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The interplay between nanostructured carbon-grafted chitosan scaffolds and protein adsorption on the cellular response of osteoblasts: Structure–function property relationship

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ABSTRACT

The rapid adsorption of proteins occurs during the early stages of biomedical device implantation into physiological systems. In this regard, the adsorption of proteins is a strong function of the nature of a biomedical device, which ultimately governs the biological functions. The objective of this study was to elucidate the interplay between nanostructured carbon-modified (graphene oxide and single-walled carbon nanohorn) chitosan scaffolds and consequent protein adsorption and biological function (osteoblast function). We compare and contrast the footprint of protein adsorption on unmodified chitosan and nanostructured carbon-modified chitosan. A comparative analysis of cell–substrate interactions using an osteoblast cell line (MC3T3-E1) implied that biological functions were significantly enhanced in the presence of nanostructured carbon, compared with unmodified chitosan. The difference in their respective behaviors is related to the degree and topography of protein adsorption on the scaffolds. Furthermore, there was a synergistic effect of nanostructured carbon and protein adsorption in terms of favorably modulating biological functions, including cell attachment, proliferation and viability, with the effect being greater on nanostructured carbon-modified scaffolds. The study also underscores that protein adsorption is favored in nanostructured carbon-modified scaffolds such that bioactivity and biological function are promoted.

1. Introduction

The rapid adsorption of proteins onto the surface of a scaffold or an implant is one of the first events that occur when they are introduced in a physiological system [1–4]. Furthermore, the proteins mediate a biological response. For instance, REDV, a peptide derived from fibronectin, facilitates the adhesion of endothelial cells, but not fibroblasts or muscle cells [5]. Thus the adsorption of proteins is a key factor in influencing cellular interactions in vivo and in vitro.

Recent studies have indicated that the presence of functional groups (oxygen, nitrogen, carboxyl, and hydroxyl) on the surface [6,7] influence protein adsorption. Functional groups such as carboxyl (–COOH), hydroxyl (–OH), epoxide, and amino ($-NH_2$) groups influence cell growth in an indirect manner through the adsorbed protein layer.

Bovine serum albumin (BSA) (molecular weight 66 kDa), fibronectin, and fibrinogen are glycoproteins proteins present in plasma, with a carbohydrate moiety covalently linked to specific amino acids, including asparagine, serine and threonine, of the protein. An important characteristic of BSA is that it does not induce physiological changes in cells to which it is attached or invoke unfavorable biological cascades. These characteristics do not interfere with cell proliferation. Additionally, the molecular structure and functions of BSA are similar to human serum albumin (HSA) and the sequence of BSA is ~76% similar to HAS [8].

Similarly, fibronectin, with a molecular weight of 550 kDa, is one of the earliest cell-binding proteins and is a constituent extracellular matrix protein (ECM) produced by osteoblasts and fibroblasts. The ECM governs cellular interactions by functioning as a bridge between cells and the artificial device [9–14]. Both fibronectin and fibrinogen when covalently cross-linked via the action of factor XIII [14,15] promote the adhesion of fibroblasts in vitro [16]. In dermal wounds fibronectin-coated fibrin forms the substratum for the in-growth of fibroblasts and endothelial cells during the formation of granulated tissue [17]. It binds to a variety of materials, including denatured collagen [14,18,19], and may act as a general opsonin in the phagocytosis of debris by the reticuloendothelial system [20]. Fibronectin is formed within platelets and is released following stimulation, when specific receptors appear on the platelet surface [21–23]. These receptors are particularly

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relevant in guiding the ability of fibronectin to promote platelet spreading [24] and clot retraction [25], irrespective of the fact that fibronectin has no apparent influence on platelet attachment per se [26].

Thus both BSA and fibronectin occupy a particularly important place among plasma proteins, with BSA representing ~50% of all plasma protein [27]. An important consequence of the adsorption of proteins is the creation of a biologically active surface. An understanding of protein adsorbed surfaces is important and constitutes the motivation of the study described here.

Factors that strongly influence protein adsorption are the chemistry of the surface, the wettability, and the topography (roughness). In this regard we have here explored the impact of different forms of biocompatible nanostructured carbon used for biomedical devices. The three different forms of nanostructured carbon that have generated significant interest in recent years are carbon nanotubes (CNTs), graphene oxide (GO) and single-walled carbon nanohorns (SWCNHs). One drawback of CNTs is the use of potentially toxic metal catalysts in their synthesis, if not removed [28], and the aggregation of CNTs due to van der Waals force is another aspect of concern. In contrast, GO and SWCNHs are soluble in water, show high dispersibility, and are biocompatible, which are necessary prerequisites for biological applications. GO was demonstrated to protect oligonuecleotides from enzymatic cleavage and effectively deliver oligonuecleotides into cells. GO was also shown to possess the ability to attach and deliver aromatic and water-insoluble drugs [29].

SWCNHs are single-walled carbon nanotubes and are an aggregate of a large number of small nanotubes of ~2–5 nm diameter and ~30–50 nm length, resembling a dahlia flower. Structurally they are similar to single-walled CNTs but are not as long as nanotubes and the tip region is closed by a conical cap with an internal cone angle of ~20°. The tip is similar to a part of fullerene [30]. More importantly, the synthesis of SWCNHs does not involve the use of a metal catalyst, which may be toxic, but are produced by laser ablation of a pure graphite target [31]. Additionally, SWCNHs are highly dispersible. Feasibility studies as a drug carrier have confirmed their non-toxic behavior [32–40]. Skin and conjunctival irritation tests confirmed them as non-irritant and non-dermal sensitizers [31]. They were observed to accelerate bone regeneration in rat calvarial bone defects [41].

Chitosan (CS) is a partially deacetylated form (poly-β(1,4)-2-amino-2-deoxy-D-glucose) of chitin, a natural polymer found in the cell wall of fungi and microorganisms. It has been widely considered for tissue engineering applications because of its biocompatibility, biodegradability, mechanical strength, hydrophilicity, good adhesion and non-toxicity [42]. In spite of these benefits, there is continued interest in enhancing the mechanical properties and biological response of CS, with a particular interest for bone tissue engineering [43]. Different approaches for the synthesis of CS derivatives with diverse chemical and molecular structure have been attempted to enhance the physico-chemical properties and cellular response [44,45]. Pegylation enhanced the water solubility of CS, while hyaluronic acid grafted onto CS improved the biological properties over those of pure CS [46,47]. As part of a continued effort to promote the application of CS for bone tissue engineering we focus on the process–structure–property relation of CS-based nanohybrid scaffolds.

The objective of the study was to elucidate the determining role of nano- or quantum-sized carbon (GO and SWCNHs) in mediating protein adsorption to CS scaffolds and their synergistic effect on cellular function. CS was grafted on two different forms of nanostructured carbon by covalently linking the carboxylic group present on GO and functionalized SWCNH to the amine group of CS. The hypothesis is that modification of CS with nanostructured carbon will favorably modulate biological function (osteoblasts function), which will be further promoted through nanostructured carbon-mediated protein adsorption. In summary, the study underscores the benefits of using nano- or quantum-size effects on biological function.

2. Experimental procedure

2.1. Materials and synthesis

Low molecular weight CS (50 kDa, 80% deacetylation) was obtained from Aldrich (St Louis, MO). GO and SWCNHs were obtained from Global Nanotechnology (Mumbai, India). Chemicals including 1-ethyl-3-(3-dimethylaminoprophy) carbondiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), sulfuric acid, hydrogen peroxide, dimethyl formamide (DMF), methylene chloride, diethyl ether, and sodium hydroxide were obtained from Aldrich (St Louis, MO). Alpha minimum essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from Gibco (Invitrogen Corp., Carlsbad, CA). Penicillin/streptomycin (10,000 IU, 10,000 μg ml^{-1}), trypsin/EDTA (0.25% trypsin/0.53 mM EDTA) in Hank's buffered salt solution, and phosphate-buffered saline (PBS) without calcium and magnesium were obtained from American Type Cell Culture Collection (ATCC Manassas, VA).

CS–GO and CS–SWCNH hybrid scaffolds were synthesized as follows. The carboxylgroups (–COOH) of nanostructured carbon (i.e. GO and SWCNH) were covalently attached with the amine group ($–NH_2$) of CS. In the case of CS–GO a GO dispersion in water was added to a solution of 0.03 M EDC and 0.06 M NHS for 1 h in order to activate the carboxyl groups (–COOH) of GO. The pH of the resulting solution was maintained at 7.0 using dilute sodium hydroxide. The activated GO solution was then added drop-wise to the CS solution (1% w/v in acetic acid) and heated at 60 °C for 30 min and allowed to cool down to room temperature, followed by lyophilization to prepare porous scaffolds.

Similarly, to synthesize CS–SWCNH scaffolds pristine SWCNH was first functionalized (f-SWCNH) by covalent attachment of CS, Briefly, SWCNH (10 mg) was added to a piranha solution (98% sulfuric acid and 30% hydrogen peroxide, 4;1) to generate carboxyl groups (–COOH) on the surface of the SWCNH. The suspension was stirred for 4 h at room temperature, and then diluted with 500 ml of distilled water, followed by filtration through a 0.1 μm isopore membrane. Subsequently, washing was carried out with water until the filtrate becomes colorless and neutral pH was obtained, followed by drying overnight under vacuum at 70 °C. Next the carboxyl groups (–COOH) of f-SWCNH were activated using EDC and NHS, filtered, washed with DMF, methylene chloride and diethyl ether, and finally dried overnight under vacuum at 70 °C. Next a 1% (w/v, in dilute acetic acid) solution of CS was added to f-SWCNH (1 wt.%), stirred overnight and then heated at 60 °C for 30 min and allowed to cool down to room temperature, followed by lyophilization to obtain porous scaffolds, as illustrated in Scheme 1.

Pure CS scaffolds were prepared in a similar manner to CS–nanostructured carbon by dissolving CS in acetic acid solution, followed by lyophilization.

2.2. Protein attachment

Scaffolds of pure CS, CS–GO, and CS–SWCNH were treated with 1 ml of a BSAprotein solution (1 mg ml^{-1} protein in PBS), and subsequently kept in a humidified incubator at 37 °C for 24 h. After 24 h the samples were removed and washed three times using PBS to remove non-adsorbed protein. The Bradford assay was used to quantify the amount of adsorbed protein. For this a 25 μl aliquot of the non-adsorbed protein solution was mixed with 400 μl of