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Multi-biofunctional polymer graphene composite for bone tissue regeneration that elutes copper ions to impart angiogenic, osteogenic and bactericidal properties

L.R. Jaidev [a], Sachin Kumar [a,b], Kaushik Chatterjee [a,*]

[a] Department of Materials Engineering, Indian Institute of Science, Bangalore 560012, India
[b] Max-Planck Institute for Polymer Research, Mainz, Germany

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ABSTRACT

Despite several recent advances, poor vascularization in implanted scaffolds impedes complete regeneration for clinical success of bone tissue engineering. The present study aims to develop a multi-biofunctional nanocomposite for bone tissue regeneration using copper nanoparticle decorated reduced graphene oxide (RGO_Cu) hybrid particles in polycaprolactone (PCL) matrix (PCL/RGO_Cu). X-ray photoelectron spectroscopy and X-ray diffraction confirmed the presence of copper oxides (CuO and Cu_2O) on RGO. Thermogravimetric analysis showed that 11.8% of copper was decorated on RGO. PCL/RGO_Cu exhibited steady release of copper ions in contrast to burst release from the composite containing copper alone (PCL/Cu). PCL/RGO_Cu exhibited highest modulus due to enhanced filler exfoliation. Endothelial cells rapidly proliferated on PCL/RGO_Cu confirming cytocompatibility. The sustained release of ions from PCL/RGO_Cu composites augmented tube formation by endothelial cells evidenced enhanced angiogenic activity. Gene expression of angiogenic markers VEGF and ANG-2 was higher on PCL/RGO_Cu compared to PCL. The osteogenic activity of PCL/RGO_Cu was confirmed by the 87% increase in mineral deposition by pre-osteoblasts compared to PCL. The bactericidal activity of PCL/RGO_Cu showed 78% reduction in viability of *Escherichia coli*. Thus, the multi-biofunctional PCL/RGO_Cu composite exhibits angiogenic, osteogenic and bactericidal properties, a step towards addressing some of the critical challenges in bone tissue engineering.

1. Introduction

Bone tissue engineering aims to develop a viable and functional synthetic graft that aids complete repair and regeneration of bone defects arising from trauma, disease or congenital disorders. Cell proliferation, organization and differentiation of osteoprogenitor cells are essential for tissue formation. Tissue graft attains its vitality by formation of blood vessels through angiogenesis, where the supply of oxygen and nutrients facilitate regeneration of bone at the defect site [1]. Thus, angiogenesis is the key to success of an implanted graft [2]. The lack of blood vessel formation in the bone fracture callus often leads to the clinical condition of fracture non-union. This condition is diagnosed with intense pain at the site of fracture, and treatment needs to facilitate angiogenesis and repeated surgeries [3]. Thus, for clinical success of engineered bone tissues, development of biomaterials for preparation of the scaffolds with angiogenic activity and osteogenic activity, is imperative.

Graphene, a two-dimensional (2D) carbonaceous material, is being widely investigated for biomedical applications owing to its unique mechanical and electrical properties in addition to its good cytocompatibility [4,5]. Among graphene derived particles, graphene oxide (GO), and reduced graphene oxide (RGO), are finding increasing usage for tissue engineering applications [6]. Reinforcement with GO or RGO enhances mechanical properties, especially modulus and strength of biodegradable polymers, thereby forming strong and stiff nanocomposites for bone tissue engineering [7]. Interestingly, GO and RGO fillers impart osteoconductivity to polymer composites [7]. Poly(ε-caprolactone) (PCL) is a biocompatible and biodegradable polymer used in various medical applications including the preparation of tissue scaffolds [8]. We recently reported that composites of PCL and graphene derived nanoparticles showed better osteogenic activity than neat PCL [9–11].

* Corresponding author.
E-mail address: kchatterjee@materials.iisc.ernet.in (K. Chatterjee).

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Table 1
Nomenclature and composition of different composite films.

Sample code	PCL (g)	RGO (g)	Cu (g)	RGO_Cu (g)	Composition		
					RGO in composite (g)	Cu in composite (g)	Filler content (wt%)
PCL	100	0	0	0	0	0	0
PCL/RGO	95.6	4.40	0	0	4.40	0	4.4
PCL/Cu	99.4	0	0.60	0	0	0.60	0.6
PCL/RGO_Cu_1	99.0	0	0	1.0	0.88	0.12	1.0
PCL/RGO_Cu_3	97.0	0	0	3.0	2.64	0.36	3.0
PCL/RGO_Cu_5	95.0	0	0	5.0	4.40	0.60	5.0

Copper ions are known to promote angiogenesis [12]. In recent years, copper chelators are being developed as anti-tumor drugs to impede angiogenesis, thereby retarding tumor growth [13]. Copper doped bioglass releases copper ions to facilitate angiogenesis [14]. Several other studies have demonstrated that copper salts imbibed into mesoporous bioactive glass scaffolds [15] or bioglass/polymer composite scaffolds [16] enhance the angiogenic ability of the biomaterial. In a recent study, copper nanoparticles were found to exhibit better angiogenic activity than copper salts [17].

Aside from its angiogenic abilities, copper is a known antibacterial and antifungal agent [18]. Copper is historically used as an antiseptic surface as it is a potent antimicrobial agent [19]. It was proposed that copper ions released from nanoparticles exhibit antibacterial activity by binding to sulfur and phosphorous groups of proteins and DNA, thereby disrupting cellular mechanisms [20,21]. In surgeries involving tissue grafts or biomedical implants, infections are a major cause of implant failure and post-surgical complications [22]. Thus, the incorporation of copper nanoparticles in the scaffold could minimize the risk of infections.

There is a need to address engineering of a new generation of multi-biofunctional materials with good mechanical properties to address various clinical challenges in bone tissue engineering. Specific aim of this work was to prepare copper decorated RGO hybrid nanoparticles (RGO_Cu). It was envisaged that graphene sheets will enhance mechanical and osteogenic properties, whereas controlled release of copper will impart angiogenic and antibacterial activity when hybrid nanoparticles are incorporated in the polymer matrix [23]. Physical, chemical, mechanical and release properties of the nanocomposites containing RGO_Cu were characterized and compared to that of neat polymer and composites containing either RGO or copper. Biological properties including angiogenic, osteogenic and bactericidal activities of the composites were characterized by measuring response of the endothelial cells, osteoblasts and bacteria, respectively.

2. Experimental

Experimental procedures in detail are available in Supplementary Information (Section 1). GO was synthesized by chemical exfoliation of graphite [24]; RGO was prepared from GO using hydrazine hydrate [24]. RGO_Cu particles were synthesized by co-reducing $CuCl_2$ with GO; the particles were characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction, X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM) and thermogravimetric analysis (TGA). Various composites were prepared by solvent precipitation technique as listed in Table 1 and films were prepared by spin coating.

The composites were characterized by SEM coupled with energy dispersive spectroscopy (EDS), AFM, optical profilometry and dynamic mechanical analysis (DMA). The release of copper ions from the films was quantified using inductively coupled plasma optical emission spectroscopy (ICP-OES).

SVEC4-10 mouse endothelial cells and MC3T3-E1 mouse pre-osteoblasts were used for assessing angiogenesis and osteogenesis, respectively. Cell proliferation was assessed by quantifying the DNA content. F-actin and cell nuclei were stained and imaged. Angiogenesis was assayed following tube formation on Matrigel in the presence of 24 h conditioned media from the composites. Cell viability was assessed using live/dead staining and the tube lengths were calculated from the images. Gene expression of known angiogenic markers including vascular endothelial growth factor (VEGF), angiopoietin-1 (ANG-1), angiopoietin-2 (ANG-2) and platelet endothelial cell adhesion molecule (PECAM) was studied by real-time PCR. Osteogenesis was evaluated from mineral deposition. Antibacterial activity was detected following direct contact with *E. coli*; viability of bacterial cells was studied by performing live/dead staining and cell morphology was studied with a SEM.

Two-way ANOVA with Tukey's test was performed to determine the difference of significance between treatments and respective time points. One-way ANOVA with Tukey test was performed to find the difference of significance between the samples. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ were checked for significance.

3. Results and discussion

3.1. Synthesis and characterization of nanoparticles

Fig. 1 illustrates the synthesis of the various nanoparticles. To prepare RGO_Cu, GO was reduced to RGO in the presence of copper chloride in solution by hydrazine in a manner similar to the other hybrid particles consisting of RGO sheets decorated with metal nanoparticles such as Sr [10], Ag [11], and Ag/Pt [25]. In this process, the metal ions tend to uniformly anchor onto the negatively-charged GO surface through electrostatic interactions. In general, oxygen sites on GO surface act as nucleating sites for anchoring metal ions [23]. After the addition of hydrazine, simultaneous reduction of metal ions (Cu^{2+}) to metal (Cu) atoms by hydrazine [26] leads to an increase in the concentration of metal atoms in solution, which further drives the growth of the copper nanocrystals on the RGO sheets. In addition, the subsequent reduction by hydrazine will also help in eliminating oxygen functional groups from the GO surface to form RGO [27]. Hence, simultaneous reduction of Cu^{2+} ions and GO was achieved resulting in the formation of a reddish black coloured precipitate, suggesting the decoration of copper nanoparticles onto RGO sheets. Hence, this unique method of simultaneous reduction and uniform decoration of nanoparticles on graphene sheets without using organic molecules, surfactant or polymers may help to minimize cytotoxicity.

Detailed description of results of physicochemical characterization of the particles is presented in Supplementary Information (Section 2.1) and is briefly summarized below. The surface morphology of RGO and RGO_Cu were characterized by SEM (Fig. 2). In the hybrid particles, spherical copper nanoparticles were uniformly dispersed on the RGO sheets. This observation was confirmed by