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Tuning nano-architectures and improving bioactivity of conducting polypyrrole coating on bone implants by incorporating bone-borne small molecules

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Abstract

Citric acid, a molecule present in fresh bone, was introduced into template-free electrochemical polymerization to form biocompatible coating made of polypyrrole (PPy) nano-cones on bone implants. It served not only as a dopant to tune the nano-architectures but also as a promoter to enhance bioactivity of the PPy-coated implants.

1D conducting polymer (CP) nano-architectures such as nanowires and nanotubes exhibit short transport path for ions and show switchable redox reversibility associated with ion transport and reversible variation of physical properties such as volume, wettability, density and conductivity.¹ They show electroactivity, biocompatibility, biomolecule affinity and environmental stability,^{2–6} and thus are ideal candidates for intelligent switching biointerface,^{6–8} drug delivery,⁹ biological sensing,^{10, 11} neural probes^{12, 13} and even tissue engineering.^{14, 15} For instance, 1D CP nano-architectures hold promise in providing bone-mimetic electrophysiological microenvironment at cellular level and thus can be decorated on bone implant for bone repair and regrowth.¹⁶ Among the numerous well-controlled nanotechnologies for constructing 1D CP nano-architectures, template-based approaches are universal and flexible strategies, however, the need of removing the template (*e.g.*, anodic

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aluminum) in these approaches result in the aggregation and damage of nano-architectures.¹⁷ Therefore, an alternative strategy without the use of templates has been sought over the last two decades. Wan *et al*¹⁸ have developed a facile, efficient, and controlled template-free method of forming 1D CP nano-architectures by using dopants such as β -naphthalene sulfonic acid (NSA) and camphorsulfonic acid (CSA). However, these sulphur-containing dopants are small organic acids, which are toxic and not biocompatible. Thus the resultant materials are unacceptable for biomedical use. Therefore, there is a pressing need in developing a new green approach to constructing 1D CP nano-architectures for biomedical use.

To fabricate nanomaterials with potential application in biomedicine through a green approach,^{19–22} it is highly desired to use environmentally benign nanotechnologies where non-toxic chemicals are used. Inspired by nature, biomolecules from organism have been employed to assist the fabrication of nanomaterials,^{23, 24} which is compatible with the green chemistry principles.²⁰ Of late years, the biomolecule-assisted fabrication of inorganic nanomaterials, ranging from elemental metals (*e.g.*, Ag, Au, Pt, Cu), and inorganic substances (*e.g.*, TiO₂, ZnO), to C-based fullerenes and their derivatives,²¹ have been studied and proven to be a promising approach.^{25–30} However, biomolecules, in particular small biomolecules, have received less attention in assisting the fabrication of polymeric nanomaterials.

Citric acid is a small biomolecule found in a lot of fruits (*e.g.*, lemon) in a large amount as well as in fresh bone with a content of 1%.³¹ It is involved in the metabolism of inorganic mineral in bone by serving as a biological ligand for Ca²⁺.^{32, 33} In the synthesis of metallic/inorganic nano-architectures, citric acid has widely been used as a reducing, complexing, or stabilizing agent.^{34–37} In this work, a citrate-assisted green approach was developed to functionalize the bone implants with PPy as an intelligent electrical platform for forming bone-mimetic electrophysiological microenvironment on bone implant since electrical cure has been proven a highly effective method in bone repair and regrowth.³⁸ Specifically, citric acid was firstly employed to assist the construction of 1D nano-architected PPy (NAPPy) on biomedical titanium (as a bone implant) in phosphate buffered saline (PBS, pH 7.4) *via* template-free electrochemical polymerization. Probably because citric acid tended to more preferentially adsorb and stabilize the free-Py than Py nano-droplet, various nano-architectures could be generated by tuning the amount of citric acid used for stabilizing the Py nano-droplets. Accordingly, a possible mechanism for citrate-assisted construction of 1D NAPPy was proposed. Furthermore, the resultant 1D NAPPy/citrate was proven to enhance the bioactivity of the implants using the *in vitro* biomineralization in simulated body fluid (SBF) and biological activities of MC3T3-E1 osteoblasts. This work represents the first-time effort in employing citric acid to promote the formation of 1D CP nanostructures for functionalizing the surface of bone implants.

By employing citric acid in constructing the nano-architected CPs in mild medium, phosphate buffered saline (PBS), *via* template-free electrochemical polymerization, a high density of conical 1D PPy/citrate nanowires, approximately 800 nm long, were successfully synthesized (Figure 1a). The architectures are cone-like nanostructures (Figure 1b) measuring about 70 nm in top diameter. These oriented nano-cones (Figure 1c) were grown

vertically on the surface of biomedical titanium implants to achieve a large specific surface area and desired electrical properties (Figure S1). Unlike NSA or CSA, citric acid is not a traditional dopant of CPs. To further understand the nano-cones, the chemical composition of 1D NAPPy/citrate was analyzed by attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy (Figure 1d). Three peaks at 1525, 1462 and 772 cm^{-1} could be assigned to the C=C stretching vibration, C–N stretching vibration, C–H out-plane ring deformation of PPy, respectively.³⁹ The relatively strong peaks at 1583 and 1249 cm^{-1} could be attributed to C=O and C–O stretching vibration of three –COO^- groups in each citric acid molecule, respectively.⁴⁰ The two peaks at 1350 and 631 cm^{-1} corresponded to –OH deformation vibration and stretching vibration in citric acid molecules, respectively,⁴¹ and the peak at 1145 cm^{-1} was associated with C–O stretching vibration of C–OH group in citric acid molecules. These results showed that citric acid was incorporated into the PPy matrix in PBS through the template-free electrochemical polymerization. In addition, the presence of citric acid in PPy matrix was further verified by XPS (Figure S2).

In the reported studies on using citric acid to synthesize the nano-architected inorganic materials such as Au and CuO, the nano-architectures were tunable by the molar ratio of citric acid to metal ion due to the complexation.^{35–37} In order to elucidate the role of citric acid in the fabrication of 1D NAPPy, the nanostructures of PPy were characterized when they were fabricated at different citric acid concentrations in conjunction with the pH value of PBS affecting the solubility of Py (Figure 2). When the citric acid concentration was doubled to 0.1 M in PBS of pH 7.4, the conical 1D nano-architectures (Figure 2a) obtained were the same as that in Figure 1a (pH 7.4, 0.05 M citrate). However, when the citric acid concentration was decreased to 0.02 M, tightly packed nanoparticles (Figure 2b) of about 100 nm in diameter were yielded. It was also found that PBS of pH 8.0 containing 0.02 M citric acid led to a higher density of short cylindrical 1D nano-architectures (Figure 2c), while a lower pH value (6.5) of PBS containing more citric acid resulted in the formation of an irregular film (Figure 2d). In our previous study,^{42, 43} Py existed in the forms of free-Py and Py nano-droplets when in PBS, and a higher pH value of PBS resulted in a lower solubility of Py and thus a higher concentration of Py nano-droplets. Therefore, it was reasonable to consider that citric acid participated in the construction of NAPPy through its interaction with Py in free-state and droplet-state.

Furthermore, the correlation between citric acid concentrations and crystallinity of NAPPy was uncovered using XRD. XRD characterization was done on three resultant PPy/citrate nanoarchitectures, formed in PBS of pH 7.4 containing citric acid with different concentrations including 0.10 M (Figure 2e1), 0.05 M (Figure 2e2) and 0.02 M (Figure 2e3), which were corresponding to those shown in Figure 2a, 1a and 2b, respectively. A peak at $2\theta = 13.0^\circ$ emerging in only Figure 3a could be assigned to the repeating unit with a periodic distance of $d = 5.9 \text{ \AA}$ between PPy chains, which could be calculated by Bragg's equation. Our data showed that the citric acid concentration reaching a specific level allowed the alignment of PPy chains, which was different from the reported amorphous (or partially crystalline) micro/nano-architected^{44–46} and 1D NAPPy/NSA (Figure 2e4 and Figure S3) and irregular PPy/Cl film (Figure 2e5). Namely, lower citric acid concentration would not favor the the alignment of polymer chains into NAPPy, suggesting that citric acid exerted a special role in constructing 1D NAPPy at both molecular and nanoscale level. It

was noteworthy that, the pH value of the PBS, which rendered the template-free electrochemical polymerization to occur in a stable medium with constant pH, was also an important factor for promoting the formation of the crystalline 1D PPy nano-architectures.

Understanding the dependence of 1D NAPPy on citric acid should help us design a better green approach to the fabrication of nanomaterials. According to the results described above, we proposed a mechanism to explain the citrate-assisted formation of 1D NAPPy (Figure 3 and supplementary information). In the PBS containing the higher citric acid concentration, there would be a higher molar ratio of citrate/Py in the electrochemical system (the case shown in Figure 2a). Consequently, a certain amount of citric acid molecules were included in Py nano-droplets, which, through the hydrogen bonding, guided the alignment of PPy chains (Figure 3d#, confirmed by Figure 2e1) during the homopolymerization in nano-droplets. It should be pointed out that, citric acid might capture the free-Py more preferentially than Py nano-droplets (Figure 3b). As such, lower citric acid concentration would not allow the citric acid to stabilize the Py nano-droplets. Hence, when citric acid concentration was lower, Py nano-droplet/citrate was not formed, leading to a random self-assembly and the subsequent formation of tightly packed nanoparticles (the case shown in Figure 2b). When the citric acid concentration was lower but the pH was higher, more Py nano-droplets were present than free Py in the PBS, indicating that after the limited citric acid molecules were preferentially used to form a complex with the free-Py, some Py nano-droplets could still be stabilized by the remaining citric acid molecules. As a result, short cylindrical 1D NAPPy was formed by the pure self-assembly of Py nano-droplet/citrate due to the negligible amount of free-Py/citrate polymerization on the outer surface (the case shown in Figure 2c). When the citric acid concentration was higher but the pH value was lower, more free-Py was present in PBS and thus more citric acid molecules were used to preferentially bind free-Py in comparison with Py nano-droplets, which caused the Py nano-droplets not to be stabilized by citrates. Hence, an irregular film was formed without the polymerization of Py nano-droplet (the case shown in Figure 2d). In one word, in the presence of citrate, 1D NAPPy was formed due to the essential role of citric acid in stabilizing the Py nano-droplets and free-Py through hydrogen bonding between citric acid and Py.

To evaluate the biocompatibility and bioactivity of 1D NAPPy constructed by the assistance of citrate, the *in vitro* biomineralization in SBF and biological activities of MC3T3-E1 osteoblasts were performed on 1D NAPPy/citrate in comparison with biomedical titanium and traditional 1D NAPPy/NSA (Figure S2). As shown in Figure 4a1, almost no hydroxyapatite (HAp) could be observed on biomedical titanium after 3 days of biomineralization. However, HAp was formed on NAPPy-coated substrates. Both the diameter and the density of HAp micro-clusters grown on 1D NAPPy/citrate (Figure 4a2) were larger than those on 1D NAPPy/NSA (Figure 4a3). Moreover, on 1D NAPPy/citrate, the nano-architecture of HAp possessed larger nano-pores and a higher porosity, and the molar ratio of Ca to P was at 1.66 (very close to 1.67 in natural bone mineral), in contrast to 1.35 on 1D NAPPy/NSA. Since mineralization on a biomaterial from SBF is an *in vitro* method to prove the bone bioactivity of the biomaterial, these results indicate that 1D NAPPy/citrate was of superior bone-bioactivity and could be used as a more bionic implant

surface for realizing osteointegration by improved osteoconductivity and even osteoinductivity. Kelvin probe force microscopy (KFM) (Figure S4) manifested that no obvious difference of the average surface potential existed between 1D NAPPy/citrate and 1D NAPPy/NSA, indicating a similar concentration of the polar group on the two 1D NAPPy surfaces. Thus it was the citric acid that made 1D NAPPy/citrate more bioactive than 1D NAPPy/NSA. Namely, since citric acid had a stronger capacity for binding Ca^{2+} by complexation than NSA, it could provide sufficient biomimetic nucleation sites for initiating the nucleation of HAp.

The number of MC3T3-E1 osteoblast adhesion was gradually increased with the culture time and stabilized at 4 h (Figure 4b). Obviously, the number of cells on 1D NAPPy/citrate was greater than that on biomedical titanium and 1D NAPPy/NSA. Moreover, the cell spreading areas on biomedical titanium and 1D NAPPy/citrate were larger than those on 1D NAPPy/NSA by fluorescence imaging of cells stained with DAPI and rhodamin-labeled phalloidin (Figure 4c). The study on the proliferation (Figure 4d) of MC3T3-E1 osteoblasts by cell counting kit 8 (CCK-8) assay revealed that 1D NAPPy/citrate more significantly promoted the cell growth than 1D NAPPy/NSA for up to 7 days. Unexpectedly, unlike adhesion and spreading, proliferation ability of MC3T3-E1 osteoblasts on biomedical titanium was slightly stronger than on 1D NAPPy/citrate. Nevertheless, the highest osteogenic differentiation rate was still found on 1D NAPPy/citrate among all substrates studied, as evidenced by the expression of osteogenic markers, including alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN), bone sialoprotein (BSP) and collagen I (COL1) by ALP assay at different time points (Figure 4e) and real time polymerase chain reaction (PCR) assay on day 7 and 14 (Figure 4f) following our published protocols.^{47, 48} These results suggested that 1D NAPPy/citrate promoted osteoblastic differentiation in comparison with other substrates. The osteogenic differentiation behaviors on the three implant surfaces were well in agreement with *in vitro* biomineralization. The higher osteogenic differentiation rate on 1D NAPPy/citrate may be due to the bone-related functional nature of citric acid. In addition, water contact angle measurements (Figure S5) show that an ideal hydrophilic surface ($64.0 \pm 2.6^\circ$) was achieved by 1D NAPPy/citrate but 1D NAPPy/NSA and biomedical titanium were hydrophobic.⁴⁹ It is known that hydrophilic surface tend to favour cell activities. Thus it is not surprising to see the enhanced biological activities of MC3T3-E1 osteoblasts on the 1D NAPPy/citrate. The improved hydrophilicity of 1D NAPPy/citrate arises from the presence of citrate on the surface of the nanostructures. NSA as a non-bio functional molecule incorporated in PPy cannot exert a positive effect on the biological response of implant surface. Hence, we can conclude that citric acid, a functional biomolecule, serves not only as a dopant to tune the nano-architectures of PPy but also as a promoter to enhance the bioactivity of the PPy nanostructures.

Conclusions

In summary, in order to form the CPs as intelligent electrical implant surface and bone-mimetic electrophysiological micro-medium, citric acid, a small biomolecule found in natural bone, was employed to develop a green fabrication approach to nano-architected CPs on bone implant for the first time. Citric acid was utilized to assist the template-free electrochemical polymerization for the construction of 1D NAPPy on biomedical titanium in

PBS. It was found that the various PPy nano-architectures could be produced by tailoring the citric acid concentrations and pH values of PBS. Citric acid probably acted as a reagent for capturing and stabilizing the Py nano-droplets by hydrogen bonding between citric acid and Py during the self-assembly polymerization of Py nano-droplets into the 1D nano-architectures. Furthermore, the *in vitro* biomineralization in SBF and biological activities (adhesion, spreading, proliferation and osteogenic differentiation) of MC3T3-E1 osteoblasts on the implants modified by the 1D NAPPy/citrate demonstrated their enhanced bioactivity, suggesting that the 1D NAPPy/citrate could be used as a more bionic implant surface. The citrate-assisted green approach can be extended to the construction of other CPs and 1D nano-structures of electro-active materials suitable for biomedical application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Field emission scanning electron microscopy (FE-SEM) (a), transmission electron microscopy (TEM) (b), atomic force microscopy (AFM) height (c) images and ATR-FTIR spectrum (d) of 1D NAPPy/citrate obtained in PBS with pH 7.4 containing 0.05 M citrate. Inset in (a) is a higher magnification view of (a).

Figure 2.

FE-SEM images of NAPPy/citrate obtained in different PBS media: (a) pH 7.4 containing 0.10 M citrate, (b) pH 7.4 containing 0.02 M citrate, (c) pH 8.0 containing 0.02 M citrate and (d) pH 6.5 containing 0.10 M citrate. (e1–e3) XRD patterns of NAPPy/citrate obtained in different PBS media: (1) pH 7.4 containing 0.10 M citrate, (2) pH 7.4 containing 0.05 M citrate and (3) pH 7.4 containing 0.02 M citrate. (e4 and e5) XRD patterns of 1D NAPPy/NSA (4) and irregular PPy/Cl film (5).

Figure 3.

Schematic illustration for citrate-assisted construction of 1D NAPPy/citrate in template-free electrochemical polymerization. (a) Py nano-droplets, free-Py and citrate dispersed in PBS on the pre-nucleated film (PNF). (a#) Size distribution of Py nano-droplets determined by dynamic light scattering (DLS) zetasizer nanoanalyzer. (b) The citrate molecules captured the free-Py and the surface of Py nano-droplets. (c) Initial PPy nano-architecture was formed by polymerization. (d) 1D conical NAPPy was formed by self-assembly of Py nano-droplet/citrate followed by polymerization. (d#) The alignment of PPy chains into nano-architectures was achieved at a higher molar ratio of citrate to Py.

Figure 4.

FE-SEM images of in vitro biomimetic mineralization in SBF (a), fluorescence images of DAPI-stained nuclei (b1–b3, 4 h) and Rhodamine-labelled phalloidin-stained cytoskeletal actin (c, 24 h) in MC3T3-E1 osteoblasts seeded on biomedical titanium (1), 1D NAPPy/citrate (2) and 1D NAPPy/NSA (3). Plot of the number of MC3T3-E1 osteoblasts nuclei versus culture time (b4). Plot of optical density (OD) value (reflecting cell proliferation rate) (d) and ALP activity (e) versus culture time. (f) Real time PCR analysis of mRNA expression levels of MC3T3-E1 osteoblasts seeded for 7 and 14 days. Insets in (a): high magnification image of

randomly selected region and its corresponding energy-dispersive x-ray (EDX) spectrum.
The * indicated significant difference ($p < 0.05$).