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Published in:
Macromolecular Bioscience

DOI:
[10.1002/mabi.201600336](https://doi.org/10.1002/mabi.201600336)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Sjollema, J., Keul, H., van der Mei, H., Dijkstra, R., Rustema-Abbing, M., de Vries, J., Loontjens, T., Dirks, T., & Busscher, H. (2017). A Trifunctional, Modular Biomaterial Coating: Nonadhesive to Bacteria, Chlorhexidine-Releasing and Tissue-Integrating. *Macromolecular Bioscience*, 17(4), [1600336]. <https://doi.org/10.1002/mabi.201600336>

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A Trifunctional, Modular Biomaterial Coating: Nonadhesive to Bacteria, Chlorhexidine-Releasing and Tissue-Integrating

Jelmer Sjollema,* Heidrun Keul, Henny van der Mei, René Dijkstra, Minie Rustema-Abbing, Joop de Vries, Ton Loontjens, Ton Dirks, Henk Busscher

Various potential anti-infection strategies can be thought of for biomaterial implants and devices. Permanent, tissue-integrated implants such as artificial joint prostheses require a different anti-infection strategy than, for instance, removable urinary catheters. The different requirements set to biomaterials implants and devices in different clinical applications call for tailor-made strategies. Here, a modular coating-concept for biomaterials is reported, which in its full, trifunctional form comprises nonadhesiveness to bacteria and antimicrobial release, combined with enhanced tissue integration characteristics. Nonadhesiveness to proteins and bacteria is accomplished by a hydrophilic brush coating (Vitrostealth). The antimicrobial release module is constituted by a chlorhexidine releasing poly(ethylene glycol) diacrylamide based-coating that continues to release its antimicrobial content also when underneath the nonadhesive top-coating. The third module, enhancing tissue integration, is realized by the incorporation of the penta-peptide Glycine-Arginine-Glycine-Aspartic acid-Serine (GRGDS) within the nonadhesive top-coating. Modules function in concert or independently of each other. Specifically, tissue integration by the GRGDS-module does not affect the nonadhesiveness of the Vitrostealth-module toward bovine serum albumin and *Staphylococcus aureus*, while the antimicrobial release module does not affect tissue-integration by the GRGDS-module. Uniquely, using this modular system, tailor-made anti-infection strategies can thus readily be made for biomaterials in different clinical applications.

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1. Introduction

Biomaterial implants are successfully applied in modern medicine to restore human function, examples being total hip arthroplasties, vascular grafts or dental implants. In addition, biomaterial devices are also used as temporary support systems, such as urinary or central venous catheters.^[1] Although successful in the majority of patient recipients, biomaterial-associated infections form a most devastating complication in on average 5% of all implant and device recipients and often lead to their replacement since these infections are recalcitrant to antibiotic treatment and impair the host immune system.^[2–4] In many cases, biomaterial-associated infections are a result of bacterial contamination occurring during surgery (the “per-operative route”) or hospitalization (the “early post-operative route”).^[5] Alternatively, biomaterial implants and devices can get bacterially contaminated any time post-surgery from haematogenous spreading of bacteria from infections elsewhere in the body.^[6] Ubiquitously effective strategies to combat biomaterial-associated infections of different implants and devices arising from any of these three routes do not yet exist, despite all research efforts.^[7]

Antibiotic- or antimicrobial-releasing coatings are amongst the few strategies that have found their way to clinical application.^[8,9] Ad libitum release however, often results in exhausted coatings before an infection occurs, particularly when occurring late post-operatively in the haematogenous route. To circumvent this drawback, a small-molecule-hosting film was constructed using layer-by-layer deposition of montmorillonite clay nanoplatelets and polyacrylic acid that releases gentamicin upon swelling induced by local pH decreases in the vicinity of adhering bacteria.^[10] Coatings that resist adhesion of bacteria have also been developed,^[11,12] but their use is limited to biomaterial implants and devices that do not require tissue integration, like contact lenses or voice prostheses. Their nonadhesiveness to tissue cells excludes their use for permanent implants and devices, since tissue integration constitutes

the best, long-term protection of such implants and devices against infection through the haematogenous route. Coatings comprised of quaternized ammonium groups have contact-bactericidal activity, directly killing bacteria after adhesion to the surface and have been applied successfully in animal models.^[13–15] However, since phagocytosis is reduced on quaternized ammonium coated surfaces and bacteria adhere to dead bacteria equally as to a biomaterials surface,^[16,17] their application is limited to situations where bacterial challenge concentrations are low or external removal forces are applied to inhibit bacterial adhesion to dead cells. The different requirements set to biomaterials implants in different clinical applications show the urgent need for tailor-made solutions.^[7] Here we report on a coating

Figure 1. Schematic presentation of the three functional modules developed. a) The VS-module is a nonadhesive coating, comprising PEG-brush coated SiO₂ nanoparticles; b) The CHX-module is a PEGDAA-based-coating containing CHX, yielding sustained release of CHX; c) The GRGDS-module adds covalently attached GRGDS-peptides (red motifs) to the VS-module, stimulating tissue integration.

concept for biomaterials, which in its full, trimodular form comprises nonadhesiveness to bacteria and antimicrobial release, combined with enhanced tissue integration characteristics when desired. Remarkably, none of the modules appeared to suppress the functionality of the others when combined in a multilayer coating. This modular independence makes the trimodular approach a promising method for preparation of high performance biomaterials. The first module, with nonadhesive properties was accomplished by applying a hydrophilic brush coating (Vitrostealth (VS), DSM, Geleen, The Netherlands, patent registered under number WO/2006/016800) on poly(ethylene terephthalate) (PET) sheets (Figure 1a). The second module is constituted by a chlorhexidine (CHX) releasing poly(ethylene glycol) diacrylamide (PEGDAA) based-coating. This PEGDAA-module is applied optionally, directly on the PET sheet prior to applying the VS-module in order to complement the nonadhesive VS-module with CHX releasing properties (Figure 1b). The third module, offering tissue integrating properties, was realized by the incorporation of penta-peptide Glycine-Arginine-Glycine-Aspartic acid-Serine (GRGDS) peptides within the top VS-module (Figure 1c).

2. Results

2.1. VS- and PEGDAA-Modules: Antiadhesiveness and Bacterial Growth Inhibition

Figure 2a shows a 97% reduction in bovine serum albumin (BSA) adsorption along with a 92% reduction in staphylococcal adhesion (Figure 2b) on PET coated with the VS-module as related to uncoated PET. In order to complement

Figure 2. a) Percentage BSA adsorption onto the VS-module as related to its adsorption on an uncoated PET surface. Error bars indicate standard deviations over five experiments. b) Percentage numbers of *S. aureus* ATCC 49230 after 2 h of flow in a parallel plate flow chamber on the VS-module as related to the staphylococcal numbers on an uncoated PET surface (4.0×10^6 bacteria cm^{-2}). Error bars indicate standard deviations over three experiments with separately grown bacterial cultures.

these nonadhesive properties with release of antimicrobials, the CHX-module was applied first, after which the VS-module was applied as an overlay (Figure 1b). Cumulative release of CHX from the CHX-module increased during a time period of 10 d, after which release leveled off (Figure 3a). Cumulative release after 16 d corresponded with the full release of the total amount of CHX loaded into the module. Note that release is hardly affected by the overlaying VS-module. Moreover, BSA adsorption remained low on the combined VS- and CHX-module, (compare Figure 2a and Figure 3b).

Staphylococcal growth was reduced by 3 log-units on the combined VS and CHX-module as related to uncoated PET (Figure 3c), while staphylococcal growth on these modules, after release of CHX, was similar to growth on uncoated PET.

2.2. Cell Adhesion and Spreading on a VS-Module Overlaying a CHX-Module in Absence and Presence of Staphylococci

U2OS osteoblast-like cells adhered and spread well on an uncoated PET surface (Figure 4a). When PET samples were coated with a VS-module, overlaying the CHX releasing module, only low numbers of adhering cells were observed, that were rounded in shape and not well spread. If cells were exposed after prior-release of CHX, a similar number of adhering cells was observed as on uncoated PET although less well spread.

When uncoated PET samples were contaminated with staphylococci (2×10^4 CFU cm^{-2}), staphylococci multiplied after adhesion and 24 h growth in the modified medium, while no adhesion and spreading of U2OS osteoblasts were observed. Contaminating staphylococci did not grow on PET surfaces coated with a combined VS- and CHX-module during release of CHX, neither was adhesion and spreading observed for U2OS osteoblasts. Finally, carrying out these experiments on this bimodular coating after prior-release of CHX, bacterial growth occurred preventing the adhesion and spreading of U2OS cells as on uncoated PET (Figure 4b).

2.3. Cell Adhesion and Spreading in Absence and Presence of Staphylococci on VS- and GRGDS-Modules Overlaying a CHX-Module

In absence of contaminating staphylococci, U2OS cells adhered and spread almost equally well on the combined VS- and GRGDS-modules as on an uncoated PET surfaces alone, regardless of prior-release of CHX (Figure 5a). Importantly however, on the GRGDS-module, U2OS cells remained adhering in presence of contaminating staphylococci during CHX-release from the CHX-module (Figure 5b). This beneficial effect required a minimal

Figure 3. a) Cumulative CHX release as a function of time from the CHX-module loaded with 10 wt% CHX, with and without an overlaying VS-module. Error bars indicate standard deviations over three experiments. b) Percentage BSA adsorption relative to an uncoated PET surface on the combined VS- and CHX-module during and after prior CHX release (10 wt% CHX loading). Error bars indicate standard deviations over five experiments. c) Percentage numbers of *S. aureus* ATCC 49230 CFU's counted on the combined VS- and CHX-module as related to numbers of CFU's on an uncoated PET surface after 48 h incubation, during and after prior CHX release (5 wt% CHX loading). Experiments were done in triplicate in a Petrifilm assay, yielding negligible standard deviations. Hundred percent bacterial growth represents an estimated number of CFUs of 10³ per sample.

GRGDS concentration of 20 wt% within the VS-module (data for lower percentages not shown).

2.4. BSA Adsorption and Staphylococcal Adhesion on the VS- and GRGDS-Modules Overlaying the CHX-Module

The GRGDS-module did not significantly increase BSA adsorption (Figure 6a) nor staphylococcal adhesion as compared to the VS-module alone (Figure 6b). When the combined VS- and GRGDS-module was applied on top of the CHX releasing CHX-module, staphylococcal growth was reduced to less than 1% as related to growth on the uncoated PET surface (Figure 6c). When experiments were

carried out on this full trifunctional coating after prior CHX release, staphylococcal growth was similar as on uncoated PET surfaces.

3. Discussion

This paper describes a modular approach toward antimicrobial surface coatings, providing tailor made solutions for different potential routes of infection of biomaterial implants and devices. This modular concept offers unique solutions for clinical applications with and without cell integration requirements, and provides combinable coatings with nonadhesive, antimicrobial-release and tissue integrating properties.

Nonadhesive properties have often been conveyed to a surface by hydrophilic polymer brush coatings, resisting not only protein adsorption,^[18,19] but also adhesion of bacteria and tissue cells.^[20] Polymer brush coatings owe their nonadhesive properties to steric hindrance from the stretched, hydrophilic molecules, densely packed in a monomolecular film,^[21] which often implies lack of mechanical robustness upon handling such as during implant surgery. More robust, nonadhesive coatings have been prepared by incorporating the hydrophilic brush structure into covalently cross-linked nanocomposites (Vitrostealth). In this robust coating, silica nanoparticles with polymerizable acrylic groups and hydrophilic PEG-chains are cross-linked yielding a thick, 500–600 nm

thick coating. These coatings withstand both protein adsorption and adhesion of bacteria, while being abrasion and scratch resistant.^[21] In our concept, the nonadhesive module makes use of this silica nanoparticle-PEG-coating (VS-module in Figure 1b). X-ray photo-electron spectroscopy (XPS) analysis confirmed that the PET-surfaces were properly coated with the VS-module, since the original double oxygen peak representing the single and double oxygen bonds (carbonyl) in PET, had disappeared, while peaks from single oxygen bonds of SiO₂ and mPEG (being the main constituents of VS) were retained (Figure S1a, Supporting Information). In addition at% of carbon were lowered with respect to oxygen after coating with the VS-module,

Figure 4. a) Phase-contrast microscopic images (image enhanced) of U2OS cell adhesion and spreading after 24 h incubation on the VS-module overlaying the CHX-module, incubated during and after prior CHX release (10 wt% CHX loading). b) Same as panel (a), now in the presence of contaminating *S. aureus* ATCC 49230 (inoculum 2×10^4 CFU cm $^{-2}$). Bars denote 50 μ m.

reflecting the lower C:O ratio of the PEG-SiO $_2$ constituents in the VS-module as compared to the C:O ratio in PET which is near to 2.5 in accordance with the molecular structure of PET (Figure S1b, Supporting Information). Moreover, the at% Si increased from its contamination level by the addition of the VS-module, yielding an Si:O ratio of 1:3.5, which is near to the expected 1:2.5 atom ratio.^[11] This indicates that mPEG is more exposed at the surface than the SiO $_2$ nanoparticles. Successful application of the VS-module was also demonstrated by the reduction of the water contact angle from 55° to 20°, due to the hydrophilic mPEG within VS (Figure S2, Supporting Information).

The nonadhesive properties of the VS in our modular concept have been extensively described with respect to adsorption of lysozyme and adhesion of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*,^[11] corroborated by the present results where BSA adsorption and adhesion of *Staphylococcus aureus* (*S. aureus*) ATCC 49230 were reduced with 97% and 92%, respectively. Yet it is known that also low numbers of bacteria adhering

on a polymer brush coating have the ability to grow over prolonged periods of time into weakly adhering microcolonies. The weak adhesion does not stimulate the transition to a biofilm mode of growth, leaving bacteria potentially in a more antibiotic susceptible state.^[22] Our modular coating concept allows to kill these bacteria as soon as they approach the surface. To this end, the VS-module was complemented with the possibility of sustained CHX release. As a first attempt to establish release, CHX was mixed into the VS-formulation. However, in this case, a high burst release was obtained and the module was exhausted within 1 h after incubation in buffer (data not shown). In order to obtain a more sustained release, a new module (CHX-module) was designed containing acidic groups to form ionic complexes with CHX to yield sustained release. In order to preserve the nonadhesiveness, the VS-module was applied on top of the CHX-module. CHX was chosen as an antimicrobial because of its strong antimicrobial efficacy and because the absence of any reports on development of bacterial resistance to date.^[23] Moreover, CHX is already applied in functionalized catheters approved by the American Food and Drug Administration.^[3] From the present results, it is again shown that CHX is a very effective antimicrobial, since the Petrifilm assay showed full inhibition (3 log-units) of bacterial growth on combined CHX- and VS-modules (Figure 3c). A 3 log-units reduction is 10 times more effective than the cut-off value for a material that can be claimed to be antimicrobial according to industrial standards, like the Japanese Industrial Standard JIS Z-2801:2000. However, the present study also shows that the release from the CHX-module can yield haemolysis (Figure S3, Supporting Information). This is not unexpected because the cationic character of CHX interferes with the cellular lipid membrane,^[24] as was also observed when using CHX releasing wound dressings.^[25] During CHX release from the CHX-module, cellular adhesion and spreading were severely hampered (Figure 4a) which may lead to apoptosis.^[26,27] In order to maintain good cellular adhesion and spreading in presence of the CHX releasing module, cell adhesion was improved (Figure 5a) by incorporating GRGDS-peptides within the VS-module. XPS and water contact angle measurements confirmed incorporation of GRGDS-peptides in the VS-module (Figures S1b and S2, Supporting Information). After application of the GRGDS-module (20 wt%), both the at% O and N increased, whereas water contact angles decreased compared with those on the VS-module, in line with literature.^[28]

Uniquely, none of the modules suppress the functionality of the others: antimicrobial properties of the CHX-module did not inhibit cell spreading stimulated by the GRGDS-module (Figure 5a) and vice versa, tissue integrating properties of the GRGDS-module did not affect antimicrobial efficacy of the CHX-module (see Figures 5b

Figure 5. a) Phase-contrast microscopic images (image enhanced) of U2OS cell adhesion and spreading after 24 h incubation on the VS- and GRGDS-module and CHX-module incubated during and after prior CHX release (5 wt% CHX loading). b) Same as panel (a), now in the presence of contaminating *S. aureus* ATCC 49230 (inoculum 2×10^4 CFU cm^{-2}). Bars denote 50 μm .

and 6b,c). In addition, protein adsorption inhibition on the VS-module was only slightly affected by the antimicrobial properties of the CHX-module (Figure 4b), probably due to small amounts of accumulated positively charged CHX, whereas no significant effect was observed by the GRGDS-module (Figure 6a). The main enabling mechanism for modular independence is that immobilizing GRGDS-peptides to the surface supersedes the negative effects of CHX on U2OS cell adhesion and spreading (Figure 5a), probably because integrin-mediated cell adhesion triggers a myriad of cell signaling inducing survival and proliferation.^[29,30] A second enabling mechanism for independence of the various modules is that the GRGDS-peptides do not function as adhesion sites for bacteria, therewith retaining the nonadhesiveness of the VS-module (see Figure 6b). This finding is in accordance with earlier observations that low adhesion of bacteria to chitosan-coated titanium was not increased by the

addition of RGD-moieties to the substrates^[10,31] and suggests that association of *S. aureus* bacteria with proteins is not mediated solely by their RGD-domains. Petrifilm assays also clearly show that growth inhibition by CHX release was independent of the concentration of GRGDS-peptides in the VS-module (Figure 6c).

4. Conclusion

We have developed tailor-made modular, nonadhesive, antimicrobial-releasing, and tissue integrating coatings. Uniquely, none of the modules suppresses the functionality of the others when combined in a multilayer coating. This modular independence makes the trimodular approach a promising method for preparation of high performance biomaterials and an attractive means toward cost-economic downward clinical application, offering control of bacterial growth in combination with tissue integration for various implants and devices with widely different requirements.

5. Experimental Section

Preparation of Coating Modules: All coatings were applied to PET surfaces, either in the form of sheets (thickness 0.2 mm), commercially obtained from Toyobo (Osaka, Japan) or PET blood collection tubes (Greiner Bio-One, Alphen aan den Rijn, The Netherlands). PET surfaces were cleaned in 1:1 vol% n-propanol/methanol followed by drying in air prior to all experiments. In addition, all samples were sterilized in 70% ethanol before and after application of the coatings.

Square samples (1.5×1.5 cm) were used for all experiments, with the exception of the cell adhesion and spreading experiments for which disk-shaped samples with a diameter of 0.8 cm were employed. BSA adsorption was studied in PET blood collection tubes (length 4.5 cm, inner diameter 9.9 mm).

The VS-module is a hydrophilic brush coating and comprises cross-linkable SiO_2 nanoparticles (60 g mol^{-1}), provided with monomethoxy poly(ethylene glycol) (mPEG, 2000 g mol^{-1}), an additional crosslinker (methylene bisacrylamide), and a photoinitiator (Irgacure 184, Ciba Specialty Chemicals Inc., Basel, Switzerland).^[31] This VS-formulation was applied on PET sheets using a 50 μm Meyer-bar at a constant application speed to yield a thickness of 500–600 nm as determined by spectral reflectance measurement (Filmetrics S20, San Diego, CA, USA). Directly after application, the coated sheets were passed five times through an UV-rig with a D-bulb (Fusion UV systems, Gaithersburg, US) at a speed of 18 m min^{-1} and a UV dose of 1 J cm^{-2} .

The CHX-releasing PEGDAA based-coating was applied directly on the PET prior to the VS-module (Figure 7a). The coating constituents were mixed with various amounts of chlorhexidine and applied using a 40 μm Meyer-bar at a constant application speed (18 m min^{-1}), after which the coating was UV cured (5 J cm^{-2}). The thickness of this PEGDAA-based CHX-module

Figure 6. a) Percentage of BSA adsorption on the combined VS- and GRGDS-modules containing various amounts of GRGDS-peptides, relative to an uncoated PET surface. Error bars indicate standard deviations over five experiments. b) Percentage of numbers of *S. aureus* ATCC 49230 after 2 h of flow in a parallel plate flow chamber on the combined VS- and GRGDS-module containing various amounts of GRGDS-peptides as related to bacterial numbers on an uncoated PET surface (100% = 4.0×10^6 bacteria cm^{-2}). Error bars indicate standard deviations over three experiments with separately grown bacterial cultures. c) Percentage of numbers of *S. aureus* ATCC 49230 CFU's counted on the combined VS- and GRGDS-modules containing various amounts of GRGDS-peptides and the CHX-module as related to numbers of CFU's on an uncoated PET surface after 48 h incubation in a Petrifilm assay during and after prior CHX release (5 wt% CHX loading). Error bars indicate standard deviations over three experiments with separately grown cultures. 100% bacterial growth represents 10^3 CFUs per sample with zero standard deviations.

amounted to 3–4 μm , as measured using spectral reflectance. Subsequently, the VS-module was applied as described above (now with a 12 μm Meyer-bar) on top of the CHX-module.

The GRGDS-module was realized by anchoring the GRGDS-peptide within the VS-module for which a specific UV-curable GRGDS-linker was developed. By coformulating these linkers with the VS-components, followed by UV curing, the GRGDS-moiety was covalently bound within the first VS-module.

For preparing the GRGDS-linker (Figure 7b), α -amino- ω -carboxy PEG hydrochloride with an average molecular weight of 3000 Da (509 mg, 0.157 mmol) and methylene bisacrylamide

(123 mg, 0.80 mmol) were dissolved in methanol (2.5 mL) under a nitrogen atmosphere. Triethylamine (NEt_3) (42 mg, 0.42 mmol) was added and the reaction mixture was stirred at 50 $^\circ\text{C}$ for 24 h. Then, the mixture was diluted with methanol (0.5 mL) and precipitated in diethylether (350 mL) twice. The resulting precipitate was dried in vacuo to yield compound 1 (MBA-PEG, see also Figure 2) (400 mg, 63%).

Compound 1 (594 mg, 0.165 mmol), 1-hydroxy-7-azabenzotriazole (HOAT) (28 mg, 0.401 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (37 mg, 0.193 mmol) were dissolved in 12 mL of dimethylformamide (DMF) under a nitrogen atmosphere. Diisopropylethylamine (DIPEA) (116 μL , 0.658 mmol) was added and the mixture was cooled on ice. After 15 min, GRGDS (171 mg, 0.175 mmol), protected by tertiary butyl (tBu) and pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) in DMF (6 mL) was added. After 48 to 60 h, the solution was diluted with 150 mL of dichloromethane (DCM). It was washed twice with 0.1 M HCl solution (150 mL) and the organic phase was dried on sodium sulfate as a desiccant to yield compound 2 (MBA-PEG-GRGDS (protected), see Figure 2).

For deprotection of the GRGDS, compound 2 was redissolved in 8.5 mL of dry DCM and 8 mL trifluoroacetic acid (TFA) under nitrogen atmosphere. 87 μL of triisopropylsilane (TIPS) was added and the mixture was stirred for 3 h. Then, the solvent was reduced in vacuo and the compound was precipitated from 400 mL ice-cooled diethylether. The resulting precipitate was dried in vacuo to yield compound 3 (443 mg, 65%) (MBA-PEG-GRGDS (deprotected), see Figure 2) which was mixed within the VS-formulation to form a GRGDS functionalized VS-coating as described above.

BSA Adsorption on the VS-Module: VS-coatings were applied to PET blood collection tubes (uncoated or precoated with the CHX-module) by completely filling them with VS-formulation (see above) and emptying them with a plastic pipette. The coated collection tubes were left to dry bottom-up under ambient conditions for 30 min. Next, the coatings were UV cured (6 J cm^{-2}) and the tubes were washed three times with demineralized water before BSA adsorption. For adsorption, 1.0 mL of a ^{125}I -BSA solution (specific activity 37 MBq/250 mL) was inserted in coated and uncoated tubes. The filled tubes were then covered with aluminum foil for overnight BSA adsorption. Finally, the ^{125}I -BSA solution was removed, tubes were washed three times with a phosphate buffered saline (PBS, 10×10^{-3} M potassium phosphate, 150×10^{-3} M NaCl)

Figure 7. a) Chemical structure of the main constituents of the CHX-releasing PEGDAA based-coating, consisting of poly(ethylene glycol) diacrylamide (PEGDAA) with chlorhexidine (CHX). b) Synthesis of α -acrylamide ω -GRGDS functionalized PEG linkers to be integrated in a VS-formulation to form the GRGDS-module.

and the tubes were transferred into glass vials, submerged in 20 mL liquid scintillation counter cocktail (Pico Fluor 15, Perkin Elmer, Waltham, USA) and radioactivity measured with a liquid scintillation counter. For coatings involving the CHX-module, BSA adsorption was studied during and after prior CHX release by keeping samples at 37 °C in an excess amount of sterile, demineralized water for 48 h, while refreshing four times daily. BSA adsorption was measured in fivefold on separate coated and uncoated tubes. In order to account for the decay of ¹²⁵I, the radioactivity measured on coated PET-tubes was always expressed as a percentage of the radioactivity measured on uncoated PET-tubes.

Chlorhexidine Release from the CHX-Module: PET sheets coated with the CHX-module, with or without an overlayer of the VS-module, were placed in a vial containing 4 mL PBS while shaking at 100 rpm and 37 °C. PBS was daily exchanged for fresh PBS and CHX release in spent PBS was determined using UV-vis spectroscopy. Absorption values at 255 nm were converted to mass concentrations of CHX via a calibration curve and plotted cumulatively against time. Experiments were arrested when absorption values were less than 0.005 (i.e., CHX concentrations below 0.035 mg L⁻¹), considered as the noise threshold.

Bacterial Adhesion and Growth Inhibition: Bacterial adhesion and growth inhibition were investigated using a *S. aureus* strain, as frequently occurring in biomaterial-associated-infections. The strain used (*S. aureus* ATCC 49230) was originally isolated from a patient with chronic osteomyelitis and routinely cultured aerobically at 37 °C on a blood agar plate. Several colonies were used to inoculate 10 mL tryptone soya broth (TSB, OXOID, Basingstoke, England) and this preculture was grown statically for 24 h at 37 °C. The preculture was diluted 1:20 in 200 mL TSB and grown statically for 16 h at 37 °C. Cultures were harvested by centrifugation (Beckman J2-MC centrifuge, Beckman Coulter, Inc. Brea, CA, USA) for 5 min at 5000 g in a JA14 rotor and the bacteria were resuspended in 10 mL PBS. Centrifugation was done twice in order to remove all traces of growth medium. To break staphylococcal aggregates, sonication at 30 W (Vibra Cell Model 375, Sonics and Materials Inc., Danbury, CT, USA) was applied (3 × 10 s), while cooling in an ice/water bath. Staphylococci were resuspended in PBS to a density of 3 × 10⁸ mL⁻¹ as determined in a Bürker-Türk counting chamber, allowing further dilution to match the inoculum requirements of the different assays carried out.

Bacterial adhesion was studied in a parallel plate flow chamber (dimensions: 76 × 38 × 0.6 mm). Coated samples

were accommodated into centrally prepared recesses in the poly(methyl)-methacrylate bottom plate of the flow chamber. The top plate of the chamber was made of glass. Prior to each experiment, in- and outlet tubes and the flow chamber itself were filled with sterile PBS, while ensuring that all air bubbles had been removed from the system. Subsequently, the system was switched to a staphylococcal suspension, which was allowed to flow through the system at a flow rate of 0.025 mL s^{-1} corresponding with a shear rate of 10 s^{-1} . Adhering bacteria were observed with a CCD- MXRi camera (High Technology, Eindhoven, The Netherlands) mounted on a phase-contrast microscope (Olympus BH-2, Olympus, Tokyo, Japan) equipped with a $\times 40$ ultralong-working distance objective (Olympus ULWD-CD Plan 40 PL). After 2 h, the flow was switched to PBS again and the number of staphylococci adhering on the bottom plate was counted by an image analyzer (MATLAB, Mathworks, Natick, MA, USA).

In order to measure growth inhibition as a result of CHX release from the CHX-module, a Petrifilm assay was executed (Petrifilm Aerobic Count Plate, 3 m Microbiology, St. Paul, MN, USA). Briefly, 50 μL droplets of a staphylococcal suspension ($2 \times 10^4 \text{ CFU mL}^{-1}$) were positioned on the centre of each sample to yield a total bacterial challenge of 10^3 CFU . Upon closing of the Petrifilm system, bacteria were spread over the area of the sample, resulting in a challenge of $\approx 400 \text{ CFU cm}^{-2}$ and after 48 h incubation at 37°C , the number of CFUs surviving CHX release were counted.

Cell Adhesion and Spreading in Absence and Presence of Bacterial Contamination: Adhesion and spreading of U2OS osteosarcoma cells (ATCC TIB-96; obtained from LGC standards, Wesel, Germany) was measured on disk-shaped samples, equipped with different combinations of modules. Samples were placed in 6 well culture plates (Greiner Cellstar dish, Sigma Aldrich, St. Louis, MO, USA) in absence and presence of low numbers of contaminating staphylococci, mimicking per-operative bacterial contamination as occurring during implantation of a biomaterial implant or device.^[32] Prior to the experiments, half of the samples were kept at 37°C in an excess amount of sterile, demineralized water for 48 h, while refreshing four times daily to stimulate complete release of CHX.

U2OS osteosarcoma cells were cultured in completed Dulbecco's Modified Eagles Medium (DMEM/LG-complete)-low glucose, supplemented with 10% fetal calf serum (FBS) and $0.2 \times 10^{-3} \text{ M}$ of ascorbic acid-2-phosphate. U2OS cells were maintained at 37°C in a humidified 5% CO_2 atmosphere and passaged at 80%–90% confluency using trypsin-EDTA. Cells with a concentration of $5 \times 10^3 \text{ mL}^{-1}$ suspended in DMEM/LG-complete were allowed to adhere to the samples for 24 h at 37°C in a humidified 5% CO_2 atmosphere, after which phase-contrast images were taken for qualitative analysis. All experiments involving cell adhesion and spreading were done in duplicate with separately grown cells and different samples.

In order to evaluate cell adhesion and spreading in presence of contaminating *S. aureus* ATCC 49230, staphylococci were cultured, as described above. In these experiments, a 50 μL droplet of a staphylococcal suspension ($2 \times 10^5 \text{ CFU mL}^{-1}$) was placed on top of each sample to yield an estimated bacterial coverage of $2 \times 10^4 \text{ CFU cm}^{-2}$ for 1 h at room temperature. Samples were then gently washed three times in sterile buffer and placed in a 24-wells

plate and U2OS cells ($3 \times 10^4 \text{ cells mL}^{-1}$) suspended in 1 ml of a modified cell culture medium (98% DMEM/LG and 2% TSB) were added to the wells.^[33] The plates were then kept at 37°C in a humidified 5% CO_2 atmosphere, after which images were taken and qualitatively analyzed.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements: This research forms part of the Project P4.01 NANTICO of the research program of the BioMedical Materials institute, cofunded by the Dutch Ministry of Economic Affairs. H.B. is also director of a consulting company SASA BV. Opinions and assertions contained herein are those of the authors and are not construed as necessarily representing views of the funding organization or their respective employer(s).

Received: August 5, 2016; Revised: September 11, 2016; Published online: October 19, 2016; DOI: 10.1002/mabi.201600336

Keywords: antimicrobial; biomaterial; coating; infection; polymer brush; release; tissue integration

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