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Evaluation of a Gelatin Modified Poly(ε-Caprolactone) Film as a Scaffold for Lung Disease

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Abstract

Lung transplantation is a necessary step for the patients with the end-stage of chronic obstructive pulmonary disease. The use of artificial lungs is a promising alternative to natural lung transplantation which is complicated and is restricted by low organ donations. For successful lung engineering, it is important to choose the correct combination of specific biological cells and a synthetic carrier polymer. The focus of this study was to investigate the interactions of

human lung epithelial cell line NCl-H292 that is involved in lung tissue development with the biodegradable poly(ε-caprolactone) before and after its chemical modification to evaluate potential for use in artificial lung formation. Also, the effect of polymer chemical modification on its mechanical and surface properties has been investigated. The poly(ε-caprolactone) surface was modified using aminolysis followed by immobilization of gelatine. The unmodified and modified polymer surfaces were characterized for roughness, tensile strength, and NCl-H292 metabolic cell activity. The results showed for the first time the possibility for NCI-H292 cells to adhere on this polymeric material. The Resazurin assay showed that the metabolic activity at 24 hours post seeding of 80% in the presence of the unmodified and greater than 100% in the presence of the modified polymer was observed. The roughness of the poly(ε-caprolactone) increased from 4 nm to 26 nm and the film strength increased from 0.01 kN to 0.045 kN when the material was chemically modified. The results obtained to date show potential for using modified poly(ε-caprolactone) as a scaffold for lung tissue engineering.

Keywords: biopolymers, immobilization, tissue cell culture, tissue engineering, viability

INTRODUCTION

64 million people suffer from chronic obstructive pulmonary disease which is a cause of morbidity and mortality worldwide (Rabe, Hurd, and Anzueto 2007). The condition worsens gradually over time, starting usually with a shortness of breath and progressing to respiratory failure and eventually ends in death. There are three common methods to treat the end-stage lung failure caused by chronic obstructive pulmonary disease. Two of them, namely mechanical ventilation and extracorporeal membrane oxygenation, only provide a temporarily solution while waiting for the third treatment - lung transplantation (Lemon et al. 2013). However a full lung

transplant is an expensive procedure, limited by availability of donor organs, and goes accompanied by a high risk of post-operational complications.

Fortunately, tissue engineering which brings together materials, cells and engineering, is offering the future possibility of alternative solution for lung transplantation. It is possible to fabricate a synthetic component composed of scaffold and biological cells which could take over natural lung functions. There are a number of materials that may be used as scaffolds for artificial lung preparation. The scaffold may be made by using the lungs from human cadavers by removing the cellular material from them and then seeding it with stem cells (Petersen et al. 2010; Weiss et al. 2011). However, this is a very laborious and complicated way of regenerating tissue. An easier alternative is to use natural or synthetic polymers to produce the scaffolds for the artificial lungs (Seal, Otero, and Panitch 2001). Natural materials such as collagen, gelatin, or laminin (Liu et al. 1992; Andrade et al. 2007) interact with cells much better compared to synthetic materials due to their bioactive properties (Moscato et al. 2008; Gamez et al. 2012). Nevertheless the poor mechanical performance of the scaffolds made from natural polymers is considered being a drawback in lung tissue engineering. Polymeric biomaterials with excellent mechanical properties, a good degree of biodegrability, and compatibility to native tissue, represent good candidates for tissue formation (Lin et al. 2006). Among the synthetic polymers (Mihai et al. 2011), polyglycolic acid and Pluronic F-127 have been shown suitable materials for lung scaffolds (Cortiella et al. 2006).

Poly(ε-caprolactone) has been commonly accepted as a biomaterial to be used in regenerative medicine because of its biodegradability properties and possible biocompatibility improvement. This biodegradable polymer has been commonly used in a number of applications such as bone regeneration (Zhou et al. 2006), trachea regeneration (Lin et al. 2011), and tissue

engineering of pulmonary artery (Matsumura et al. 2013). It has also been used as a drug carrier for drug delivery (Aqil et al. 2012; Li et al. 2012; Xu et al. 2014) and as a plain film in wound healing (Ng et al. 2007; Fu et al. 2014). A poly(ε-caprolactone) scaffold has been shown to support fibroblasts (Mei et al. 2005), human umbilical arterial smooth muscle cells (Hu et al. 2014), endothelial cells (Guex et al. 2014), and mesenchymal stem cells (Zhao et al. 2014). Poly(ε-caprolactone) is known to produce less acidic products after hydrolysis under physiological conditions compared to other poly(ε-caprolactone) or polyglycolide polymers. This feature makes poly(ε-caprolactone) an attractive substrate for lung tissue engineering.

The important requirement for the successful artificial lung development is the high degree of cell adhesion on the scaffold surface. Because of the hydrophobic nature of most of the polymers including poly(ε-caprolactone), modification of the material to improve its favorability for cell growth is necessary. One of the characteristics needed for scaffolds to support cell growth is biocompatibility (Gamez et al. 2012). Biocompatibility refers to the capability of a material to perform under physiological conditions without inducing toxic effects in the biological system (Lin et al. 2006; Moscato et al. 2008). To increase the biocompatibility of poly(ε-caprolactone), various methods have been developed (Ng et al. 2007; Yuan et al. 2012; De Luca, Terenghi, and Downes 2014) while at the same time the mechanical properties were maintained. Among these methods is aminolysis, which is easy to carry out and seems to bring many advantages (Zhu et al. 2002; Wang et al. 2009; Shaneh et al. 2015) such as reduced hydrophobicity of the surface, easy attachment of the biomolecules and consequently the possibility of cell growth on the surface, their adhesion, proliferation, and differentiation (Mei et al. 2005; Mota et al. 2014).

The presence of amine groups on the surface of polymer enables bonding of gelatin and later cells attachment. Gelatin is a naturally occurring protein which is derived from denaturation of collagen (Moscato et al. 2008; Tripathi, Kathuria, and Kumar 2009). It is a molecule which is combined with synthetic polymers to allow enhanced cell growth (Moscato et al. 2008). The reasons gelatin is selected for this purpose include the presence of functional groups (carbonyl groups) enhancing cell adhesion (Moscato et al. 2008); high viscosity and plasticity (Tripathi, Kathuria, and Kumar 2009; Dhandyuthapani et al. 2011) improving mechanical properties of the final product; biodegradability (Tripathi, Kathuria, and Kumar 2009; Dhandyuthapani et al. 2011); biocompatibility (Moscato et al. 2008; Dhandyuthapani et al. 2011); and relatively low cost (Tripathi, Kathuria, and Kumar 2009; Lim et al. 2011). Gelatin is naturally hydrophilic and its compatibility to natural tissue has been examined (Zhu et al. 2002). Most research indicates that the presence of gelatin increases cytocompatibility of the material.

The material-cell interaction is influenced by surface topography. Atomic force microscopy (AFM) is a common technique for roughness measurement. The technique is based on obtaining a topographic image of the sample surface by scanning its surface in the x and y plane with a probe at the controlled distance (Raposo, Ferreira, and Ribeiro 2007). The quality of the AFM images are optimized with the software and quantitative analysis of the surface topography can be performed. The most common parameter used in characterizing the surface topography is the average roughness. It is the arithmetic average of the values of the deviations of the surface height taken from the mean plane. Another parameters used in quantitative analysis of atomic force microscopy data image very well described can be found here (Raposo, Ferreira, and Ribeiro 2007). The roughness is widely used in the field of bioengineering. Zhang et al. reported that cell response was improved by increasing the roughness of the poly(lactic-co-

glycolic) acid surface from 0.62 to 36.9 nm (Zhang, Chun, and Webster 2010). The other study showed that the adhesion and proliferation of human fetal osteoblasts was enhanced when the roughness of the material reached 15 nm (Lim et al. 2007).

In vitro investigation of binding interactions between cells and carrier materials is a first step to help to assess biocompatibility before in vivo study. Compared to cells with limited lifetime, immortalized cell lines have indefinite possibility to be grown in culture which makes them suitable for in vitro analysis of biocompatibility of synthetic polymers (Altman, Randers, and Rao 1993; Zange, Li, and Kissel 1998; Huhtala et al. 2007; Singh, Wu, and Dunn 2011). Mammalian cell lines are used for such biocompatibility testing; normally a cell line of either human or mouse origin is used. The in vitro environment offers a setting to test the effects of the synthetic material on cell adhesion, proliferation, and viability (Huhtala et al. 2007). From this study, any cytotoxic effects related directly to the presence of the polymer may be assessed (Altman, Randers, and Rao 1993). In vitro models offer a relatively inexpensive, rapid method to provide initial biocompatibility screening that reduces the number of animal studies to be carried out (Zange, Li, and Kissel 1998; Huhtala et al. 2007). In this study, the cell line of choice was the human epithelial cell line NCI-H292. This line was derived from a lymph node metastasis of a pulmonary mucoepidermoid carcinoma.

This paper investigates the interaction of human lung epithelial cell line NCl-H292 with a biodegradable poly(e-caprolactone) and poly(ε-caprolactone) modified with gelatin to consider its use as an implantable material for lung scaffold for treating chronic obstructive pulmonary disease. The cell compatibility of poly(ε-caprolactone) with the NCI-H292 cell line has not previously characterized in the literature. Similarly, the influence of polymer modification on

cell proliferation of the proposed material has not been assessed. The mechanical and structural properties of poly(ε-caprolactone) before and after modification are also be discussed.

EXPERIMENTAL

Preparation and Modification of Poly(ε-Caprolactone) Films

Poly(ε-caprolactone) was prepared using a method previously reported by Yuan et al. (2012). Briefly, 1 ml of poly(ε-caprolactone) solution (1.5×10^{-3} mol/dm^3) in dichloromethane was formed and deposited on a glass substrate. After removal of the solvent at room temperature, the glass substrates coated with thin film of poly(ε-caprolactone) were dried in a vacuum oven for 24 hours at 35°C. The samples were then washed with deionized water and isopropanol followed by drying at room temperature for 12 hours in a vacuum oven. Then a well established surface modification method was used based on an aminolysis procedure described previously (Zhu et al. 2002). A 10% 1, 6-hexanediamine solution in isopropanol was prepared and heated at 40°C with poly(ε-caprolactone) films for 1 hour. The samples were then rinsed with deionized water followed by drying in a vacuum oven over night at 25°C. Having amine groups on the surface, the next step was to attach gelatin to them. The samples were placed in 10 ml of 2.5 wt % glutaraldehyde solution in a phosphate buffer (pH 8) and the reaction was allowed to proceed for 12 hours at room temperature. The samples were rinsed with deionised water and the films were placed in 50 ml of 3 mg/ml gelatin in phosphate buffer with continuous stirring for 24 hours at room temperature. The samples coated with gelatin were washed with deionized water and phosphate buffer for 14 hours.

Surface Characterisation of the Poly (ε-Caprolactone) Films

In this study we have used a Veeco multimode V atomic force microscope in tapping mode to investigate the morphology before and after the surface modification with gelatin via aminolysis process. The surface properties of the material influence the interactions between the cells and the material (Lim et al. 2007; Zhang, Chun, and Webster 2010). Infrared spectroscopy was used to verify the presence of amine groups and gelatin on the surface of the poly(ε-caprolactone) films using a Spectrum One Fourier transform spectrometer, (PerkinElmer, USA).

Mechanical Characterization

The mechanical strength of poly(ε-caprolactone) before and after modification with gelatin was investigated. The comparison between mechanical properties of unmodified and modified poly(ε-caprolactone) was analysed. Tensile tests were done using an Instron model 5565 Universal Electromechanical test system instrument. The test was done at a constant extension rate of 1 mm/min, and the tensile force applied to the specimens (with 10 mm diameter disk shape) was recorded by a 500 N load cell. The test was complete upon sample failure.

Preparation of Polymers for Cell Culture

The polymers were immersed in isopropanol and placed in a sonicator for twenty minutes. The isopropanol was removed and the polymers were rinsed 8 to 10 times with deionized water. The twenty minute sonication was repeated using deionized 18.2 Ω water. The polymers were dried under nitrogen prior to being oxygen plasma (Harrick Plasma) treated at 100 W for twenty minutes.

Cell Culture

NCI-H292 cells were cultured in sterile growth medium. The basal media RPMI- 1640 media was used. This was supplemented with 10% fetal bovine serum (Sigma, Ireland) and stored at 4°C for up to six weeks. Hank's balanced salt solution without calcium and magnesium (Sigma, Ireland) was used in the culturing process as a rinsing agent. Trypsin /EDTA (Sigma, Ireland) was used to detach the cells from the surface of the tissue culture flask. The process first involved removal of spent media that was discarded. The flask was rinsed gently with 10mls warmed Hank's balanced salt solution twice. 3 ml of warmed trypsin-EDTA were added and the flask was incubated for twenty minutes at 37ºC. An excess of warmed routine media (7 ml) was added and centrifuged at 1000 rpm for five minutes. Supernatant was separated and discarded. The pellet was resuspended in routine media and transferred to new flask with a subcultivation ratio of 1/3 to 1/8. A final volume of 30 ml media was required. Media renewal was performed every two to three days. The cells were grown at 37 °C in a humidified 95% air/5% CO_2 environment.

Cell Count-Trypan Blue Exclusion Assay

The Trypan blue exclusion assay is employed to verify the percentage of viable cells that a suspension contains. The basis for this test is that live cells due to an intact cell membrane will exclude Trypan blue dye, whereas dead cells have lost their membrane permeability barrier and so do not exclude the dye (Altman, Randers, and Rao 1993). After exposure to Trypan blue, live cells appear white and dead cells appear blue upon microscopic examination (Altman, Randers, and Rao 1993). Equal volumes of Trypan blue stain and cell suspension were mixed. Cells were counted using a hemocytomer. This is used to determine the quality of the cell suspension prior to use in an experiment. NCI-H292 cells used showed a cell viability of ≥95%.

Exposure Assay

NCI-H292 cells were seeded in a twenty four well plates at 70,000 cells/well in the presence and absence of the test polymers (modified and unmodified polymers were tested). The plate was placed in the incubator for 1 hour to allow cell attachment prior to the volume in each well being made up to a final volume of 1ml in each well. The plate was incubated for an additional 24 hours and the cell viability was determined spectrophotometrically. Excitation was performedat 445 nm and emission at 590 nm with the Resazurin assay and cell morphologic changes were analyzed microscopically.

Optical Analysis-Cell Morphology

The images of the cells were taken using an inverted phase microscope (Olympus CKX41) with a 100× magnification. Qualitative biocompatibility analysis was carried out by comparing the morphology of NCI-H292 cells cultured in the presence of modified and unmodified polymers to control samples.

RESULTS AND DISCUSSION

Preparation and Characterisation of the Functionalised Poly(ε-Caprolactone) Film

The natural hydrophobic properties of poly(ε-caprolactone) and absence of a bioactive functional group (Domb et al. 1997) on its surface restricts its application for tissue engineering by decreasing its biocompatibility. To enable attachment of gelatin to the surface of poly(ε-caprolactone), it was necessary to provide polymer surface with functional groups; in this case, amine groups to which gelatin could bond.

Figure 1 presents infrared spectra obtained of poly(ε-caprolactone) surfaces before and after amine attachment and gelatin immobilization. Poly(ε-caprolactone) included characteristic peaks at 2800 and 2900 cm -1 due to symmetric and asymmetric CH_2 stretching. The absorption at 1726 cm-1 represents the presence of carbonyl groups while the peak at 1299 cm^{-1} is from C-O and C-C groups (Yuan et al. 2012). **Figure 1b** includes absorption at 3521, 1650, and 1550 cm^{-1} characteristic for amide I (N–H), C=O, and amide II N-H. **Figure 1c** includes shifts in the amide I peak to 1650 cm-1 and amide II band to 1550 cm^{-1}, indicating immobilization of gelatin on the surface of the poly(ε-caprolactone)-NH_2. These results are comparable to those obtained by Yuan et al. (2012) and confirm the surface modification of poly(ε-caprolactone) with gelatin.

Further investigation of the poly(ε-caprolactone) film before and after chemical modification was carried out using atomic force microscopy to characterize the surface roughness. The biological response of cells to polymeric material is influenced by number of factors including roughness of the material (Chang 2011) (Yuan et al. 2012). It is important to note that the cell response to roughness varies depending on the cell type.

Figure 2 shows the representative atomic force micrographs of the unmodified and modified poly(ε-caprolactone) surfaces. The uniform and smooth surface has been observed in the unmodified poly(ε-caprolactone) film. The surface roughness was 4 nm (**Figure 2a**). The surface roughness value increased to 26 nm (**Figure 2b**) after aminolysis treatment and gelatin immobilization. The observation that this chemical modification caused an increase in the surface roughness was also reported by Yuan et al. (2012). The increased surface roughness is expected to enhance the performance of the material due to increased surface area available for cell interaction (Lim et al. 2007; Zhang, Chun, and Webster 2010)

Mechanical Properties

The material for lung scaffolds should be mechanically strong and flexible to assure successful transplantation and functioning. One of the reasons that poly(ε-caprolactone) is widely used for tissue engineering is the favorable mechanical properties of the material. Most reported work on mechanical properties of poly(ε-caprolactone) involves poly(ε-caprolactone) composites (Niu et al. 2014; Cocca et al. 2015; Kim et al. 2015) or poly(ε-caprolactone) modified with gelatine without early introduction of amine groups (Zhang et al. 2005; Zhoua et al. 2011; Duan et al. 2013). Although aminolysis is often employed to increase the biocompatibility of poly(ε-caprolactone) as reported in the literature (Zhu et al. 2002; Wang et al. 2009; Yuan et al. 2012; Shaneh et al. 2015), there is no study characterizing the influence of this process on the mechanical properties of the poly(ε-caprolactone). Also, the effects are unknown when gelatine is immobilized on its surface. It is important to ensure that poly (ε-caprolactone) does not lose its strength after modifications to its surface. For this reason, tensile tests were performed before and after modification.

The difference in mechanical behavior between unmodified and modified poly(ε-caprolactone) is shown in **Figure 3**. The modified poly(ε-caprolactone) requires a higher force to break the film than the unmodified poly(ε-caprolactone) with a difference of 6 N. However, while the modification makes the poly(ε-caprolactone) stronger, its stretching ability is reduced compared to the unmodified poly(ε-caprolactone). The modified poly(ε-caprolactone) was observed to be stiff, brittle, and less strong compared to unmodified poly(ε-caprolactone) which is more flexible and ductile and breaks at less than half the strength (0.0045 kN) of modified poly(ε-caprolactone) . Ideally, a compromise should be obtained, with reduced strength but with

more flexibility. Mechanical strength of the material is an advantage for scaffolds in artificial lung engineering, while flexibility accommodates the breathing action of the lungs.

In Vitro Cell Culture

The biocompatibility of a material indicates that the material after contact with a living organism is not toxic. The biocompatibility of modified and unmodified poly(ε-caprolactone) was evaluated in vitro using the resazurin assay and by cell morphology investigation of the cells cultured in direct contact with poly(ε-caprolactone). The resazurin assay was performed to measure the reduction of the blue non-fluorescent 1,2,3 resazurin dye (7-hydroxy-3H-phenoxazin-3-one-10-oxide) to pink fluorescent resorufin by metabolically active cells indicating cell viability. The reduction of resazurin correlates with the number of live mammalian cells present (Zange, Li, and Kissel 1998; Strober 2001; Weaver et al. 2010; Cavet et al. 2012). The reduction was quantified by spectrophotometrical measurements at 445 nm and emission at 590 nm. The results for the poly(ε-caprolactone) cell proliferation test, modified and unmodified, are presented in **Figure 4**. When the cells were incubated with modified poly(ε-caprolactone), the viability of the sample (103%) slightly exceeded the control (100%) and unmodified poly(ε-caprolactone) (80%). Due to the nature of the assay, it was possible to have viability greater than 100% when comparing with the positive control. The control was used as an indication of cell growth and a benchmark for ensuring that the cells were healthy and responding properly to their environment. If the control was affected by the assay, it would signal that something else was causing the cells to undergo a cytotoxic response as opposed to the material or surface that the cells where being cultured on. This test indicates that the modified poly(ε-caprolactone) samples did not induce cytotoxic effects and increase cell proliferation. Both poly(ε-caprolactone) samples provided acceptable biocompatibility compared with the control. The modied poly(ε-

caprolactone) caused a slight increase in cell viability compared to the control and a larger increase in viability when compared to the unmodified sample. The results confirmed the biocompatibility of the modified poly(ε-caprolactone) and the positive effect on cell behaviour. These results also showed for the first time that human lung epithelial type NCI-H292 cells may adhere to and proliferate on the poly(ε-caprolactone) material.

To analyze the cell morphology, images were taken from modified and unmodified poly(ε-caprolactone), as shown in **Figure 5**. The results showed that the cells demonstrated the same characteristics after incubation with both poly(ε-caprolactone) samples. No obvious morphological differences between the unmodified poly(ε-caprolactone) and modified poly(ε-caprolactone) samples were apparent using thane inverted phase microscope with a 100× magnification. The results confirmed biocompatibility of the modified poly(ε-caprolactone) and positive effects on cell behavior and that human lung epithelial cells may adhere to and proliferate on poly(ε-caprolactone).

CONCLUSIONS

Poly(ε-caprolactone) is widely used in regenerative medicine with many applications in tissue engineering. Here an application has been added with a successful evaluation to use poly(ε-caprolactone) as a lung scaffold for chronic obstructive pulmonary disease.

The aminolysis, as previously reported (Zhu et al. 2002), is an easy to employ chemical process that has been used to increase the biocompatibility of poly(ε-caprolactone). Accordingly, integration between the scaffold material and the lung cells is crucial for tissue formation. Here, a poly(ε-caprolactone) modification (Zhu et al. 2002) was employed for the human epithelial cell line NCI-H292. Unlike previous work on poly(ε-caprolactone) material modified with gelatin by

aminolysis, this work focused on structural and mechanical properties, and showed the possibility of using the material for the chronic obstructive pulmonary disease.

Gelatin was immobilized on poly(ε-caprolactone) surface with the amine groups. The modification of poly(ε-caprolactone) increased the strength of the material, but with the loss of flexibility, and increased the biocompatibility of the material. Both poly(ε-caprolactone) samples did not present cytotoxic effects and the cells spread well as observed by optical microscopy. Cell proliferation on the modified poly(ε-caprolactone) increased compared to the unmodified film. **Table 1** summarizes the properties of modified and unmodified poly(ε-caprolactone).

Poly(ε-caprolactone) modification with gelatin was shown for the first time to not negatively affect the spreading of human epithelial cell line NCI-H292 cells and increased metabolic activity at 24 hours post seeding to 103% compared to control samples. The results show that poly(ε-caprolactone) modified with gelatin is a promising material for lung scaffold engineering. In order to provide a reliable polymeric material for successful artificial lung development, the lifetime prediction of the polymer in natural environment must be investigated. This parameter will be evaluated in future work.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

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Table 1. Summary of poly(ε-caprolactone) properties before and after chemical modification with amine groups and gelatin

Parameter	Unmodified poly(ε-caprolactone)	Modified poly(ε-caprolactone)
Biocompatibility	Yes	Yes
Applied force at which the film breaks	0.006 kN	0.012 kN
Surface roughness	4 nm	26 nm
Cytotoxicity	No	No
Viability	80%	103%

Figure 1. Infrared spectra of (a) poly(ε-caprolactone), (b) poly(ε-caprolactone) after aminolysis, and (c) poly(ε-caprolactone) after aminolysis and immobilization with gelatin.

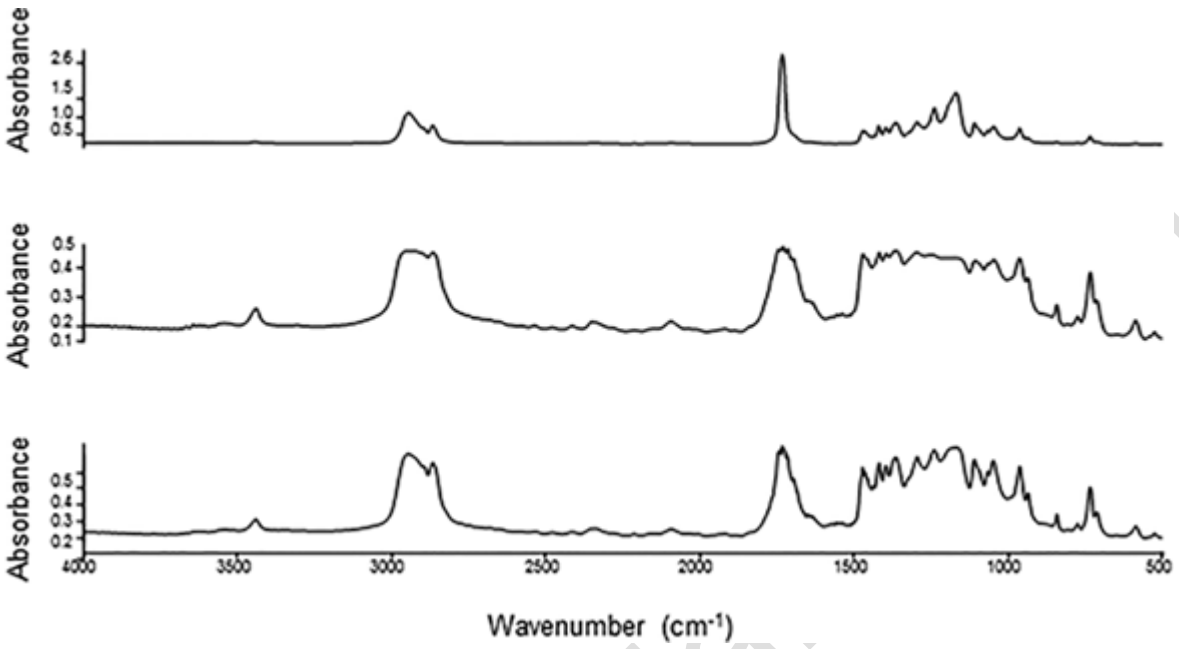

Figure 2. Atomic force micrographs of (a) unmodified poly(ε-caprolactone) and (b) modified poly(ε-caprolactone).

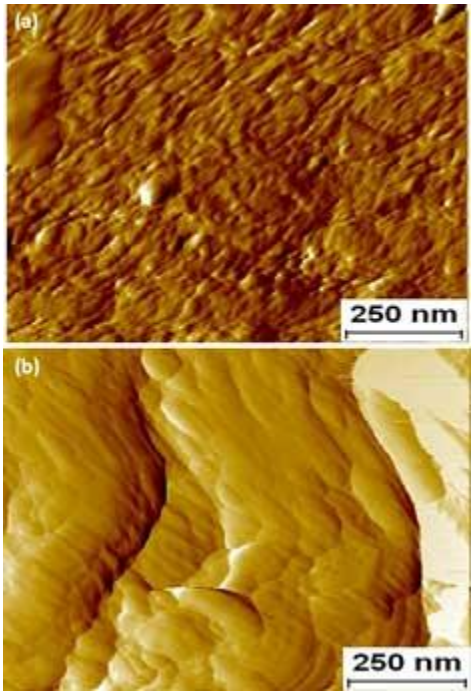

Figure 3. Load-extension curves of unmodified and modified poly(ε-caprolactone).

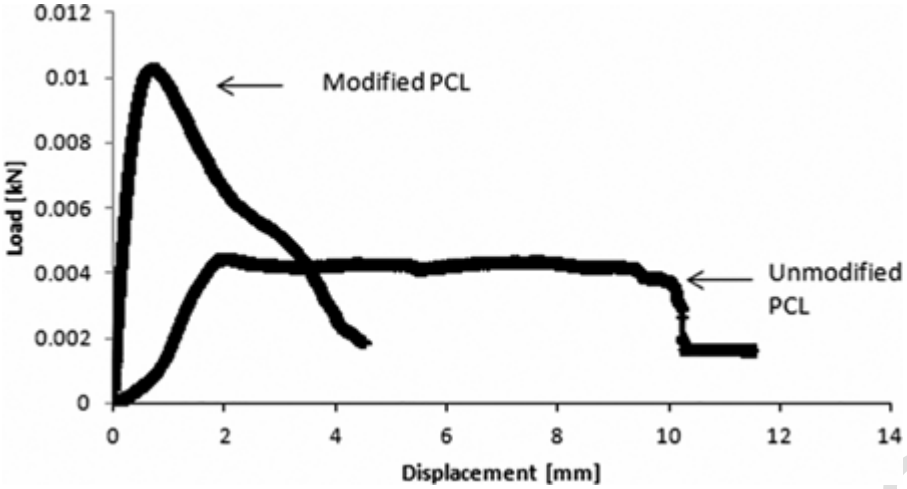

Figure 4. Cell viability in presence of unmodified and modified poly(ε-caprolactone) extracted after 24 hours of culture. For modified samples, $n = 3$; for unmodified samples $n = 4$. Statistical comparison was made using a one sample Student's t-test. A value of $p < 0.05$ was considered to indicate statistical significance. All results were shown to be statistically significant; $p < 0.001$.

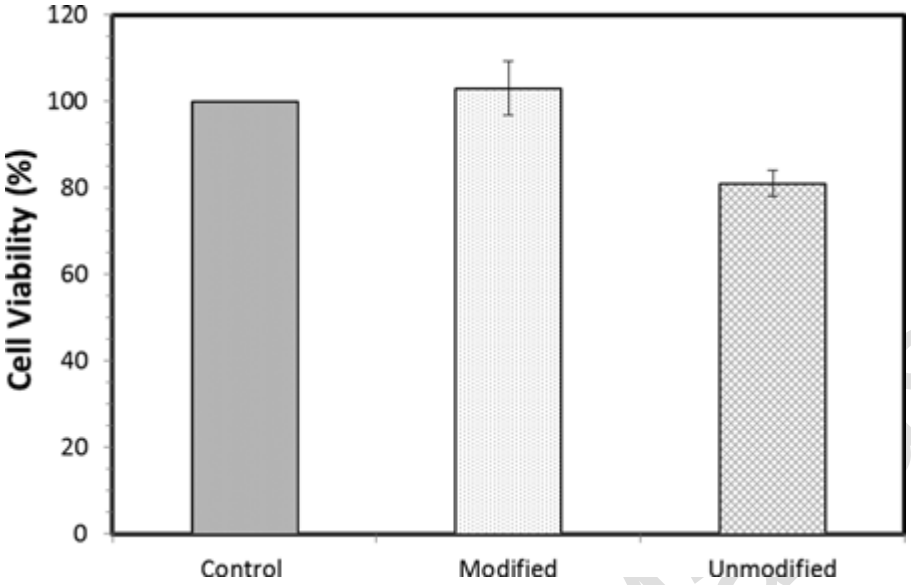

Figure 5. Images of cell cultures obtained following 24 hours for (a) unmodified poly(ε-caprolactone) and (b) modified poly(ε-caprolactone) using an inverted phase microscope with 100× magnification.