ADVANCED FUNCTIONAL MATERIALS
www.afm-journal.de

A Single Component Conducting Polymer Hydrogel as a Scaffold for Tissue Engineering

Damia Mawad, Elise Stewart, David L. Officer, Tony Romeo, Pawel Wagner, Klaudia Wagner, and Gordon G. Wallace**

Conducting polymers (CPs) have exciting potential as scaffolds for tissue engineering, typically applied in regenerative medicine applications. In particular, the electrical properties of CPs has been shown to enhance nerve and muscle cell growth and regeneration. Hydrogels are particularly suitable candidates as scaffolds for tissue engineering because of their hydrated nature, their biocompatibility, and their tissue-like mechanical properties. This study reports the development of the first single component CP hydrogel that is shown to combine both electro-properties and hydrogel characteristics. Poly(3-thiopheneacetic acid) hydrogels were fabricated by covalently crosslinking the polymer with 1,1′-carbonyldiimidazole (CDI). Their swelling behavior was assessed and shown to display remarkable swelling capabilities (swelling ratios up to 850%). The mechanical properties of the networks were characterized as a function of the crosslinking density and were found to be comparable to those of muscle tissue. Hydrogels were found to be electroactive and conductive at physiological pH. Fibroblast and myoblast cells cultured on the hydrogel substrates were shown to adhere and proliferate. This is the first time that the potential of a single component CP hydrogel has been demonstrated for cell growth, opening the way for the development of new tissue engineering scaffolds.

1. Introduction

Conducting polymers (CPs) are impacting on a wide variety of bioapplications such as neural interfaces,[1] biosensors,[2] and as drug delivery,[3] bioerodible[4] or biodegradable platforms.[5] In particular, CPs have exciting potential as scaffolds for tissue engineering, typically applied in regenerative medicine applications.[6] Regenerative medicine holds the promise of treating or curing a wide range of conditions such as burned skin,[7] damaged muscle tissue[8] and spinal cord injury.[9]

The optimal physical and biological characteristics of a scaffold for tissue engineering include tissue-like mechanical properties, a hydrated environment, biocompatibility (preferably enhancing cell growth) and biodegradability or bio-erodibility. Many of these characteristics are found in hydrogels rendering them excellent candidates for tissue scaffolds.[10,11] However, scaffolds for muscle and nerve cell regeneration would also benefit from electrical conductivity, which has been shown to provide enhanced cell growth and differentiation.[12,13] Electrically conductive hydrogels (ECHs) could fulfill this role. Largely, however, ECHs are polymeric composites fabricated from two components; a hydrogel component that provides a highly hydrated environment and a CP component that provides electrical conductivity. Gilmore et al.[14] were the first to report the fabrication of such a composite, a material based on polypyrrole directly electropolymerised on a preformed polyacrylamide hydrogel. Since this proof of principle, ECHs have been the subject of growing attention and a variety of composite hydrogels have been developed.[15]

Table 1 lists examples of ECHs composites for which conductivity has been reported to date.[16–24] While there is no defined level of conductivity required for tissue engineering scaffolds, it can be seen that all these materials exhibit, at best, moderate conductivities. As stated by Bendrea et al.,[25] despite the low conductivity, CP-based materials should still be able to pass the low currents that are potentially required for cell stimulation (in the range of 10 to 100 μA[26]). Although these composite ECHs have been promoted as suitable tissue scaffolds, they have a series of limitations. The reported conductivities were not measured at physiological pH (7.4) or physiological temperature (37 °C); for instance, the conductivity of polyaniline (PANI) would significantly drop at physiological pH because it turns back to its neutral state. As the CP is typically physically entrapped in the hydrogel matrix, it can leach out as the hydrogel network swells[27] leading to a drop in the conductivity of the system as well as possible toxic side effects. In cases where the CP is ionically bound to the non-conductive polymeric component, for instance PANI polycation with the residual acrylic acid units in polyacrylamide, the ionic interaction dissociates as the hydrogel swells at physiological pH due to the neutralization of the PANI chains.[18]

The development of a single component non-hybrid conductive hydrogel could overcome the aforementioned limitations.

Dr. D. Mawad, Dr. E. Stewart, Prof. D. L. Officer, T. Romeo, Dr. P. Wagner, Dr. K. Wagner, Prof. G. G. Wallace
ARC Centre of Excellence for Electromaterials Science
Intelligent Polymer Research Institute
University of Wollongong
Wollongong, NSW, Australia
E-mail: damia.mawad@unswalumni.com; gwallace@uow.edu.au

DOI: 10.1002/adfm.201102373

Table 1. Reported conductivities of ECHs composites.

ECHs	Conductivity [S/cm]	Refs
Pani-PAMPS[a)]	1.3×10^{-3}	[16]
Pani-polyacrylate	2.33×10^{-3}	[17]
Pani-PAAm	0.62	[18]
Aniline tetramer-PLA-PEG-PLA	4.69×10^{-7} - 1.05×10^{-4}	[19]
Pani-PVA	97.45×10^{-6}–130.3×10^{-6}	[20]
pPy-pHEMA composite[b)]	76×10^{-3}	[21]
PEDOT_PSS	4.1×10^{-3}	[22]
PEDOT-S	10^{-2}–10^{-1}	[23]
PTAA-ADP	10^{-2}	[24]

[a)]Pani: poly(aniline); PAMPS: poly(2-acrylamido-2-methyl propane sulphonic acid);[b)]pPy: polypyrrole;pHEMA: poly(hydroxyethylmethacrylate).

To date, there are only three single component hydrogels reported in the literature.[22–24] While all these studies reported effective approaches for the fabrication of conducting polymer hydrogels and reasonable conductivities were reported (Table 1), none of these reported on the hydrogel characteristics appropriate for a tissue scaffold as discussed above. In particular, conductivities were not measured at physiological pH, the ability of these hydrogels to maintain their three dimensional (3D) integrity at physiological pH was not determined, mechanical properties were not explored and the potential of the hydrogel to promote cell adhesion and growth was not investigated.

Herein we report for the first time the development of a single component CP hydrogel characterized for potential use as a scaffold in biomedical applications. The hydrogel is based on PTAA, covalently crosslinked using 1,1′-carbonyldiimidazole (CDI) as represented in **Figure 1**. PTAA was chosen for the simplicity of its synthesis; however the crosslinking method has the potential to be applied to other CPs with the appropriate functional groups. The benefits of covalently crosslinking the polymer are increased stability and enhanced mechanical strength. In contrast to the condensing agent, DCC, employed previously for fabrication of PTAA hydrogels,[24] CDI and its decomposition products are water soluble and they could be easily washed from the network. Additionally, the anhydride bond we employ has been shown to be biodegradable.[28]

Hydrogels were fabricated with three different crosslinking densities by varying the molar ratio of DCI to the polymer repeating unit. Their swelling behavior and mechanical properties were assessed as a function of the crosslinking density. The electroactivity and conductivity of these hydrogels, at physiological pH, were also determined. The feasibility of using the hydrogel surface for adhesion and proliferation of two cell types, fibroblast L929 and C2C12 myoblast cells, and the response of primary myoblasts to the polymer solution was investigated.

2. Results and Discussion

A single component PTAA hydrogel was successfully fabricated by covalently crosslinking the polymer with DCI. Hydrogel properties as well as the electronic properties of the system were characterized.

2.1. Hydrogel Fabrication

PTAA hydrogels were fabricated by reacting poly(3-thiopheneacetic acid) with DCI in dimethyl sulfoxide (DMSO, Figure 1). DCI was used as a coupling agent; it is extensively used for the activation of carboxylic groups resulting in a reactive *N*-acylimidazole intermediate and the release of carbon dioxide (CO_2).[29] The carboxylic groups of the polymer reacted with DCI as indicated by the evolution of CO_2 resulting in an activated intermediate. Over time, the intermediate reacted with the available free carboxylic groups causing crosslinking of polymer chains by anhydride bond formation.[30]

Gels were removed from the vials; they were dark red in color and cylindrical in shape. Specimens of each crosslinking density were transferred into 20 liter deionized water (DI-H_2O) and left to soak overnight. The DMSO was exchanged with water

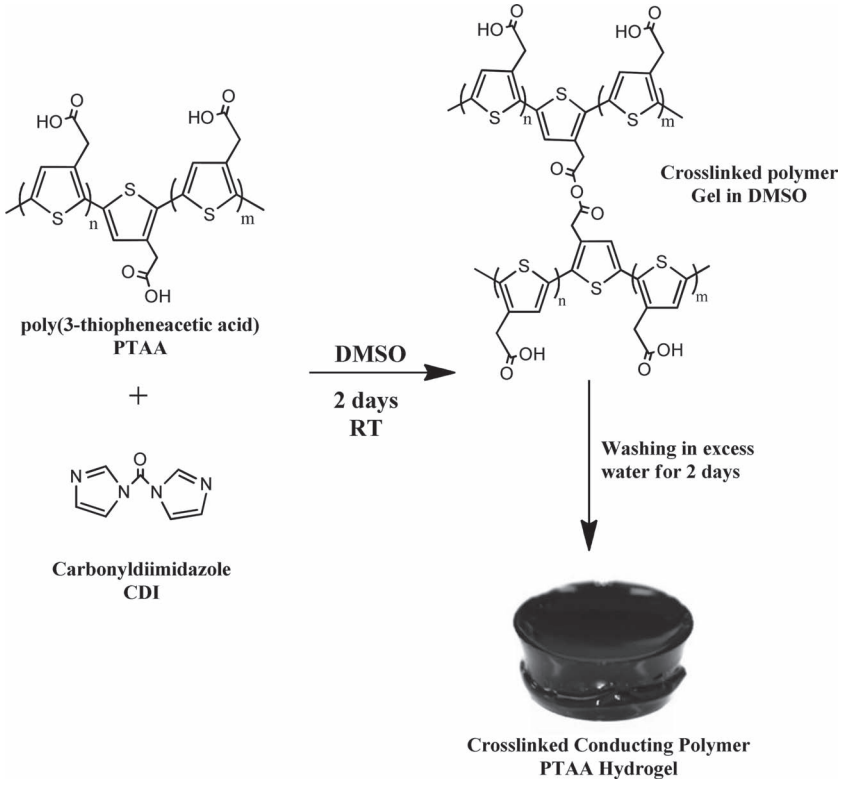

Figure 1. Schematic illustration of hydrogel fabrication.

resulting in the hydrogels characterized in this study. It was observed that the water turned yellow after the first wash mainly due to unreacted polymeric chains that had leached out of the gel. The washing procedure was repeated twice for 3hrs before no peaks corresponding to either DMSO or DCI was detected in the GC. Hence, it could be concluded that two washes in water are effective in exchanging the DMSO with water in the hydrogel. Notably, the use of DMSO in the hydrogel fabrication should not impose any limitation on their biocompatibility because of the effectiveness of the purification procedure.

Previously, PTAA hydrogels were prepared by crosslinking PTAA polymer using adipoyl dihydrazide (ADH) as the crosslinking agent and *N,N′*-dicyclohexylcarbodiimide (DCC) as a condensation agent.[24] An advantage of the fabrication method we describe here over the carbodiimide agent previously used to produce the gels is that the byproducts of the current protocol (carbon dioxide and imidazole) are readily separated from the gels by simple repeated washing in water.

2.2. Hydrogel Characterization

The swelling behavior of these hydrogels, which influences their application in drug delivery or tissue engineering, was gravimetrically determined as a function of time (**Figure 2**). All hydrogels swelled and reached equilibrium within 1 day incubation (Figure 2a). The equilibrium swelling ratio is an important parameter reflecting the amount of water in the network at equilibrium and is determined by parameters such as crosslinking density, hydrophilicity, and the degree of ionization of functional groups within the gel. There was a significant difference ($p < 0.0001$) in the equilibrium swelling ratio (q) of the hydrogels of various crosslinking density. As shown in Figure 2b, q decreased from 9.35 ± 1.12 to 3.97 ± 0.13 as the ratio of the crosslinker was increased from 0.5 to 2. Similarly, hydrogels with the lowest crosslinking density (LXL-PTAA) exhibited significantly higher amount of water uptake ($p < 0.0001$, Figure 2a). The swelling of PTAA gels is due to the dissociation of the carboxylic groups at pH = 7.4.[31] The ionized carboxylic groups result in electrostatic repulsion between the polymeric chains leading to water uptake and swelling of the network.

CP hydrogels exhibited a low to moderate degree of swelling (see **Table 2**). This result is to be expected due to the hydrophobic nature of the PTAA polymer backbone. In comparison to widely used hydrogels, the swelling degree of the HXL-PTAA hydrogels (~344%) is comparable to that of the poly(HEMA) based hydrogels that are widely known to display low levels of swelling.[32,33] Upon decreasing the crosslinking density, the degree of swelling was dramatically increased to 879%. In comparison to some reported ECHs,[34,35] these hydrogels exhibit a significantly higher swelling ratio which is an advantage for bioapplications including tissue engineering and drug delivery. Equilibrium swelling studies demonstrated that the structural properties of the CP hydrogels can be controlled by varying the crosslinking density of the network.

The compressive moduli of hydrogels were determined as function of their crosslinking densities (Table 2). The compressive modulus significantly varied as function of the crosslinking density ($p < 0.0001$) increasing from 1.22 kPa for LXL-PTAA hydrogel to 17.88 kPa for HXL-PTAA hydrogel. This significant change in the mechanical behavior can be attributed to the higher crosslink density in HXL-PTAA. This result demonstrates the ability to control the mechanical properties of these

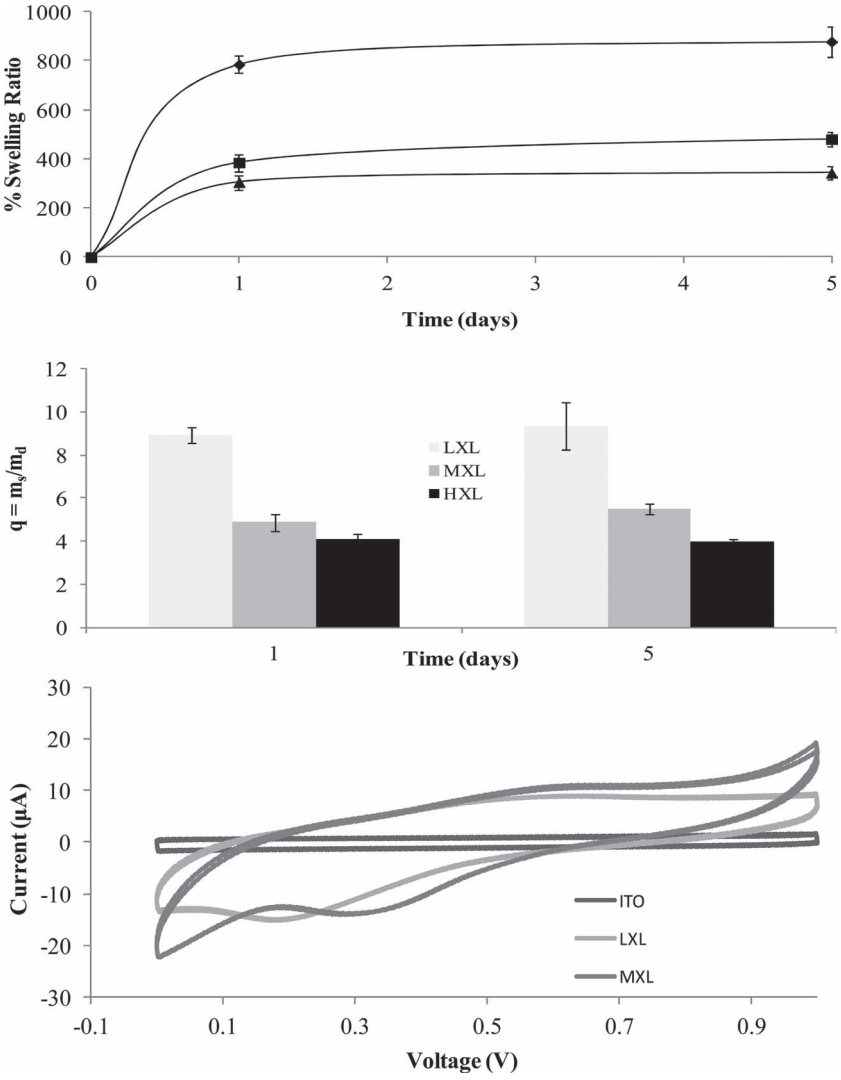

Figure 2. a) Percentage swelling ratio of PTAA hydrogels with different crosslinking densities. ◆) LXL; ▲) MXL; ■) HXL. b) Swelling ratio (q) as a function of time. Mean ± S.D, n = 9. c) Cyclic voltammograms (100 mV s^{-1}) of ITO bare electrode, LXL, and MXL hydrogels in 0.1 M PBS.

Table 2. Hydrogel: label, crosslinking density, % swelling ratio and mechanical properties.

PTAA Gel Label[a)]	Crosslinking Density Moles of DCI/ Moles of repeating unit of polymer	% Swelling ratio	Compressive Modulus [kPa]
LXL	0.5	878.55 ± 61.04	1.225 ± 0.685
MXL	1	480.97 ± 30.99	12.820 ± 0.701
HXL	2	343.36 ± 27.88	17.875 ± 1.473

[a)]LXL = low crosslinking; MXL = medium crosslinking; HXL = high crosslinking.

hydrogels by merely varying the crosslinking density during fabrication of the gels.

Given the importance of porosity for tissue scaffolds, the internal 3D structure of the hydrogels was examined by SEM. **Figure 3** shows cross-sectional micrographs of the networks. All hydrogels had porous interior structures and interconnected pores, with the pore size distributions and wall structures varying with the cross-linking density used. In agreement with the physical and structural properties described earlier, the thin sheet-like wall structure for LXL-PTAA (Figure 3a) implied a low mechanical strength. Conversely, hydrogels with the highest crosslinking density (HXL-PTAA, Figure 3c) possessed a thick wall structure and displayed higher mechanical strength.

The electroactivity of perchlorate doped hydrogels anchored on ITO was investigated using cyclic voltammetry (CV) in 0.1 M PBS. Figure 2c shows the CVs of ITO bare electrode and that of the doped LXL and MXL-PTAA hydrogels. A significant increase in the peak currents can be observed in comparison to the ITO bare electrode indicating an increase in the effective surface area of the electrode due to the hydrogel coating. The CVs of the hydrogels show similar responses exhibiting an anodic peak at 0.57 V and a broad reduction peak between 0.20 and 0.30 V. While the crosslinking density does not appear to affect the overall charge carrying capability of the hydrogel, the reduction potentials of the oxidised hydrogels are affected; the less crosslinked hydrogel is more difficult to reduce. This maybe due to the fact that the oxidised MXL-PTAA is more conductive in PBS than LXL-PTAA hydrogel as described below. However, it is clear that effective charge transport can occur through the crosslinked hydrogels even in their swollen state.

The electrical conductivities of the hydrogels were determined using *I–V* curves and the results are listed in **Table 3**. Following the washing procedure in water, conductivities of the water swollen neutral hydrogels in the order of 10^{-6} S/cm were obtained and these low conductivities are attributed to the fact that the polymer used for hydrogel fabrication is undoped. After swelling in PBS, conductivities of all hydrogels increased by an order of magnitude (10^{-5} S/cm). This increase is likely due to the ionic conductivity of the buffer. To confirm whether the conductivity being recorded was merely ionic, we prepared a poly(acrylic acid) (PAA) hydrogel and attempted to measure its conductivity in the swollen state with the 2-probe setup used for CP hydrogels. However, we could not record a current response. This result suggests that the conductivities being recorded for the CP hydrogel are mainly accounted for by the presence of the conducting polymer component.

Upon doping the hydrogels with perchlorate (water swollen $HClO_4$ doped, Table 3), conductivities increased significantly (from 10^{-6} to 10^{-3} S/cm). These values are comparable and in some instances higher than other reported ECHs (Table 1). Of particular interest, the conductivity of the doped hydrogels falls in the range of reported conductivities for muscle tissue (10^{-3} to 10^{-2} S/cm, frequency dependent)[36] suggesting that these scaffolds could potentially be used as platforms for stimulating muscle cells.

Since the hydrogels are intended as scaffolds for tissue engineering, we then measured the conductivity of the perchlorate doped hydrogels re-swollen in PBS at 37 °C. Although the conductivities of reswollen MXL and HXL doped hydrogels were comparable to those of the water swollen $HClO_4$ doped hydrogels, they were an order of magnitude higher than the un-doped PBS swollen materials (10^{-4} vs. 10^{-5} S/cm respectively). It is worth noting that the hydrogels do shrink slightly after doping in acid. The shrinkage of the network may cause the conducting polymeric chains to be in closer proximity facilitating electron transfer in the network. There is little difference, however, between the MXL and HXL $HClO_4$ doped hydrogels and their re-swollen analogues. This is likely due to limited water uptake and minimal dedoping. In contrast, the drop in conductivity of re-swollen LXL $HClO_4$ doped hydrogels could be explained by the higher degree of swelling of these hydrogels, which in turn causes expansion of the network resulting in separation of the polymeric chains and/or possible loss of dopant. Further, more detailed studies are required in order to elucidate the specific causes of these conductivity changes.

Alternative aqueous-based dopants/oxidants such as $Fe(ClO_4)_3$ and KI/I_2 were selected because they are reported to be biocompatible to a certain concentration.[37,38] LXL-PTAA hydrogels doped in $Fe(ClO_4)_3$ and KI/I_2 aqueous solutions showed conductivities in the order of 10^{-4} S/cm comparable to $HClO_4$ doped hydrogels re-swollen in PBS. While we determined in the first instance the conductivity of these hydrogels as they were incubated for one day in PBS at physiological temperature, it remains important to assess the electrical stability of these hydrogels over incubation times appropriate for

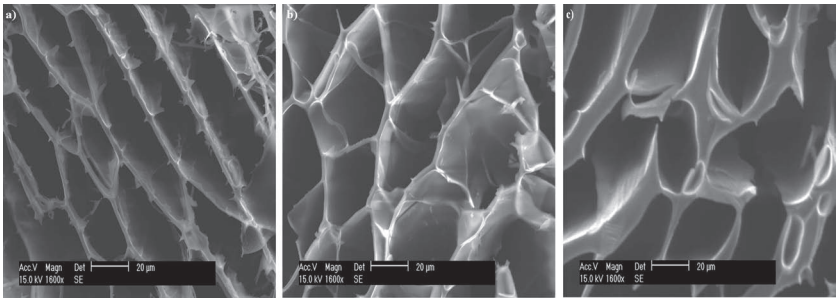

Figure 3. SEM microscopy images of a) LXL, b) MXL, c) HXL- PTAA hydrogels.

Table 3. Conductivity [S/cm] of PTAA hydrogels in their various states.

Hydrogel State	LXL	MXL	HXL
Water swollen neutral	4.96×10^{-6}	2.86×10^{-6}	2.15×10^{-6}
PBS swollen	8.35×10^{-5}	2.90×10^{-5}	1.26×10^{-5}
Water swollen $HClO_4$ doped	1.45×10^{-3}	1.18×10^{-3}	4.85×10^{-4}
PBS re-swollen $HClO_4$ doped	4.15×10^{-4}	1.04×10^{-3}	3.46×10^{-4}
Doped in KI/I_2 aqueous solution	3.41×10^{-4}	N/A	N/A
Doped in $FeClO_4$ aqueous solution	2.92×10^{-4}	N/A	N/A

the application. However, the applications envisaged in this study (nerve/muscle regeneration) do not require long term conductivity but one matched to the tissue repair and polymer degradation process. The question over longer term stability of organic conducting polymers in aqueous environments should not become a limiting factor here.

It is worth noting that reported conductivities of the pristine PTAA undoped and doped polymer are in the order of 10^{-8} and 10^{-7} S/cm respectively.[39] It is evident that the hydrogel conductivity is thus limited by the low conductivity of the polymer itself.

2.3. Cell Studies

Since the PTAA hydrogels are intended for use as scaffolds in tissue engineering, fibroblast and C2C12 myoblasts were cultured on the hydrogel substrate and their growth and differentiation were qualitatively assessed. The response of the myoblast cells was of particular interest given the modulus range of the crosslinked hydrogel material and its comparability to that of muscle tissue,[40] as well as the conducting nature of the material. Also, the cytotoxicity of the PTAA polymer itself was determined in case any polymeric chains leach out after soaking the hydrogels in the culture media.

2.3.1. Cytotoxicity of PTAA Polymer Solution

The cytotoxicity of the PTAA polymer was tested against L929 (rat fibroblast) and primary muscle cells. The effect of 48 h exposure to a dilution series of the PTAA polymer in cell culture media was determined by comparing cytosolic LDH activity of exposed cells to control cells. Results are shown in **Figure 4**. Cell numbers were also compared following exposure to the polymer solutions using a PicoGreen cell number assay (data not shown). Results obtained utilising the LDH activity and the cell number based on PicoGreen correlated well in determining the IC_{50} of the polymer for both cell lines tested. The L929 cell line showed a higher tolerance with a 50% decrease in cytosolic LDH activity and 50% reduction in cell number at 1 mg/mL. There was no significant difference observed in cell activity below 0.5 mg/mL. The primary cells were more susceptible to the presence of the polymer with a 50% decrease in cytosolic LDH activity and 50% reduction in cell number at 0.75 mg/mL. No significant difference in cell activity was seen below 0.25 mg/mL. This increased susceptibility to the polymer is likely due to the primary nature of the myoblasts compared to the immortalized nature of the fibroblast cell line. However, both IC_{50} values indicate a high degree of tolerance for polymer solution in vitro.

Cell morphology was examined by phase contrast microscopy following exposure to the polymer. At high polymer concentrations, the cells did not spread well on the TCP substrate and appeared smaller than control cells. At low concentrations of PTAA polymer, the cells appeared similar in morphology to the control cell populations.

2.3.2. Cell Studies on the Hydrogel Substrate

To determine the suitability of the fully hydrated hydrogel structure to support cell attachment and growth, the C2C12 (mouse myoblast) cell line was chosen as it has previously been utilised to examine the effect of substrate modulus on cell behaviour.[41] It is also a potential model to examine the effect of electrical stimulation on these electroresponsive cells. The preswollen hydrogel was seeded at high density and cells were assessed after 72 h incubation on the surfaces for viability and also internal cytoskeletal structure following fixation and staining. Cytoskeletal staining showed the morphology of the cells clearly indicating that the cells had adhered and spread on the surface of the hydrogel (**Figure 5**a: a–c). Calcein staining revealed that each of the gels with varying crosslinking density promoted cell viability on its surface with no obvious difference between softer and stiffer hydrogels (Figure 5a: d–f). SEM imaging also showed the undulating surface topography of the hydrogel with cells showing an adherent morphology on the surface (Figure 5b). No significant differences were seen between the hydrogels of varying crosslinking density. However cell viability and morphology were the only parameters examined in this study as preliminary indicators of the suitability of these materials for tissue engineering applications. Future studies will include measurement of cell differentiation as this parameter appears to be highly influenced by substrate modulus.[41]

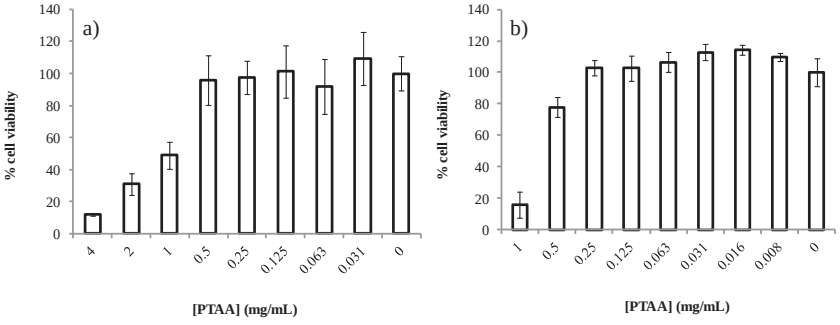

Figure 4. Representative LDH cytotoxicity assay expressed as a percentage of control untreated cells for a) fibroblast and b) primary myoblasts treated with a series of dilutions of PTAA polymer in culture media

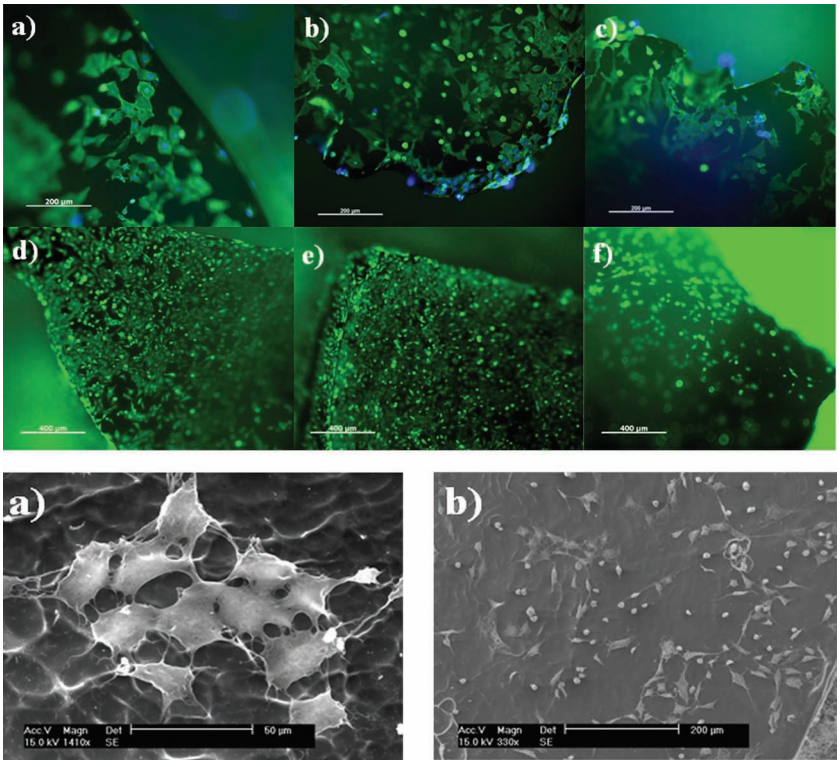

Figure 5. Top: Fluorescent images of myoblast cells on hydrogel substrates following 72h incubation. a–c) Cells are stained with Phalloidin-Alexa 488 (green) to visualise F-actin filaments, and DAPI (blue) to visualise nuclei. d–f) Cells are stained with CalceinAM to visualise metabolically active cells. Scale bars represent 200 µm (a–c) or 400 µm (d–f). Bottom: Representative SEM images of myoblast cells adhered to the hydrogel substrates following 72 h incubation.

3. Conclusions

We have successfully developed a single component conducting polymer hydrogel with promising properties that suggest it may be useful as a scaffold for tissue engineering. The CP hydrogel was prepared by covalently crosslinking PTAA with DCI, leading to the formation of anhydride bonds. Since polymers with anhydride linkages have been shown to undergo hydrolysis, this PTAA hydrogel could be expected to biodegrade over time. Current studies are being conducted to investigate the biodegradation of these hydrogels. Despite the hydrophobic nature of PTAA backbone, the system exhibited all the requisite hydrogel characteristics for a good scaffold material; notable swelling ratio while maintaining the 3D integrity of the hydrogel, good mechanical properties comparable to muscle tissue, and a porous internal structure. Notably, PTAA CP hydrogels with tailored structural properties could be fabricated using the crosslinking method we employed. Additionally, the CP hydrogels exhibited conductivities in the order of 10^{-3} S/cm and were electroactive at physiological pH and temperature.

Furthermore, this study has established that the PTAA polymer solution had an IC_{50} of 1 and 0.75 mg/mL for L929 fibroblasts and primary myoblasts respectively indicating a good level of tolerance for the polymer solution. C2C12 myoblast cells were shown to adhere and proliferate on the hydrogel substrate. The additional dimension of electrical stimulus delivery presents an exciting material for the study of not only muscle cell behaviour but also neural engineering applications. This is the first time that the potential of a single component CP hydrogel has been demonstrated for cell growth, opening the way for the development of new tissue engineering scaffolds that have potential application for nerve and muscle regeneration.

4. Experimental

Materials: 3-Thiopheneacetic acid, ferric chloride ($FeCl_3$), 1,1′-carbonyldiimidazole (DCI), ferric perchlorate ($Fe(ClO_4)_3$) and perchloric acid ($HClO_4$) were purchased from Sigma. Potassium iodide (KI), iodine (I_2) and dimethyl sulfoxide (DMSO) were purchased from Ajax Finechem. All chemicals were used without further purification. DMSO was stored over molecular sieves (Scharlau, 4Å, 2-3mm). Phosphate buffer solution (PBS, pH = 7.4) was prepared by dissolving PBS tablet (Calbiochem) in de-ionized water (DI-H_2O, 1 liter). Poly(3-thiopheneacetic acid) (PTAA) was synthesised as described in the literature [42] by chemical oxidation using $FeCl_3$ in dry chloroform. PTAA M_w (13235 Da) was determined by gel permeation chromatography (GPC). Its UV-vis spectral characterization in aqueous solution gave a broad absorbance band at 420 nm.

Hydrogel Fabrication: PTAA gels were fabricated by dissolving PTAA in DMSO to a final concentration of 10 wt% polymer. DCI was added to the polymer solution at various molar ratio (with respect to the repeating unit of the polymer) to fabricate gels with diffrerent crosslinking densities (detailed in Table 2). The solutions were vortexed for 2 minutes and left for 2 days at room temperature. Gelation of the solutions was confirmed by the test tube inverting method.[43] The vials containing the mixture were inverted and gelation was regarded to have occurred when no flow of liquid was observed. Gels were crosslinked into disc shapes using a 5 mL glass vial as a mould.

Crosslinked gels were taken out and immersed in excess deionized water (DI-H_2O). The water was changed daily and the washing procedure was monitored by gas chromatography (GC-2010, Schimadzu). The GC was equipped with an HP-INNOWAX column (length: 30 m, inner diameter: 0.32mm, film thickness: 0.32µm) and a flame ionization detector (FID). The initial GC oven temperature was 70 °C. After 5 min, the temperature was raised to 150 °C at a rate of 5 °C/min, held for 10 min, then followed by a second increase to 200 °C at 10 °C/min. It was then held at 200 °C for 2 min. Liquid samples taken from the washing medium were analyzed by GC for the detection of DMSO and DCI residues. The washing procedure was repeated until no peaks corresponding to the impurities were detectable. The hydrogels were then taken out of the water bath and dried in a vacuum oven at 50 °C.

Swelling Studies: The dried weight of the hydrogels was measured (m_d), then hydrogels were incubated in PBS (pH 7.4) at 37 °C and left to swell until equilibrium was reached. The wet weight of swollen samples ($m_{s,t}$) was determined at different time points after gently blotting the hydrogels with filter paper. When the swollen samples reached a constant equilibrium weight (m_e), they were dried in a vacuum oven and their dry weight recorded ($m_{d,e}$). The percentage swelling ratio[44] was calculated as follows:

$$\%Swelling\ Ratio = \frac{m_{s,t} - m_d}{m_d} \times 100 \quad (1)$$

and their equilibrium swelling ratio (q) [45–47] calculated as:

$$q = \frac{m_e}{m_d} \quad (2)$$

Three experiments were performed for each crosslinking density and each experiment included nine specimens.

Mechanical Testing: The compression modulus of the swollen hydrogels was measured at room temperature using an EZ-S Shimadzu model Testing Instrument equipped with a 10 N load cell. The sample disks were removed from PBS and their thickness and radius (r) measured. The samples were placed between parallel plates and compressed at a constant rate of 0.5 mm s^{-1} until complete deformation. The stress was calculated as follows:

$$stress = \frac{load}{\pi x r^2} \quad (3)$$

where r is the initial unloaded radius. The strain under compression was defined as the change in the thickness relative to the thickness of the freestanding specimen. The compression modulus was calculated from the slope of the linear region of the stress-strain plot at less than 15% deformation. Results are reported for data collected from 6 specimens for each crosslinking density.

Statistical Analysis: Samples were statistically analyzed using one-way ANOVA at a significance level of 0.05. Tukey's multiple comparison post test was used to compare pairs of group means.

Scanning Electron Microscopy (SEM): To investigate the internal structure of the swollen hydrogels, a Philips XL30 SEM was used. Swollen specimens were frozen for 40 secs in liquid nitrogen (LN2) and fractured with a LN2 cooled razor blade before being placed in the SEM for viewing. The SEM was operated at 15 kV and the gels viewed in secondary electron mode.

Electroactivity and Conductivity Measurements: Cyclic voltammetry measurements were recorded using an eDAQ potentiostat system controlled by eDAQ EChem software (v.2.0.7). The counter electrode was a platinum mesh and the reference electrode was Ag/AgCl in saturated KCl aqueous solution (Bioanalytical System). The working electrode was ITO coated glass (Delta Technologies Limited) on which the hydrogel was anchored. Electrochemical measurements were performed at 100 mV/sec using 0.1 M PBS aqueous electrolyte solutions.

Conductivity of hydrogels was estimated from their resistance values measured by an Agilent multimeter/waveform generator. A 2-probe connection was inserted in the hydrogels and connected to the instrument. *I–V* characteristics were recorded by sweeping the applied voltage (V) from –0.05 to 0.05V. Conductivity was measured for hydrogels in different states: water and PBS swollen un-doped and doped hydrogels. Doping the hydrogels was achieved by dipping the samples in 60 wt% $HClO_4$ aqueous solution for 30 min.[48] Samples were removed and their surfaces wiped gently with a filter paper to remove excess $HClO_4$. Conductivity of these samples was measured in air (water swollen $HClO_4$ doped hydrogels). The samples were then incubated in PBS for one day and the conductivity of the re-swollen samples (PBS re-swollen doped hydrogels) was measured at 37 °C. To explore the possibility of doping these hydrogels in aqueous media at a pH similar to that of physiological fluid, LXL-PTAA hydrogels were doped either in 0.1 M $FeClO_4$ aqueous solution or in phosphate buffer solution containing 0.2 M KI and 0.1 M I_2. To confirm that the recorded conductivity is due to the conductive polymer and not just ionic conductivity, poly(acrylic acid) (PAA) hydrogels were fabricated and their conductivities measured by the same technique. PAA was chosen because it contains carboxylic groups that ionize in PBS similarly to the investigated CP hydrogels.

Cell Culture: Mouse fibroblast (L929) and skeletal muscle myoblast (C2C12) cell lines, were originally sourced from the ATCC (American Type Culture Collection) and cultured in DMEM (Dulbecco's Modified Eagle's Medium, Invitrogen, Sydney, Australia) containing 4 mM L-glutamine and 10% Fetal Bovine Serum (FBS). Primary skeletal muscle myoblasts, isolated from 5–6 weeks old mice according to the protocol described by Todaro et al.,[49] were donated by the Centre for Clinical Neuroscience and Neurology Research, St. Vincent's Hospital, Melbourne, Australia. Primary myoblasts were cultured in Hams F10 (Sigma, Sydney, Australia) containing 20% Foetal Bovine Serum (FBS, Thermo, Noble Park, Victoria), 2 mM L-glutamine, 2.5 ng/ml bFGF (Peprotech) and used at or beyond preplate 7 for experimentation. Antibiotics (penicillin/streptomycin, P/S) were added to culture media for cell growth experiments utilising the polymers. Greiner cell culture plasticware was used throughout (Interpath, Sydney, Australia). Cells were cultured at 37 °C in a humidified, 5% CO_2 atmosphere and were subcultured twice weekly.

Cytotoxicity of PTAA Polymer Solution: Two cytotoxicity assays were used to assess the short term cytoxicity of the PTAA aqueous polymer solutions: PicoGreen cell number assay and cytosolic lactate dehydrogenase cytotoxicity assay (LDH).

PicoGreen Cell Number Assay: The PTAA polymer was dissolved in culture media at a concentration of 8 mg/mL and a 2-fold dilution series was prepared and sterilised by filtration (0.2 mm, Millex PES, Millipore). Cells were seeded at 15×10^2 cells/cm^2 in 96-well plates and allowed to adhere for 24 h at 37 °C in a humidified 5% CO_2 environment, followed by transfer of PTAA polymer serial dilutions to corresponding wells. Cells were exposed to polymer solutions for 48 h. Changes in the cell morphology and adhesion were assessed in parallel using phase contrast imaging using an Axiovert microscope equipped with an AxioCAM IcM camera (Zeiss). After the incubation period, polymer solutions were removed and cells washed 3 times with PBS to remove non-adherent cells and any polymer residues. Remaining cells were lysed by the addition 100 µL of 0.1% (w/v) Triton X-100 in Tris-EDTA buffer per well, followed by a freeze/thaw cycle (-80 °C/37 °C). Cell lysate was then collected and transferred to fresh 96 well microtitre plates along with cell number standards prepared by the same method. Lysate was then incubated with PicoGreen QuantIT reagent (Invitrogen) according to the manufacturer's instructions for 15 min, and the fluorescent signal was read utilising a FluoStar Omega with an excitation of 480 nm and an emission of 520 nm. The fluorescent signal obtained was converted to cell number based on cell number standards. The IC_{50} was calculated as the polymer concentration that causes 50% reduction in cell number relative to that of un-treated cells

Cytosolic Lactate Dehydrogenase Cytotoxicity Assay: LDH activity in the remaining adherent cells was assayed by using the Promega Cytotox 96 assay kit (Promega, Sydney, Australia) according to the manufacturer's instructions. The total LDH activity for the remaining adherent cells was compared to that of control cells with no added PTAA (media only). The relative cell viability was calculated from culture-media corrected absorbance as (absorbance/control cell absorbance) × 100. The IC_{50} was calculated as the polymer concentration that inhibits 50% cytosolic enzyme activity relative to that of un-treated cells. Positive control cells were treated with 7.5% ethanol over the same time period, resulting in approximately 90% reduction in enzyme activity. The cytotoxicity of the PTAA polymer was assessed using triplicates of each polymer dilution in each of 4 (L929) or 3 (primary myoblast) replicate experiments.

Cell Studies on the Hydrogel Substrate: Cell adhesion on the swollen PTAA hydrogels was also investigated. Hydrogels were cross-linked at 3 different densities and extensively washed to remove unincorporated polymer before the fully swollen networks were prepared for cell culture. The PTAA hydrogel was soaked in 70% (v/v) EtOH for 30 min before rinsing in serum free media. This was removed and the PTAA hydrogel was then soaked in fresh serum free media for 1h prior to cell seeding. C2C12 myoblast cells were seeded at high density in DMEM supplemented with 10% (v/v) FBS and penicillin/streptomycin. Media was changed after 48 h and cells were imaged after 4 days on the surface of the PTAA hydrogel. Cells were stained for metabolic activity by addition of 1µM CalceinAM to the media followed by incubation at 37 °C in a humidified 5% CO_2 environment for 15 min. The media was then removed and replaced with fresh PBS prior to imaging with an AxioImager equipped with an AxioCAM Mrm camera (Zeiss).

Cell samples were also fixed with 4% paraformaldehyde in PBS for 20 min at room temperature before permeabilisation with 0.1% (v/v) Triton X-100 for 5 min at room temperature. Phalloidin-Alexa 488 was then incubated with the cells for 20 min at a dilution of 1/200 in PBS, followed by the addition of DAPI at 1 μg/mL and a further 5 min incubation. The solution was then replaced with fresh PBS and the cells imaged as above with overlays prepared using AxioVision software (Zeiss).

Fixed cells on the hydrogel surfaces were also imaged by SEM to further investigate qualitatively their density and growth pattern following the same technique described in section 2.2.4. above.

Acknowledgements

This work was supported by the ARC Centre of Excellence for Electromaterials Science. We also gratefully acknowledge funding from the Australian Research Council and National Health and Medical Research Council for continued financial support. We would like to thank Nick Roach for synthesizing large batches of the PTAA polymer. We would like to thank the Centre for Clinical Neuroscience and Neurology Research, St. Vincent's Hospital, Melbourne, Australia for donating the primary myoblasts.

Received: October 4, 2011
Revised: January 9, 2012
Published online: April 12, 2012

[1] S. M. Richardson-Burns, J. L. Hendricks, B. Foster, L. K. Povlich, D. H. Kim, D. C. Martin, *Biomaterials.* **2007**, *28*, 1539.
[2] B. D. Malhotra, A. Chaubey, S. P. Singh, *Anal. Chim. Acta.* **2006**, *578*, 59.
[3] M. Gizdavic-Nikolaidis, J. Travas-Sejdic, G. A. Bowmaker, R. P. Cooney, C. Thompson, P. A. Kilmartin, *Current Applied Physics.* **2004**, *4*, 347.
[4] D. Mawad, K. Gilmore, P. Molino, K. Wagner, P. Wagner, D. L. Officer, G. G. Wallace, *J. Mater. Chem.* **2011**, *21*, 5555.
[5] T. J. Rivers, T. W. Hudson, C. E. Schmidt, *Adv. Funct. Mater.* **2002**, *12*, 33.
[6] L. Ghasemi-Mobarakeh, M. P. Prabhakaran, M. Morshed, M. H. Nasr-Esfahani, H. Baharvand, S. Kiani, S. S. Al-Deyab, S. Ramakrishna, *J. Tissue Eng. Regen. Med.* **2011**, *5*, e17.
[7] V. Barbier-Chassefière, S. Garcia-Filipe, X. L. Yue, M. E. Kerros, E. Petit, P. Kern, J. L. Saffar, D. Papy-Garcia, J. P. Caruelle, D. Barritault, *J. Biomed. Mater. Res. A.* **2009**, *90*, 641.
[8] A. D. Bach, J. P. Beier, J. Stern-Staeter, R. E. Horch, *J. Cell. Mol. Med.* **2004**, *8*, 413–22.
[9] L. N. Novikov, L. N. Novikova, A. Mosahebi, M. Wiberg, G. Terenghi, J. O. Kellerth, *Biomaterials* **2002**, *23*, 3369.
[10] C. R. Nuttelman, D. J. Mortisen, S. M. Henry, K. S. Anseth, *J. Biomed. Mater. Res.* **2001**, *57*, 217.
[11] S. H. Gehrke, J. P. Fisher, M. Palasis, M. E. Lund, *Ann. N. Y. Acad. Sci.* **1997**, *831*, 179.
[12] D. M. Pedrotty, J. Koh, B. H. Davis, D. A. Taylor, P. Wolf, L. E. Niklason, *Am. J. Physiol. Heart Circ. Physiol.* **2005**, *288*, H1620.
[13] Y. Kawahara, K. Yamaoka, M. Iwata, M. Fujimura, T. Kajiume, T. Magaki, M. Takeda, T. Ide, K. Kataoka, M. Asashima, L. Yuge, *Pathobiology* **2006**, *73*, 288.
[14] K. Gilmore, A. J. Hodgson, B. Luan, C. J. Small, G. G. Wallace, *Polym. Gels Netw.* **1994**, *2*, 135.
[15] A. Guiseppi-Elie, *Biomaterials.* **2010**, *31*, 2701–16.
[16] S. K. Siddhanta, R. Gangopadhyay, *Polymer.* **2005**, *46*, 2993.
[17] Q. Tang, J. Wu, J. Lin, *Carbohydr. Polym.* **2008**, *73*, 315.
[18] Q. Tang, J. Wu, H. Sun, J. Lin, S. Fan, D. Hu, *Carbohydr. Polym.* **2008**, *74*, 215.
[19] B. Guo, A. Finne-Wistrand, A. C. Albertsson, *Chem. Mater.* **2011**, *23*, 1254.
[20] T. S. Tsai, V. Pillay, Y. E. Choonara, L. C. du Toit, G. Modi, D. Naidoo, P. Kumar, *Polymer* **2011**, *3*, 150.
[21] A. Guiseppi-Elie, *Anal. Bioanal. Chem.* **2011**, *399*, 403.
[22] T. Dai, X. Jiang, S. Hua, X. Wang, Y. Lu, *Chem. Commun.* **2008**, *36*, 4279.
[23] R. Du, Y. Xu, Y. Luo, X. Zhang, J. Zhang, *Chem. Commun.* **2011**, *47*, 6287.
[24] L. Chen, B. S. Kim, M. Nishino, J. P. Gong, Y. Osada, *Macromolecules.* **2000**, *33*, 1232.
[25] A. D. Bendrea, L. Cianga, I. Cianga, *J. Biomater. Appl.* **2011**, *26*, 3.
[26] A. S. Rowlands, J. J. Cooper-White, *Biomaterials.* **2008**, *29*, 4510.
[27] J. Lin, Q. Tang, J. Wu, Q. Li, *J. Appl. Polym. Sci.* **2010**, *116*, 1376.
[28] D. J. Quick, K. K. Macdonald, K. S. Anseth, *J. Controlled Release.* **2004**, *97*, 333.
[29] G. W. Anderson, R. Paul, *J. Am. Chem. Soc.* **1958**, *80*, 4423.
[30] H. A. Staab, G. Walther, W. Rohr, *Chem. Ber.* **1962**, *95*, 2073.
[31] B. S. Kim, Y. Osada, *Korea Polym. J.* **1999**, *7*, 350.
[32] L. Wu, C. S. Brazel, *Int. J. Pharm.* **2008**, *349*, 1.
[33] W. F. Lee, C. F. Chen, *J. Polym. Res.* **1998**, *5*, 105.
[34] J. H. O. Owino, O. A. Arotiba, P. G. L. Baker, A. Guiseppi-Elie, E. I. Iwuoha, *Reactive and Functional Polymers.* **2008**, *68*(8), 1239.
[35] T. Dai, X. Qing, Y. Lu, Y. Xia, Polymer. **2009**, *50*, 5236.
[36] C. Gabriel, S. Gabriel, E. Corthout, *Phys. Med. Biol.* **1996**, *41*, 2231.
[37] Y. Li, K. G. Neoh, L. Cen, E. T. Kang, *Langmuir.* **2005**, *21*, 10702.
[38] L. Jie, D. Ying, S. Yuyu, *J. Bioact. Compat. Polyms.* **2010**, *25*, 185.
[39] P. Mukherjee, A. Dawn, A. K. Nandi, *Langmuir.* **2010**, *26*, 11025.
[40] A. J. Engler, S. Sen, H. L. Sweeney, D. E. Discher, *Cell.* **2006**, *126*, 677.
[41] A. J. Engler, M. A. Griffin, S. Sen, C. G. Bonnemann, H. L. Sweeney, D. E. Discher, *J. Cell. Biol.* **2004**, *166*, 877.
[42] B. S. Kim, L. Chen, J. Gong, Y. Osada, *Macromolecules.* **1999**, *32*, 3964.
[43] W. Zhu, J. Ding, *J. Appl. Polym. Sci.* **2006**, *99*, 2375.
[44] T. Caykara, S. Kiper, G. Demirel, *J. Appl. Polym. Sci.* **2006**, *101*, 1756.
[45] L. Vervoort, G. van den Mooter, P. Augustijns, R. Kinget, *Int. J. Pharm.* **1998**, *172*, 127.
[46] D. Mawad, J.L.J.R. Foster, A. Lauto, *Int. J. Pharm.* **2008**, *360*, 231.
[47] D. Mawad, R. Odell, L. A. Poole-Warren, *Int. J. Pharm.* **2009**, *366*, 31.
[48] B. S. Kim, Y. Osada, *Korea Polym. J.* **1999**, *7*, 350.
[49] M. Todaro, A. Quigley, M. Kita, J. Chin, K. Lowes, A. J. Kornberg, M. Cook, R. Kapsa, *Hum. Mutat.* **2007**, *28*, 816.