as received. GRGDS peptide was purchased from Bachem. All solvents were AR grade unless otherwise stated and were used as received. Ethyl acetate, dichloromethane, THF were purchased from Ajax Finechem. Acetone-d_6 and CD_3OD were purchased from Cambridge Isotope Laboratories. PBS buffer at pH 7.4 was prepared in our laboratory.

Polymer Characterization. PCL-based polymers were characterized by NMR on a Varian Unity Plus 400 Spectrometer operating at 400 MHz for proton NMR. GPC was used to determine polymer molecular weight (number and weight average) and polydispersity on a Shimadzu system equipped with a Wyatt DAWN DSP MALLS detector and a Wyatt OPTILAB EOS interferometric refractometer. THF was used as the eluent, and three Phenomenex phenogel columns (500, 10^4 and 10^6 Å) were operated at 1 mL/min.

Synthesis of NPC-Activated Polymer. The NPC-functional polymer was synthesized as described previously.[15] In brief an ϵ-CL monomer containing a pendent ketone functional group was synthesized (**1** in Figure 1) and copolymerized with commercially available ϵ-caprolactone (**2**) to produce copolymer **3**. A spacer was then added to the ketone groups by reacting hydroxyethyl hydrazine with the ketone groups to form a hydrazone bond in polymer **4**. Finally the pendent hydroxyl group resulting from this step was activated by reaction with an excess of 4-nitrophenyl chloroformate to afford NPC-functionalized PCL (**5** in Figure 1). GPC-MALLS (THF) $M_n = 9.6 \times 10^3$g/mol, PD = 1.12. ^{1}H NMR (acetone-d_6, 400 MHz): δ (($(CD_3)_2CO$ = 2.05) 8.28 (m, 2H, $CHCNO_2CH_{NPC}$), 7.40 (m, 2H, $CHCOCH_{NPC}$), 4.05 (t, 4H, $CH_2O_{\epsilon-CL+OPD}$), 2.30 (t, 4H, $C{=}OCH_{2\epsilon-CL+OPD}$), 1.65 (m, 8H, $C{=}OCH_2CH_2CH_2CH_2CH_2O_{\epsilon-CL}$ + $C{=}OCH_2CH_2CNCH_2CH_2O_{OPD}$), 1.38 (m, 2H, $CH_2CH_2CH_2O_{\epsilon-CL}$).

Synthesis of Fluorescent Molecule. 9-Anthracenecarboxylic acid chloride (**7**) was first synthesized by adding 9-anthracenecarboxylic acid (4.5mmol) and an excess of thionyl chloride (13.5mmol) to a reaction flask. The reaction was then allowed to stir under an inert argon atmosphere at 60 °C for 16 h. Unreacted thionyl chloride was removed by evaporation to obtain solid 9-anthracenecarboxylic acid chloride. To synthesize the amine derivative of anthracene, tris(2-aminoethyl)amine (12mmol) was dissolved in 10 mL dichloromethane. To this was added dropwise anthracenecarboxylic acid chloride (3.12mmol) in 3 mL of dichloromethane and 2 mL of THF, at 0 °C. The reaction was allowed to warm to room temperature and proceed under stirring for 16 h. The solvent was removed and the solid redissolved in methanol. Precipitation into ethyl acetate allowed for recovery of the amine anthracene product (**8** in Figure 3). ^{1}H NMR (D_2O, 400 MHz): δD_2O = 4.80, 8.55 (s, 1H, arom), 8.12 (d, 2H, arom), 8.04 (d, 2H, arom), 7.56 (m, 4H, arom), 3.33 (s, 2H, $C{=}ONHCH_2CH_2$), 2.91 (s, 4H, $NH_2CH_2CH_2$), 2.63 (s, 6H, $NH_2CH_2CH_2$).

Casting of Thin Films. Thin films for ATR-FTIR and fluorescence microscope analysis were cast by pipetting a 200 μL aliquot of a polymer solution (either 5 or 10%w/v) onto a glass cover slip. This was then allowed to dry before being stored in a desiccator until use. Films used for fluorescent spectrophotometry analysis were spin cast onto glass cover slips from a 5%w/v solution of polymer in dichloromethane. The spin coater was operated at 500 rpm for 15 s, and 200 μL of polymer solution was dropped onto the glass slide. Once removed from the machine, these films were allowed to dry in a fume-hood before further drying and storage in a desiccator until use. Films for cell adhesion studies were prepared as described below.

Attachment of Fluorescent Molecule to the Polymer Films. The amine-functional anthracene was covalently attached to the film surface by first dissolving the molecule in PBS at pH 7.4. Glass cover slips coated with a thin polymer film were submerged in the solution and left for the desired time period. Following this they were removed and rinsed with PBS followed by copious amounts of distilled water to remove any noncovalently linked molecules. The films were then left to dry overnight in a desiccator before analysis.

Polymer Film Characterization. The film surfaces were analyzed by ATR-FTIR (Varian 7000 FTIR equipped with a horizontal ATR attachment) to confirm the presence of NPC functional groups.

Fluorescent microscopy (Olympus IX71 Digital Widefield) was used to visualize the attachment of the fluorescent anthracene to the films, and fluorescence spectrophotometry (Fluorolog Horiba) was used to quantify the level of attachment of the anthracene derivative. Water contact angle analysis (DataPhysics OCA20 Tensiometer) was used to determine the change in surface hydrophobicity and hence the success of the surface reactions. The sessile drop method was used and at least five measurements were taken and averaged for each film surface.

Preparation of Substrates for Cell Culture. Glass slides (13 mm diameter, ProSciTech) were dip-coated into 5%w/v polymer solutions in dichloromethane and allowed to dry overnight in a desiccator. They were then submerged in a 2 mL solution of 0.1 mg/mL of either poly-(L-lysine) (PLL) or GRGDS in PBS pH 7.4 for 4 h. They were washed thoroughly with distilled water and placed into a 24 well plate and allowed to dry. They were then UV sterilized for 15 min prior to cell seeding. The reaction solutions for each film were analyzed for 4-nitrophenol content by UV−vis spectrophotometry (Shimadzu UV-2101PC Scanning Spectrophotometer). This equates to the amount of PLL or GRGDS conjugated to the polymer film.

Cell Adhesion Studies. Mouse 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) in a humidified incubator at 37 °C and 5% CO_2. Cells were counted using a hemocytometer, and approximately 10 000 cells (in 1 mL media) were added to each well. After incubation for 24 h, the cells were fixed with 3% paraformaldehyde for 10 min. The films were washed with PBS, and cell adhesion was visualized using microscopy (Olympus inverted microscope). Several images of each film were taken at 10× and 4× magnification to determine cell density and spreading.

Results and Discussion

The ketone-containing polymer, poly(ϵ-caprolactone-*co*-2-oxepane-1,5-dione) (poly(ϵ-CL-*co*-OPD)), was synthesized and subsequently conjugated with a spacer molecule, hydroxyethyl hydrazine, followed by activation of the pendent hydroxyl groups with 4-nitrophenyl chloroformate as described previously[15] and shown in the reaction scheme in Figure 1.

Using peak integration in NMR analysis, it was found that the polymer used in the following experiments contained 4.2% NPC-functional groups which were reactive toward primary amines. This is considered feasible, as previous studies have shown that polymers with similar levels of reactive functionality, ∼6%[14] or ∼3−4%,[20] have achieved positive cellular responses once modified with bioactive molecules. The polymer had a number average molecular weight (M_n) of 9600Da which equates to four NPC units per polymer chain.

This NPC-activated polymer was cast into thin films either by spin casting, solution casting, or dip coating onto glass coverslips, with the appropriate method chosen depending on the analysis technique to be used. Control films were cast from purchased poly(ϵ-caprolactone) with a molecular weight (M_n) of 80 000Da. In order to prove that the NPC-active groups were present on the surface of the film ATR-FTIR analysis was used, as it typically penetrates only a few microns into the film. Films were produced by spin-casting for this technique. Figure 2 shows the relevant portion of the spectra obtained for both the control PCL film and the NPC-activated film.

It can be seen that while the traces are largely the same, indicating the presence of poly(ϵ-caprolactone), the NPC-activated film trace contains extra peaks at 1520, 1590, 1615, and 1765 cm^{-1} (indicated by the arrows in Figure 2) which are consistent with those expected for the hydrazone bond between hydroxyethyl hydrazine and the polymer backbone, as well as the nitrophenol group of the NPC-activated hydroxyls. This indicates that there are indeed functional groups present on the surface of the NPC-activated PCL films. To further confirm this, as well as provide a model reaction for later coupling with bioactive groups, a fluorescent compound was chosen to react with the films. To closely model the subsequent reaction between a bioactive molecule and the film, which was to be carried out under aqueous conditions, a water soluble fluorescent molecule was required. A primary amine-functional water-soluble anthracene derivative was thus synthesized by first synthesizing the acid chloride of 9-anthracenecarboxylic acid before reacting it with an excess of tris(2-aminoethyl)amine as shown in Figure 3. The presence of multiple amine groups imparts the characteristic of water solubility on the otherwise hydrophobic anthracene.

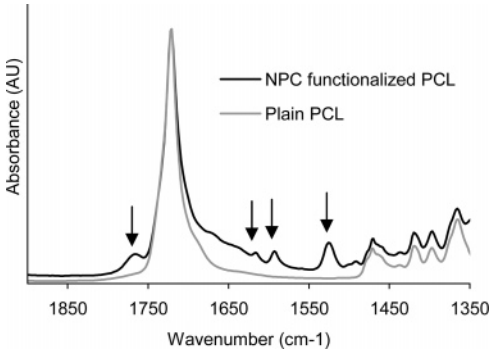

Figure 2. ATR-FTIR spectra of the NPC-functionalized PCL film and the commercially obtained PCL film. Extra peaks at 1520 cm^{-1} (nitrophenyl), 1590 cm^{-1} (amine N−H), 1615 cm^{-1} (hydrazone C═N), and 1765 cm^{-1} (carbonate C═O) (indicated by the arrows) are due to the additional hydrazone bond from hydroxyethyl hydrazine conjugation and the NPC-activating group.

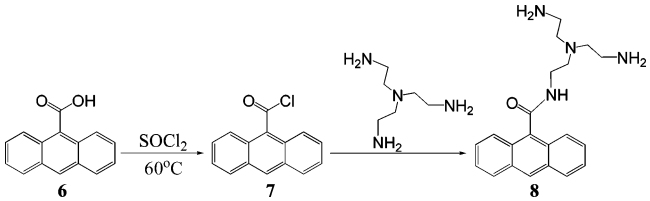

Figure 3. Synthetic scheme for water soluble amine-functionalized anthracene **8**. Reaction proceeds from 9-anthracenecarboxylic acid (**6**) via the acid chloride **7** to the product **8**.

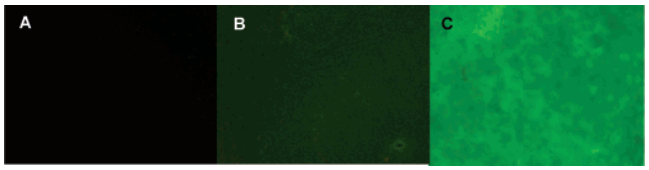

Figure 4. Fluorescent microscope images of poly(ϵ-caprolactone) films after immersion into a solution containing 10 mg/mL amine-functional anthracene in PBS buffer at pH 7.4. (A) Control: purchased PCL, (B) NPC-functional PCL cast from 5%w/v solution, (C) NPC-functional PCL cast from 10%w/v solution.

The chemical conjugation of the amine-anthracene compound to the NPC-functional PCL films was carried out by immersing thin polymeric films into a 10 mg/mL solution of amine-anthracene in phosphate buffered saline (PBS) at pH 7.4. The films were removed from the solution after 24 h and extensively rinsed with PBS followed by distilled water. They were then viewed by fluorescent microscopy with the images shown in Figure 4.

Panel A in Figure 4 shows the control film cast from purchased PCL, immersed in the amine-anthracene solution, and then thoroughly rinsed. Little to no fluorescence can be seen, indicating that the amine-anthracene is not adsorbing onto the

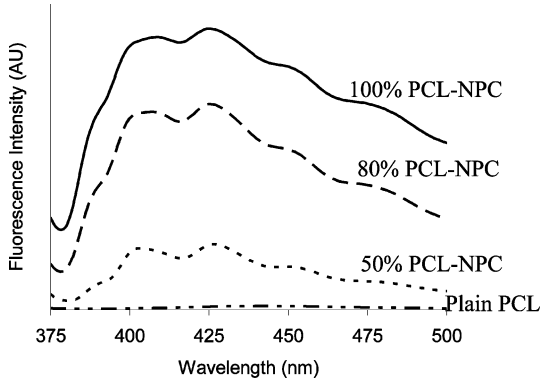

Figure 5. Fluorescence intensity measurements for plain PCL film and films containing various concentrations of PCL-NPC following reaction (4 h) with amine-functional anthracene (all films were spin-cast); excitation wavelength = 355 nm. Characteristic anthracene peaks can be seen at 400−450 nm in all samples containing the NPC-functional polymer, indicating successful anthracene attachment. The fluorescence intensity increases with increasing concentration of NPC functionality in the film.

film surfaces; hence, the washing steps are adequate and future positive results can be solely attributed to successful chemical conjugation. Panels B and C show films cast from solutions containing different concentrations of NPC-activated polymer, 5 and 10%w/v respectively, followed by submersion in the amine-anthracene solution. It can be seen that the brightness of the fluorescence increases in these two films, as would be expected if the number of NPC-active groups present in the film is increasing. These fluorescent microscope images, while visually demonstrative of the success of the reaction, are not quantitative. Therefore, fluorescent spectrophotometry was used to further characterize the surface reaction between the NPC-functional polymer film and primary amine molecules in PBS at pH 7.4. For this analytical technique, films were produced by spin-casting onto glass slides in order to significantly reduce the effect any variability in film thickness would have on the results. Figure 5 shows fluorescence intensity data, using an excitation wavelength of 355 nm, for films of different functional PCL-NPC polymer concentration. All films were cast from a 5%w/v solution of polymer in dichloromethane with the ratio of functional PCL-NPC to plain PCL in each film being varied.

Figure 5 demonstrates that the plain PCL film has no fluorescence, as would be expected from its lack of fluorescent groups, and as was shown by the lack of color in the microscopy images (Figure 4). The NPC-functional films that have been immersed in the amine-anthracene solution display fluorescence intensity peaks corresponding to those inherent to anthracene (400−450 nm) indicating the presence of anthracene and hence the success of the chemical conjugation reaction. It can also be seen that as the concentration of PCL-NPC in the polymer film increases (from 50%, to 80%, to 100%), the intensity of the fluorescence signal also increases. This trend matches that expected with the increase in the number of NPC groups available, leading to an increased number of anthracene molecules coupling to the film and hence a stronger fluorescence signal.

The next point of interest was the time needed for complete reaction between NPC on the surface and a primary amine and therefore whether bioactive functionality could be introduced to the films by simply "dipping" them into a solution containing the desired molecule. These films were spin-cast from a solution containing 5%w/v polymer which consisted of 80% NPC-functional polymer and 20% purchased PCL, the latter of which

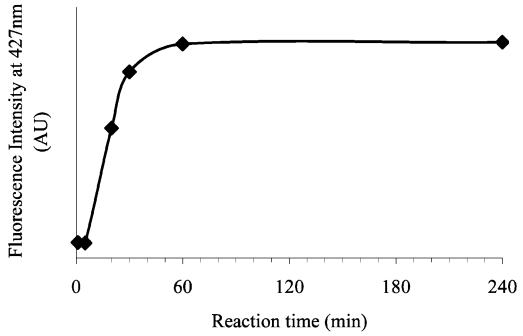

Figure 6. Plot of fluorescence intensity at 427 nm for films containing 80% NPC-functional PCL reacted with amine-anthracene for different reaction lengths. The data for the 24 h reaction is not shown but was of equal intensity as that after 4 h.

Table 1. Composition of ϵ-Caprolactone-Based Polymer Films Used in Poly(L-lysine) and GRGDS Studies. Contact Angle Measurements (Sessile Drop) and Density of Bioactive Groups Attached Are Shown

film no.	composition	contact angle (deg)	bioactive group attached (nmol/cm^2)
1	PCL (100%)	79 ± 1	0
2	PCL-NPC (20%)	75 ± 1	0
3	PCL-NPC (80%)	74 ± 1	0
4	PCL-NPC (20%) + PLL	69 ± 1	0.53
5	PCL-NPC (80%) + PLL	66 ± 1	1.00
6	PCL-NPC (20%) + GRGDS	72 ± 1	0.32
7	PCL-NPC (80%) + GRGDS	71 ± 1	1.08

was included in order to improve the mechanical strength of the film. These films were then immersed into the amine-anthracene-containing solution for a specified period of time ranging from 10 s to 24 h. Following removal they were thoroughly washed with PBS followed by distilled water and dried in a desiccator until analysis. Figure 6 shows the results from fluorescence spectrophotometry with the intensity maxima at 427 nm plotted against reaction time up until 4 h. The data for 24 h is not shown, but the intensity was found to be at the same level as that obtained for the sample reacted for 4 h.

It can be seen that while the coupling reaction is relatively quick, it does require more time than just a simple "dip". Figure 6 shows that the reaction nears completion after around 1 h, with the 4 and 24 h samples demonstrating the same level of fluorescence as that at 1 h.

Following the successful results from the fluorescent study, the NPC-functional PCL films were reacted with bioactive groups, either poly(L-lysine) or GRGDS, in order to investigate the cell adhesion behavior after modification. Films were produced by dipping a round glass slide (13 mm diameter) into a 5%w/v solution of polymer in dichloromethane and withdrawing it slowly to allow for smooth film coverage over the entire glass slide. Control films were cast from solutions containing commercially available PCL, while the NPC-functional films were cast from solutions containing varying concentrations of the commercially available PCL and the synthesized NPC-functional PCL. These films were then submerged in a solution containing 0.1 mg/mL of either poly(L-lysine) or GRGDS in PBS at pH 7.4 with the different film compositions shown in Table 1. After a reaction time of 4 h, the films were removed and washed thoroughly with PBS and distilled water. Previous studies have found that surface topography and casting solvent seem to have little effect on cell adhesion results,[17,21] while it has been suggested that the film thickness might have an effect on cell adhesion in that it modifies the mechanical properties of the films.[21] As all films used in the cell studies reported here

were cast using the same casting method and conditions, it is expected that there will be little difference in these properties across the films.

Poly(ϵ-caprolactone) is a hydrophobic polymer, while poly(L-lysine) and GRGDS are hydrophilic; therefore, a good measure of the success of the surface conjugation reaction is whether any change in surface hydrophobicity can be detected. This was analyzed by using the sessile drop method to measure the water contact angle on the surface of the films, with the results shown in Table 1. The contact angle of the plain PCL film 1 was 79°, agreeing with previously published values for similar films,[19] while the films containing NPC-functional groups (films 2 and 3) showed slightly lower contact angles of 75° and 74°, respectively. This was expected, as the pendent nitrophenol groups increase the hydrophilicity of the polymer. Following conjugation of the hydrophilic poly(L-lysine) to the polymer surface the contact angles reduce significantly to 69° and 66° for films 4 and 5, respectively, indicating successful conjugation. Likewise, conjugation of GRGDS to the film surface reduces the contact angle when compared to the unmodified films, 72° and 71°, respectively, for films 6 and 7. The lesser reduction in contact angle for films conjugated with GRGDS compared to poly(L-lysine) is due to GRGDS being a smaller molecule than PLL, hence having a smaller impact on surface hydrophobicity.

The density of bioactive groups on the surface of the polymer film could be determined by measuring the amount of 4-nitrophenol that had been released during reaction. This was done by measuring the UV absorbance of the reaction mixtures and using a standard curve to calculate the number of moles of 4-nitrophenol released. This was then converted to the number of moles of bioactive group attached by assuming that for every mole of 4-nitrophenol released, one mole of bioactive group had covalently linked to the film surface. This could be converted to the densities shown in Table 1 by dividing the number of moles by the area of the glass slide onto which the polymer film was cast (2.66 cm^2).

It can be seen from analysis of the nitrophenol released that a relatively similar amount of GRGDS has attached to the film when compared to the PLL-modified films (0.32 cf. 0.53 for films containing 20% PCL-NPC and 1.08 cf. 1.00 nmol/cm^2 for films containing 80% PCL-NPC). This indicates the reactions have all gone to the same extent, probably completion. It is known that only a small amount of GRGDS is required to enhance cellular response on polymer surfaces with various reports of $\sim 4 \times 10^{-4}$ nmol/cm^2 being sufficient to result in cell proliferation.[22,23] Various other studies have used peptide densities 6−7 magnitudes higher than this minimum level, a similar amount to that in these modified films, and also reported positive cellular responses.[24]

Cell adhesion studies were carried out by first sterilizing the films, before incubation for 24 h with mouse 3T3 fibroblast cells at a density of 10 000 cells per well. Following incubation, the adhered cells were fixed with paraformaldehyde and visualized using optical microscopy with the resultant microscope images shown in Figure 7.

Fibroblast adhesion and spreading on unmodified PCL, film 1, was limited, with some cells adhering and partially spreading, but the majority remaining rounded, indicating that cellular attachment is not favored on this surface. Films cast from 80% NPC-functionalized polymer which did not undergo any further surface modification reactions (film 3) showed some cells adhering and partially spreading on the surface, indicating that the NPC-functional groups are not completely hindering cell adhesion, or particularly increasing it. This means the cell adhesion results obtained for later films modified with a bioactive molecule are entirely due to the bioactive groups attached to the film surface, not the PCL film itself or the presence of any unreacted NPC groups.

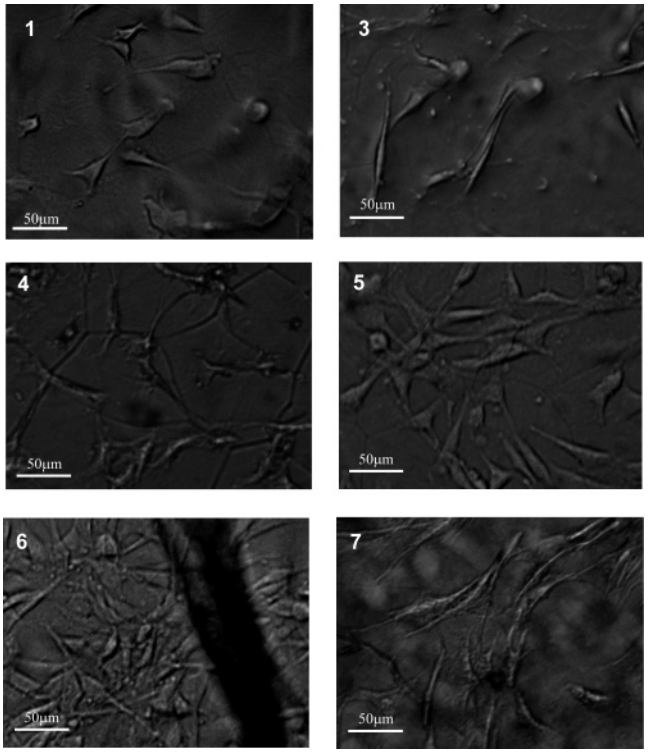

Figure 7. Microscope images from cell adhesion studies on PCL-based films modified with GRGDS. Film 1: plain PCL no reaction; Film 3: PCL-NPC (80%) no further reaction; Film 4: PCL-NPC(20%) coupled with PLL; Film 5: PCL-NPC (80%) coupled with PLL; Film 6: PCL-NPC (20%) coupled with GRGDS; Film 7: PCL-NPC (80%) coupled with GRGDS.

Modification of films with poly(L-lysine) resulted in an increase in cellular adhesion and spreading. Film 4, containing 20% PCL-NPC and immersed in a solution of 0.1 mg/mL PLL in PBS, shows only a slight increase in the number of cells that have adhered to the surface; however, they are better spread than the control films, indicating that they slightly prefer this modified surface. When the proportion of PCL-NPC incorporated into the film was increased to 80% and the film immersed in a PLL solution of the same concentration, film 5 in Figure 7, an increase in the number of cells attached to the surface over the control films (1 and 3) was seen, as well as improvement over the film containing less PCL-NPC (film 4). The cells are also better spread on film 5, indicating that the attachment of poly(L-lysine) to the film surface causes a positive increase in cellular adhesion. However, the cell studies showed only a minor improvement in cellular adhesion and spreading over the control films. This result correlates well with literature where various reports demonstrate that surfaces modified with poly(L-lysine) do not show a significant improvement in cellular adhesion over unmodified surfaces.[3,4,25]

Films modified with GRGDS showed an increased level of cellular adhesion and spreading when compared to the PLL-modified films. Film 6, containing 20% NPC-functional polymer, shows a significant increase in the number of cells attaching to the surface over the control films (1 and 3), and they are elongated, indicating that cellular attachment is favored on the GRGDS-modified film surface. Increasing the level of incorporation of PCL-NPC in a film to 80% before immersing it

into the GRGDS solution (film 7) also invoked a positive cellular response with an increase in the number of cells adhering to the surface when compared to the control films, as well as a significant increase in the elongation of the cells. However, there does not appear to be a large increase in cellular adhesion on film 7 (80% NPC) compared to film 6 (20% NPC); this could be due to the previously reported effect of overpresentation of surface RGD resulting in suboptimal cellular adhesion and migration.[3,26]

These cell adhesion studies demonstrate that only a low incorporation of the NPC-containing polymer in the film is necessary to elicit a positive cellular response following conjugation with GRGDS, and in fact a surface density even lower than that obtained in this study could be sufficient to produce positive results. This means only a low level of incorporation of the NPC-functional polymer is required in order to invoke enhanced cellular responses, reducing the cost and difficulties associated with synthesizing and incorporating the functional polymer into the final substrate.

Conclusions

Casting the previously synthesized NPC-functionalized poly(ϵ-caprolactone) into thin films has enabled straightforward covalent attachment of primary amine-containing molecules as demonstrated by an amine-functional fluorescent anthracene, poly(L-lysine) and GRGDS. Cell adhesion studies with 3T3 fibroblasts showed a slight improvement in cell adhesion and spreading on films modified with poly(L-lysine) and a significant increase in adhesion and spreading on films modified with GRGDS. This method therefore provides a new means of adding covalently attached biological functionality to polymeric surfaces, a technique that could be further utilized in applications such as tissue engineering, drug delivery, and biosensors.

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