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Abstract

Two synthesis routes to graphene/polycaprolactone composites are introduced and the properties of the resulting composites compared. In the first method, mixtures are produced using solution processing of polycaprolactone and well dispersed, chemically reduced graphene oxide and in the second, an esterification reaction covalently links polycaprolactone chains to free carboxyl groups on the graphene sheets. This is achieved through the use of a stable anhydrous dimethylformamide dispersion of graphene that has been highly chemically reduced resulting in mostly peripheral ester linkages. The resulting covalently linked composites exhibit far better homogeneity and as a result, both Young's modulus and tensile strength more than double and electrical conductivities increase by 14 orders of magnitude over the pristine polymer at less than 10 per cent graphene content. In vitro cytotoxicity testing of the materials showed good biocompatibility resulting in promising materials for use as conducting substrates for the electrically stimulated growth of cells.

Keywords

engineering, linked, tissue, covalently, composites, polycaprolactone, graphene, biocompatible

Disciplines

Engineering | Physical Sciences and Mathematics

Publication Details

Sayyar, S., Murray, E., Thompson, B. C., Gambhir, S., Officer, D. L. & Wallace, G. G. (2013). Covalently linked biocompatible graphene/polycaprolactone composites for tissue engineering. *Carbon*, 52 (February), 296-304.

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Covalently linked biocompatible graphene/polycaprolactone composites for tissue engineering

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Two synthesis routes to graphene/polycaprolactone composites are introduced and the properties of the resulting composites compared. In the first method, mixtures are produced using solution processing of polycaprolactone and well dispersed, chemically reduced graphene oxide and in the second, an esterification reaction covalently links polycaprolactone chains to free carboxyl groups on the graphene sheets. This is achieved through the use of a stable anhydrous dimethylformamide dispersion of graphene that has been highly chemically reduced resulting in mostly peripheral ester linkages. The resulting covalently linked composites exhibit far better homogeneity and as a result, both Young's modulus and tensile strength more than double and electrical conductivities increase by ≈ 14 orders of magnitude over the pristine polymer at less than 10 % graphene content. *In vitro* cytotoxicity testing of the materials showed good biocompatibility resulting in promising materials for use as conducting substrates for the electrically stimulated growth of cells.

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1. Introduction

Polymer nanocomposites with nanosized carbonaceous fillers have shown large improvements in mechanical, conductivity and barrier properties over the pristine polymers. For example, the storage modulus and conductivity of polyimide are greatly improved by integrating carbon nanotubes into the polymer [1, 2] and the addition of a small amount of fullerene materials has led to a 30-40 % increase in Young's modulus and the tensile strength of polyamides. [2]

Graphene is a single layer two-dimensional graphitic carbon material packed densely in a honeycomb crystal structure, with promising mechanical, electrical, optical, thermal and magnetic properties. It has also been shown to be biocompatible and even beneficial in the growth of cells.[3-5] It is reported that graphene-based polymer nanocomposites show much improved mechanical and electrical properties when compared to other carbon filler based nanocomposites.[6-9] These unique properties have attracted researchers to investigate graphene as a reinforcing agent in biocompatible composite materials in order to improve mechanical, thermal and electrical properties.[10-14]

Poly(ϵ -caprolactone) (PCL) is a biodegradable and biocompatible aliphatic polyester with good resistance to water, solvents and oil, synthesized by the ring opening polymerization of ϵ -caprolactone. Polycaprolactone's unique mechanical and chemical properties have resulted in its extensive commercial development for biomedical and materials applications.[15-17] The addition of an electrically conducting filler, such as graphene, opens up a number of further possible applications, such as conducting substrates for the electrically stimulated growth of cells.

Recently, a number of authors [19, 20] have shown that the addition of non-conducting graphene oxide as a filler in polycaprolactone results in improvements in the mechanical

properties of PCL. Composites of PCL and graphene oxide (GO) have also been synthesised by ring opening polymerization of ϵ -caprolactone using GO as an initiator [19] and have shown that GO has an excellent nucleating effect on the crystallization of PCL. To the best of our knowledge there have been no previous reports on the use of conducting reduced graphene nanosheets in polycaprolactone composites.

In this work, we use highly reduced graphene dispersions to prepare graphene/polycaprolactone composites. Highly reduced chemically converted graphene exhibits far greater conductivity than graphene oxide resulting in highly conducting composites. Composites are prepared using two different methods; a mixing method producing binary mixtures of graphene in a polymer matrix and a chemical method resulting in polymer that is covalently linked to the remaining functionalities on the periphery of the graphene sheet. The resulting graphene/biopolymer materials retained the biocompatibility and processability of the polymer but with conductivities and mechanical properties enhanced by orders of magnitude due to the graphene filler.

2. Experimental

2.1 Materials

ϵ -caprolactone (97 %), N,N-dimethylformamide, N,N'-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), Dulbecco's modified Eagle's medium (DMEM), methanol, dichloromethane, tin (II) ethylhexanoate (95 %), polycaprolactone (MW 80,000) and triethylamine were sourced from Sigma-Aldrich and used as received. Graphite powder was obtained from Bay Carbon. Milli-Q water with a resistivity of $18.2 \text{ m}\Omega \text{ cm}^{-1}$ was used in all preparations.

2.2 Preparation of graphene oxide (GO)

Graphene oxide was synthesized from natural graphite powder using a modified Hummers' method in two steps using $\text{K}_2\text{S}_2\text{O}_8$, P_2O_5 and H_2SO_4 followed by H_2SO_4 , KMnO_4 and H_2O_2 to achieve better oxidation of graphite (20, 21).

2.3 Preparation of chemically converted graphene (CCG) dispersions

Graphene oxide (62.5 g) was diluted with deionised water (2 L) and sonicated for 80 min. Then, hydrazine (400 μL) and ammonia (4 mL) were added and the solution heated at 90 $^\circ\text{C}$ for 1 hr. A further aliquot of hydrazine (3 mL) was added to the solution and the mixture was heated and kept at 90 $^\circ\text{C}$ for two hours under constant stirring. After cooling to the room temperature, the solution was acidified with H_2SO_4 (aq. 30%), then the agglomerated graphene powder was filtered and washed until the waste water was at a neutral pH. The agglomerated graphene powder was filtered and dried in vacuum oven at 50 $^\circ\text{C}$ for 2 days.

To form a stable suspension, dried CCG (300 mg) was added to dimethylformamide (DMF) (150 ml, moisture content ≤ 350 ppm by Karl-Fischer). Triethylamine (50 μl) was added and the solution was extensively sonicated with continuous cooling under a dry nitrogen purge. DMF (300 ml) and triethylamine (500 μl) were then added and the suspension was further sonicated under nitrogen. The dispersion was centrifuged to separate any agglomerated graphene sheets and the resulting supernatant (0.5 mg ml^{-1}) was stable for several months without any agglomeration.

2.4 Preparation of polycaprolactone (PCL)

In a standard synthesis for a chain length of $\approx 40,000$, caprolactone (5 ml) was mixed with tin 2-ethylhexanoate (50 μl) in a flask that was evacuated then purged with nitrogen. The mixture was reacted using microwave irradiation (100 W max) at a constant temperature of 140 $^\circ\text{C}$ for 1 hour. The crude solid product was dissolved in dichloromethane and precipitated in cold methanol, filtered and dried in a vacuum oven at 50 $^\circ\text{C}$. The chain length could be easily controlled by varying the reaction time.

2.5 Preparation of PCL-CCG composites

Graphene/polycaprolactone binary mixtures (mixPCL-CCG) were prepared by mixing polycaprolactone in an appropriate amount of a 0.5 mg ml⁻¹ stable solution of DMF-dispersed graphene at 75 °C for 3 hours before cooling to room temperature. The polymer blends were then precipitated in cold methanol, filtered and dried in a vacuum oven at 50 °C.

Covalently linked graphene/PCL composites (cPCL-CCG) were prepared by mixing polycaprolactone in an appropriate amount of a 0.5 mg ml⁻¹ stable solution of DMF-dispersed graphene at 75 °C for 1 hour. 50mg of DMAP was added followed by the slow addition of DCC and stirred at 75 °C for a further 4 hours before cooling to room temperature. The polymer composites were then precipitated in cold methanol, filtered and dried in a vacuum oven at 50 °C.

2.6 Characterisation

Microwave-assisted polymerization was carried out in a CEM Discover 2.45-GHz microwave oven. Raman spectra were recorded on a Jobin Yvon Horiba HR800 Raman microscope using a 632 nm laser line and a 300-line grating. Scanning electron microscopy (SEM) images were collected with a field-emission SEM instrument (JEOL JSM-7500FA). Samples were sputter-coated (EDWARDS Auto 306) with a thin layer of platinum (≈3 nm thickness). X-ray powder diffraction (XRD) analysis was performed using GBC MMA diffraction equipment (GBC Scientific Equipment Pty Ltd, Australia) equipped with Cu- $\kappa\alpha$ radiation. Thermal gravimetric analysis (TGA) was performed in using TGA Q500, TA Instruments with a heating rate of 10 °C under nitrogen atmosphere. Differential scanning calorimetric (DSC) analysis was performed on a DSC Q100, TA Instruments. 5 to 8 mg of the sample was presealed into an aluminum pan and first heated to above the melting temperature (T_m) of the polymer (100 °C), then cooled to 0 °C at 10 °C min⁻¹, the temperature increased to above 100 °C at 10 °C min⁻¹. All sonication was done using a Brandson Digital Sonicator (S450D, 500

W, 40 % amplitude). Mechanical properties were tested using an *Instron 5566* Universal Testing Machine (USA). To prepare samples for mechanical properties tests, the samples were hot pressed at 100 °C to obtain a 0.1 mm thick film. The film then was cut to strips with a width of 3 mm and a length of 20 mm. The tensile properties of the samples were measured with a constant rate of 10 mm min⁻¹.

2.7 Cell culture

Materials were prepared for cell culture by attachment of chamber slides directly onto the surface using silicon glue, and sterilisation by soaking the surfaces with 70% ethanol before air drying in a biological safety cabinet. The sterilised materials were soaked in DMEM with penicillin/streptomycin for 24 hours before the cells were plated. For live/dead cell imaging, L-929 cells (a mouse fibroblast cell line) were seeded in DMEM supplemented with 10% foetal bovine serum (FBS) at 5000 cells cm⁻² of PCI-CCG composite, and incubated at 37 °C in 5 % CO₂ for 48 hours. After 48 hours, the cells were removed from the incubator and stained with calcein AM and propidium iodide for 10 minutes before imaging with a Zeiss AxioImager microscope. Image analysis for cell counting was performed using Image Pro Plus, and cell numbers were averaged over at least 3.6 mm² of the culture surface, with microscope images taken from random, separate areas of the culture well. For growth curves of cells on the materials, three cell lines were used. L-929 cells (in DMEM + 10% FBS), PC-12 cells (rat phaemocrycytoma cells, in DMEM + 5% FBS plus 10% horse serum) and C2C12 (mouse myoblast, in DMEM + 10% FBS) cells were seeded onto the materials at 25000 cells/mL (approx 8000 cells/cm²), and incubated for up to 4 days on the materials. At 2, 24, 48, 72 and 96 hours, cells were harvested by removal of media and lysis of the cells in 50 µL 0.1% sodium dodecyl sulfate/TE (tris ethylenediaminetetraacetic acid) buffer. A Pico Green assay was performed on 10 µL of the lysed cell solutions diluted in 90 µL TE buffer to quantify the amount of DNA in the solutions by addition of 100 µL of Pico Green reagent

(diluted 1:200 in TE buffer), subsequent incubation for 5 min and reading of the fluorescence of each well using a 485 nm excitation and 520 nm emission on a FLUOstar Omega (BMG Labtech) microplate reader.

3. Results and Discussion

The introduction of highly reduced graphene into many polymers is challenging given that stable graphene dispersions are typically prepared in water, precluding the effective introduction of exfoliated graphene nanoplatelets into water-insoluble polymers. Removal of the water leads to graphene aggregates, which are difficult to redisperse in either organic solutions of the polymer or the pristine polymer itself. This has prompted us to prepare an anhydrous dispersion of chemically converted graphene (CCG) in dimethylformamide (DMF) that is more highly reduced than our previously prepared aqueous CCG dispersion [29] and stable for more than a year at a concentration of 0.5 mg ml^{-1} . The full details of this DMF CCG dispersion will be published elsewhere (*ref: S. Gambhir, E. Murray, D.L Officer, manuscript in preparation*). The stable DMF dispersion of CCG then allowed us to readily prepare a range of covalently linked and solution mixed polycaprolactone-graphene composites (0.1-10 wt.% graphene)

The covalently linked graphene/polycaprolactone composites visually showed excellent dispersion of graphene in the polymer matrix even on hot-pressing and could be easily resuspended in a suitable solvent. On the other hand, composites produced using solution mixing showed washing-out of polymer during the precipitation and washing step, resulting in poor control of graphene content (initial addition of 5 wt.% graphene resulted in a composite graphene content of almost 17 %). They also exhibited visible agglomerates in a hot-pressed thin film and in solution.

3.1. Thermal Analysis

Thermal gravimetric analysis (TGA) shows that the addition of graphene, whether in a binary mixture or a covalently linked composite, has very little effect on the decomposition temperature of polycaprolactone. The pure polymer (MW $\approx$ 80,000) shows a monotonic weight loss at approximately 380 °C assigned to the decomposition of the polymer chains (Fig. 1). The graphene/polycaprolactone composites show a similar weight-loss profile and, as CCG weight losses are minimal in this temperature range, the residual weight after full decomposition of the polymer can be assigned to the graphene content. The graphene percentage calculated from TGA analysis of the composites prepared by covalent attachment method (cPCI-CCG) is very consistent with the percentage of graphene added to the reaction initially and indicates good attachment of the polymer. However, the graphene percentage in the mixtures (mixPCI-CCG) is very different to that added to the reaction mixture. This is consistent with the observation of polymer being washed out of the composite during precipitation.

Figure 1. Thermal gravimetry curves of PCI-CCG composites. The residual weight after full decomposition of the polymer indicates the amount of graphene in composites

Differential scanning calorimetry (DSC) showed that the addition of graphene to polycaprolactone either covalently or as a mixture did not significantly affect the melting point of the PCI composites, which remains at 55-60 °C (Fig. 2). However, the addition of graphene increases the crystallization temperature for all of the composites. Addition of just

0.1 % graphene results in very large increases in the crystallization point temperature from 19 °C in pristine PCI to 33 °C in the composite. This is likely to be due to the nucleating effect of graphene on PCI crystallization. Increasing addition of graphene further increases the crystallization temperature and broadens the crystallization peak, indicating confined mobility of polymer chains in the composite.

The nucleating effect of graphene nanosheets on the crystallisation of PCI is also confirmed by polarised optical microscopy (figure 3) on cooling materials below the crystallisation temperature which shows that spherulites of pure PCI are much larger in size and less densely packed than the graphene doped material.

Figure 2. Differential scanning calorimetry curves of (a) cPCI-CCG and (b) mixPCI-CCG composites. The addition of graphene generally has little effect on the melt temperature but increases the crystallization temperature of the composites. Polarised optical microscopy images taken below the crystallisation temperature of pure PCI (c) and cPCI-CCG (d) showing the difference in spherulite size and density.

3.2. Material Composition and Morphology

In the scanning electron microscopy images of the graphene/PCI composites in figure 3, the two samples have clearly different fracture surfaces, which reflect underlying differences in morphology agglomeration, alignment and bonding of the graphene nanosheets to the matrix.[6,7] The graphene filler appears dispersed more homogenously inside the polymer matrix in the cPCI-CCG composite. This ordering is due to the covalently linked polymer on the graphene sheet forcing the retention of sheet separation even on precipitation. In the mixed composite (mixPCI-CCG), the polymer can be washed out from between sheets resulting in the observed agglomeration and poor alignment of graphene sheets inside the polymer matrix.

Figure 3. Cross-sectional scanning electron microscopy images of (a) mixPCI-CCG and (b) cPCI-CCG composites

Figure 4 shows the X-ray diffraction patterns of polycaprolactone and graphene/PCI composites with varying graphene contents. The polymer diffraction pattern is typical of PCI with two major peaks at 21.3° and at 23.7° 2θ [20, 21]. Addition of graphene did not significantly affect the XRD patterns, except for a slight decrease in the peak at $2\theta = 21.3^\circ$ indicating a slight decrease in the crystallinity of the graphene/PCI composites similar to the effect observed by DSC. As such, it can be assumed that the chemical structure of the polymer is not significantly altered with increasing graphene content.

Figure 4. X-ray diffraction patterns of mixPCI-CCG and cPCI-CCG composites. The peaks can be attributed to the polymer component of the composite.

The Raman spectrum of PCI shows the characteristic peaks at 1726 cm^{-1} ($\nu\text{ C=O}$), 1060 cm^{-1} and 1106 cm^{-1} (skeletal vibration) and $1281\text{--}1305\text{ cm}^{-1}$ attributable to CH_2 groups.[27, 28]

Figure 5 shows the effect of increasing graphene content on the Raman spectra of graphene/PCI composites synthesized by covalent attachment and mixing methods. On increasing addition of CCG, the peaks due to PCI are less visible as the intensity of the characteristic D and G bands of graphene are greater than that of PCI. In samples with highest graphene contents, only the D and G band graphene peaks are visible. The ratio of the intensities of the graphene D and G bands (I_D/I_G) decreases slightly with increasing graphene

content. This can be attributed to a decrease in the crystalline size of the composite on increasing the graphene content.[30, 31]

Figure 5. Raman spectra of PCI-CCG composites. The two significant peaks at 1337 and 1602 cm^{-1} correspond to D and G band of graphene sheets.

3.3. Material Properties

Polycaprolactone shows conductivity of less than $10^{-15} \text{ S cm}^{-1}$ as a pristine polymer.[23] As expected, increasing the graphene content results in much improved conductivity (Fig. 6),

with conductivity increasing by more than 13 orders of magnitude on addition of less than 5 % graphene. At low graphene contents (< 1 %), mixPCI-CCG samples show better conductivity than covalently linked cPCI-CCG. This is probably due to the agglomerated nature of the graphene nanosheets in the mixed blend resulting in a shorter conducting path length conducting domains surrounded by non-conducting regions. An increase in graphene content results in an increase in the size and number of these domains resulting in a flatter increase in conductivity. On the other hand, above the percolation threshold the conductivity of the well dispersed cPCI-CCG samples increases rapidly and consistently, with the addition of less than 10% graphene resulting in a conductivity of almost $10^{-2} \text{ S cm}^{-1}$.

Figure 6. Conductivity measurements of PCI-CCG composites showing large increase in conductivity above the percolation threshold (<0.1 % graphene content).

In general, the addition of graphene improves the strength of the composites but decreases the elongation at break as the interaction between graphene and the matrix restricts the

movement of the polymer chains. Figure 7a shows the increase in tensile yield strength in both covalently linked and mixed method materials. In covalently attached composites, the addition of just 0.5 % graphene increased specific strength by almost 50 % and trebled it on addition of 5 %. Similarly the specific modulus increases from 88 MPa in pristine PCI to 252 MPa to 591 MPa across the same range.

Figure 7. Stress-strain curves of (a) cPCI-CCG and (b) mixPCI-CCG composites showing the large increase in tensile strengths and reductions in elongation at break.

In the composites prepared by the mixing method (Fig. 7b), the improvement in the tensile strength on the addition of graphene is less than that shown by the covalently linked composites, while the elongation at break decreased far more significantly. For a mixture with 5 % graphene, the elongation at break is decreased to just 69 % while the specific tensile strength is increased to 19.9 MPa, as compared to 160 % and 39.5 MPa for a similar covalently linked material. The increased strength and plasticity of the covalently-linked material is due to the far better dispersion of graphene nanosheets throughout the sample. (Table 1)

Table 1. Mechanical properties of polycaprolactone, covalently-linked composites cPCI-CCG and binary mixtures mixPCI-CCG.

	Graphene Content (%)	Specific Strength (KN.m/kg)	Specific Modulus (KN.m/kg)	Elongation at Break (%)
Polycaprolactone	0	13.5 (± 3)	88.7 (± 17)	1212 (± 86)
Covalent	0.1	12.3 (± 2)	83.0 (± 12)	842 (± 57)
	0.5	21.9 (± 4)	252.7 (± 34)	788 (± 38)
	1	24.3 (± 4)	356.6 (± 50)	286 (± 18)
	5	39.5 (± 7)	591.1 (± 103)	166 (± 12)
Mixture	0.1	12.6 (± 3)	130.1 (± 9)	321 (± 53)
	0.5	17.5 (± 1)	256.0 (± 48)	282 (± 28)
	5	19.9 (± 3)	366.1 (± 57.6)	69.5 (± 11)
	17	63.4 (± 14)	1147.1 (± 232)	48.4 (± 12)

3.4. Biocompatibility

Polycaprolactone is a well-established biocompatible and biodegradable material having found use in many applications including drug delivery, sutures and as a scaffold for tissue engineering. Fibroblast cells adhered to and proliferated on films cast from PCI, cPCI-CCG 0.5 % and cPCI-CCG 5 %, indicating that the material surface were suitable for attachment of cells, and did not leach any toxic compounds through the culture period. After 48 hours in culture media, cells were imaged (Fig. 8) and the images analysed for cell density on the surfaces and proportion of dead cells (cells that were permeable to propidium iodide). On PCI, an average of 400 ± 100 cells mm^{-2} were observed on the surface, with 1.8 ± 0.8 % dead cells. On cPCI-CCG 0.5 % L-929 cells were at 380 ± 70 cells/ mm^2 with 1.1 ± 0.1 % dead cells, and on cPCI-CCG 5 % the cell density was 450 ± 90 cells/ mm^2 with 2 ± 1 % dead cells. The cell density across the surfaces of all materials was inconsistent leading to a somewhat variable cell density across difference microscope fields, so the slight increase in average cell

density on cPCI-CCG 5 % was not statistically significant. No differences were observed in the growth of L-929 cells on mixPCI-CCG compared to cPCI-CCG materials. Cell density increased approximately 8 times from the seeding density on all materials (including tissue culture plastic – data not shown), indicating that the proliferation of cells was not significantly modified by the PCI or PCI-CCG materials.

Figure 8. Live L-929 cells stained with calcein (green) and prodidium iodide (red) on sheets of a) PCI, b) cPCI-CCG 0.5% and c) cPCI-CCG 5%. The scale bars indicate 100 μ m.

The growth of three different cell types was assessed on the cPCI-CCG materials and compared to growth on pristine PCI and tissue culture plastic. Fibroblast (L-929), neural (PC-12) and muscle (C2C12) cell lines were seeded onto the materials, and the cell number was assessed at various time points over 4 days by Pico Green Assay. The fluorescence generated by the Pico Green reagent is proportional to cell number in each well of a 96-well plate. The results are shown in Figure 9, indicating that all cell lines proliferated on PCI, cPCI-CCG 0.5% and 5% in a similar way to tissue culture plastic, although the cell number was consistently slightly greater on tissue culture plastic up to 48 hours across all cell lines. Both L-929 and C2C12 showed typical growth curves on the materials, with a small increase in the cell number between seeding and 48 hours, then a log phase of cell proliferation between 48-72 hours. The decrease in cell density at 96 hours is attributed to cells becoming overgrown after 3 days, and depleting the nutrients in the media. Overall, a 7-8 fold increase in fluorescence was seen between seeding and the maximum cell density at 72 hours. PC-12

cells grown on PCI or cPCI-CCG 0.5% were slightly less adhered at 24 h than the original seeding density. However, after 48 hours the adhesion of the cells was similar to tissue culture plastic. Interestingly, cPCL-CCG 5% showed the same adhesion of cells at 24 hours as tissue culture plastic. Over the 4 days of culture a 2-3 fold increase in fluorescence was observed from PC-12 cells grown on all PCI-containing materials, identical to that on tissue culture plastic. PC-12 cells often adhere poorly to surfaces without collagen coating (which was not performed here), and so these results are a good indication that the materials are suitable to support the adhesion and proliferation of even cells lines that typically exhibit poor materials compatibility.

Figure 9. Growth curves of three cell lines on PCI, tissue culture plastic and cPCI-CCG materials. Fibroblasts (a, L-929 cell line), muscle cells (b, C2C12 cell line) and neural cells (c, PC12 cell line) all adhered to and proliferated on the materials for 72-96 hours. Each point represents the average of three measurements, and the error bars indicate one standard deviation.

4. Conclusions

In this paper we have presented two methods for the synthesis of polycaprolactone graphene composites. Previous work on graphene oxide [20, 21] had introduced a mixing method for the production of graphene oxide polymer materials. Adapting this method using highly reduced, well dispersed graphene resulted in graphene/polycaprolactone composites with good mechanical and conducting properties but poor graphene dispersion in the matrix. Covalently linking the polymer to the graphene chains resulted in improved mechanical properties, conductivity and very homogeneously dispersed graphene nanosheets. The excellent processability and solubility of these covalently linked materials makes them good candidates for use in a number of applications including conducting biodegradable systems for tissue engineering.

Acknowledgements

This work has been supported by the Australian Research Council under the Superscience fellowship. We also acknowledge the Australian National Fabrication Facility (ANFF) for providing both materials and instrumentation. We thank Prof. Geoff Spinks for helpful scientific discussion.

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