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Multilayered DNA coatings: *In vitro* bioactivity studies and effects on osteoblast-like cell behavior

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Abstract

This study describes the effect of multilayered DNA coatings on (i) the formation of mineralized depositions from simulated body fluids (SBF); and (ii) osteoblast-like cell behavior with and without pretreatment in SBF. DNA coatings were generated using electrostatic self-assembly, with poly-D-lysine or poly(allylamine hydrochloride) as cationic polyelectrolytes, on titanium substrates. Coated substrates and non-coated controls were immersed in SBF with various compositions. The deposition of calcium phosphate was enhanced on multilayered DNA coatings as compared with non-coated controls, and was dependent on the type of cationic polyelectrolyte used in the build-up of the DNA coatings. Further analysis showed that the depositions consisted of carbonated apatite. Non-pretreated DNA coatings were found to have no effect on osteoblast-like cell behavior compared with titanium controls. On the other hand, SBF-pretreatment of DNA coatings affected the differentiation of osteoblast-like cells through an increased deposition of osteocalcin. The results of this study are indicative of the bone-bonding capacities of DNA coatings. Nevertheless, future animal experiments are required to provide conclusive evidence for the bioactivity of DNA coatings.

Keywords: DNA coating; SBF; Calcium phosphate; Osteoblast-like cell; Layer-by-layer deposition

1. Introduction

The insertion of orthopedic and dental implants to replace malfunctioning articulating joints and lost teeth is a common surgical procedure. For the construction of orthopedic and dental implants, many types of metals (or metallic alloys), ceramics and polymers, and combinations thereof, have been used [1,2]. In order to remain functional, the material properties must meet the requirements for a given situation. For instance, functional orthopedic and dental implants require a tight conjunction with the surrounding bone tissue.

Amongst several materials, calcium phosphate (CaP) ceramics such as hydroxyapatite (HA) have the capacity to form a chemical bond with bone tissue at the interface and can induce a continuous transition from bone tissue to the implant surface [3]. In view of this, the formation of bone-like apatite on the surface is proposed as the essential requirement for an artificial material to bond to living bone [4]. However, due to their brittleness, the *in vivo* use of these so-called "bioactive" materials for mechanically loaded applications is limited to coatings on a mechanically stronger implant base material, such as titanium.

The most commonly used methods to provide CaP coatings on implants, including plasma spraying and sputter techniques, have limitations regarding implant geometry and porosity, and the incorporation of biologicals. Hence,

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studies have been undertaken to elucidate the mechanism responsible for the apposition of bone tissue to bioactive materials in order to find methods to increase surface bioactivity that do not entail these limitations. In view of this, the role of surface functional groups has been studied using *in vitro* experiments with simulated body fluids (SBF), which are solutions that are compositionally similar to human blood plasma. Such studies, in which *in vivo* apatite formation can be reproduced [5], indicated that negatively charged groups, and phosphate-containing groups in particular, are the most potent inducers of the CaP nucleation process [6,7].

A recently proposed implant coating material with high phosphate content is DNA [8]. In addition to the proposed beneficial effects on CaP nucleation, DNA is an immunologically interesting material [9–11] and has the capacity to incorporate and bind other compounds [12]. In view of this, we recently used electrostatic self-assembly (ESA) to fabricate multilayered DNA coatings [8], consisting of either poly-D-lysine (PDL) or poly(allylamine hydrochloride) (PAH) as the cationic counterpart of anionic DNA. The build-up of such coatings depends on the adsorption of the first polyelectrolyte to a substrate, after which electrostatic forces between oppositely charged polyelectrolytes allow the formation of multilayered coatings [13,14]. Both types of DNA coating were shown to contain 15 μg DNA cm^{-2}, as demonstrated using radiolabeled DNA [8]. Furthermore, these DNA coatings displayed a polycation-dependent surface roughness, as studied using atomic force microscopy [8]. Finally, both types of DNA coating were demonstrated (i) to be cyto- and histocompatible [15,16]; (ii) to decrease the secretion of the major pro-inflammatory cytokine (tumor necrosis factor alpha, TNF-α) by macrophages [17]; and (iii) to be eligible for functionalization with the osteoinductive factor bone morphogenetic protein 2 [18].

In the present study, we aimed at evaluating potential effects of the phosphate groups in both types of DNA coating on CaP nucleation and growth in SBF immersion experiments. For this purpose, DNA coatings were prepared on titanium substrates and immersed in SBF of various compositions. Non-coated titanium substrates served as controls. After immersion in SBF for 1, 2 and 4 weeks, the surfaces of the substrates were evaluated morphologically (scanning electron microscopy, SEM) and chemically (X-ray diffraction, XRD; Fourier transform infrared spectroscopy, FTIR; energy dispersive spectroscopy, EDS). Subsequently, titanium substrates with or without DNA coatings and subsequent SBF immersion pretreatment were seeded with primary rat bone marrow-derived osteoblast-like cells to study cell proliferation, differentiation and morphology.

2. Materials and methods

2.1. Materials

Polyanionic salmon DNA (±300 bp $molecule^{-1}$; sodium salt) was kindly donated by Nichiro Corporation (Kawasaki-city, Kanagawa prefecture, Japan). Potential protein impurities in the DNA were checked using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA), and were measured to be below 0.20% w/w (data not shown). The polycationic polyelectrolytes PDL (MW 30,000–70,000) and PAH (MW ~70,000) were purchased from Sigma (Sigma–Aldrich Chemie B.V., Zwijndrecht, The Netherlands). All materials were used without further purification.

2.2. Generation of multilayered DNA coatings

DNA coatings were generated using the ESA technique on disc-shaped titanium substrates (12 mm diameter, 1.5 mm thickness; commercially pure titanium, as-machined), as described previously [8]. Briefly, the substrates were immersed in an aqueous solution of either PDL (0.1 mg ml^{-1}) or PAH (1 mg ml^{-1}) for 30 min, allowing sufficient time for the adsorption of the first cationic polyelectrolyte layer onto the substrates. Subsequently, substrates were washed in ultra-pure water (5 min; continuous water flow) and dried using a pressurized airstream. Thereafter, substrates were immersed alternately in an anionic aqueous DNA solution (1 mg ml^{-1}) and the respective cationic polyelectrolyte solution for 7 min each, with intermediate washing in ultra-pure water and subsequent drying using a pressurized airstream to obtain final coating architectures of $[PDL/DNA]_5$ or $[PAH/DNA]_5$ (the number indicates the total number of double layers).

2.3. SBF immersion experiments

2.3.1. Simulated body fluid

The recipe for the preparation of SBF was adopted from Kokubo et al. [4,19]. Immersion experiments were performed with SBF containing physiological (SBF_1) and twofold increased calcium- and phosphate-ion concentrations (SBF_2) compared to human blood serum (Table 1). The final pH value of both solutions was set at 7.4. Immersion studies were performed in 15 ml tubes (Greiner Bio-One B.V., Alphen aan de Rijn, The Netherlands), using one substrate per tube in 4 ml of SBF. Tubes were placed in a water bath at 37 °C under continuous shaking. The SBF solution in the tubes was refreshed on a weekly basis. After

Table 1
Concentrations of ionic species (mM) in SBF of different Ca^{2+} and PO_4^{3-} concentrations and human blood plasma (HBP)

Species	SBF_1	SBF_2	HBP
Na^+	142.0	142.0	142.0
K^+	5.0	7.0	5.0
Ca^{2+}	2.5	5.0	2.5
Mg^{2+}	1.5	1.5	1.5
Cl^-	148.0	147.0	103.0
CO_3^{2-}	4.2	4.2	27.0
PO_4^{3-}	1.0	2.0	1.0
SO_4^{2-}	0.5	0.5	0.5

ID	Title	Pages
2038	Multilayered DNA coatings: In vitro bioactivity studies and effects on osteoblast-like cell behavior	10