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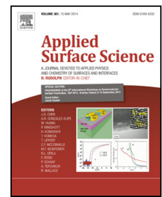

Flexible bactericidal graphene oxide–chitosan layers for stem cell proliferation

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ABSTRACT

Graphene oxide (GO)–chitosan composite layers with stacked layer structures were synthesized using chemically exfoliated GO sheets (with lateral dimensions of ~1 μm and thickness of ~1 nm), and applied as antibacterial and flexible nanostructured templates for stem cell proliferation. By increasing the GO content from zero to 6 wt%, the strength and elastic modulus of the layers increased ~80% and 45%, respectively. Similar to the chitosan layer, the GO–chitosan composite layers showed significant antibacterial activity (>77% inactivation after only 3 h) against *Staphylococcus aureus* bacteria. Surface density of the actin cytoskeleton fibers of human mesenchymal stem cells (hMSCs) cultured on the chitosan and GO(1.5 wt%)–chitosan composite layers was found nearly the same, while it significantly decreased by increasing the GO content to 3 and 6 wt%. Our results indicated that although a high concentration of GO in the chitosan layer (here, 6 wt%) could decelerate the proliferation of the hMSCs on the flexible layer, a low concentration of GO (i.e., 1.5 wt%) not only resulted in biocompatibility but also kept the mechanical flexibility of the self-sterilized layers for high proliferation of hMSCs.

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1. Introduction

Human mesenchymal stem cells (hMSCs) are thought to be multipotent cells, which can replicate as undifferentiated cells having the potential to differentiate to lineages of mesenchymal tissues including bone, cartilage, fat, tendon, muscle, and marrow stroma [1]. hMSCs are critical for numerous groundbreaking therapies in the field of regenerative medicine. Combination of stem cells with biomaterial scaffolds provides a promising strategy for engineering tissues and cellular delivery [2–4]. Some natural biomaterials such as polysaccharides play important role in maintaining the structure of the extracellular matrix and have been investigated for use as a potential scaffold material for stem cell transplantation [3]. Chitosan (poly (1,4-*b*-D-glucopyranosamine)) is a *N*-deacetylated cationic polysaccharide that has attracted much attention for various biomedical applications, mainly because of its excellent antimicrobial [5], non-toxicity, high biocompatibility and bioadsorbility properties [6,7]. Positive surface charges of chitosan and its biocompatibility possess an ability to support the cell growth effectively [8]. Nevertheless, chitosan has some drawbacks and its mechanical properties are not good enough for some biomedical applications. To improve the mechanical properties of chitosan and other biopolymers, various inorganic fillers have been utilized [9–11].

Meanwhile, carbon nanostructures such as carbon nanotubes [11,12], graphene [13], graphene oxide (GO) [14,15], nanodiamonds [16], and fullerene [15] have been the focus of recent studies due to their promising properties. Particularly, GO is very attractive due to its extraordinary mechanical properties (high Young's modulus and hardness, and excellent flexibility) together with lower cost compared to other carbon nanostructures [17]. GO consists of graphene sheets which are chemically functionalized with hydroxyl and epoxy groups [17,18]. Carbonyl groups are also present as carboxylic acids along the sheet edges. The existence of oxygen-containing groups makes GO hydrophilic and dispersive into some polar solvents forming intercalated composites with polar molecules through the strong interaction. Epoxide, carboxyl, and hydroxyl groups present on the basal plane and edges of GO enable greater interactions with proteins through covalent, electrostatic, and hydrogen bonding [19,20]. On the other hand, GO have been proved to exhibit better biocompatibility than reduced graphene oxide [20,21]. Thin GO sheets can potentially serve as a

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biocompatible, transferable, and implantable platform that have the potential to mediate stem cell lineage specification for tissue regeneration [19]. Recent studies have also shown that GO can be effective preconcentration platforms for accelerated stem cell growth and differentiation through molecular interactions [19,22,23]. Thus, GO is a promising nanofiller for improving the properties of chitosan without hampering its biocompatibility.

Fabrication of graphene–chitosan composite layers has been the focus of recent studies. Hu et al. [24] used microwave irradiation to prepare chitosan modified graphene sheets. The results indicated that chitosan is covalently grafted onto the surface of graphene sheets by amido bonds. Ganesh et al. [25] and Yang et al. [26] showed that the graphene or graphene oxide sheets prefer to disperse well within the chitosan matrix, improving its mechanical strength. He et al. [27] fabricated porous graphene oxide–chitosan materials with a high adsorbing ability to metal ions having enhanced compressive strength. On the other hand, Bush et al. [28] demonstrated that ultra-low graphene loading in chitosan-based composite causes a dramatic increase in the wettability of chitosan. Fan et al. [29] showed that the graphene–chitosan composites are biocompatible to L929 cells. Albeit the potential applications and advantages of GO–reinforced chitosan layers for biomedicine, little work has been performed on their biocompatibility and interactions with stem cells. So, the present work aimed at preparing chitosan layers contained GO to get more insight into the effect of GO content in chitosan matrix on the stem cell proliferation, antibacterial and mechanical properties.

2. Experimental

2.1. Reagents

Chitosan with a medium molecular weight of 90–150 kDa and a degree of deacetylation above 80% was prepared from Sigma-Aldrich (Munich, Germany). The graphite used in this study was obtained from Qingdao Haida Graphite Co. (Qingdao, China) and other reagents and chemicals were purchased from Merck Co. (Darmstadt, Germany).

2.2. Synthesis of graphene oxide

GO was synthesized using the modified Hummers' method [30]. Briefly, 0.5 g graphite was vigorously stirred for 10 min in 50 mL concentrated H_2SO_4 in an ice-water bath. Then, 0.5 g $NaNO_3$ and 3 g $KMnO_4$ were slowly added into the solution and stirred for 2 h in the ice-water bath. After removing the bath, 100 mL deionized (DI) water was added into the flask during ∼1 h while the solution was stirred and its temperature was kept at 98 °C. The resultant mixture was further stirred for 2 h at 98 °C. The temperature was reduced to 60 °C and then 3 ml H_2O_2 (30 wt% aqueous solution) was added. The mixture was cooled to room temperature, diluted with DI water and left overnight. The obtained GO was filtered (grade No.40 filter paper, Whatman, Kent, UK) and washed with HCl (10 vol%) solution and then DI water to remove the residual acid. GO sheets were obtained by ultrasonication of the filtered product in DI water at power of 600 W for 1 h. The obtained dispersion was centrifuged 2 cycles at 5000 rpm for 20 min to remove unexfoliated GO.

2.3. Preparation of GO–chitosan composites

A chitosan aqueous solution was prepared by dissolving 1 g chitosan powder in 1.0 v/v% acetic acid solution. Then, GO suspension (0.1 mg/mL) was slowly added into the chitosan aqueous solution during a vigorous stirring. The obtained solution was agitated in an ultrasonic bath for approximately 10 min and stirred at room temperature for an additional 2 h to obtain a homogeneous solution. The GO–chitosan layers with various GO contents (0, 1.5, 3 and 6 wt%) were fabricated by a solution-casting method. The composite layers were dried at 50 °C overnight to completely remove acetic acid.

2.4. Material characterization

Topography of the GO sheets and the GO–chitosan composites was characterized by atomic force microscopy (AutoProbe CP-Research, Veeco, Plainview, NY, USA). For atomic force microscopy (AFM), the samples were prepared by drop-casting a diluted GO suspension on a freshly cleaved mica substrate. Raman Spectroscopy (Senterra, Bruker, Leipzig, Germany) of the GO sheets was carried out at room temperature using a 528 nm laser excitation source. The mechanical properties of the GO–chitosan layers were investigated using a universal tensile test instrument (STM20, Santam, Tehran, Iran). Specimens for the tensile test were cut into strips of 10.0 mm wide and 60 mm long from the layers with thickness of 0.3 mm. The tensile tests were performed in the air using a 50 N load cell at a crosshead speed of 2 mm/min. Five tests were performed for each composition and the average of the results were reported as the mechanical properties with standard deviation. Scanning electron microscopy (SEM, Mira, TESCAN, Cranberry TWP, USA) operating at 2.0 kV was used to study the surface morphology and fractured cross-section of the layers.

2.5. Antibacterial test

Antibacterial performance of the chitosan and GO–chitosan layers was investigated against *Staphylococcus aureus* (*S. aureus*, BBRC 10050[1]) as a Gram-positive bacterium. Before antibacterial assay, all samples were sterilized by 70% ethanol spray and dried under a sterile hood. The bacteria were cultured on a nutrient broth at 37 °C for 24 h. Then, a portion of the bacterial suspension was diluted to 10^7 CFU/mL. For antibacterial drop-testing, each sample was placed into a sterilized Petri dish. Then, 100 μL of the bacterial broth culture was seeded onto the samples at 37 °C. After 3 h, the bacteria were washed out in 9.9 mL saline solution. After sonication for 30 s, each bacterial suspension was spread on a nutrient agar plate and incubated at 37 °C for 24 h to count the surviving bacterial colonies by using an optical microscope. The total number of the colonies was determined by area based estimation. The reported data were the average value of three separate tests ± standard deviations.

2.6. Cell viability assay

The hMSCs were isolated from umbilical cord blood (UCB) of an infant with informed consent. The UCB single-nuclear cells were obtained by negative immunodepletion using a commercial kit (RosetteSep, StemCell Technologies, USA). The cells were centrifuged at 8000 rpm for 10 min to discard the supernatants. Cells were cultured in an environment under 5% CO_2 at 37 °C and fed with Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, 10 ng/mL basic fibroblast growth factor and 2 mM L-glutamine. The viability of the cells exposed to the layers (∼1000 cells/cm^2) was evaluated by using a fluorescence staining cell method after 7 days of incubation. In this method, the cells were fixed in 5% paraformaldehyde, stained with rhodamin–phalloidin (RhP) for staining the actin cytoskeleton fibers of the cells (red color) and 4′,6-diamidino-2-phenylindole

[1] Biochemical and Bioenvironmental Research Center Culture Collection (a local culture collection).

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