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Biadhesive Peptides for Assembling Stainless Steel and Compound Loaded Micro-Containers

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Biadhesive peptides (peptesives) are an attractive tool for assembling two chemically different materials—for example, stainless steel and polycaprolactone (PCL). Stainless steel is used in medical stents and PCL is used as a biodegradable polymer for fabrication of tissue growth scaffolds and drug delivering micro-containers. Biadhesive peptides are composed of two domains (e.g., dermaseptin S1 and LCI) with different material-binding properties that are separated through a stiff peptide-spacer. The peptesive dermaseptin S1-domain Z-LCI immobilizes antibiotic-loaded PCL micro-containers on stainless steel surfaces. Immobilization is visualized by microscopy and field emission scanning electron microscopy analysis and released antibiotic from the micro-containers is confirmed through growth inhibition of *Escherichia coli* cells.

The application of implants requires an improved biocompatibility and the possibility of interactions with complex biological environments.[1] Common stents consist of bare metal alloys such as stainless steel (316L), cobalt-chromium or nickel-titanium and are often functionalized by chemical and physical surface functionalization techniques to increase biocompatibility (e.g., as porous drug eluting layers, with magnetic silicon or carbon nanoparticles or with self-assembled monolayers).[2,3] Biological surface functionalization is an economic and versatile tool for the construction of functional implant coatings. Biological coating technologies are essential for biocompatible interfaces and are based on adsorption, entanglements, or covalent linkages.[4]

Adhesive peptides found in nature (e.g., fibrin, fibronectin, and mussel foot proteins) are applied since the early 1990s as tissue adhesives (glues or sealants) in surgery to promote wound closure and healing.[5,6] Adhesive peptide coatings can stimulate re-endothelialization of implants, can minimize implant-associated bacterial infections and reduce biofilm formation.[7] One example is the WKYMVm peptide, which acts as selective formyl peptide receptor agonist; WKYMVm was dispersed in hyaluronic acid polymers and has been applied by dip-coating to bare-metal stents as a primary coat. The immunosuppressive and antiproliferative sirolimus—plus poly lactic-glycolic acid, 50:50; in tetrahydrofuran—was consecutively sprayed onto the primary coat. In this bare-metal stent coating, WKYMVm promoted the homing of endothelial colony-forming cells whereas restenosis was inhibited by sirolimus.[8] Re-endothelialization of polystyrene (PS) has been improved by employing a pro-healing stent on which a bifunctional peptide was used as coating (PS-binding motif and a human endothelial cell-binding motif). The cell-binding motif binds specifically to endothelial cells but not platelets and reduces inflammatory responses.[9]

Anchor peptides are naturally occurring adhesion promoting peptides that offer efficient and "easy"-handling possibilities for surface functionalization.[10,11] Tachystatin A2[12] (TA2) and LCI[13] show strong binding affinity to biologically inert polymers (e.g., PS and polypropylene (PP)) in aqueous solutions and at ambient temperature.[14] After binding of the anchor peptide LCI to PP surface, a dense monolayer of 4.1 nm was formed.[15] Binding strength and specificity of anchor peptides is tunable to application conditions by employing the Peptide-Polymer evolution (PeP*evo*) protocol[14] in KnowVolution campaigns.[16]

Herein, we report the design and application of biocompatible biadhesive peptides (peptesives) to immobilize polycaprolactone (PCL) micro-containers loaded with active compounds on a stainless steel surface. Five potential adhesion promoting peptides were fused to the fluorescent reporter protein eGFP (enhanced green fluorescent protein) and probed for binding onto a stainless steel surface and PCL. Binders were identified based on eGFP fluorescence intensity by fluorescence microscopy. Finally, a selective bifunctional fusion peptide binding to

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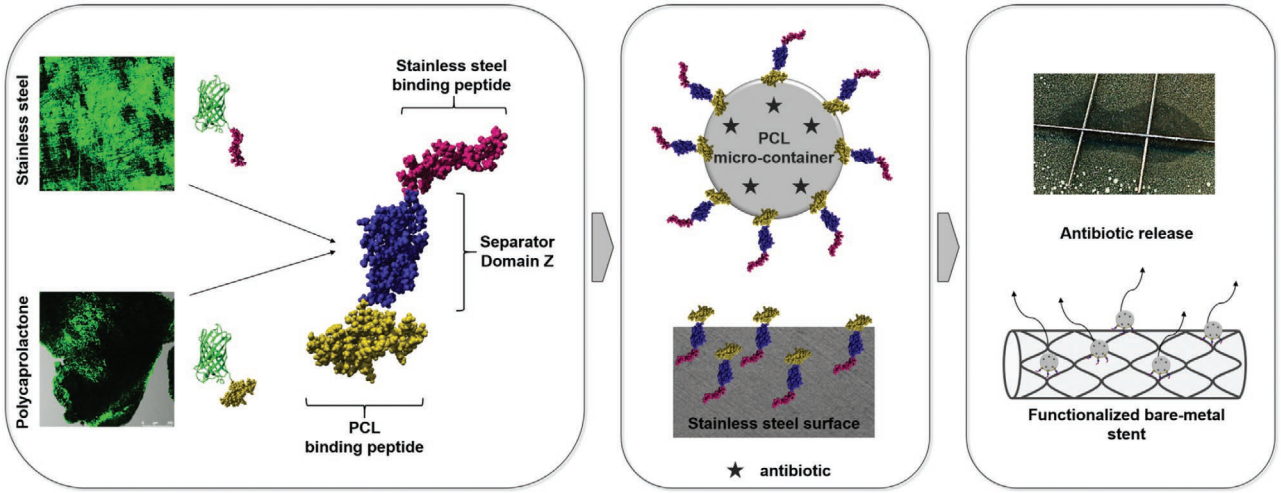

Figure 1. Schematic immobilization of PCL particles on stainless steel. One stainless steel binding anchor peptide and one polycaprolactone (PCL) binding anchor peptide were genetically fused to a biadhesive peptide (peptesive) with a separating domain Z. The peptesive assembled PCL micro-container was loaded with the antibiotic kanamycin and immobilized on stainless steel. The release of kanamycin was confirmed by bacterial growth inhibition (*Escherichia coli* DSM 498).

stainless steel and PCL was generated with functional separation of both units by the spacer peptide domain Z. Immobilizing of PCL micro-containers on stainless steel was mediated by the latter peptesives and visualized by Field Emission Scanning Electron Microscopy (FE-SEM) analysis and fluorescent microscopy. Antimicrobial properties of kanamycin releasing micro-containers were proven by growth inhibition of *Escherichia coli* (*E. coli*) DSM 498 cells surrounding coated steel wires (**Figure 1**).

The binding of anchor peptides to stainless steel or PCL surfaces was determined by the detection of the reporter protein eGFP. eGFP and the selected anchor peptides were separated by a stiff spacer helix (AEAAAKEAAAKEAAAKA)[17] with an incorporated TEV cleavage site (ENLYFQG) (Figure S1 and Table S1, Supporting Information).[18] LCI (47 aa), TA2 (44 aa), and THA (Thanatin, 21 aa)[19] already proved to have a high potential as polymer- (PS and PP) and leaf surface-binders when C-terminally fused to eGFP (referred to as C-anchors).[14,15,20,21] Material binding properties of DS1 (dermaseptin S1, 29 aa)[22] have not yet been reported; therefore, eGFP fusions with an N-terminal anchor (DS1-eGFP) as well as a C-terminal anchor (eGFP-DS1) orientation were investigated.

Binding of DS1-eGFP, eGFP-DS1, eGFP-LCI, eGFP-TA2, and eGFP-THA to stainless steel as well as PCL were analyzed by confocal microscopy (**Figure 2**). DS1-eGFP showed the strongest binding to stainless steel whereas the C-terminal anchor peptide DS1 (eGFP-DS1) was removed almost completely after washing. eGFP-LCI fluorescence was distinctly decreased in comparison to DS1-eGFP. Binding analysis of PCL revealed that eGFP-LCI and eGFP-TA2 bound strongest after washing with the anionic surfactant sodium dodecylbenzenesulfonate

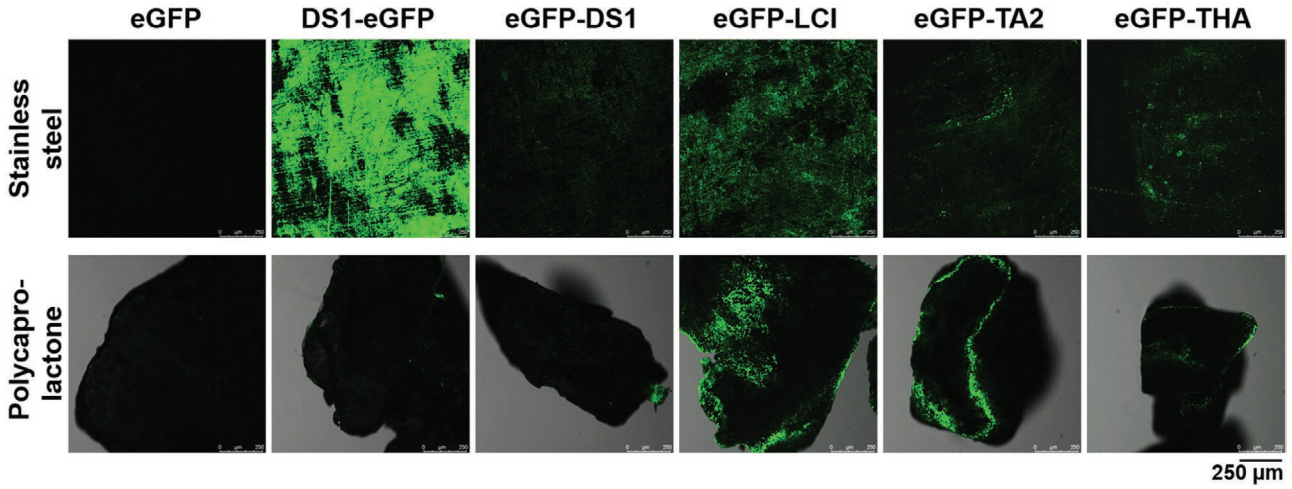

Figure 2. Binding of eGFP-anchor peptides to stainless steel and PCL. Binding of negative control eGFP and eGFP-anchor peptides (DS1, LCI, TA2, and THA) was investigated by incubation (10 min, ambient temperature) on stainless steel or PCL particles. In three successive washing steps (1 mL ddH_2O each) followed by a washing step with LAS (0.5 mM, 0.5 mL, 5 min prevention of non-specific eGFP binding) superfluous peptides were removed. Binding of eGFP and anchor peptide fusion proteins was analyzed by confocal microscopy.

(LAS) (0.5 mM in 50 mM Tris/HCl pH 8.0). LAS was reported to reduce unspecific binding of eGFP and was therefore used to reduce background fluorescence.[14] DS1-eGFP and eGFP-DS1 were not detectable on the PCL surface after washing. Consequently, DS1 was selected as a stainless steel-binder and LCI, TA2 as well as THA as suitable C-terminal PCL binders for generating bifunctional peptesives.

Peptesives consisted of two functional anchor peptides, separated by the stiff staphylococcal protein A domain Z (DZ, 58 aa). DZ is an α-helical protein forming three antiparallel helices and therefore the N- and C-termini of the protein are located on opposite sides of the domain.[23] Generated peptesives were composed of the N-terminal anchor peptide DS1, the separator DZ, and the C-terminal anchor peptide (LCI, TA2, or THA).

The production of selected anchor peptides (<15 kDa) can be challenging, since they belong to the class of antimicrobial peptides and possess a potential toxicity to the production host.[24,25] Many antimicrobial peptides are problematic to produce in soluble form and associate after cell lysis to bacterial membrane fractions.[26] The spacer protein DZ was used to separate both anchor peptide units and to increase solubility of peptesives.[27] The expression of DS1-DZ-LCI, DS1-DZ-TA2, and DS1-DZ-THA in *E. coli* BL21-Gold (DE3) was optimized by varying expression temperature (20, 30, 37 °C) and time (16 and 48 h) (finally used: 30 °C, 16 h, 200 rpm, 70% humidity, 50 mL LB medium). An expression temperature of 30 °C was chosen as suitable balance between soluble peptesive expression and cell viability. DS1-DZ-TA2 could not be produced in detectable amounts at any temperature (Figure S2, Supporting Information). As a negative control, *E. coli* cells transformed with an empty vector (pET28-EV) were used for production. The pET28-EV control represents the protein background of *E. coli* cells. The peptesives DS1-DZ-LCI and DS1-DZ-THA were enriched from culture broth and dialyzed against water using Amicon Centrifugal Filter Units (EMD Millipore Amicon Ultra-0.5 Centrifugal Filter Units, MWCO 10 kDa, Thermo Fisher Scientific—Darmstadt, Germany). The enriched peptesives were used for binding studies without further purification steps.

Antibiotic-eluting stents are used in treatments of the urinary tract to reduce bacterial infections after surgery. The synthesized PCL micro-containers were loaded with kanamycin sulfate as a model antibiotic, which is active against gram positive and negative cocci and bacteria.[28]

PCL (FDA-approved) is an inexpensive, biodegradable, and biocompatible polymer[29] that is often used for implants or stents[8,30,31] and is especially suited for long term drug delivery in cancer therapies.[32] Kanamycin-loaded PCL micro-containers (20% w/w kanamycin sulfate) were synthesized via extrusion and subsequently dispersion in absolute ethanol. Kanamycin is not soluble in absolute ethanol which prevents leaching from PCL loaded micro-containers.[28] PCL has a low melting temperature of 60 °C[31] and extrusion is not limited by the thermal resistance of kanamycin (decomposition temperature >250 °C).

The size distribution of PCL micro-containers was determined by confocal microscopy and dynamic light scattering (DLS) (**Figure 3**A) and showed a size distribution of <5–25 µm using a 63-fold magnification of the device. DLS measurements indicated a highly polydisperse product (Mastersizer 2000, Malvern Panalytical GmbH, Kassel, Germany). A further purification through size exclusion was not performed since the current manuscript focused on the development of bifunctional

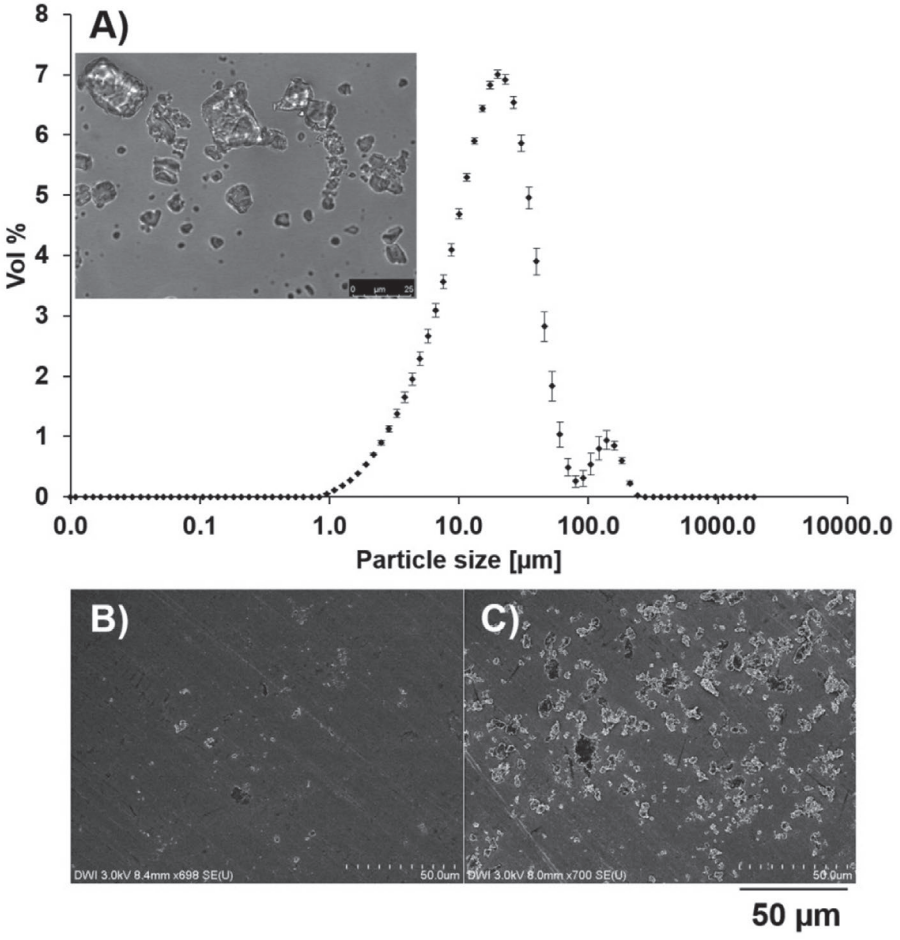

Figure 3. Peptesives mediated assembling of PCL particles and stainless steel. A) Morphology and size distribution of PCL micro-containers in ethanol determined by confocal analysis (Leica TCS SP8 microscope; 63-fold magnification, zoom 2, PMT Trans detector, gain 300, Leica Microsystems GmbH—Wetzlar, Germany) and DLS analysis (Mastersizer 2000; Size range 0.020–2000 µm, Malvern Panalytical GmbH—Kassel, Germany). B,C) FE-SEM analysis of assembly. PCL micro-containers assembled on stainless steel with negative control—pET28-EV (B). PCL micro-containers assembled on stainless steel with DS1-DZ-LCI (C).

adhesion promoters to immobilize kanamycin-loaded PCL micro-containers on bare metal stents.

The ability of peptesives (DS1-DZ-LCI, DS1-DZ-TA2, and DS1-DZ-THA) as well as the negative control (pET28-EV) to function as "assembling" promotor was investigated by confocal microscopy (Figure S3, Supporting Information) and confirmed by FE-SEM analysis (Figure 3B,C).

The highest amount of immobilized PCL micro-containers on stainless steel was achieved using DS1-DZ-LCI as adhesion promoter (confocal analysis; Figure S3A, Supporting Information). DS1-DZ-THA (confocal analysis; Figure S3B, Supporting Information) showed a lower binding strength in comparison to DS1-DZ-LCI. The negative control (confocal analysis; Figure S3C, Supporting Information) did not show any PCL micro-container immobilization on the stainless steel surface.

A detailed analysis of assembly was performed by FE-SEM using the negative control pET28-EV (Figure 3B) and DS1-DZ-LCI (Figure 3C). After stainless steel coating, the surface was sputtered with a 4 nm layer of Au-Pd to avoid electrostatic effects. FE-SEM analysis confirmed that only the peptesive DS1-DZ-LCI was able to immobilize efficiently PCL micro-containers on stainless steel. Micro-containers detected on the surface ranged in size from <5 to 15 µm. Immobilization using the negative control pET28-EV showed as expected that only few PCL micro-containers (<10 µm) were bound on the stainless steel surface. In summary, it can be concluded, that peptesives immobilize efficiently particles of up to 15 µm on the stainless steel surface. Larger particles are removed during the washing process.

The antimicrobial properties of PCL micro-containers on stainless steel wires were determined by growth inhibition of *E. coli* DSM 498 cells (**Figure 4**). The peptesive DS1-DZ-LCI (fivefold concentrated supernatant) was used to immobilize kanamycin-loaded PCL micro-containers on stainless steel wires. As controls, stainless steel wires were incubated with pET28-EV (fivefold concentrated peptesive free supernatant), the peptesive DS1-DZ-LCI only (potential antimicrobial effect of the applied peptesive), kanamycin-loaded PCL micro-containers (PCL control; potential adherent effect of kanamycin to stainless steel), and kanamycin positive control (additionally added kanamycin after washing). Liquid agar containing a cell density of OD_{600} = $1 \times 10^{-5/-6}$ (200 000 or 20 000 colony forming units (cfu)) was poured onto the coated stainless steel wires and cells were cultivated overnight (37 °C, 200 rpm, 70% humidity; Multitron Pro, Infors AG) (Figure S4, Supporting Information).

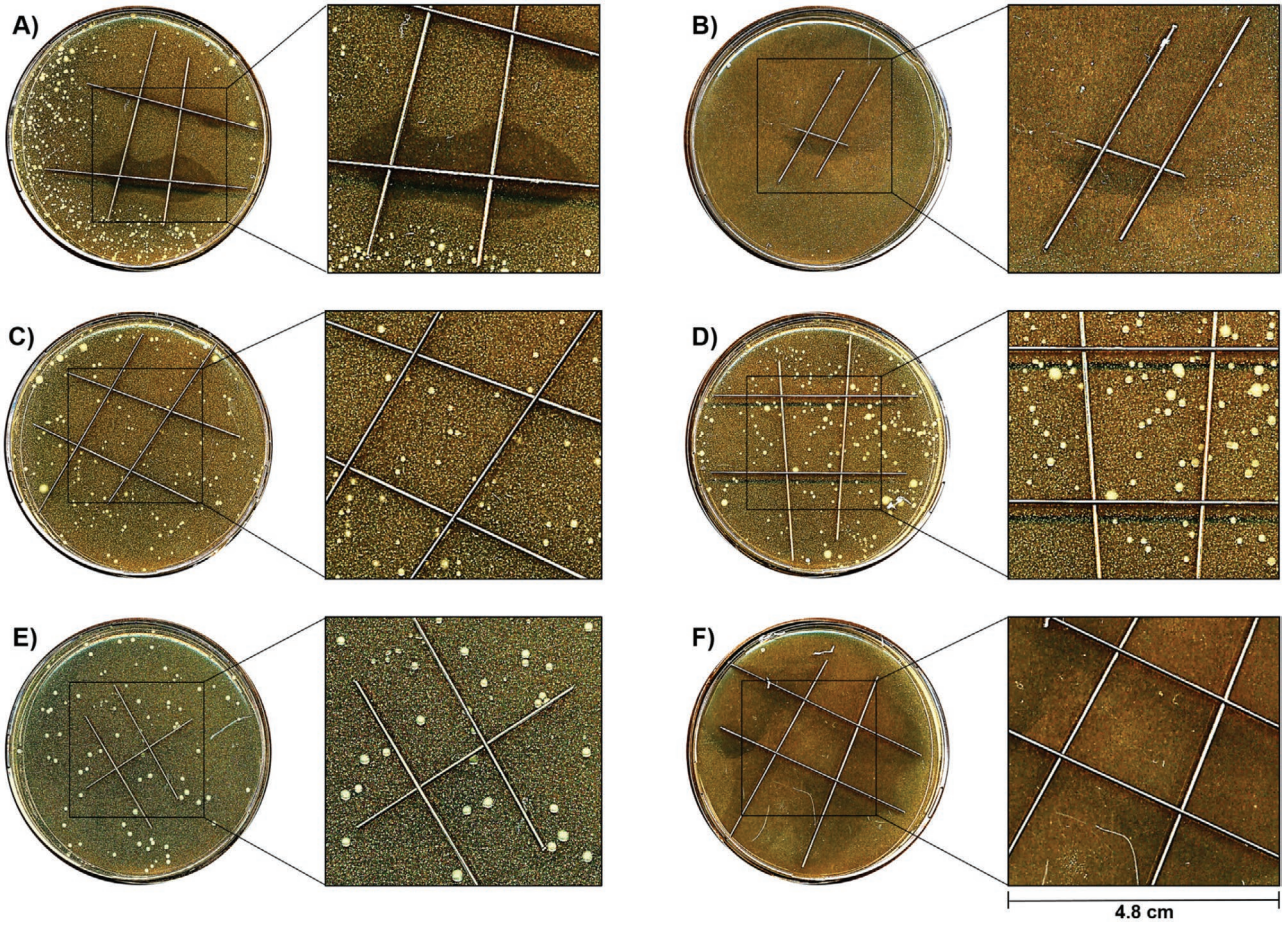

Figure 4. Antibiotic effect of PCL coated stainless steel. Kanamycin-loaded PCL micro-containers were immobilized on stainless steel wires and incubated with *E. coli* DSM 498 cells (200 000 cfu). Halo formation indicates growth inhibition of bacterial cells by released kanamycin. A) peptesive (DS1-DZ-LCI; 200 000 cfu), B) peptesive (DS1-DZ-LCI; 20 000 cfu), C) pET28-EV control, D) PCL control, E) peptesive control, and F) kanamycin positive control.

Kanamycin-loaded PCL micro-containers that were immobilized with DS1-DZ-LCI on stainless steel wires inhibited the growth of *E. coli* DSM 498 cells (200 000 cfu) by forming a halo around the coated wires (Figure 4A). At a cell number of 20 000 cfu, the growth of *E. coli* DSM 498 was inhibited almost completely (Figure 4B). None of the performed control experiments (Figure 4C–E) showed any antimicrobial effect on *E. coli* DSM 498 cells. The kanamycin positive control inhibited the cell growth completely as expected (Figure 4F).

Biadhesive peptides provide exciting possibilities for immobilization of compound loaded micro-containers on medically important materials such as stainless steel. The broad range of material binding peptides and broad range of metal surfaces (e.g., platinum, gold, and zinc/titanium oxide) renders it very likely that the peptesive immobilization technology can be extended to a broad range of medical stents, implants, or catheters. Micro-containers can be loaded with a broad range of pharmaceuticals, which makes the peptesives technology likely to be of general medicinal interest.

Supporting Information

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

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

antibiotic delivery, bare-metal stents, biadhesive peptide, drug-eluting system, polycaprolactone

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