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Aspects of Multimodal Hybrid Biomaterials

Abstract: Immobilizing a single growth factor such as rhBMP-2 on Implant materials can significantly increase the integration of implants into bone in animals. Paradoxically the effective osteoinductive dose of rhBMP-2 in humans is ca. 100-1000-fold higher than in animals and may induce serious side effects in spine fusion surgery. In physiological bone healing at least 10 separate growth and differentiation factors are sequentially involved in the first two healing phases leading to a callus and/or woven bone. It is therefore hypothesized that a significant dose reduction for rhBMP-2 applications in humans may be achieved by a combination of rhBMP-2 with other growth factors in a biomimetically oriented manner. Some aspects involved in *multimodal bioactive hybrid carriers* with spatio-temporal release kinetics and a new phenomenon ("protein interference") are presented.

Keywords: biohybrid, biomolecular material, bimodal release, BMP-2, cooperativity, polyelectrolyte multilayers (PEM), protein interference, titanium, Aligpore, VEGF

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1 Introduction

State-of-the-art immobilization of a single growth factor such as bone morphogenetic protein 2 (rhBMP-2) on Implant materials can significantly increase the integration of implants in bone in animals [1,2]. Unphysiologically high doses, essential in humans, have led to side effects and serious complications in spine fusion surgery [3]. A necessary dose reduction for rhBMP-2 applications in humans may be achieved by a combination of rhBMP-2 with a second essential mediator such as vascular endothelial growth factor (rhVEGF₁₆₅). In physiological secondary bone healing at least 10 different growth and differentiation factors are involved in the first two healing phases leading to a callus and/or woven bone. To this end a new generation of bi- or *multimodal bioactive hybrid carriers* with specific temporal or spatial release kinetics for each factor is our engineering aim e.g. to release rhVEGF with a shorter half-life than rhBMP-2, based on the physiological bone healing time-scale.

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A versatile material for developing such a carrier is poly-(D,L)-lactide (PDLLA), where a growth factor can either be immobilized on the surface or inside the bulk material e.g. by foaming technologies [4] or electrospinning [5]. RhBMP-2- and VEGF-containing foamed tablets or electrospun fleeces have shown sustained release half-lives ranging between 85 and 350 days.

2 Materials and Methods

Material Titanium miniplates (10 mm x 5 mm x 1 mm; MorphoPlant GmbH, Bochum) with ultrahydrophilic sand-blasted, acid etched (μ SLA) surface [6] (= μ SLA-Ti), were employed. Poly-L-lysine hydrobromide (PLL) ($m \sim 30$ -70 kDa), chondroitin sulfate from shark cartilage (CS) ($m \sim 15$ -40 kDa) were from Sigma, Munich. Aligpore (98% HA; AlgOss 100, 1.3×10^4 Particles/g) was obtained from AlgOss Bio-technologies GmbH, A-1180 Vienna, Austria [7]. **Growth factors.** RhBMP-2 and rhVEGF₁₆₅ prepared in *E. coli*, according to [8] and [9] respectively, were from MorphoPlant GmbH, Bochum) and radioactively labeled by the iodine monochloride method, i.e. ¹²⁵I-BMP-2, ¹²⁵I-VEGF (specific radioactivity: 8000-10000 cpm/ μ g), according to [10,11]. The *biological activity equivalents* were determined for rhBMP-2 to $K_{0.5} \sim 5$ -20 nM) and for rhVEGF₁₆₅ to $K_{0.5} \sim 5$ -20 pM [11]. **Buffers.** As buffers HEPES-NaCl-**buffer A** (25 mM HEPES and 137 mM NaCl, pH 7.4), PBS-**buffer B** (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, pH7.4) and NaAc-**buffer C** (20 mM sodium acetate pH 4.5) were employed. **Polyelectrolyte multilayers.** Polyelectrolyte multilayers (PEMs) [12] composed of PLL and CS (25 bilayers) were prepared by dipping the μ SLA miniplates at ~ 22 °C for 15 min alternately into polyelectrolyte solutions of PLL or CS in **buffer A** ($c = 1$ mg/ml each) composing 1 bilayer. After the final layer had been adsorbed (PEM- μ SLA = Ti-(PLL-CS)₂₅-PLL), the discs were washed with HEPES-NaCl-**buffer A** and then dried in a N₂-stream and stored dry at 5 °C. For adsorption equilibrium the μ SLA-PEM-Ti-miniplates were incubated with 0.5 ml either ¹²⁵I-labelled or unlabelled BMP-2 or ¹²⁵I-VEGF₁₆₅ (0.1 mg/ml, in 20 mM NaAc-**buffer C**, pH 4.5) over night at room temperature [12]. For desorption the ¹²⁵I-BMP-2- or ¹²⁵I-VEGF-PEM- μ SLA-Ti-miniplates were transferred to vials with 1.5 ml PBS-**buffer B** (batch method, see [4,12]) for the indicated times up to 40-50 hours. Before measurements the miniplates were washed in PBS buffer and after measurements in the γ -counter trans-

ferred to fresh PBS-**buffer B** for next desorption. For simultaneous adsorption a 1:1 mixture (each factor 0.1 mg/ml, in 20 mM NaAc, pH 4.5) of cold rhBMP-2 and hot ^{125}I -VEGF₁₆₅ were incubated with $\mu\text{SLA-PEM-Ti}$ -miniplates and desorbed as above. **Algipore**. The incubation mixture contained: 2.0 g Algipore in 5 ml NaAc-**buffer C** with ^{125}I -rhBMP-2 (initial concentration: 0.1 mg/ml) or ^{125}I -rhVEGF₁₆₅ solution (initial concentration: 10 $\mu\text{g/ml}$) for 15 hours at room temperature, leading to 0.81 ± 0.01 ($n = 3$) mg/g for rhBMP-2 and to 86.5 ± 4.7 ($n = 3$) $\mu\text{g/g}$ for rhVEGF₁₆₅. For desorption the protein loaded particles (1.0-2.0 g) were washed 3 times with 2 ml of Tris-**buffer D** 50 mM Tris/HCl, 150 mM NaCl, pH 7.4 and transferred to fresh 2 ml PBS-**buffer B** for desorption at room temperature. At specified times the protein loaded Algipore samples are retrieved washed 3 times with 2 ml Tris-**buffer D** and transferred to fresh 2 ml buffer PBS-**buffer B** for the next time period up to 24 days. The immobilized amounts of ^{125}I -rhBMP and ^{125}I -rhVEGF on the particles are determined in a gamma-counter ($n = 3$). In all cases the calculated half-lives of desorption were corrected for the spontaneous decay of ^{125}I ($t_{1/2} = 60$ d) [13].

3 Results and Discussion

3.1 Biomaterials and Hybrids

In the medical sciences hybrid materials are composed of biomaterials (**Fig. 1**) necessitating a brief definition of terms. “A Biomaterial is a fabricated state of non-viable, condensed matter for the production of biocompatible medical devices with a defined structure, surface and function for a reproducible interaction with cells, tissues, and organs and/or integration in humans and animals” [14,15].

Fig. 1.

Scheme of the four classes of biomaterials as suggested previously [16] and in ref.[15].

“Biomolecular materials correspond to all materials and molecules of biological origin, that are produced by genetic engineering and/or biotechnology”, e.g. recombinant human proteins such as rhBMP2 and rhVEGF [15,16]. The term hybrid used here is a working definition taken from ref. [17]: “A hybrid material is a combination of two or more materials in a predetermined geometry and scale, optimally serving a specific engineering purpose”. In our case the hybrid should serve a medical purpose, namely a curative intent. Thus, the combination of one of the three biomaterial classes e.g. metal, polymer, and ceramic with a biomolecular material is termed a biomolecular hybrid, in short a biohybrid.

3.2 Release Modalities

PDLLA nanofibers constitute a versatile biocompatible base material for bioactive factors allowing multiple simple immobilization and release technologies (see example in **Fig. 2**).

Fig. 2

Scheme of immobilizing three familiar growth factors with a PLLA-nanofiber. BMP-2 is an inclusion within the fiber and VEGF and SDF1 α are adsorbates (see also [5]).

3.2.1 Bimodal temporal hybrids

A bimodal biomolecular hybrid biomaterial is capable of releasing two bioactive molecules from a single area of the implant in a controlled manner at distinctly different rates. Such a biomaterial is shown schematically in (**Fig. 3A**) with VEGF being released at a higher rate i.e. earlier than BMP-2. In this way blood vessels would be induced first for transporting oxygen to the implant surface, where the initial partial pressure of oxygen is zero. The later release of BMP-2 would induce bone, when the oxygenation of tissue is assured.

3.2.2 Multimodal temporal and spatial hybrids

The synthesis of a trimodal temporal hybrid is schematically shown in **Fig. 2**. A Multimodal hybrid is shown schematically in **Fig. 3B**. In that case of **Fig. 2** the sequence of events would be to first release VEGF for provision of oxygen, followed by SDF1 α release to initiate a homing of stem cells to the implant site, thus paving the way for BMP-2 release and the induction of periimplant bone.

Fig. 3

Models exemplifying Release modalities.

A. Bimodal model for consecutive release of VEGF and BMP-2.

B. Multimodal spatio-temporal release model with ten virtual proteins (Shekel-function). (modified from ref. [18])

In more complex multimodal systems another variable, i.e. space, is added, for constructing a multimodal spatio-temporal biomolecular hybrid material displaying a controlled release of bioactive molecules e.g. from 10 separate areas of a hypothetical implant (Fig. 2B).

3.3 Bimodal Release

3.3.1 Independent release

Fig. 4

Release of ^{125}I -rhBMP-2 and ^{125}I -rhVEGF₁₆₅ from algipore particles (24 days).

Two separate experiments of ^{125}I -rh-BMP-2 (■) and ^{125}I -rhVEGF₁₆₅ (▲) release from Algipore respectively are superimposed. The data has been fitted to two-phase exponential release kinetics yielding half-lives for the burst and sustained release phases. For further details see Methods and ref. [11].

First desorption experiments of ^{125}I -rhBMP-2 were described from metal surfaces in 2002 [13]. A sustained exponential first-order decay was found with half-lives of 100-138 days [13]. On hydroxyl-apatite wafers it was found that first-order release is not always monophasic but may be of biphasic nature, consisting of a first-order burst phase and a first-order sustained-release phase [19]. These observations also apply

Fig. 5

Separate (A & B) and simultaneous (C) adsorption and release of (^{125}I -rhBMP-2 and of ^{125}I -rhVEGF₁₆₅ on $\mu\text{SLA-Ti-(PLL-CS)}_{25}\text{-PLL}$ $\mu\text{SLA-PEMs}$ (2 Days).

A. Adsorption and release of ^{125}I -rhBMP-2 on Ti-PEMs (blue line) (●); on PEM-free control titanium (= green line) (○)
 B. Adsorption and release of ^{125}I -rhVEGF₁₆₅ on Ti-PEMs (red line) (●); on PEM-free control titanium (= green line) (○)
 C. Simultaneous adsorption and release of a 1:1 mixture of cold (unlabelled) rhBMP-2 and ^{125}I -rhVEGF₁₆₅ on Ti-PEMs. Only the release of ^{125}I -rhVEGF₁₆₅ from Ti-PEMs

(red line) (•) is shown; release from Ti-control (= green line) (o). (Madenci, S. & Jennissen, H.P., unpublished) to the experiments in **Fig. 4**, which were performed on the bone-replacement material Aligpore (see Methods and [11]). After adsorption of ^{125}I -rhBMP-2 (810 $\mu\text{g/g}$) the factor is released with a burst-phase half-life of 2.3 days and a sustained release half-life of 101 days. In contrast ^{125}I -rhVEGF₁₆₅, adsorbed in a ~ 100 -fold lower amount of 86 $\mu\text{g/g}$ is released with a burst-phase half-life of 1.7 days and a 5-fold lower sustained release half-life of 23 days, i.e. at a 5-fold higher rate. Thus, in principle we have a bimodal release with distinctly different rates for the two proteins.

3.3.2 Interference, a new form of non-independence

What happens, if the two above proteins are mixed and simultaneously equilibrium adsorbed and released? This is shown for polyelectrolyte multilayers (see [12]) in **Fig. 5**. Under equilibrium binding (0.1 mg/ml, over night, room temperature) the non-covalent binding capacity of μSLA -PEMs for ^{125}I -rhBMP-2 was determined to 8.6 $\mu\text{g/cm}^2$ (**Fig. 5A**, $t = 0$). On Release of ^{125}I -rhBMP-2 a short burst phase is followed by a sustained release phase ($t_{1/2} > 100$ d; total release both phases 13%, 2 d). Adsorption of ^{125}I -rhBMP-2 on the PEM-free Ti-control yields a low capacity of ~ 1.3 $\mu\text{g/cm}^2$. In contrast (**Fig 5B**), the corresponding binding capacity of μSLA -PEMs for ^{125}I -VEGF₁₆₅ is only 0.40 $\mu\text{g/cm}^2$, i.e. 20-fold lower than that for rhBMP-2. A sustained release phase is absent with 86% of the adsorbed protein being released in a burst within 60 min ($t_{1/2} \sim 20$ min). The PEM-free Ti-control has a 3-fold higher capacity (than the μSLA -PEMs) of 1.3 $\mu\text{g/cm}^2$ ($t = 0$) and a long sustained release phase ($t_{1/2} > 100$ d). In **Fig. 5C** a mixture of rhBMP2/ ^{125}I -rhVEGF₁₆₅ is adsorbed and released from the μSLA -PEMs. Since rhBMP-2 is unlabelled only the release of ^{125}I -rhVEGF₁₆₅ is observed. In the combination with unlabelled rhBMP-2 the capacity of μSLA -PEMs for ^{125}I -rhVEGF₁₆₅ increases 2.7-fold to 1.1 $\mu\text{g/cm}^2$. In addition the release curve now exhibits a burst phase and a sustained release phase, the latter on a 20-fold higher level than in **Fig. 5B** (2-phase exponential decay, $t_{1/2} \sim 20$ -40 d, total release 25%, 2 d). RhBMP-2 thus interferes with the release of ^{125}I -rhVEGF₁₆₅, reducing the release rate dramatically from $t_{1/2} \sim 1.4 \times 10^{-2}$ days (20 min) for ^{125}I -rhVEGF₁₆₅ alone to a $t_{1/2} \sim 20$ -40 d for the combination with rhBMP-2. This we may call "adsorbate protein-protein interference", or *protein interference* for short. In protein interference two proteins interfere with one another for binding on a surface in such a manner, that either the initial preferred surface-valency, or the conformational, orientational, steric or lateral interaction preferences of one molecule are altered by the presence and

preferences of a second molecule in reaching a final kinetic steady state and surface coverage for both. A ^{125}I -rhVEGF₁₆₅-rhBMP-2 complex may also have been formed. More work is pertinent, to determine the exact mechanism of this effect.

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Author's Statement

The Author states no conflict of interest.

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