Bioactive Coating of Titanium Surfaces with Recombinant Human β -Defensin-2 (rHu β D2) May Prevent Bacterial Colonization in Orthopaedic Surgery

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Background: A promising strategy to prevent infections around orthopaedic titanium implants is to use naturally occurring cationic antimicrobial peptides (CAMPs) such as the human β -defensin-2 as antibacterial coatings. Human antimicrobial peptides represent a part of the innate immune system and have a broad antimicrobial spectrum against bacteria, fungi, and viruses.

Methods: In the present study, titanium surfaces were functionalized by four different self-assembled monolayers (SAMs) forming methoxy silanes: (1) hexadecyltrimethoxysilane, (2) dimethoxymethyloctylsilane, (3) allyltrimethylsilane, and (4) 3-aminopropyltrimethoxysilane. In addition, calf skin type-I collagen was cross-linked to the SAM surface 3-aminopropyltrimethoxysilane by means of two different treatments: (1) N-hydroxysuccinimide and (2) glutaraldehyde. The functionalized titanium surfaces were coated with recombinant human β -defensin-2 (rHu β D2), an antimicrobial peptide, and were tested for antibacterial activity against *Escherichia coli*. The release of rHu β D2 was quantified by means of enzyme-linked immunosorbent assay (ELISA).

Results: The coating of functionalized titanium surfaces with rHu β D2 was successful. Recombinant Hu β D2 was eluted from the titanium surfaces continuously, yielding antimicrobial activity up to several hours. Antimicrobial activity with a killing rate of 100% was observed for all functionalized titanium surfaces after two hours of incubation. The dimethoxymethyloctylsilane-functionalized titanium surface delivered 0.65 μ g of rHu β D2 after six hours with a 60% bacterial killing rate. The silane-functionalized surfaces exhibited a faster release of antimicrobially active rHu β D2 compared with collagen modifications.

Conclusions: Natural antibiotics such as rHu β D2 integrated into the metal surface of titanium implants may be a promising tool to prevent and control infections around orthopaedic implants.

Clinical Relevance: This kind of titanium surface modification may provide an alternative treatment of serious, life-threatening infections related to prosthetic implant surgery.

The insertion of titanium implants during orthopaedic surgery is a common and relatively safe procedure today. However, bacterial infections around an implant remain difficult to control. *Staphylococcus aureus* and several other strains of *Staphylococcus* are frequently associated with the colonization of orthopaedic implants and are responsible for

subsequent infections. Peri-implant infections may be dependent on the microbial flora at the operative site, and hospital-acquired multi-drug-resistant bacteria play an increasing role in causing these infections^{1,2}.

Antibiotics such as vancomycin, when covalently attached onto titanium surfaces, reduce in vitro colony formation of

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gram-positive bacteria³, but the prophylactic use of local antibiotics during implant placement is controversial. Treatment with antibiotics may trigger allergic reactions⁴ and could promote the induction of multi-drug-resistant bacteria^{5,6}. Thus, the development of surfaces and coatings that can actively kill bacteria remains a challenge. Probably the oldest and most widespread coatings are silver ions, which have been successfully applied against methicillin-resistant *Staphylococcus aureus* (MRSA)⁷. However, a drawback of this approach is the cytotoxicity of silver ions toward mammalian cells⁸.

More recently, surface functionalizations such as adsorption, coating, or covalent binding with artificial antimicrobial peptides and several cationic, polycationic, and steroid antibiotics have been described⁹⁻¹². The hydrophobic and positively charged side groups of these small molecules can interact with the microbial membrane, leading to its disintegration and the metabolic breakdown of the microbe. However, the production of such surfaces for medical use is still expensive. In addition, medical acceptability of these molecules is low because of their unknown interactions with mammalian cells. Therefore, a promising strategy to prevent infection around orthopaedic implants is the use of naturally occurring cationic antimicrobial peptides (CAMPs) for bioactive coatings.

CAMPs represent a part of the innate immune system and have a broad antimicrobial spectrum¹³. The cationic nature of CAMPs mainly contributes to their cell selectivity because the surface of bacterial membranes is more negatively charged than that of mammalian cells. Therefore, CAMPs exhibit a strong antimicrobial activity but no cytotoxicity to mammalian cells¹⁴. This class of antimicrobial peptides is active even against multi-drug-resistant bacteria such as MRSA. As the mechanism of their action differs from that of classical antibiotics, the formation of resistant microbes has not been observed¹⁵⁻¹⁸. The CAMP used in the present in vitro study is recombinant human β -defensin-2 (rHu β D2), a molecule that is expressed in human epithelial cells after stimulation by injury or different inflammatory factors¹⁹. Moreover, rHu β D2 takes part in the recruitment of cells of the adaptive immune response in chemoattractant macrophages and mast cells promoting wound-healing²⁰⁻²³. Recombinant Hu β D2 exhibits antimicrobial activity against both gram-positive and gram-negative bacteria, fungi, and viruses²⁴⁻²⁶.

The purpose of the present study was to test the antimicrobial activity of rHu β D2 coated on different functionalized titanium surfaces. Furthermore, the release behavior in relation to the antimicrobial properties of rHu β D2 on two different collagen titanium scaffolds was investigated.

Materials and Methods

Titanium Pins

One hundred custom-designed oxidized titanium pins were used in the present study. Cylindrical pins measuring 1 mm in diameter and 5 mm in height were made from pure titanium (ASTM Grade 4) according to ISO (International Organization for Standardization) 5832-2. The surfaces were produced by means of a sandblasting process with corundum particles, followed by a strong acid-etching procedure (a mixture of HCl/H₂SO₄) at room

temperature for several minutes. This led to fine micropits of 2 to 4 μ m superimposed on the sand-blasted surface.

Preparation of the Titanium Surface

Prior to surface functionalization, all of the pins underwent ultrasonic treatment (Sonorex Super 10P; Bandelin, Berlin, Germany) for ten minutes in 5-M KOH, for ten minutes in 69% HNO₃, and finally for fifteen minutes in a 2:1 H₂SO₄/H₂O₂ mixture at room temperature. Titanium pins were then extensively rinsed with distilled water. Next, the pins were incubated for one hour at 65°C in an oxidizing solution (NH₄OH/H₂O₂/distilled water in a ratio of 1:1:1) and were stored for sixteen hours at 4°C in 70% ethanol.

Functionalization of the Titanium Surface

Prior to coating of rHu β D2 on the titanium pins, the surfaces of the pins needed to be functionalized. Four different self-assembled monolayers (SAM1 through SAM4) were produced by means of direct silanization of the hydrophilized pin surfaces²⁷. Thus, twenty titanium pins were incubated in 250 μ L toluene consisting of a 10% solution of hexadecyltrimethoxysilane (SAM1), dimethoxymethyloctylsilane (SAM2), or allyltrimethoxysilane (SAM3). In addition, forty pins were incubated in 250 μ L toluene consisting of a 10% solution of 3-aminopropyltrimethoxysilane (SAM4). Both silanization procedures were performed for twenty-four hours at room temperature. Then the pins were extensively rinsed with toluene and were dried at room temperature. Additional carboxylic acid (COOH) groups on the SAM3 titanium pins were generated by oxidation of the CH=CH₂ functional group from allyltrimethoxysilane by incubation with 5% KMnO₄ acid aqueous solution and finally were washed with distilled water.

Collagen Binding to the Functionalized Titanium Surface (SAM4)

To bind collagen in a stable manner onto the functionalized titanium oxide surface, the protein fibrils were cross-linked with the 3-aminopropyltrimethoxysilane (SAM4) layer in two different ways.

First, forty SAM4 titanium pins were pre-incubated in 200-mM 2-(N-morpholino)ethane sulfonic acid (MES) (pH 5.5) for one hour at room temperature. Twenty-five microliters of collagen solution from calf skin type I (0.1%, C8919; Sigma-Aldrich, St. Louis, Missouri) were applied by pipette to the pins, and the pins were dried at room temperature. Cross-linking with N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC) was performed by incubating twenty pins in a solution of 10-mM NHS and 30-mM EDC in 200-mM MES (pH 5.5) for six hours at room temperature. The surface modification was referred to as SAM4::Col-NHS.

Second, the dried collagen fibrils on the SAM4 titanium pin surface were cross-linked by incubating the remaining twenty pins in a 25- μ L glutaraldehyde solution (25%) for one hour at room temperature. Subsequently, the pins were rinsed in a 0.1% w/v bovine serum albumin (BSA) and 200-mM phosphate-buffered saline (PBS) solution for twenty minutes, followed by incubation in 100-mM Na₂HPO₄ for one hour, and finally were washed in distilled water, dried in a desiccator, and stored at 4°C. The surface modification was referred to as SAM4::Col-Glu. The successful binding of collagen was monitored with a collagen-specific stain after treatment with 0.1% w/v DirectRed 80 in 0.5% v/v acetic acid. All chemicals were purchased from Sigma-Aldrich if not described otherwise.

Coating of rHu β D2 to the Functionalized Titanium Surfaces

Five different groups of functionalized titanium pins were produced and investigated in this study: three silanized titanium surfaces (SAM1, SAM2, SAM3) and two SAM4::collagen titanium surfaces (SAM4::Col-NHS, SAM4::Col-Glu). Recombinant human β -defensin-2 (rHu β D2) was produced by PLANTON (Kiel, Germany) as described previously²⁵. Coating of this peptide was performed by directly adding 10 μ L of a 1 mg/mL or an 8 mg/mL rHu β D2 solution (in 0.01% acetic acid) onto the functionalized titanium surfaces. Finally, the pins were dried in a desiccator and were stored at 4°C prior to use.

In this way, two sets of SAM1, SAM2, and SAM3 layers were generated by coating either 10 μ g or 80 μ g of rHu β D2 on each surface. The

TABLE I Antimicrobial Activity of Functionalized Titanium Pins in Correlation with the Applied and the Determined Amount of rHuβD2*

	rHuβD2 Controls					
	NC	PC1	PC2	PC3	PC4	PC4/ab
Amount of applied rHuβD2 (μg)	0	0.02	0.20	2.00	10.00	10.00
Amount of rHuβD2 determined with ELISA† (μg)	0	0.02 ± 0.01	0.18 ± 0.01	1.70 ± 0.14	9.01 ± 0.82	8.89 ± 0.72
Antimicrobial activity, bacterial killing† (%)	0	14.80 ± 2.78	32.38 ± 2.93	92.80 ± 3.91	100	0

*NC = negative control; PC1, PC2, PC3, and PC4 = positive controls 1, 2, 3, and 4, respectively; PC4/ab = positive control 4 with monospecific anti-rHuβD2 antibodies; SAM1-n = self-assembled monolayer of hexadecyltrimethoxysilane without rHuβD2; SAM1 = self-assembled monolayer of hexadecyltrimethoxysilane coated with rHuβD2; SAM2-n = self-assembled monolayer of dimethoxymethyloctylsilane without rHuβD2; SAM2 = self-assembled monolayer of dimethoxymethyloctylsilane coated with rHuβD2; SAM3-n = self-assembled monolayer of allyltrimethoxysilane without rHuβD2; and SAM3 = self-assembled monolayer of allyltrimethoxysilane coated with rHuβD2. †The values are given as the mean and the standard deviation.

SAM4::collagen functionalized titanium surfaces were coated with 80 μg of rHuβD2.

Antibacterial Assays

The antibacterial activity of the rHuβD2-coated pins was tested by means of a microdilution assay as described recently²⁷, but with the following modifications. *Escherichia coli* strain DB3.1 (Cat. No. 11782-018; Invitrogen, Carlsbad, California) was used. *Escherichia coli* was streaked from a glycerol stock onto a Brain Heart Infusion (BHI) agar plate (3.7% w/v BHI bouillon, 1.5% w/v agar), was grown overnight at 37°C, and subsequently was used to inoculate 40 mL of 3.7% w/v BHI bouillon without any antibiotics in 100-mL flasks. Fresh cells were harvested by means of centrifugation and were washed twice with 10-mM sodium phosphate buffer (pH 7.2). The optical density was adjusted with 3.7% w/v BHI bouillon to 10⁴ cells/mL. For antibacterial testing, a single pin coated with rHuβD2 was incubated in one well of a microtiter plate containing 100 μL of the bacterial suspension.

Functionalized titanium pins without rHuβD2 served as negative controls (SAM1-n, SAM2-n, SAM3-n, SAM4::Col-NHS-n, SAM4::Col-Glu-n). In addition, five independent control reactions consisting of rHuβD2 in a final amount of 0 μg (NC), 0.02 μg (PC1), 0.2 μg (PC2), 2 μg (PC3), and 10 μg (PC4) were examined in parallel. To determine the specificity of the antibacterial activity of rHuβD2, 10 μL of polyclonal monospecific anti-rHuβD2 antibodies (10 μg/mL in PBS) were co-incubated with 10 μg of rHuβD2 and bacterial cells (PC4/ab) (Table I).

A single microtiter plate for each experiment, including the control reaction and the five different functionalized pins, was incubated for two hours at 37°C. The kinetic studies were performed by replacing the bacterial suspension, after two hours of cultivation, with a freshly precultured *Escherichia coli* solution, which contained the same bacterial concentration, and cultivating for another two hours, and so on. Colony-forming units (CFU) were determined after plating 100 μL of a 1:100 dilution of the bacterial suspension on Petri dishes with BHI medium and incubating them overnight at 37°C. For every group of the functionalized titanium pins and the controls, five independent antibacterial experiments were performed. A control (NC) of bacterial growth was examined for every independent assay. The number of colony-forming units on the control (consisting of a single Petri dish) was set to 0% bactericidal activity. The reduction in the colony-forming units resulting from the antibacterial activity of rHuβD2 was related to the absolute colony-forming units of the control within every independent assay. This value, given as a percentage of the bacterial "killing" activity, was used to describe the antibacterial activity. Normalization of the antibacterial data was performed for every experimental group.

Quantification of rHuβD2 with ELISA

In a parallel reaction, the amount of rHuβD2 released into the BHI medium was determined by means of an enzyme-linked immunosorbent assay (ELISA). BHI

medium was prepared as described above without bacterial cells. Titanium pins were incubated in the BHI medium for two hours at 37°C, corresponding to the antibacterial assay. The same set of controls was examined as described above. Microtiter plates were coated with 50 μL of polyclonal monospecific rabbit anti-rHuβD2 antibodies (100 ng/mL) in 0.05-M Na₂CO₃ (pH 9.6) and then were blocked with 0.5% BSA in PBS for two hours at 37°C. After each step, the wells were washed three times with PBS (pH 7.4) containing 0.1% v/v Tween 20. Fifty microliters of the sample, consisting of BHI medium that contained either the titanium pins or the control reactions, diluted in PBS/0.1% v/v Tween 20, was added in duplicate and incubated for sixty minutes at 37°C. After washing, 50 μL of the rabbit biotinylated anti-rHuβD2 antibody (100 ng/mL) was added, followed by incubation for sixty minutes. After washing, horseradish peroxidase (HRP)-conjugated streptavidin was added and incubated for forty-five minutes at 37°C. Finally, the reaction was visualized by adding 50 μL of tetramethylbenzidine (TMB) substrate for ten to twenty minutes. The reaction was stopped with 0.5-M H₂SO₄, and the absorbance was determined at 450 nm with use of an ELISA plate reader. As a reference for quantification, a standard protein curve was established by a serial dilution of rHuβD2 (45 pg/mL to 1 μg/mL).

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Results

To determine the antibacterial activity of functionalized and subsequently coated titanium surfaces, an antibacterial assay was established. The antibacterial activity data were generated by normalizing the colony-forming units of every independent assay to the control assay. The resulting percentage of the killing activity of five independent assays was averaged. Thus, the variability of the colony-forming units of the individual antibacterial assays was reduced.

The results confirmed the efficacy and the specificity of the recombinant human β-defensin-2 (rHuβD2) as an antibacterial agent (Table I). The bacteria were completely killed at a concentration of 10 μg of rHuβD2. In contrast, the entire bactericidal activity was blocked when a specific anti-rHuβD2-antibody was added to the assay. The amount of rHuβD2 detected by means of ELISA in the control assays (NC, PC1 through PC4, and PC4/ab) corresponded with the amount initially applied to the anti-bacterial assay (Table I).

Titanium oxide surfaces were functionalized by self-assembled monolayers (SAM) of common silanes. In the first

TABLE I (continued)

Titanium:SAM1		Titanium:SAM2		Titanium:SAM3	
SAM1-n	SAM1	SAM2-n	SAM2	SAM3-n	SAM3
0	10.00	0	10.00	0	10.00
0	6.77 ± 1.72	0	8.05 ± 2.14	0	5.35 ± 1.32
0	93.20 ± 3.78	0	95.63 ± 4.39	0	89.83 ± 3.98

experiment, titanium pins functionalized by hexadecyltrimethoxysilane (SAM1), dimethoxymethyloctylsilane (SAM2), and oxidized allyltrimethoxysilane (SAM3) were coated with 10 µg of rHuβD2. The SAM surfaces alone (SAM1-n, SAM2-n, SAM3-n) did not exhibit any antimicrobial activity prior to coating with rHuβD2 (Table I). In contrast, functionalized titanium pins coated with rHuβD2 (SAM1, SAM2, SAM3) exhibited an antibacterial activity killing rate of >90% (Table I). The biological activity of the coated titanium pins corresponded with the positive rHuβD2 control (PC4) with the highest concentration (10 µg) and demonstrated successful coating of the pins with rHuβD2. Furthermore, the ELISA data presented in Table I confirmed (1) that coating with rHuβD2 of the functionalized pins was nearly 100%, (2) that biologically active rHuβD2 was eluted from the pins, and (3) that the discrepancy between the amount of rHuβD2 applied for coating and the amount eluted from the pins indicated a storage function of the SAM layers for rHuβD2.

To examine the release behavior of rHuβD2 from the different functionalized titanium pins, a time kinetic experiment was performed. As observed in the previous experiment, coating with 10 µg of rHuβD2 was not sufficient to achieve complete bacterial killing, so the titanium pins were coated with an excess of rHuβD2 (80 µg). Thus, a high antimicrobial activity with a killing rate of 100% was observed for all SAMs after two hours of incubation in the antibacterial assay (Fig. 1). ELISA quantification revealed that, at this time point, about 74 to 79 µg of rHuβD2 was eluted from the different titanium pins into the medium (Table II). The time-dependent cultivation of the titanium pins was performed by incubating the same pin after the first two-hour cultivation in a freshly prepared *Escherichia coli* culture containing the same amount of bacteria for another two hours, and so on. After the second cultivation step (four hours), the antimicrobial activity still reached killing rates of 52% (corresponding to 0.41 µg of

Fig. 1

Bar graph showing the kinetics of rHuβD2 release from silanized titanium surfaces as a percentage of bacteria killed. The antimicrobial activity of different silanized titanium pins coated with 80 µg of rHuβD2 (hexadecyltrimethoxysilane [SAM1, black bars], dimethoxymethyloctylsilane [SAM2, fasciated bars], and oxidized allyltrimethoxysilane [SAM3, white bars]) after two, four, six, and eight hours of cultivation in an *Escherichia coli* suspension is shown. Bacterial killing activity is shown as the mean percentage of five independent antimicrobial assays, along with the standard error of the mean. The test of significance was performed with the Student t test. An asterisk indicates a significant difference ($p < 0.001$) between groups.

TABLE II Quantification of rHuβD2 Release with ELISA *

	rHuβD2 Release (μg)				
	SAM1	SAM2	SAM3	SAM4::Col-Glu	SAM4::Col-NHS
2 hr	76.18 ± 1.72	74.28 ± 1.11	78.85 ± 1.12	17.20 ± 4.30	14.31 ± 1.72
4 hr	0.61 ± 0.24	1.29 ± 0.38	0.41 ± 0.16	6.62 ± 2.54	0.46 ± 0.22
6 hr	0.08 ± 0.03	0.65 ± 0.08	0	0	0
8 hr	0	0	ND	ND	ND

*The values are given as the mean and the standard deviation. SAM1 = self-assembled monolayer of hexadecyltrimethoxysilane coated with rHuβD2, SAM2 = self-assembled monolayer of dimethoxymethyloctylsilane coated with rHuβD2, SAM3 = self-assembled monolayer of allyltrimethoxysilane coated with rHuβD2, SAM4::Col-Glu = calf skin collagen type I cross-linked on 3-aminopropyltrimethoxysilane by glutaraldehyde and coated with rHuβD2, SAM4::Col-NHS = calf skin type-I collagen cross-linked on 3-aminopropyltrimethoxysilane by N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl and coated with rHuβD2, and ND = not determined.

rHuβD2 in the medium) to 69% (corresponding to 1.29 μg of rHuβD2 in the medium) (Table II). At this time point, a stronger killing activity of rHuβD2 of the SAM2 pins compared with the other functionalized surfaces could be observed ($p < 0.001$) (Fig. 1). After six hours, the killing rate for SAM2 was still 60% (corresponding to 0.65 μg of rHuβD2 in the medium), that for SAM1 was about 29% (corresponding to 0.08 μg of rHuβD2 in the medium), and that for SAM3 dropped significantly, below 5% ($p < 0.001$) (Fig. 1). These findings indicated that different functionalized titanium pins exhibited a different elution profile in a defined time span.

As it is known that collagen has a positive impact on wound-healing, functionalized titanium pins with collagen were investigated. Two different cross-linking strategies for collagen on SAM4 were applied: (1) a covalent binding strategy with the NHS/EDC cross-linking system, and (2) the use of glutaraldehyde. Successful binding of collagen was monitored with collagen-specific DirectRed staining. Functionalized collagen titanium pins without rHuβD2 did not show any antibacterial activity (Fig. 2). After two hours of incubation, pins of both cross-linking strategies generated killing rates of 100% (Fig. 2). However, ELISA quantification of these pins revealed a

Fig. 2

Bar graph showing the antimicrobial activity of collagen titanium pins coated with rHuβD2 as a percentage of bacteria killed. The antimicrobial activity of two different collagen titanium pins coated with 80 μg of rHuβD2 (SAM4::Col-Glu [black bars] and SAM4::Col-NHS [white bars]) after two, four, six, and eight hours of cultivation in an *Escherichia coli* suspension is shown. Bacterial killing activity is shown as the mean percentage of five independent antimicrobial assays, along with the standard error of the mean. The test of significance was performed with use of the Student t test. An asterisk indicates a significant difference ($p < 0.001$) between groups.

release of only 14 to 17 µg of rHuβD2 into the medium (Table II). After four hours of cultivation, the killing rate dropped to 53% (corresponding to 0.46 µg of rHuβD2 in the medium) for the NHS/EDC system whereas collagen pins treated with glutaraldehyde exhibited a killing rate of almost 92% (corresponding to 6.6 µg of rHuβD2 in the medium). After six hours, the antimicrobial activity of both systems dropped below 10% of the killing rate and no rHuβD2 was detectable in the medium with use of ELISA.

Discussion

Previous reports have shown that recombinant human β-defensin-2 (rHuβD2) exhibited antibacterial activity at a minimal bactericidal concentration of >10 mg/L against gram-positive bacteria^{28,29} and at a minimal bactericidal concentration of <12 mg/L against gram-negative bacteria²⁵. These findings make rHuβD2 a very good candidate as a naturally occurring antimicrobial peptide for bioactive implant coating. The antibacterial tests in the present study were performed with use of a sensitive indicator organism, *Escherichia coli*, in order to establish the concept of an effective antimicrobial drug delivery system using rHuβD2 on titanium surfaces. The results corroborated the efficient antibacterial activity of rHuβD2 and, in addition, showed its bioactivity when coated on metallic surfaces. The present study demonstrated that titanium surfaces functionalized by different SAM (self-assembled monolayers) could store and deliver antimicrobially active rHuβD2.

The aliphatic SAM layers (SAM1 and SAM2) and also the negatively charged SAM3 layer could store and release sufficient rHuβD2 for antimicrobial activity in vitro (Table I). However, there were differences between the three functionalized surfaces. The rHuβD2 coating exhibited continuous release of rHuβD2 for several hours (Fig. 1). Hexadecyltrimethoxysilane (SAM1) and dimethoxymethyloctylsilane (SAM2) differ in the length of their exposed aliphatic chains. The aliphatic chain of dimethoxymethyloctylsilane (SAM2) is shorter and might exhibit a better rHuβD2 delivery system, as indicated by an antimicrobial activity of 60% killing after three cultivation steps (six hours). The other hydrophobic layer (SAM1) exhibited a killing rate of only 29%.

Both hydrophobic surfaces (SAM1 and SAM2) exhibited a higher capacity for storage and peptide release compared with the SAM3 pins (Fig.1). In addition, the functionalized SAM3 surface exhibited the fastest decrease of activity over time. After six hours, almost no killing of *Escherichia coli* was observed and no rHuβD2 was detected with ELISA (Fig. 1 and Table II). The reason for this finding may be an ionic interaction between the SAM3 surface and rHuβD2. However, excessive and unbound rHuβD2 was released immediately, resulting in the delivery of most of the rHuβD2 during the first two hours of cultivation.

Bacterial contamination of implants and subsequent infections often occur immediately after device implantation. Recent protocols have recommended short-term antibiotic prophylaxis^{30,31}. The silane-functionalized titanium

surfaces enable the immediate release of rHuβD2 in a short time in vitro, which may prevent early postoperative infections around the implants in clinical use.

A positive effect of tissue regeneration occurred when surfaces of biomaterials were treated with collagen. Morra et al.³² reported that biochemical alteration of titanium surfaces by collagen can increase the healing rate. From this point of view, an experiment was designed to cover the titanium specimen with bovine type-I collagen prior to rHuβD2 coating.

The direct comparison of collagen-treated surfaces and pure silane monolayers revealed that less rHuβD2 was eluted into the medium from collagen pins (Table II). This effect might be associated with a strong and irreversible binding of rHuβD2 onto the collagen matrix. Within the first two hours of the antibacterial assay of the collagen titanium pins, only 14 to 17 µg of rHuβD2 was detected with ELISA, which led to a complete killing of the bacteria. However, after the second cultivation step (four hours), a significant difference was observed between rHuβD2 release from the glutaraldehyde cross-linked collagen surface (SAM4::Col-Glu) and the other collagen surface (SAM4::Col-NHS) (Table II and Fig. 2) ($p < 0.001$). SAM4::Col-Glu delivered about 6.6 µg of rHuβD2 and caused 92% of bacterial killing. In contrast, SAM4::Col-NHS released only 0.46 µg of rHuβD2 with an antibacterial activity of 53% (Table II and Fig. 2).

It was not possible to determine the bioactive rHuβD2 remaining in the collagen matrix, but we speculate that as a consequence of gradual collagen catabolism in the surrounding tissue in vivo, bioactive rHuβD2 might be slowly released. On the basis of this hypothesis, a peptide delivery system that will yield defined antimicrobial dosages in vivo could be developed in the future by using different collagen binding systems.

Various approaches have been described for bioactive coating onto titanium surfaces^{3,10,11,33,34}. However, most antibiotic substances are difficult to apply because of their toxicity, their putative promotion of bacterial resistances, or their lack of availability. Here, we demonstrated, for the first time, that the use of a recombinant human β-defensin-2 as an antimicrobial substance is suitable for bioactive coating of titanium surfaces.

Future studies will be performed to investigate these types of antibacterial coatings on orthopaedic implants and their effects on osseointegration in vivo. ■

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