Journal of Materials Chemistry B

COMMUNICATION

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

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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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 absence[5] or presence[6] of an electrical field, commonly finding improved osteogenesis for the electrically stimulated samples. 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-based[9] 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. Polymers displaying amines, carboxylates or sulfonates (Figure 1) facilitate mineralization of silica or calcium carbonates or phosphates. These conducting bone tissue scaffolds enable electrical stimulation of human mesenchymal stem cells which promotes their differentiation towards osteogenic outcomes.

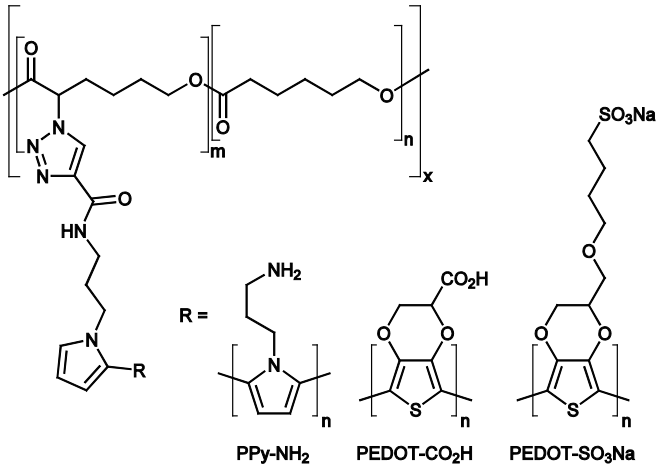

Figure 1. Conducting polymers enabling biomineralization with silica (R-NH_2), or calcium carbonate/phosphate (R-CO_2H or R-SO_3Na).

Propiolic acid was coupled to aminopropylpyrrole[11,12] by carbodiimide-mediated peptide coupling (Scheme S1), and these were coupled to PCL derivatives displaying azide moiteties[13] by

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Electronic Supplementary Information (ESI) available: experimental details, supplementary schemes and figures. See DOI: 10.1039/x0xx00000x

Cu(I)-mediated triazole formation[13] (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-NH_2, Figure 1) or carboxylate displaying poly(3,4-ethylenedioxythiophene) derivative (PEDOT-CO_2H, 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-NH_2 films were biomineralized with silica and those of the blue-grey PEDOT-CO_2H were biomineralized 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 Kα emissions of carbon and oxygen, respectively, and the very weak emission at 0.392 keV is the Kα 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 Kα emission lines of chlorine and iron, the peak at 0.705 keV is the Lα emission line of iron (Figure 2B-E), and the peak at 2.307 keV is the Kα emission line of sulphur present in the backbone of the PEDOT-CO_2H (Figure 2D and 2E). The successful biomineralization of the PPy-NH_2 films (Figure 2B) with silica is clear from the appearance of the Kα emission peak of silicon at 1.739 keV (Figure 2C). Likewise, the successful biomineralization of the PEDOT-CO_2H 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 Kα 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 biomineralized films (Figure 2B-E).

The electrical sheet resistance of the biomineralized samples was measured in accordance with the method described by Schmidt[11,16] and Zhang.[17] The PPy-NH_2 films biomineralized with silica had sheet resistances of 31.6 ± 9.1 kΩ, and those of PEDOT-CO_2H biomineralized with calcium phosphate had sheet resistances of 248.6 ± 71.8 kΩ, which is of a similar order of magnitude to interpenetrating networks of polypyrrole and polystyrenesulfonate in PCL (68.0 ± 18.1 kΩ).[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]

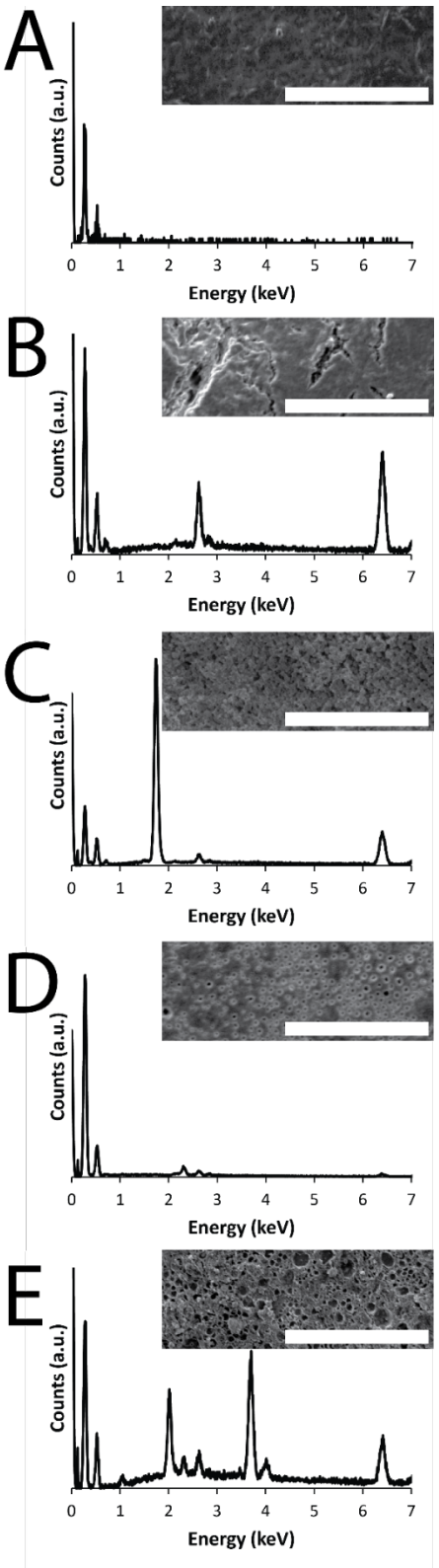

Figure 2. Physicochemical analysis of conductive materials. A) EDX analysis of PCL-triazole-Py functionalized films, inset SEM image. B) EDX analysis of PPy-NH2 functionalized films, inset SEM image. C) EDX analysis of PPy-NH2 functionalized films biomineralized with silica, inset SEM image. D) EDX analysis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

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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-coated film without electrical stimulation. D) Conducting silica-coated film with electrical stimulation. E) Conducting calcium phosphate-coated film without electrical stimulation. F) Conducting calcium phosphate-coated film with electrical stimulation. Scale bars represent 100 µm.

To investigate the potential of the biomineralized 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. We seeded six different systems: 1) cells seeded on TCP controls; 2) cells seeded on PCL (80 kDa); 3) cells seeded on silica-coated PPy-NH_2 films without electrical stimulation; 4) cells seeded on silica-coated PPy-NH_2 films with electrical stimulation; 5) cells seeded on silica-coated PEDOT-CO_2H films without electrical stimulation; 6) cells seeded on silica-coated PEDOT-CO_2H 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 stimualtion, and no stimulation thereafter).

After 3 weeks in 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 biomineral coatings on the biomineralized 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 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 biomineralized scaffolds 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 Langer[4] 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 enhances levels of ALP activity which is a hallmark of bone tissue formation.

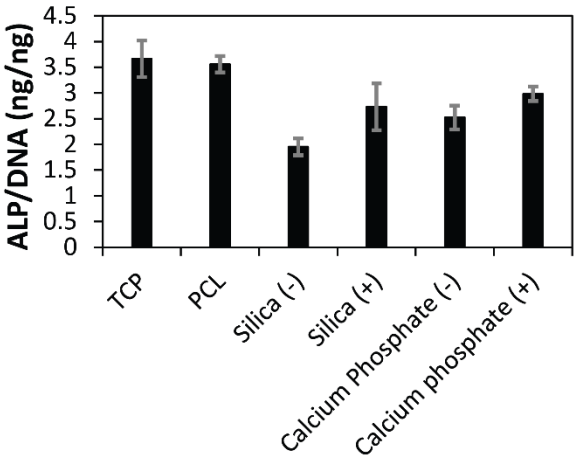

Figure 4. Biochemical analysis of in vitro cell culture experiments. A) ALP activity. TCP, Tissue-culture treated Corning® Costar® tissue culture plate controls. PCL, PCL control. Silica (-), conducting silica-coated film without electrical stimulation. Silica (+), conducting silica-coated film with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

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. We report the first examples of biomineralized conductive bone tissue scaffolds and show that the electrical stimulation of HMSCs residing thereon enhances levels of ALP activity, which represents an important step towards the formation of bone tissue.

Calcium carbonate is increasingly interesting in biomedicine as a novel scaffolds for bone tissue engineering,[22] and it is possible to biomineralize PEDOT-CO_2H films with calcium carbonate (Figure S2). While it is possible to biomineralize analogous materials incorporating interpenetrating networks of sulfonate displaying PEDOT-SO_3Na (Figure 1, Scheme S5)[23] with calcium-based biominerals we found them to be mechanically unstable during long term cell culture experiments. PEDOT-SO_3Na 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,[16] and allow the growth of calcium-based biominerals such as calcium carbonate (Figure S3).

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. FHRRIKA),[24] and potentially also peptides

that control other aspects of cell behaviour (e.g. RGD, YIGSR or KRSR for cell adhesion, and NSPVNSKIPKACCVPTELSAI for osteoinduction),[24] thereby allowing us to tailor the properties of the scaffold to specific niche applications (and potentially specific patients).

Acknowledgements

We thank the University of Texas at Austin for financial support of David J. Mouser and Rushi C. Sukhavasi in the form of Undergraduate Research Fellowships. We thank Professor Christopher W. Bielawski for access to the GPC and Dr. Robert J. Ono and Dr. C. Daniel Varnado for training. SEM and EDX were carried out at the Texas Materials Institute and we thank Dr. Shouliang Zhang for training. We thank the University of Florida for financial support in the form of startup resources.

References

1 A. Atala, *J. Tissue Eng. Regen. Med*., 2007, **1**, 83; J. O. Hollinger, S. Winn and J. Bonadio, *Tissue Engineering*, 2000, **6**, 341; C. T. Laurencin, A. M. A. Ambrosio, M. D. Borden and J. A. Cooper, Jr., *Annu. Rev. Biomed. Eng.*, 1999, **1**, 19; J. R. Porter, T. T. Ruckh and K. C. Popat, *Biotechnol. Prog.*, 2009, **25**, 1539; M. A. Fernandez-Yague, S. A. Abbah, L. McNamar, D. I. Zeugolis, A. Pandit and M. J. Biggs, *Adv. Drug Deliv. Rev.*, 2014, http://dx.doi.org/10.1016/j.addr.2014.09.005; D. Marolt, M. Knezevic and G. Vunjak-Novakovic, *Stem Cell Res. Ther.*, 2010, **1**, 10; W. L. Grayson, T. P. Martens, G. M. Eng., M. Radisic and G. Vunjak-Novakovic, *Semin. Cell Dev. Biol*., 2009, **20**, 665; M. Fröhlich, W. L. Grayson, L. Q. Wan, D. Marolt, M. Drobnic and G. Vunjak-Novakovic, Curr. *Stem Cell Res. Ther*., 2008, **3**, 254.

2 N. M. Alves, I. B. Leonor, H. S. Azevedo, R. L. Reis and J. F. Mano, *J. Mater. Chem.*, 2010, **20**, 2911; S. V. Dorozhkin and M. Epple, *Angew. Chem. Int. Ed*., 2002, **41**, 3130; A. R. Boccaccini, M. Erol, W. J. Stark, D. Mohn, Z. Hong and J. F. Mano, *Comp. Sci. Technol.*, 2010, **70**, 1764; F. C. Meldrum and H. Cölfen, *Chem. Rev.*, 2008, **108**, 4332; S. Heinemann, T. Coradin and M. F. Desimone, *Biomater. Sci*., 2013, **1**, 688; C. Li and D. L. Kaplan, *Curr. Opin. Solid State Mater. Sci.*, 2003, **7**, 265; N. M. Alves, I. B. Leonor, H. S. Azevedo, R. L. Reis and J. F. Mano, *J. Mater. Chem.*, 2010, **20**, 2911; A. Dey, G. de With and Nico A. J. M. Sommerdijk, *Chem. Soc. Rev.*, 2010, **39**, 397; A. J. Salinas, P. Esbrit and M. Vallet-Regí, *Biomater. Sci.*, 2013, **1**, 40; E. Ko and S.-W. Cho, *Int. J. Stem Cells*, 2013, **6**, 87.

3 B. Guo, L. Glavas and A. C. Albertsson, *Prog. Polym. Sci*., 2013, **38**, 1263; M. Muskovich and C. J. Bettinger, *Adv. Healthcare Mater*., 2012, **1**, 248; M. Berggren and A. Richter-Dahlfors, *Adv. Mater*., 2007, **19**, 3201; R. Balint, N. J. Cassidy and S. H. Cartmell, *Acta Biomaterialia*, 2014, **10**, 2341; R. A. Green, N. H. Lovell, G. G. Wallace and L. A. Poole-Warren, *Biomaterials*, 2008, **29**, 3393; Mihai Irimia-Vladu, *Chem. Soc. Rev*., 2014, **43**, 588; N. K. Guimard, N. Gomez and C. E. Schmidt, *Prog. Polym. Sci*., 2007, **32**, 876; J. G. Hardy, J. Y. Lee and C. E. Schmidt, *Curr. Opin. Biotechnol*., 2013, **24**, 847; D. Svirskis, J. Travas-Sejdic, A. Rodgers and S. Garg, *J. Control. Release*, 2010, **146**, 6; J. G. Hardy, D. J. Mouser, N. Arroyo-Currás, S. Geissler, J. K. Chow, L. Nguy, J. M. Kim and C. E. Schmidt, *J. Mater. Chem. B*, 2014, **2**, 6809; T. F. Otero and J. G. Martinez, *J. Mater. Chem. B*, 2013, **1**, 26; R. Gracia and D. Mecerreyes, *Polym. Chem*., 2013, **4**, 2206; T. H. Qazi, R. Rai and A. R. Boccaccini, *Biomaterials*, 2014, **35**, 9068; J. Zimmerman, R. Parameswaran and B. Tian, *Biomater. Sci.*, 2014, **2**, 619.

4 V. P. Shastri, N. Rahman, I. Martin and R. Langer, *Mat. Res. Soc. Symp. Proc.*, 1999, **550**, 215.

5 C. Rincón and J. C. Meredith, *Macromol. Biosci*., 2010, **10**, 258; C. Rincón, C.-C. Chen and J. C. Meredith, *Macromol. Biosci*., 2010, **10**, 1536; E. De Giglio, S. Cometa, C.-D. Calvano, L. Sabbatini, P. G. Zambonin, S. Colucci, A. Di Benedetto and G. Colaianni, *J. Mater. Sci. Mater. Med*., 2007, **18**, 1781; B. Lakard, L. Ploux, K. Anselme, F. Lallemand, S. Lakard, M. Nardin and J.Y. Hihn, *Bioelectrochemistry*, 2009, **75** 148; J. S. Moreno, S. Panero, S. Materazzi, A. Martinelli, M. G. Sabbieti, D. Agas and G Materazzi, *J. Biomed. Mater. Res*., 2009, **88A**, 832; H. Castano, E. A. O'Rear, P. S. McFetridge and V. I. Sikavitsas, *Macromol. Biosci*. 2004, **4**, 785.

6 J. Cao, Y. Man and L. Li, *Biomedical Reports*, 2013, **1**, 428; L. Liu, P. Li, G. Zhou, M. Wang, X. Jia, M. Liu, X. Niu, W. Song, H. Liu and Y. Fan, *J. Biomed. Nanotechnol*., 2013, **9**, 1532; W.-W. Hu, Y.-T. Hsu, Y.-C. Cheng, C. Li, R.-C. Ruaan, C.-C. Chien, C.-A. Chung and C.-W. Tsao, *Mater. Sci. Eng. C*, 2014, **37**, 28; G. Jin and G. Kim, *J. Mater. Chem. B*, 2013, **1**, 1439; J. Zhang, K. G. Neoh, X. Hu, E.-T. Kang and W. Wang, *Biotechnol. Bioeng.*, 2013, **110**, 1466; Y. Liu, H. Cui, X. Zhuang, Y. Wei and X. Chen, *Acta Biomaterialia*, 2014, **10**, 5074; H. Cui, Y. Liu, M. Deng, X. Pang, P. Zhang, X. Wang, X. Chen and Yen Wei, *Biomacromolecules*, 2012, **13**, 2881; H. Cui, Y. Wang, L. Cui, P. Zhang, X. Wang, Y. Wei and X. Chen, *Biomacromolecules*, 2014, **15**, 3146; S. Meng, M. Rouabhia and Z. Zhang, *Bioelectromagnetics*, 2013, **34**, 189; S. Meng, Z. Zhang and M. Rouabhia, *J. Bone Miner. Metab.*, 2011, **29**, 535.

7 S. Yala, H. Khireddine, D. Sidane, S. Ziani and F. Bir, *J. Mater. Sci*., 2013, **48**, 7215; Y. Liu, H. Cui, X. Zhuang, P. Zhang, Y. Cui, X. Wang, Y. Wei and X. Chen; *Macromol. Biosci.*, 2013, **13**, 356.

8 D. Gopi, S. Ramya, D. Rajeswari, M. Surendiran and L. Kavitha, *Coll. Surf. B: Biointerfaces*, 2014, **114**, 234.

9 J. Liao, H. Pan, C. Ning, G. Tan, Z. Zhou, J. Chen and S. Huang, *Macromol. Rapid Commun.*, 2014, **35**, 574; J. Liao, Y. Zhu, Z. Yin, G. Tan, C. Ning and C. Mao, *J. Mater. Chem. B*, 2014, **2**, 7872; E. De Giglio, M. R. Guascito, L. Sabbatini and G. Zambonin, *Biomaterials*, 2001, **22**, 2609; E. De Giglio, L. Sabbatini and P. G. Zambonin, *J. Biomater. Sci. Polymer Edn*., 1999, **10**, 845; E. De Giglio, L. Sabbatini, S. Colucci and G. Zambonin, *J. Biomater. Sci. Polymer Edn*., 1999, **10**, 1073; E. De Giglio, L. De Genarro, L. Sabbatini and G. Zambonin, *J. Biomater. Sci. Polymer Edn*., 2001, **12**, 63; E. De Giglio, S. Cometa, C.-D. Calvano, L. Sabbatini, P. G. Zambonin, S. Colucci, A. Benedetto and G. Colaianni, *J. Mater. Sci. Mater. Med*., 2007, **18**, 1781.

10 S. Sirivisoot, R. A. Pareta and T. J. Webster, *Solid State Phenomena*, 2009, **151**, 197; S. Sirivisoot, R. A. Pareta and T. J. Webster, *Nanotechnology*, 2011, **22**, 085101.

11 J. Y. Lee, C. A. Bashur, A. S. Goldstein and C. E. Schmidt, *Biomaterials*, 2009, **30**, 4325

12 J. Y. Lee and C. E. Schmidt, *J. Biomed. Mater. Res. Part A*, 2014, DOI: 10.1002/jbm.a.35344.

13 S. Lenoir, R. Riva, X. Lou, C. Detrembleur, R. Jérôme and P. Lecomte, *Macromolecules*, 2004, **37**, 4055; R. Riva, P. Lussis, S. Lenoir, C. Jérôme, R. Jérôme and P. Lecomte, *Polymer*, 2008, **49**, 2023; R. Riva, S. Schmeits, F. Stoffelbach, C. Jérôme, R. Jérôme and P. Lecomte, *Chem. Commun.*, 2005, 5334.

14 M. Malkoch, R. Vestberg, N. Gupta, L. Mespouille, P. Dubois, A. F. Mason, J. L. Hedrick, Q. Liao, C. W. Frank, K. Kingsbury and C. J. Hawker, *Chem. Commun.*, 2006, 2774.

15 R. H. Karlsson, A. Herland, M. Hamedi, J. A. Wigenius, A. Åslund, X. Liu, M. Fahlman, O. Inganas and P. Konradsson, *Chem. Mater*., 2009, **21**, 1815.
16 J. G. Hardy, R. C. Cornelison, R. C. Sukhavasi, R. J. Saballos, P. Vu, D. L. Kaplan and C. E. Schmidt, *Bioengineering*, 2015, **2**, 15.
17 X. P. Jiang, D. Tessier, L. H. Dao and Z. Zhang, *J. Biomed. Mater. Res.*, 2002, **62**, 507.
18 A. Kros, N. A. J. M. Sommerdijk and R. J .M. Nolte, *Sensors and Actuators B*, 2005, **106**, 289.
19 P. M. Mountziaris and A. G. Mikos, *Tissue Eng. Part B*, 2008, **14**, 179; L. J. Raggatt and N. C. Partridge, *J. Biol. Chem*., 2010, **285**, 25103; S. Hofmann, M. Hilbe, R. J. Fajardo, H. Hagenmuller, K. Nuss, M. Arras, R. Muller, B. von Rechenberg, D. L. Kaplan, M. P. Merkle and L. Meinel, *Eur. J. Pharm. Biopharm*., 2013, **85**, 119; D. J. Hadjidakis and I. I. Androulakis, *Ann. N. Y. Acad. Sci*., 2006, **1092**, 385; J. P. Santerre, R. S. Labow and E. L. Boynton, *Can. J. Surg*., 2000, **43**, 173; N. A. Sims and T. J. Martin, *BoneKEy Reports*, 2014, **3**, 481; J. C. Crockett, M. J. Rogers, F. P. Coxon, L. J. Hocking and M. H. Helfrich, *J. Cell Sci*., 2011, **124**, 991.
20 G. G. Wallace, M. J. Higgins, S. E. Moulton and C. Wang, *Nanoscale*, 2012, **4**, 4327; M. J. Higgins, P. J. Molino, Z. Yue and G. G. Wallace, *Chem. Mater*., 2012, **24**, 828; A. Gelmi, M. K. Ljunggren, M. Rafat and E. W. H. Jager, *J. Mater. Chem. B*, 2014, **2**, 3860.
21 Y.-R. V. Shih, Y. Hwang, A. Phadke, H. Kang, N. S. Hwang, E. J. Caro, S. Nguyen, M. Siu, E. A. Theodorakis, N. C. Gianneschi, K. S. Vecchio, S. Chien, O. K. Lee and S. Varghese, *PNAS*, 2014, **111**, 990.
22 C. Combes, B. Miaoa, R. Bareilleb, C. Rey, Biomaterials, 2006, 27, 1945; W. Schneiders, A. Reinstorf., W. Pompe, R. Grass, A. Biewener, M. Holch, H. Zwipp and S. Rammelt, *Bone*, 2007, *40*, 1048; K. Sariibrahimoglu, S. C. G. Leeuwenburgh, J. G. C. Wolke, L. Yubao, J. A. Jansen; *J. Biomed. Mater. Res. Part A*, 2012, **100A**, 712; A. H. Dewi, I. D. Ana, J. Wolke, J. Jansen, *J. Biomed. Mater. Res. Part A,* 2013, **101A**, 2143; H. Zhu, J. Schulz, H. Schliephake, *Clin. Oral Implants Res.*, 2010, **21**, 182.
23 O. Stéphan, P. Schottland, P.-Y. Le Gall, C. Chevrot, C. Mariet and M. Carrier, *Electroanal. Chem.*, 1998, **443**, 217; M. Yamada, N. Ohnishi, M. Watanabe and Y. Hino, *Chem. Commun*., 2009, 7203.
24 K. G. Sreejalekshmi and P. D. Nair, *J. Biomed. Mater. Res., Part A*, 2011, **96A**, 477; H. Shin, S. Jo and A. G. Mikos, *Biomaterials*, 2003, **24**, 4353.