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A comparative study of simulated body fluids in the presence of proteins

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ABSTRACT

Simulated body fluid (SBF) is widely used as part of an *in vitro* method to evaluate implant materials such as their apatite forming ability (AFA), a typical indication of potential bone-bonding ability *in vivo*. We report the use of carbonate-buffered SBFs as potential solutions for implant evaluation and the effect of proteins, represented by bovine serum albumin (BSA) in SBFs on the formation of hydroxyapatite (HA). These solutions are buffered by the thermodynamic equilibrium with 5% CO_2 in an incubator, and result in a deposition of carbonated HA. Using several titanium-based surfaces, these solutions were studied in comparison with the widely-used SBF (ISO 23317). The presence of BSA strongly inhibited the formation of HA in traditional SBF, while HA can still be observed in carbonate-buffered SBFs. A kinetic study reveals that the inhibitory effect is concentration dependent with 0.1 g/L and 1 g/L of BSA having little effect on HA growth but a complete inhibition of HA formation at 5 g/L of BSA, as tested using NaOH treated titanium with a known positive AFA. The decrease in solution pH and free calcium concentrations in SBFs due to the addition of BSA is not significant, suggesting other causes for the strong inhibitory effect.

Statement of Significance

The successful use of simulated body fluids (SBFs) to evaluate potential bioactive implants relies on the better understanding of the heterogeneous nucleation and growth of hydroxyapatite in solution. Although a standardized recipe for SBF was developed over a decade ago, a few key issues remain to be understood, i.e. the behavior of carbonate-buffered SBFs having similar buffering mechanism as human blood, and the effect of proteins on hydroxyapatite formation on bioactive materials. This paper addresses these two issues and would help the reader better understand the subtleties in this domain and better interpret the results generated using SBFs.

1. Introduction

The discovery of bone-bonding glass in the 70s by Hench et al. opened up new possibilities in bioactive implant materials, attracting many research activities and leading to the developments of various commercial products [1,2]. Instead of being passively implanted and often encapsulated by fibrous tissues, these materials can actively form a direct bonding with the surrounding bone, thus significantly increasing the implant fixation and the long-term stability. The rapid development of new potential implant materials presents both scientific and technical challenges for the design of a fast assessment method other than traditional animal experiments for implant evaluation.

Kokubo et al. proposed an *in vitro* method using an acellular solution with inorganic ion composition and pH similar to biological fluids called simulated body fluid (SBF) to test new implants [3,4]. The method is based on the observations that a bonelike apatite layer is often found between bioactive implants and the surrounding bone, acting as a key component for osseointegration [5]. Thus the question of material bioactivity (which hereafter refers to the bone-bonding ability), reduces to the apatite forming ability (AFA) of a material *in vivo*. It is further assumed that the apatite forming behavior *in vivo* can be reproduced using an artificial solution supersaturated with respect to hydroxyapatite (HA) *in vitro*, meaning that a material exhibiting HA deposition in SBF would indicate its bone-bonding ability. In 2007, the testing protocol of this *in vitro* method was standardized by the International Organization for Standardization (ISO 23317) and since then has been widely used as a method to evaluate various implant materials [4,6–8]. Despite its success, the simplifications of using

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a solution to mimic the body fluid and determining the bioactivity by the AFA also result in several limitations, which should be pointed out.

1. The bone bonding/formation process is essentially determined by osteoblasts, the activity of which is regulated by various local factors including both inorganic ions as well as organic growth factors. Although hydroxyapatite is widely used as a coating on implant surfaces to increase the osteoconductivity and the *in vivo* formation of HA would also likely result in enhanced osseointegration [9,10], it should be noted that the osseointegration is a complex process involving platelet activation, blood clot and recruitment and migration of osteogenic cells [11]. Thus other possible mechanisms of bioactivity also exist, which could include bioactivity from specific interactions with osteoblasts/stem cells of biofunctional materials by either releasing certain biomolecules or possessing special surface topographical features [12,13]. The existence of other possible mechanisms might explain some cases of inconsistency between *in vitro* predictions and *in vivo* results [14].
2. Assuming the bone-bonding is achieved by the formation of an apatite layer, a qualitative correlation between a material's apatite forming ability *in vivo* and its bone-bonding strength is still difficult to establish. That is, a higher AFA *in vivo* does not necessarily lead to a stronger bonding. This is due to many other factors determining the final bonding strength including surface mechanical properties (adhesion between surface porous layer and bulk implant), and surface roughness, which is typically observed to be a critical factor in animal experiments [15,16].

If we limit the discussion to the chemical route to bioactivity (through the formation of HA) and focus on the *in vitro* method to predict *in vivo* AFA, there are still some issues that remain to be understood. The major criticism of the method is the lack of organics, especially proteins, which are abundant in human blood plasma (roughly 70 g/L in human blood plasma [17]) and are well-known to have specific/non-specific adsorption on various surfaces, which could affect the nucleation & growth of apatite in SBF [18–23]. In fact, proteins, along with other organic molecules, are also active players in the regulation of the biomineralization processes *in vivo* [24]. Despite the existence of a number of *in vitro* studies on the effect of proteins, their role as promotors or inhibitors for calcium phosphate nucleation and growth is still unclear. The reported discrepancies come from varying experimental methodologies used in the literature, as well as other factors including protein concentration and conformation [25,26]. In terms of the heterogeneous nucleation/growth of HA on Ti-based implants in SBF, only a few papers can be found but no clear conclusions can be made due to the use of different SBF compositions and operating procedures [27,28].

Also, the current SBF (ISO 23317) uses tris(hydroxymethyl)aminomethane ($(CH_2OH)_3CNH_2$, Tris) to buffer the solution pH, while Tris is not a component of human blood plasma and might have an effect on calcium phosphate nucleation [29]. The human blood is buffered by the equilibrium between carbonates and 5% partial pressure of CO_2 in blood serum [30]. Due to the high structural variability of apatite crystals, the presence of carbonate species in solution will result in carbonate incorporation into the apatite lattice by either substituting the hydroxyl groups (type A) or occupying the phosphate position (type B) [31–33], as it is the case for apatite in natural bone [34]. Furthermore, it has also been demonstrated that carbonate complexes could lead to an increased apparent solubility of HA [35]. Despite being proposed in a review by Bohner and Lemaitre [18], unfortunately, so far there has been very limited studies using carbonate-buffered SBFs for implant evaluation.

This paper aims to fill this gap and address the above-mentioned issues, namely the performance of a carbonate-buffered SBF versus the SBF proposed by Kokubo [8] and the effect of proteins on the nucleation and growth of apatite from SBF. Two new solution compositions are proposed and their ability to nucleate apatite on different representative titanium-based surfaces are compared with Kokubo's SBF, with or without the addition of protein, represented by bovine serum albumin (BSA). The effect of proteins on the kinetics of HA formation is also investigated at different protein concentrations. The changes in solution pH and free calcium ion concentrations in the presence of proteins are measured to help interpret the results. These studies aim to address the remaining questions in the field, the answers of which could lead to a better understanding of the system and help better interpret the results produced using SBFs.

2. Materials and methods

2.1. Sample preparation

Four different titanium-based surfaces were chosen for this study: i) sandpaper-polished titanium, ii) titanium – polished and heat-treated in air, iii) titanium – polished and treated with NaOH solution, and iv) titanium – polished, NaOH treated and heated in air. Commercial pure (CP) grade 1 titanium discs (Hempel Special Metals, Switzerland) of 19 mm × 1.5 mm were all machine polished before any chemical treatment. The polishing was applied on both sides of the discs using silicon carbide P320, P1200, and 1200/4000 abrasive paper consecutively, followed by washing with acetone, water and ethanol in an ultrasonic bath. The NaOH treatment was conducted using 5 M NaOH solution for 24 h at 60 °C. Afterwards, the discs were washed using flowing de-ionized water for about 30 s and then dried. The heat treatment was applied at 600 °C (heating rate 100 °C/h, natural cooling) for an hour in ambient atmosphere.

2.2. Design and preparation of simulated body fluids

A thermodynamic model was used to design SBFs with a target pH of 7.40 at testing conditions. More details about this model are given in the supplementary information (SI-1). Two new SBFs are proposed and the compositions in comparison with human blood plasma ionic concentration are given in Table 1. SBF-BCS1 was prepared by mixing equal volumes of two stock solutions A (containing NaCl, $NaHCO_3$, $Na_2HPO_4{\cdot}2H_2O$ and Na_2SO_4) and B (containing NaCl, $MgCl_2{\cdot}6H_2O$, $CaCl_2$ and HCl). SBF-BCS2 was prepared in a similar manner with additional KCl in solution B and a slightly different ionic concentration. The detailed recipe is given in the supplementary information (SI-2). The purpose of using two stock solutions is to separate calcium and phosphate ions before the AFA test to avoid undesired nucleation in a solution supersaturated with respect to several calcium phosphate phases. Both SBF-BCS1 and SBF-BCS2 (hereafter abbreviated as BCS-1 and BCS-2) were designed to be used under 5% CO_2 at 36.5 °C. Kokubo's corrected SBF (c-SBF) was prepared by strictly following the procedures of ISO 23317:2014 [8]. The solution was prepared using NaCl, $NaHCO_3$, KCl, $K_2HPO_4{\cdot}3H_2O$, $MgCl_2{\cdot}6H_2O$, $CaCl_2$, and Na_2SO_4 and buffered by Tris and HCl solution at 36.5 °C. The final ionic concentrations are given in Table 1.

In the study of proteins in SBF, bovine serum albumin (lyophilized powder, ⩾96%, Sigma-Aldrich) was added to BCS-1, BCS-2 (both in solution B) and c-SBF with 0.2 g antibacterial agent NaN_3 per 1 L SBF. The pH values of solution BCS-1 and BCS-2 were measured using a pH meter (FiveEasy Plus, Mettler Toledo) under 5% CO_2 at 36.5 °C four times at each BSA concentration.