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biomineralized conducting polymers enhances their
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Electrical stimulation of human mesenchymal stem cells on biomineralized conducting polymers enhances their differentiation towards osteogenic outcomes

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Tissue scaffolds allowing the behaviour of the cells that reside on them to be controlled are of particular interest for tissue engineering. Herein we describe biomineralized conducting polymer-based bone tissue scaffolds that facilitate the electrical stimulation of human mesenchymal stem cells, resulting in enhancement of their differentiation towards osteogenic outcomes.

Bone conditions requiring surgical intervention are of growing importance in societies with populations in which life expectancies are increasing, motivating the development of pro-regenerative biomaterials.¹ Non-biodegradable materials (e.g. titanium), biodegradable materials (e.g. biopolymers, calcium phosphate cements) and multifunctional materials that combine habitats for the cells with the capability to deliver drugs, have been investigated as potential bone tissue scaffolds.¹ Biomineralized materials are commonly investigated as bone tissue scaffolds, because the presence of the biomineral in the scaffold may promote osteogenesis.²

Conducting polymer (CP)-based biomaterials (such as derivatives of polyaniline, polypyrrole or polythiophene), have potential for both long term biomedical applications (e.g. electrodes) and short term biomedical applications (e.g. drug delivery or tissue engineering).³ CP-based scaffolds have been developed for the regeneration of bone, muscle and nerve tissue.³ Langer and coworkers first reported the use of CP-based materials for their application as bone tissue scaffolds.⁴ The application of a potential difference of 20 mV mm⁻¹ over 2-dimensional polypyrrole films encouraged bone marrow-derived stromal cells to differentiate towards osteogenic outcomes (assayed as an increase in alkaline phosphatase (ALP) activity per cell relative to non-stimulated control substrates).⁴

A variety of research groups have reported further developments in conducting polymer-based materials for bone tissue engineering in the absence⁵ or presence⁶ of an electrical field, commonly finding improved osteogenesis for the electrically stimulated samples *in vitro*. Moreover, the success of inorganic bone substitutes in the clinic has led researchers to develop conducting polymer-based coatings for calcium phosphate-based,⁷ steel-based,⁸ and titanium-based⁹ biomaterials which offer a method of directly electrically stimulating cells residing on the materials, or delivering a drug from such a coating upon the application of an electrical stimulus.¹⁰ Here we describe the preparation of polycaprolactone (PCL) derivatives displaying pyrrole moieties from which conducting polymers (such as polypyrrole or polythiophene derivatives) can be grown (Figure 1).

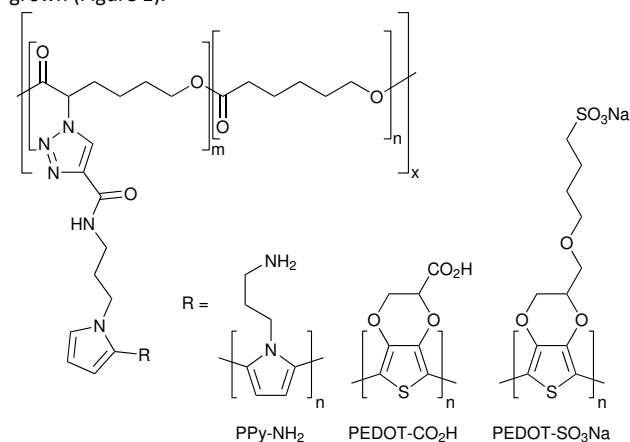


Figure 1. Conducting polymers enabling biomineralization with silica (R-NH₂), or calcium carbonate/phosphate (R-CO₂H or R-SO₃Na).

PCL derivatives displaying amines (e.g. PPy-NH₂, Figure 1) facilitate the mineralization of silica through interactions between the amine which is positively charged under physiological conditions and negatively charged silicic acid precursor to silica; whereas those PCL derivatives displaying carboxylates or sulfonates (e.g. PEDOT-CO₂H or PEDOT-SO₃Na, Figure 1) facilitate the mineralization of calcium carbonates or phosphates through interactions of the negatively charged carboxylate/sulfonate moieties with Ca²⁺ (which

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subsequently encourages the deposition of carbonates or phosphates). Biomaterialized biomaterials have been shown to promote osteogenic differentiation of stem cells,² as indeed have conducting biomaterials,⁶ yet this is to the best of our knowledge the first report of biomaterialization of conducting polymer-based bone tissue scaffolds which yield biomaterials that combine these attractive features. We demonstrate that such composite biomaterials can be used as bone tissue scaffolds that facilitate electrical stimulation of human mesenchymal stem cells which promotes their differentiation towards osteogenic outcomes *in vitro*.

In this study we report a variety of new conducting PCL derivatives. We began by synthesizing PCL derivatives displaying azides to facilitate the coupling of an alkyne-displaying pyrrole derivative which may act as a site from which to grow conducting polymers. Propionic acid was coupled to aminopropylpyrrole^{11,12} by carbodiimide-mediated peptide coupling (Scheme S1), and these were coupled to PCL derivatives displaying azide moieties¹³ by Cu(I)-mediated triazole formation¹³ (Scheme S2), after which the copper was removed by incubation in a solution of ethylenediaminetetraacetic acid (EDTA).¹⁴ The material was extensively washed to remove traces of EDTA and vacuum dried yielding pyrrole-displaying PCL derivative (depicted in Figure 1) with $M_n = 5.0$ kDa and M_w/M_n of 1.95 (Figure S1) in the form of a light brown powder. Films of the resulting polymer were solution cast on either commercially available tissue-culture treated Corning® Costar® tissue culture plates (TCP) or glass. An interpenetrating network of either amine displaying polypyrrole derivative (PPy-NH₂, Figure 1) or carboxylate displaying poly(3,4-ethylenedioxythiophene) derivative (PEDOT-CO₂H, Figure 1) were generated by incubation of the pyrrole-functionalized PCL films in aqueous solutions of the appropriate pyrrole and EDOT derivatives in the presence of the initiators ammonium persulfate and ferric chloride (Scheme S3 and S4, respectively).¹⁵ Films of the amine or carboxylate derivative displaying films were washed thoroughly with water to remove the by-products (e.g. initiators, monomers, oligomers and polymers) and vacuum dried. The brown-black PPy-NH₂ films were biomaterialized with silica and those of the blue-grey PEDOT-CO₂H were biomaterialized with calcium phosphate. Energy dispersive X-ray (EDX) analysis of the films confirms that their surface chemistry is different. Peaks in the EDX spectra of the PCL derivatives displaying pyrrole moieties have lines at 0.277 and 0.525 keV that are the characteristic K α emissions of carbon and oxygen, respectively, and the very weak emission at 0.392 keV is the K α emission of nitrogen (Figure 2A-E). The peaks in the spectra of the films after the polymerization reactions at 2.621 and 6.398 keV are characteristic K α emission lines of chlorine and iron, the peak at 0.705 keV is the L α emission line of iron (Figure 2B-E), and the peak at 2.307 keV is the K α emission line of sulphur present in the backbone of the PEDOT-CO₂H (Figure 2D and 2E). The successful biomaterialization of the PPy-NH₂ films (Figure 2B) with silica is clear from the appearance of the K α emission peak of silicon at 1.739 keV (Figure 2C). Likewise, the successful biomaterialization of the PEDOT-CO₂H films (Figure 2D) with calcium phosphate is clear from the appearance of the peaks at 2.013 and 3.690 keV, that are characteristic of the K α emissions of phosphorous and calcium, respectively (Figure 2E). The inset SEM images show the surface

morphologies of the films (Figure 2A-E), with nanometer to micrometer scale pores present on the surface of the biomaterialized films (Figure 2B-E).

The electrical sheet resistance of the biomaterialized samples was measured in accordance with the method described by Schmidt^{11,16} and Zhang.¹⁷ The PPy-NH₂ films had sheet resistances of 8.8 ± 3.6 k Ω which increase to 31.6 ± 9.1 k Ω after biomaterialization with silica; whereas the sheet resistances of PEDOT-CO₂H films was 2.7 ± 0.4 M Ω which decreased to 248.6 ± 71.8 k Ω after biomaterialization with calcium phosphate; and these values are of a similar order of magnitude to interpenetrating networks of polypyrrole and polystyrenesulfonate in PCL (68.0 ± 18.1 k Ω) that we have previously used to electrically simulate cells.¹⁶ While the electrochemical stability of the polypyrrole and PEDOT derivatives are known to decrease over long periods of time which may be problematic for biointerfaces intended for long term use,¹⁸ we and others have found them to be acceptable for the short term stimulation of cells residing in tissue scaffolds such as those reported here.^{3,4,6,11,16,17}

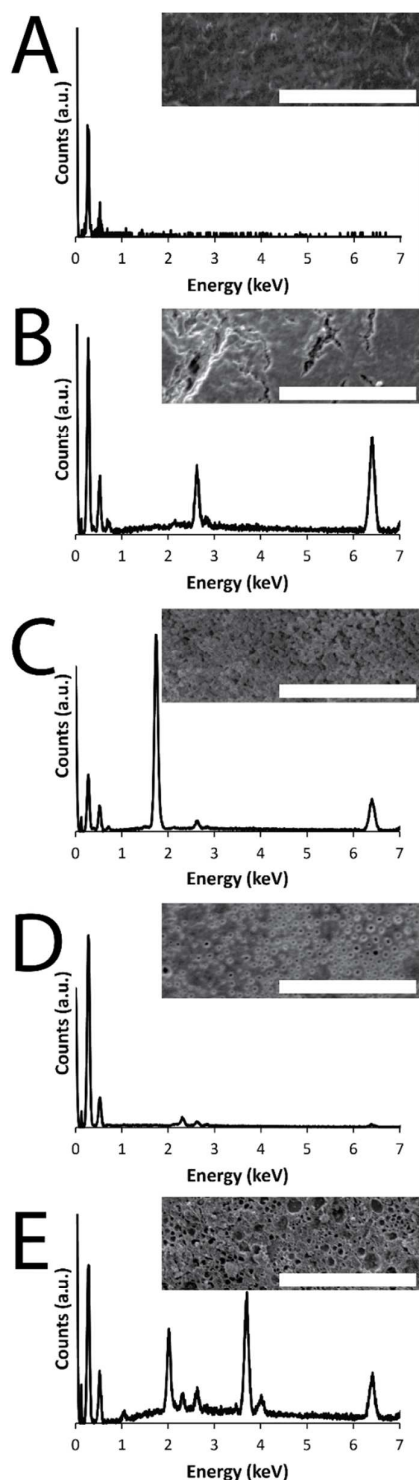


Figure 2. Physicochemical analysis of conductive materials. A) EDX analysis of PCL-triazole-Py functionalized films, inset SEM image. B) EDX analysis of PPy-NH₂ functionalized films, inset SEM image. C) EDX analysis of PPy-NH₂ functionalized films biom mineralized with silica, inset SEM image. D) EDX analysis of PEDOT-CO₂H functionalized films, inset SEM image. E) EDX analysis of PEDOT-CO₂H functionalized films biom mineralized with calcium phosphate, inset SEM image. Scale bars represent 50 µm.

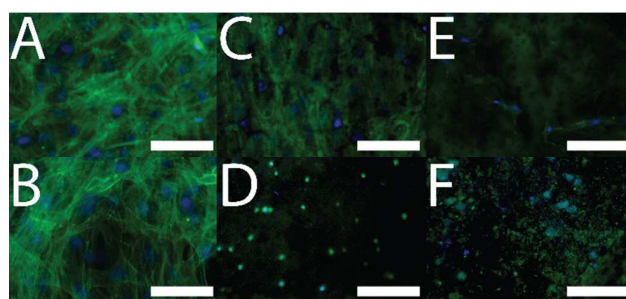


Figure 3. Fluorescently stained cells cultured on various substrates. DAPI-stained nuclei are blue and Alexa Fluor® 488-stained actin is green. A) Tissue-culture treated Corning® Costar® tissue culture plate controls. B) PCL control. C) Conducting silica-biom mineralized film without electrical stimulation. D) Conducting silica-biom mineralized film with electrical stimulation. E) Conducting calcium phosphate-biom mineralized film without electrical stimulation. F) Conducting calcium phosphate-biom mineralized film with electrical stimulation. Scale bars represent 100 µm.

EDITORS: figure 3 should be 2 columns in width!

To investigate the potential of the biom mineralized CPs to act as bone tissue scaffolds, we seeded human mesenchymal stem cells (hMSCs) on their surfaces and cultured them in osteogenic medium for 3 weeks *in vitro* (testing necessary prior to *in vivo* testing). We seeded six different systems: 1) cells seeded on TCP controls; 2) cells seeded on PCL (80 kDa); 3) cells seeded on silica-biom mineralized PPy-NH₂ films without electrical stimulation; 4) cells seeded on silica-biom mineralized PPy-NH₂ films with electrical stimulation; 5) cells seeded on silica-biom mineralized PEDOT-CO₂H films without electrical stimulation; 6) cells seeded on silica-biom mineralized PEDOT-CO₂H films with electrical stimulation. Those samples that were electrically stimulated were cultured for 2 days without stimulation, followed by four periods of stimulation at 10 mV mm⁻¹ for 8 hours then 40 hours without stimulation, and no stimulation thereafter; a stimulation paradigm analogous to that of Langer and coworkers⁴ and indeed ourselves¹⁶).

After 3 weeks of *in vitro* culture, cells were fixed with paraformaldehyde and cell nuclei and actin filaments within cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and Alexa Fluor® 488 Phalloidin, respectively. We observed that cells were homogeneously distributed on the TCP and PCL controls, and that cells had infiltrated the biom mineralized CP films (Figure 3) which is promising for their integration in the body where infiltration of cells such as macrophages and osteoclasts facilitates remodelling of implanted biomaterials.¹⁹ The differentiation of the cell population towards osteogenic fates *in vitro* was shown using a biochemical assay for alkaline phosphatase (ALP) activity which is a characteristic marker of bone formation. To within experimental error, ALP activity of cells cultured on the TCP and PCL control substrates was the same (Figure 4). ALP activity for cells cultured on the conductive biom mineralized scaffolds *in vitro* was reduced relative to the TCP and PCL control substrates, which is likely to be because of subtle differences in cell-matrix interactions as observed for analogous systems.²⁰ Interestingly, ALP activity of cells cultured on the scaffolds mineralized with calcium phosphate was slightly higher than for cells cultured on the scaffolds mineralized with silica, which is likely to be because the calcium phosphate acts as a

source of calcium and phosphate ions enabling the production of calcified extracellular matrix.²¹ Furthermore, the ALP activity of cells cultured on the conductive biomineralized scaffolds was increased after electrical stimulation (four periods during which a potential step of 10 mV mm⁻¹ was applied across the conductive substrates for 8 hours), which is in line with reports by Langer⁴ and others.⁶ Therefore, our biochemical analysis reveals that while the non-conductive scaffolds support differentiation of HMSCs towards osteogenic outcomes, the application of an electrical stimulus to HMSCs residing in a conductive scaffold *in vitro* enhances levels of ALP activity which is a hallmark of bone tissue formation.

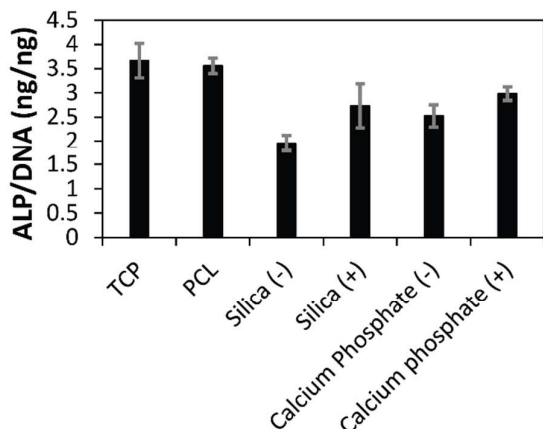


Figure 4. Biochemical analysis of *in vitro* cell culture experiments. A) ALP activity. TCP, Tissue-culture treated Corning® Costar® tissue culture plate controls. PCL, PCL control. Silica (-), conducting silica-biomineralized film without electrical stimulation. Silica (+), conducting silica-biomineralized film with electrical stimulation. Calcium phosphate (-), conducting calcium phosphate-biomineralized film without electrical stimulation. Calcium phosphate (+), conducting calcium phosphate-biomineralized film with electrical stimulation

In the long term we believe it should be possible to prepare a variety of conductive biomineralized tissue scaffolds by chemical modification of the scaffolds with peptides directing the mineralization (e.g. FRRRIKA),²² and potentially also peptides that control other aspects of cell behaviour (e.g. RGD, YIGSR or KRSR for cell adhesion, and NSPVNSKIPKACCVPTLSAI for osteoinduction),²² thereby allowing us to tailor the properties of the scaffold to specific niche applications (and potentially specific patients).

Conclusions

Pro-regenerative biomaterials for the treatment of bone conditions and disorders that require surgical intervention are of growing importance in modern societies in which life expectancies are increasing. Bone tissue scaffolds that control the behaviour of cells residing on them are particularly interesting for such applications.

Calcium carbonate is increasingly interesting in biomedicine as a novel scaffolds for bone tissue engineering,²³ and it is possible to biomineralize PEDOT-CO₂H films with calcium carbonate (Figure S2). While it is possible to biomineralize analogous materials incorporating interpenetrating networks

of sulfonate displaying PEDOT-SO₃Na (Figure 1, Scheme S5)²⁴ with calcium-based biominerals we found them to be mechanically unstable during long term *in vitro* cell culture experiments. PEDOT-SO₃Na is the most hydrophilic/water soluble of the conducting polymers tested, which is likely to increase rates of enzymatic degradation of the PCL matrix as we have observed for interpenetrating networks of PCL with water insoluble polyplexes of polypyrrole/polystyrenesulfonate.¹⁶ Moreover, we know that such PCL/polypyrrole/polystyrenesulfonate-based materials are stable to long term cell culture *in vitro*,¹⁶ and allow the growth of calcium-based biominerals such as calcium carbonate (Figure S3).

Herein we report the first examples of bone tissue scaffolds that combine the attractive properties of biomineralized substrates and electrical conductivity. Controlling the surface chemistry of the substrate enables us to impart electrical conductivity to a biodegradable polymer-based substrate (i.e. PCL) by functionalizing it with conducting polymers (e.g. polypyrrole and poly(3,4-ethylenedioxythiophene) derivatives. Appropriate choice of monomers constituting the conducting polymers enables us to display moieties that facilitate the deposition of specific biominerals; conducting polymers displaying amines that are positively charged under physiological conditions interact with silicic acid which is a precursor to silica formation, whereas those displaying carboxylates or sulfonates which are negatively charged under physiological conditions interact with Ca²⁺ encouraging their mineralization with calcium phosphate or indeed calcium carbonate, all of which have been shown to be beneficial for bone tissue engineering. Finally, we show that the electrical stimulation of HMSCs residing on our biomineralized conducting polymer-based substrates *in vitro* enhances levels of ALP activity, which represents an important step towards the formation of bone tissue. We believe that such materials represent useful prototypes for conducting bone tissue scaffolds and warrant further development.

Acknowledgements

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