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Polyelectrolyte multilayer films functionalized with peptides for promoting osteoblast functions

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Abstract

Layer-by-layer deposition of polyelectrolyte multilayer (PEM) thin films has recently been applied to biomaterial applications. This simple and versatile technique provides a wide variety of potential utilization by insertion of biomolecules such as cell adhesion peptides. In this work dual peptides containing RGD (a cell-binding domain) and LHRRVKI (a heparin-binding domain) were immobilized onto polystyrene by the PEM technique and the effects on osteoblast cell culture were investigated. These peptides were conjugated to the amino groups of poly(allylamine hydrochloride) and then adsorbed onto the top of a 10 layer poly(allylamine hydrochloride)/poly (acrylic acid) film assembled at either pH 2.0 or pH 6.5. Osteoblasts, isolated from neonatal rat calvariae, were then seeded and cultured on the peptide-conjugated surfaces. We found that the cells adhered and grew better on the RGD-conjugated PEM films. The osteoblasts exhibited a better differentiated phenotype on the pH 2.0 films than the pH 6.5 films with respect to calcium deposition. The incorporation of LHRRVKI did not support cell adhesion, growth and matrix mineral deposition. Our results showed that the efficacy of RGD conjugation on osteoblast behavior was affected by the base PEM film.

Keywords: RGD; Polyelectrolyte multilayer; Osteoblasts; Calcium deposition; Cell adhesion

1. Introduction

Cell attachment to biomaterials is an important step in many biomedical applications, such as implants, tissue engineering and cell-based sensors [1–3]. Cells in native tissues adhere to the surrounding extracellular matrix (ECM) via cell membrane receptors (e.g. integrins [4]) that specifically bind to ECM adhesion proteins such as fibronectin, vitronectin or laminin. Since synthetic biomaterials lack the natural mechanisms that mediate cell attachment, there is a broad need to couple bioactive molecules to artificial substrates that render artificial materials biologically functional.

Among the strategies to improve cellular affinity of synthetic surfaces, the most popular one is adsorption or conjugation of ECM adhesion proteins onto biomaterial surfaces [5–7]. Alternatively, conjugation of peptides containing the cell-binding sequences of ECM adhesion proteins also supports cell adhesion to biomaterials [8,9]. The advantages of peptides include cost effectiveness and less vulnerability to denaturation in comparison with intact adhesion proteins. The most commonly applied cell-binding peptide is the tri-amino acid sequence arginine–glycine–aspartic acid (Arg–Gly–Asp or RGD), which is found in many ECM adhesion proteins [2,8,10]. Several types of integrins bind fibronectin and vitronectin via the RGD domain so as to mediate cell adhesion.

Besides the integrin-binding domains, many adhesion proteins contain heparin-binding domains that support cell

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adhesion. Such domains consist of clustered positively charged basic amino acid residues, electrostatically interacting with negatively charged carboxylate and sulfate groups found in glycosaminoglycans (GAGs) such as heparan sulfate, chondroitin 4-sulfate and dermatan sulfate [11]. The GAGs associated with the extracellular sides of the cell membrane can bind the heparin-binding domains of cell adhesion proteins [12]. The heparin-binding domains act as a cofactor in promoting cell adhesion and spreading [13,14]. Many studies have indicated that peptides derived from the heparin-binding domains of adhesion proteins such as fibronectin and vitronectin enhance cell adhesion and the formation of stress fibers and focal contacts [13,15,16]. Such a mechanism is inhibited by the addition of heparin and chondroitin sulfate to culture media [13].

Methods for grafting peptides onto substrates include physical adsorption and covalent coupling. Physically adsorbed peptides are liable to desorption from the surfaces. Alternatively, peptides can be coupled to substrates containing a reactive functionality such as carboxyl or amino groups via suitable chemical reactions. However, such an approach is not applicable for chemically inert surfaces. Plasma discharge or γ-irradiation treatment can be applied to chemically inert surfaces to create surface free radicals for surface grafting. An alternative approach to generate peptide-containing biointerfaces is to synthesize polymer–peptide hybrid copolymers [17–19]. Recently, a simple and versatile method based on electrostatic attraction between oppositely charged polyelectrolytes has shown the potential for peptide immobilization. This technique of layer-by-layer (LBL) polyelectrolyte multilayer (PEM) deposition is based on the alternate adsorption of positively and negatively charged polyelectrolytes to build an ultra-thin film onto a substratum [20]. This simple LBL deposition technique possesses several advantages for peptide conjugation to biomaterials. First, this method is not restricted to the type and size of the substratum and can be used to coat arbitrarily shaped objects. Second, many polyelectrolytes possesses functional groups, such as amino and carboxyl groups, that can conjugate biomolecules including peptides. Third, the electrostatic attraction between multiple oppositely charged polyelectrolytes provides "covalent bond-like" interactions to prevent immobilized peptide desorption. Previous studies have shown that conjugation of RGD peptides to PEM films enhances osteoblast adhesion [21,22] and can be used to spatially control cell adhesion [23].

Rezania and Healy previously studied osteoblast behavior on surfaces that were chemically conjugated with heterogeneous peptides, RGD (a cell-binding domain derived from fibronectin) and FHRRIKA (a putative heparin-binding domain derived from bone sialoprotein) [24,25]. They found that the mixed peptides of the cell- and heparin-binding domains promoted cell spreading, strength of attachment, focal contact and cytoskeletal organization and mineralization of the deposited ECM compared with homogeneous RGD or FHRRIKA. In this study we investigate the effects of both types of peptides conjugated to substrates by the LBL technique on osteoblast behavior. Peptides were first conjugated to poly(allylamine hydrochloride) (PAH) and then adsorbed onto PEM films based on poly(allylamine hydrochloride) and poly(acrylic acid) (PAA). The internal and surface compositions of multilayer films of weak polyelectrolytes are strongly affected by the pH of deposition solutions due to pH-dependent ionization of these polymers [26–28]. It has been shown that PEM films from the same polyelectrolyte pair at different pH values affect cell behavior differently [26]. Therefore, we also studied the impact of the underlying PEM films on peptide conjugation and cell behavior. Short-term adhesion and growth of primary rat calvaria osteoblast-like cells and long-term mineralization were determined to evaluate the feasibility of peptide-conjugated PEM deposition in orthopedic applications.

2. Materials and methods

2.1. Materials

PAH (mol. wt. 70,000), PAA (mol. wt. 10,000) and 3-mercaptol-propanesulfonic acid, sodium salt (MPS) were purchased from Aldrich. *N*-Hydroxysuccinimide (NHS), 2-mercaptoethanol, trypsin–EDTA solution, 2-amino-2-methyl-1propanol (AMP), 4-nitrophenyl phosphate disodium salt hexahydrate (PNPP), *p*-nitrophenol (PNP), phalloidin–TRITC, paraformaldehyde, dexamethasone, Alizarin red S and L-ascorbate were purchased from Sigma. ε-Maleimidocaproic acid (EMCA) was obtained from Pierce. *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) was purchased from Fluka.

The osteoblast culture medium contained α-minimum essential medium (α-MEM) (HyClone), 10% fetal bovine serum (JRH), 0.0679% (v/v) 2-mercaptoethanol, 200 μg ml^{-1} gentamicin (Gibco) and 25 μg ml^{-1} fungizone (Gibco), pH 7.4. The osteoblast culture medium supplemented with 1 mM solidum glycerophosphate, 0.1 μM dexamethasone and 50 μg ml^{-1} L-ascorbate constituted the osteogenic culture medium. Phosphate-buffered saline (PBS) contained 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4.

2.2. Conjugation of peptides to PAH

The RGD-containing peptide (*N*-acetyl-GCRGYGR GDSPG-amide) and the LHRRVKI-containing peptide (*N*-acetyl-CGGYGLHRRVKI-amide) were synthesized by the batchwise fmoc–polyamide method and purified by reverse-phase HPLC [29]. The purity of the peptides was more than 99.9%, analyzed by analytical HPLC. The molecular weight of the peptides was verified by matrix-assisted laser desorption and time-of-flight mass spectrometry. Peptide concentration was determined according to

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