Antibacterial, Cytocompatible, Sustainably Sourced:

Cellulose Membranes with Bi-functional Peptides for Advanced Wound Dressings

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Abstract

Progressive antibiotic resistance is a serious condition adding to the challenges associated with skin wound treatment and antibacterial wound dressings with alternatives to antibiotics are urgently needed. Cellulose-based membranes are increasingly considered as wound dressings, necessitating further functionalisation steps. A bi-functional peptide, combining an antimicrobial peptide (AMP) and a cellulose binding peptide (CBP) is designed. AMPs affect bacteria via multiple modes of action, thereby reduce the evolutionary pressure selecting for antibiotic resistance. The bi-functional peptide is successfully immobilised on cellulose membranes of bacterial origin or electrospun fibres of plant-derived cellulose, with tight control over peptide concentrations ($0.2 \pm 0.1 \mu\text{g}\cdot\text{mm}^{-2}$ to $4.6 \pm 1.6 \mu\text{g}\cdot\text{mm}^{-2}$). With this approach, new materials with antibacterial activity against *Staphylococcus aureus* (log4 reduction) and *Pseudomonas aeruginosa* (log1 reduction) are developed. Further, membranes are cytocompatible in cultures of human fibroblasts. Additionally, a cell adhesive CBP-RGD peptide is designed and immobilised on membranes, inducing a 2.2-fold increased cell spreading compared to pristine cellulose. The versatile concept provides a tool-box for the functionalisation of cellulose membranes of different origins and architectures with a broad choice in peptides. Functionalisation in Tris-buffered saline avoids further purification steps, allowing for translational research and multiple applications outside the field of wound dressings.

1. Introduction

Nosocomial infections and bacterial colonisation of the wound bed constitute one of the most serious secondary complications following skin wounding. Depending on the clinical scenario, biomaterials or implant-associated infections in combination with biofilm formation can further aggravate the problem.^[1] If not appropriately addressed, persistent infections become chronic and lead to tissue inflammation, necrosis, and, in the worst case, systemic infection and sepsis which can become exceedingly hard to treat.^[2] Broadband administration and abuse of conventional antibiotic therapies have contributed to the progressive emergence of antibiotic resistance, demanding for the development of alternative treatment strategies.^[3] Antimicrobial peptides (AMP) are considered as promising candidates and currently evaluated for clinical use to treat for example infected skin wounds or non-healing diabetic foot ulcers.^[4] AMPs comprise host defensive peptides, with multiple targets on pathogenic microbes and are thus effective by several routes of action.^[3a, 5] This diversification reduces the selective pressure that drives the evolution of bacterial resistivity.^[6] AMPs also mediate pro- and anti-inflammatory cascades, and induce enhanced wound healing via mechanisms other than antibacterial properties.^[7] Furthermore, specific AMPs were shown to penetrate biofilms to target bacteria, providing a possible solution to treat chronic infections.^[6, 8] However, their high sensitivity to the local environment, entailing short blood-plasma half-life, susceptibility to degrading proteases, and often poor solubility in aqueous environments still taint their clinical use.^[7] Some of these limitations can be bypassed by tethering AMPs to delivery matrices (e.g. polymer or lipid capsules), or wound dressings, a strategy that confers the additional benefit of site specific effects, avoiding systemic administration.^[7, 9] In this direction, custom design methods and synthetic strategies should be investigated and developed in order to achieve controlled action at the site of interest. Bacterial cellulose (BC) has gained increasing interest for the use in wound healing applications based on excellent interaction with soft tissues with much reduced fibrotic encapsulation and

minimised inflammatory response in vivo.^[10] BC is produced by acetic bacteria of the *Acetobacteraceae* family in highly defined fibrillary 3D-networks as a component of their natural biofilm environment.^[11] The high purity and defined nanoscale structure, in combination with a distinct hydrophilicity that reduces protein adsorption and material-tissue interactions, are essentially responsible for the success of BC in biomedical research.^[12] Origin and availability of resources as well as methods to produce and functionalise wound dressings need to be addressed to meet the requirements for clinical translation. With some advantages over BC, such as a high abundance in nature, and cost-efficient production not being limited by low yield, plant-derived cellulose presents a valid alternative for cellulose-based substrates in this respect.^[13] Blending of materials with distinct chemical or mechanical characteristics has proven useful to combine advantageous properties within one material. Electrospinning in particular comes forward as a promising one-step technique to produce fibres from polymer blends to achieve specific surface functionalities, and / or distinct mechanical properties.^[14] Electrospun scaffolds based on cellulose were developed for skin wound applications.^[15] Enhanced mechanical properties were achieved by layer-by-layer assemblies of alternating polyurethane or cellulose fibres, or direct blending of cellulose and polyurethane, or cellulose and poly(caprolactone).^[16] Cellulose, of plant-derived or of bacterial origin, presents very poor solubility in organic solvents, which renders the material difficult to electrospin. Therefore, the more soluble form, cellulose acetate, is used for the production of electrospun membranes, followed by deacetylation.^[16c, 17] Plant-derived and bacterial cellulose share identical chemical structures and the hydrophilic surface and absence of functional entities, other than hydroxyl groups, restricts targeted interactions with other (bio)molecules. While this can be of interest to reduce foreign-body interactions upon implantation, it requires special functionalisation strategies to induce targeted, material-induced responses.^[10c, 10f] Direct immobilisation of biomolecules through a site-directed interaction based on moieties with specific bioaffinity to cellulose has

arisen as a powerful approach. In nature, several proteins have been identified that have a specific affinity for carbohydrate substrates, including cellulose, and are referred to as carbohydrate-binding modules (CBM) which are domains in carbohydrate-active proteins.^[18] Specific biomolecules that have been covalently linked to a CBM, thereby creating a bi-functional molecule of two fused units, can thus be immobilised spontaneously and in an oriented way to pristine cellulose substrates.^[19] This allows introducing bioactive functionalities to cellulose substrates without affecting their inherent biocompatible properties through excessive chemical surface modification.^[10b, 20] In previous work, T4 lysozyme or AMPs were fused to cellulose-active proteins to decorate cellulose wound dressings.^{[21] [22]} Additionally, cellular adhesion was enhanced by functionalising cellulose membranes with fusion proteins of the cell adhesive peptide RGD and a protein containing a CBM.^[23] The use of proteins is, however, associated with considerable limitations related to production and purification, immunogenic responses, and high susceptibility to conformational and functional changes within different buffers. Peptides on the other hand can be synthesised with defined chemistry and specific modifications to induce targeted cell responses when conjugated to materials.^[24] Within cellulose binding proteins, the presence of a minimal consensus amino acid sequence (WHWTYYW) has been identified, allowing for the design of synthetic cellulose binding peptides (CBP) which opened new avenues for biomaterial development.^[25] Extending this preserved amino acid sequence with additional peptide units of known biological activity to design a bi-functional peptide seems a logic continuation of the approach. Nevertheless, substrate functionalisation with CBP, and in particular bi-functional peptides, is still in its infancy, and several challenges with respect to controlled reaction, stability, efficiency, and long term effect, to mention a few only, request further research. In this paper, we describe the functionalisation of cellulose surfaces with a multi-functional peptide that combines a CBP with an AMP. The design was further shown to be translatable to other bi-functional peptides, such as CBP-RGD, which provides a versatile

model peptide to study cell spreading as a specific readout. We are reporting on a functionalisation strategy that overcomes previous limitations of harsh chemical conditions requiring washing and purification steps and captivates by its versatility to introduce application-specific functionalities in a one-step process. Functionalisation was successfully achieved on nanofibrous films of bacterial cellulose and on microfibrillar electrospun membranes of plant-derived cellulose blended with polyurethane. This functionalisation strategy could provide a versatile and effective method for the prevention of bacterial wound infection by use of cytocompatible, antibacterial cellulose wound dressings of renewable sources.

2. Results and Discussion

2.1 Peptide design and antibacterial efficacy

A bi-functional peptide was developed by combining the antimicrobial ligand tet009,^[26] a 12-amino-acid long linear peptide (sequence RRWKIVVIRWRR), with a CBP (WHWTYYW)^[25] via a flexible linker (GSGSGGS).^[27] To increase the solubility in aqueous solutions, an affinity tag (GKKKK) was added to the C-terminal (**Scheme 1**). In order to quantify the amount of membrane-immobilised peptides, the bi-functional peptide was labelled with a fluorescent FAM unit (AMP-CBP-FAM)

Scheme 1 a) Cellulose-based membranes were modified with bi-functional peptides. A cellulose binding peptide (CBP, green) was linked to an antimicrobial peptide (AMP, red) or a cell adhesive peptide (RGD, blue). b) The versatile design concept allows for coupling of peptides of interest (light blue) to the amino or the carboxy terminus of CBP for custom designed material development. CBP binds with high selectivity and strong affinity to cellulose via a combination of interactions.

Tet009 has been shown to be highly active against important wound colonisers like *Pseudomonas spp.*, *Staphylococcus spp.*, and *Candida spp.*^[28] Prior to membrane functionalisation, an initial susceptibility test was used to assess whether the antimicrobial efficacy of the bi-functional peptide was retained. AMP-CBP-FAM was added to the culture media and tested against *Staphylococcus aureus* (*S. aureus*) or *Pseudomonas aeruginosa* (*P. aeruginosa*), respectively. The bi-functional peptide exhibited an inhibitory effect at a concentration of $0.25 \mu\text{g}\cdot\text{mL}^{-1}$ and higher against *S. aureus*, while concentrations of $4 \mu\text{g}\cdot\text{mL}^{-1}$ were needed to reduce the growth of *P. aeruginosa* (**Figure S1**). For the same AMP (tet009, not linked to CBP) Hilpert et al. reported a MIC of $2 \mu\text{M}$ (corresponding to $3.4 \mu\text{g}\cdot\text{mL}^{-1}$) against *P. aeruginosa*, which lies in agreement with our observations.^[28] The MIC is generally very AMP specific, sensitive to environmental conditions, and can differ significantly among various peptides and/or against different bacterial strains. For melittin or nisin for example, concentrations of 2 to $6 \mu\text{g}\cdot\text{mL}^{-1}$ or 2 to $32 \mu\text{g}\cdot\text{mL}^{-1}$, respectively, were reported to inhibit the growth of methicillin-resistant *S. aureus* (MRSA).^[29] In an extensive study, evaluating 13 different MRSA strains, MIC of 1 up to $16 \mu\text{M}$, depending on the bacterial strain were reported for the antimicrobial peptides WR-12 or D-IK8.^[30] Of paramount importance, cytotoxicity of new materials or compounds intended for clinical application needs to be taken into account early during the design process. To this end, the cytotoxicity of the newly designed bi-functional peptide was assessed in vitro. Monolayers of normal human dermal fibroblasts (NHDF) were treated with increasing concentrations ranging from 0.02 to $0.1 \text{ mg}\cdot\text{mL}^{-1}$ AMP-CBP-FAM dispersed in cell culture media. These concentrations were chosen to be orders of magnitude higher than the MIC in order to account for the higher concentration that will be experienced by cells in direct contact when seeded on AMP-CBP-FAM-functionalised membranes. For all concentrations, a cell metabolic activity of more than 70% (threshold according to ISO 10993-5), relative to the control group (no bi-functional peptide, cell culture media only), was found. Furthermore, Hoechst analysis revealed similar DNA contents for NDHF treated with or without AMP-CBP-FAM without significant

differences among the conditions (**Figure S2**). It was therefore concluded that the bi-functional peptide did not have any measurable cytotoxic effect against human dermal fibroblasts. Taken together, these results demonstrate the antibacterial activity of the designed bi-functional peptide in media against two bacterial strains with no cytotoxic effects in the chosen in vitro system.

2.2 Material development and characterisation

The use of bacterial derived cellulose (BC) for various applications has been known for decades and its production and harvesting have been well documented.^[31] The BC used here contains fibrillary structures with diameters in the nanometer range (measured on SEM images, 69 ± 26 nm), (**Figure 1**). The fibres are flattened, very densely packed, and appear to be fused to each other on many locations. This essentially presents a 2-dimensional network with a fibrous surface structure, rather than a porous material. Different to this, plant derived cellulose, in its acetylated form, was processed by electrospinning into porous fibrous membranes.^[17b, 17c, 32] In our hands, electrospun membranes of pure cellulose acetate were highly brittle and could not be processed for use in cell culture experiments. Furthermore, at various cellulose acetate (CA) concentrations in different solvents systems (mixtures of DMF and acetone, acetic acid at various concentrations, pure DMF), bead formation occurred. Addition of the polyurethane Elastollan C95A55 (PUR) to the spinning solution tremendously improved the mechanical properties, resulting in the production of non-brittle, free-standing membranes, at the additional benefit of facilitating a continuous electrospinning process (formation of CAPU membranes). Fibres with a mean diameter of 0.80 ± 0.41 μm , assessed based on SEM images, were produced.

Figure 1. Scanning Electron Microscopy (SEM) images of membranes of varying cellulose origin and properties. a) electrospun fibres of cellulose acetate / polyurethane blends (CAPU), b) deacetylated electrospun fibres of cellulose/polyurethane (CPU). SEM images acquired before and after deacetylation do not reveal any differences in fibre morphology. Electrospun fibres are in the sub-micrometer range, displaying mean diameters of $0.80 \pm 0.41 \mu\text{m}$ ($n = 200$). c) Bacterial cellulose (BC) displays fibres with a mean diameter of $69 \pm 26 \text{ nm}$ ($n = 150$). d) On all membranes, water in air contact angles were measured. Data are represented as mean value $\pm$ SD of $n = 3$ measurements per membrane. Complete wetting was observed on CPU membranes.

In line with previous work, we expected a blend concentration-dependent surface chemical compositions, with a phase separation of cellulose or PUR enriched phases, respectively.^[14a, 14b, 14d] Indeed, XPS analysis of the as-spun membranes showed a reduction in the nitrogen signal, selective for PUR, at higher CA to PUR ratios, indicative for the formation of a cellulose enriched sheath (**Figure 2**). As highlighted in the insert (magnification of the signal around 400 eV), a concentration of 5% w/w CA to PUR was not sufficient for a complete coverage of CA on the outside and a nitrogen peak stemming from PUR was still apparent. A concentration of 10% w/w CA was thus the optimal formulation to produce electrospun membranes for further

evaluations. These findings lie in strong agreement with previous studies on co-continuous CA/PUR fibres by Tang et al.^[16b] For pure CA solutions, beads-on-a-string morphologies were determined, with homogeneous fibre production at increasing PUR concentrations. Furthermore, the authors reported changes in mechanical properties by blending CA with PUR, with an increase in tensile strength from 0.98 ± 0.09 MPa to 7.46 ± 0.64 MPa for a pure CA membrane or a 20:80 CA:PUR membrane, respectively. In their work, selective dissolution of CA or PUR resulted in fibres without apparent pore formation within the fibres. The authors concluded that core-sheath/co-continuous fibres rather than aggregation of phase separated polymer islands took place.^[16b] The process of core-sheath formation during electrospinning of polymer blends is highly complex, depending on many different parameters, and has not been fully elucidated up to date. Factors affecting phase separation are attributed to the characteristics of the individual polymers such as molecular weight, solubility, evaporation rate under electrohydrodynamic conditions, viscosity in solution, electrical conductivity, surface tension, surface energy and crystalline regions.^[14a, 14b, 33] Furthermore, interactions between the individual polymers, such as hydrogen bonds, significantly contribute to the miscibility or phase separation of individual polymers within the blend.^[34] We are suggesting that the phase separation in the CA:PUR system is mainly driven by the lower molecular weight of CA compared to PUR, resulting in a lower viscosity and a surface aggregation of CA during spinning jet formation. To fully elucidate and understand the process, further studies are needed, comparing different blend ratios, the use of CA with various degrees of acetylation or polymer blends of different PUR. By using this approach, the formation of fibres with cellulose enriched surfaces, at the benefit of free-standing membranes with mechanical integrity was achieved. The deacetylation process (resulting in cellulose:PUR membranes, CPU) did not have measurable effects on fibre morphology, as confirmed by SEM analysis (Figure 1). XPS characterisation, in particular the changes observed in the O-C=O peak at a binding energy of 289.00 ± 0.14 eV were indicative

for successful deacetylation (Figure 2d). Correlating with differences in surface chemical composition, the wetting behaviour changed dramatically, from highly hydrophobic membranes with a contact angle of $127.0 \pm 6.8^\circ$, to hydrophilic ones after deacetylation where complete wetting was observed (Figure 1d). Measuring the contact angle of CAPU or CPU membranes gives an additional indication for successful deacetylation and could further confirm the changes in chemical composition from hydrophobic acetyl groups to the hydrophilic properties of non-acetylated cellulose.

Figure 2. Chemical surface composition of substrates characterised by X-ray photoelectron spectroscopy (XPS). a) Chemical structure of cellulose acetate and deacetylated cellulose. b) Survey spectra of bacterial cellulose (BC), cellulose acetate (CA), polyurethane (PU), 5:95 cellulose acetate:polyurethane (5 % CAPU), 10:90 cellulose acetate:polyurethane (10 % CAPU), and deacetylated 10:90 cellulose:polyurethane (CPU). Insert: zoom in at 400 eV. The presence of nitrogen in PUR and 5% CAPU is apparent by a peak at 400 eV. c) High resolution spectra of the C1s chemical environment and corresponding fitting of C-C, C-O, O-C-O, and O-C=O of i) BC ii) 10% CAPU, iii) deacetylated 10% CPU. The O-C=O peak was used to monitor

deacetylation of electrospun membranes. d) Quantification of acetyl groups. After deacetylation in Na/MetOH for three hours, a decrease in at% of O-C=O was observed. Data are presented as mean $\pm$ SD of $n = 7$ (CAPU) or $n = 5$ (CPU) individual membranes. Statistical significance was assessed in a non-parametric Mann-Whitney test with $*p < 0.05$.

2.2.1 Peptide binding

Control over peptide concentration was achieved by varying the immobilisation parameters, specifically incubation time and initial AMP-CBP-FAM concentration in Tris-buffered saline (TBS) (**Figure 3**, quantification described in supporting information, **Figure S3**). A higher initial AMP-CBP-FAM concentration, in combination with longer incubation time led to an increased concentration of bound peptides on BC films. Based on these results, four conditions that resulted in significant differences of peptide concentration on the membranes were selected (red circles in Figure 3a). In detail, AMP-CBP-FAM was immobilised at concentrations of $6 \text{ mg} \cdot \text{mL}^{-1}$ for 6 h, $1 \text{ mg} \cdot \text{mL}^{-1}$ for 6 h, $0.6 \text{ mg} \cdot \text{mL}^{-1}$ for 1 h or $0.06 \text{ mg} \cdot \text{mL}^{-1}$ for 3 h for all further experiments. These conditions resulted in an apparent peptide concentration between $0.2 \pm 0.1 \text{ } \mu\text{g} \cdot \text{mm}^{-2}$ to $1.3 \pm 0.5 \text{ } \mu\text{g} \cdot \text{mm}^{-2}$ on BC films, and $0.3 \pm 0.2 \text{ } \mu\text{g} \cdot \text{mm}^{-2}$ to $4.6 \pm 1.6 \text{ } \mu\text{g} \cdot \text{mm}^{-2}$ on CPU membranes (Figure 3b and c).

Figure 3. a) Peptide quantification on bacterial cellulose films (BC). To control the apparent, final peptide concentration on the cellulose membranes, different immobilisation conditions were evaluated. Four conditions that differ significantly in their final peptide concentration were selected for in vitro evaluation in bacterial as well as mammal cell culture (red labels). Data are represented as mean $\pm$ SD of $n = 4$ membranes per condition. b) Quantification of peptide concentration on cellulose substrates in function of immobilisation conditions on bacterial cellulose (BC), and c) electrospun cellulose: polyurethane membranes (CPU). At similar immobilisation parameters, a higher amount of peptides could be tethered to electrospun membranes. All individual membranes used in this study are plotted. Horizontal lines represent the mean value. Statistical significance was assessed in a non-parametric Kruskal-Wallis test, followed by pairwise comparison in a Mann-Whitney test. Mean values among the conditions in b) or c) differed significantly among each other with $P < 0.05$ unless indicated by n.s.

Previous work in our group reported on enhanced antimicrobial activity of a nanocellulose-nisin biocomposite, exposed to bacteria in suspension compared to nisin alone.^[35] The study indicated no direct correlation of the activity of material-tethered AMP nisin with the activity, or MIC, of nisin in suspension. It is suggested that the increased activity can arise from controlled orientation and thus increased availability of peptides on the nano-carriers. Similar observations on a structure-function relationship were reported by other groups with BC-entrapped AMP being more active than peptides in suspension or composites of nanocrystalline cellulose and nisin presenting a prolonged activity over 24 hours as compared to the peptide in suspension.^[36]

Due to these discrepancies between MIC of free peptides in culture media and material tethered peptide concentrations, we evaluated membranes of different amounts of immobilised peptides. CPU membranes are highly porous, with an interconnected fibre network. BC films on the other hand display fibres in the nanometer range that are assembled into a non-porous, 2-dimensional film (Figure 1). These differences in morphology most likely resulted in the higher loading capacity of peptides on CPU membranes. Furthermore, complete wetting of CPU was observed, which additionally facilitates immersion in the peptide solution and thus peptide immobilisation. At AMP-CBP-FAM concentrations of $0.06 \text{ mg} \cdot \text{mL}^{-1}$, the differences between BC and CPU were not significant, which suggests that at higher concentrations a saturation is achieved on BC films, but not on CPU membranes. The high variations in peptide concentrations on CPU membranes can be attributed to small changes in fibre morphology as well as varying degrees of deacetylation which did occur despite standardised protocols (Figure 2d, standard deviations in the O-C=O content of 31%). Nevertheless, by using a material of significantly different architecture, and adapted immobilisation protocols, degree of functionalisation could be steered by using a bi-functional peptide. Up to date, antimicrobial properties of cellulose membranes were mainly restricted to the addition of releasing components such as silver ions, curcumin,

dextran, acetate-zein, or propolis coatings.^[16c, 32c, 37] Electrospun membranes of a variety of other polymers have been functionalised with AMPs that were directly added to the spinning solution, physisorbed, or covalently bound to the fibres by introducing active groups such as thiol entities, or surface initiated by use of radical polymerisation. A review by Felgueiras and colleagues provides insight into the various approaches.^[38] Despite promising antibacterial effects, physisorbed peptides were shown to be released over time, often in an uncontrolled manner, which should be avoided for targeted local contact killing.^[39] Furthermore, organic solvents used during the spinning process could, affect the peptide structure and/or function. Post-functionalisation with an affinity peptide in aqueous TBS does thus provide advantages in this regard. Cellulose binding motives or proteins are known from naturally occurring enzymes and their substrate specificity and binding strengths to cellulose substrates have been subject of research for more than two decades, and have been investigated by molecular dynamics, principle component analysis, and binding free energy calculations.^[18, 40] Binding to cellulose strongly depends on the specific amino acid sequence, and various research approaches resulted in the identification of shorter units, the CBP.^[25, 41] The affinity is governed by combined non-covalent interactions that involve e.g. binding of tyrosine to single cellulose polymer chains *via* CH/ π -stacking-, hydrogen bonding, and hydrophobic interactions with the glucose rings.^[25, 42] These interactions in concert facilitate the site-directed immobilisation of CBPs on cellulose substrates with high selectivity and (bio-)affinity constants as high as 10^5 M^{-1} .^[25] In addition, the polarity or charge of peptides can furthermore interact with the substrate and significantly contribute to the binding strength, specificity or overall interaction with a material.^[26]

Work by Hsu *et al.* reports on the functionalisation of PUR, PLLA or PLGA membranes by use of protein-based cellulose binding domains fused to RGD (CBD-RGD).^[43] Despite the lack of a cellulose substrate, the authors report on successful immobilisation of RGD, evidenced by increased cell adhesion. To assess the robustness of the here presented functionalisation strategy prior to in vitro evaluation, we have investigated the binding specificity and stability of AMP-

CBP-FAM in TBS towards different materials and over a time frame of three weeks. Results of this preceding experiment are presented in Supporting Information in **Figure S4**. On BC and CPU membranes, no significant decrease in fluorescent signal was observed over 21 days, indicating stable immobilisation of the peptide. Exclusive binding to cellulose-based materials was not as pronounced as expected and PUR electrospun membranes also retained non-neglectable amounts of peptides. Comparing PUR fibres to PUR 2D films (drop-casted) indicates that on this polymer, binding is linked to the fibrous structure. We are suggesting that increased peptide retention is associated with a higher surface to volume ratio and thereby larger accessible surface for non-specific physisorption. Furthermore, investigations should focus on the specificity of CBP and the stability of formed interactions under different environmental conditions, such as temperature (e.g. 4°C for long term storage, 37°C upon application), or the ionic strength of the buffer. Interestingly, BC entrapped AMP were shown to be more efficient at 37°C than the free AMP in suspension, while at 4°C, both forms resulted in similar antibacterial efficiency.^[36a]

In summary, these observed effects warrant further research and should be carefully considered for the design of cellulose specific functionalisation strategies. Within this study, we were interested in the biological response and have thus evaluated the antibacterial efficiency as well as the cytocompatibility of the developed membranes.

2.3 Biological evaluation

2.3.1 Antibacterial properties

The developed membranes were evaluated against a gram⁽⁺⁾ and a gram⁽⁻⁾ strain. On AMP-CBP-FAM loaded BC films, all conditions resulted in a significantly reduced number of *S. aureus* compared to the control group with a reduction of log2 (mean value) at an AMP-CBP-FAM concentration of $1.7 \pm 0.5 \mu\text{g}\cdot\text{mm}^{-2}$ (**Figure 4**). In line with an increased loading efficiency on CPU membranes and overall increase in peptide concentration on electrospun

membranes, the same immobilisation conditions ($6 \text{ mg} \cdot \text{mL}^{-1}$ or $1 \text{ mg} \cdot \text{mL}^{-1}$ for 6 h) resulted in higher antibacterial efficiency against *S. aureus* compared to BC films. A mean log4 or log3 reduction was observed on electrospun CPU membranes of $4.5 \pm 1.8 \text{ } \mu\text{g} \cdot \text{mm}^{-2}$ or $2.4 \pm 1.3 \text{ } \mu\text{g} \cdot \text{mm}^{-2}$, respectively.

Against *P. aeruginosa*, AMP-CBP-FAM concentrations up to $1.7 \pm 0.5 \text{ } \mu\text{g} \cdot \text{mm}^{-2}$ on BC films were not sufficient to induce the targeted log3 reduction in CFU compared to the control. Nevertheless, a statistically significant reduction in CFU compared to pristine BC films was observed. On electrospun CPU membranes, an apparent peptide concentration of $6.0 \pm 0.4 \text{ } \mu\text{g} \cdot \text{mm}^{-2}$ led to a mean log1 reduction of *P. aeruginosa*, which resulted in significantly less CFU compared to the control group. In the three other conditions, where membranes had lower apparent peptide concentrations of 1.0 ± 0.2 , 0.7 ± 0.2 , or $0.2 \pm 0.1 \text{ } \mu\text{g} \cdot \text{mm}^{-2}$, respectively, no significant antibacterial activity against *P. aeruginosa* compared to the control group was observed. Bacterial colonisation of wounds can not only delay wound healing, but may also lead to chronic wounds under certain circumstances and the formation of biofilms that are highly resistant to antimicrobial intervention.^[44] The microbiome of chronic wounds often consists of *S. aureus*, while burn wounds are more likely populated by *P. aeruginosa*.^[45] Depending on the application, wound dressings are thus required to be effective against a variety of bacterial strains. Hypothetically, an even more pronounced antibacterial effect could be induced by higher peptide loading. On BC membranes, a saturation of peptide concentration was observed, while adapted immobilisation parameters are subject of future investigations to augment the degree of functionalisation on CPU membranes. The antimicrobial activity of AMPs as well as binding strength of CBP to cellulose, have been shown to be influenced by the linker length^[46] and the specific amino acid sequence.^[26] This structure-function relationship can be further influenced by the material or substrate the peptides are tethered to, their resulting spatial conformation, and concomitant accessibility to bacterial cells. This so-called substrate effect can additionally change the antibacterial efficiency (MIC) of AMPs in response to the underlying material.^[35]

Despite considerable differences in the macroscopic and microscopic morphology between BC films and CPU membranes, our data do not give evidence for such a material-induced effect. This can be attributed to the architectural structure in the micrometer to sub-micrometer range that is by magnitudes larger than the dimensions of nanofibrillated cellulose (NFC, 10 to 20 nm) where such an effect was observed.^[35] To fully elucidate the effect of the underlying substrate on the antimicrobial activity, further studies are necessary, in particular focusing on cellulose substrates of different architectural dimensions in the nanometer range.

Figure 4. Antibacterial activity of AMP-CBP-FAM functionalised membranes. Colony forming units (CFU) in response to peptide concentration were determined by a regrowth assay. Measured mean peptide concentrations of the actual membranes used for bacteria incubation

are displayed on the x-axis. a) *S. aureus*; Non-significant differences among conditions are highlighted with n.s. All other conditions display statistically significant differences among each other. b) *P. aeruginosa*; * $P < 0.05$ compared to the control group.

In a) and b), individual data points are presented, horizontal lines represent mean values, yellow background highlights control groups without immobilised peptides. Statistical significance was assessed in a non-parametric Kruskal-Wallis test, followed by pairwise comparison in a Mann-Whitney test. Significant differences among the mean value were accepted for $P < 0.05$. N=3 individual experiments with n=3 to 5 technical repeats in each experiment.

2.3.2 Cytocompatibility

Preceding experiments with CBP-AMP-FAM added to cell culture media (**Figure S2**) confirmed the cytocompatibility of the here designed bi-functional peptide which was further validated on functionalised BC or CPU membranes. Metabolic activity of NHDFs was determined after one or three days of culture and presented relative to pristine BC films or CPU membranes, respectively (**Figure 5**). On modified BC and CPU membranes no cytotoxic effects compared to the control group were observed. At the two highest peptide concentrations on BC, differences to control membranes on day one were significant, levelling out after prolonged culture for three days. On CPU membranes, cell metabolic activity was significantly higher on functionalised membranes. Increased cell metabolic activity indicates an increased number of adherent cells after one day and proliferation over three days. Confocal laser scanning microscopy (CLSM) images (representative conditions displayed in **Figure 6**; additional images in **Figure S5**) are supportive of this data, demonstrating an increased cell density on functionalised membranes compared to pristine ones. On these, BC or CPU, fibroblasts formed high density cell-aggregates with minimal cell-material interaction, while increased cell-material interactions on AMP-CBP-FAM functionalised ones was observed. Surface functionalisation altered the overall chemical surface properties of the membranes (introduced by the positive charge and chemical groups of the bi-functional peptide), which has an effect on protein adsorption from culture media and subsequent cell adhesion. BC itself has been recognised as a material of low bioactivity, and its hydrophilicity and inertness towards adsorption of proteins makes it an ideal candidate for biomedical application, i.e. reducing the risk for and level of fibrotic encapsulation.^[10f, 12] Not surprisingly, cell adhesion is thus also restricted on BC membranes compared to other polymeric cell culture substrates. At very few spots, cell monolayers were formed, comprising spread cells with an apparent actin cytoskeleton formation. Hypothetically, individual fibroblasts did adhere to defects on the BC membrane, secreting their own extracellular matrix and thereby further triggering more cells to adhere next to these initially adhering ones.

However, regions of aggregated, rounded cells are more abundant. The relatively large standard deviations recorded for cell metabolic activities can thus be explained by heterogeneous cell coverage on individual membranes.

Figure 5. In vitro cytotoxicity of functionalised membranes. a) Bacterial cellulose (BC), b) electrospun cellulose:polyurethane membranes (CPU). Human dermal fibroblasts were seeded on functionalised membranes of different peptide concentrations and assessed for cell metabolic activity after one or three days (d1, d3). Box plots are representing the 25th and 75th percentile, whiskers are representing min. and max. values, black squares are representing outliers, mean value shown as horizontal line. Measured mean peptide concentrations for the membranes used are displayed on the x-axis. Values are displayed relative to non-functionalised membranes (control group, 0, yellow background) on day one. * $P < 0.05$ compared to the control group of d1, # $P < 0.05$ compared to the control on d3. $N = 3$ individual experiments with $n = 3$ technical repeats. Statistical significance was assessed in a non-parametric Kruskal-Wallis test, followed by pairwise comparison in a Mann-Whitney test.

Figure 6. CLSM images of NHDF cultured on AMP-CBP-FAM functionalised membranes. NHDF were cultured for 3 days prior to imaging. Images of two different functionalisation conditions are displayed: low peptide concentration (0.2 ± 0.1 or $0.3 \pm 0.2 \mu\text{g}\cdot\text{mL}^{-1}$ on BC or CPU, respectively) or high peptide concentration ($1.1 \pm 0.4 \mu\text{g}\cdot\text{mL}^{-1}$ or $4.6 \pm 1.5 \mu\text{g}\cdot\text{mL}^{-1}$ on BC or CPU, respectively). Pristine membranes, without any peptides served as controls. Left row: 3x3 tiles image, presenting an overview of the respective membranes; right row: 20x magnification of a representative spot. NHDF were stained for actin (red) and nuclei (blue).

Depending on the chosen application, enhanced cell adhesion is desired and can be achieved by surface functionalisation. The RGD peptide, derived from fibronectin, has been widely used as a minimal amino acid sequence to induce cell adhesion.^[47] In previous work, proteins consisting of RGD fused to a cellulose binding protein were shown effective in promoting adhesion and spreading of fibroblasts, chondrocytes, osteoblasts, or endothelial cells.^[23, 43] Following our design concept of fusing peptides of interest, an RGD unit has been linked to CBP to functionalise

BC films. CLSM images of NHDFs seeded on RGD decorated membranes were acquired after one day in serum-free culture, and after an additional day in serum-supplemented media, respectively. Cell metabolic activity was assessed on both days (**Figure 7**). The presence of RGD enhanced the number of cells adhering to the films and cell spreading. In line with these findings, and indicative for increased cell number, metabolic activity was significantly enhanced on RGD functionalised membranes compared to pristine ones (increased by 1.5 times on day one and 2.0 on day 2). Determination of cell spreading was based on image analysis by quantifying the area that was covered by the cell body, positively stained for actin. A significantly enhanced cell spreading was found at both time points for RGD-functionalised membranes compared to the control group. For wound healing scenarios, cell adhesion to bandages is usually not desired and the here presented approach thus consists in a proof of concept to underpin the robustness of the functionalisation strategy with bi-functional peptides. The cell response to RGD, in the form of cell spreading, can be used as a readout for successful peptide immobilisation. These results provide evidence for the versatility of our design concept that cellulose membranes can be functionalised by use of bi-functional peptides. In the future, other scenarios could be envisaged by coupling peptides of interest to CBP.

Figure 7. BC membranes were functionalised with a CBP-RGD bi-functional peptide. NHDF were cultured on RGD-functionalised or control membranes without any peptides for a) one day (d1) or b) two days (d2). NHDF on RGD-functionalised membranes are larger and more spread compared to NHDF on pristine BC membranes (control). c) Cell metabolic activity assessed with alamarBlue. Values are normalised to BC on day one. An increased cell metabolic activity was observed for cells on RGD-functionalised BC films compared to pristine ones. d) Cell spreading area was quantified based on CLSM images and normalised to cell counts (area per cell). On RGD-functionalised membranes, cell spreading was significantly larger compared to the control group. N=3 individual experiments with n=3 technical repeats. Cell spreading was quantified based on n = 8 representative images per condition. Statistical significance was assessed in a non-parametric Kruskal-Wallis test, followed by pairwise comparison in a Mann-Whitney test with $*P < 0.05$.

3. Conclusion

Antimicrobial, cytocompatible membranes were designed by use of naturally derived materials and exploiting bi-functional peptides with a cellulose binding unit that spontaneously binds to

cellulose substrates. Our results provide evidence for a concentration-dependent antibacterial effect against *S. aureus* and *P. aeruginosa*. In vitro evaluation in cultures of human dermal fibroblasts confirmed the cytocompatibility of the designed membranes in the chosen model system. The concept was successfully transferred to an RGD moiety coupled to CBP, which resulted in enhanced cell spreading.

The modular approach induced multiple degrees of freedom to control the final peptide concentration, including origin (bacterial or plant) and architectural properties of cellulose membranes, and tightly controlled reaction conditions (peptide concentration and incubation time). In future studies, peptide sequences could furthermore be designed to entail an additional cleavage site (e.g. enzyme sensitive units), allowing for substrates with controlled release of biomolecules. Overall, the here reported strategic functionalisation route provides a molecular toolbox for custom-modification of cellulose-based membranes with broad choice over functional peptides. This could guide the design of alternative materials to circumvent the administration of antibiotics to treat and prevent wound infections.

4. Experimental Section

Materials If not noted differently, all chemicals were purchased from Sigma-Aldrich, Switzerland, and used as received. Solvents were of analytical grade and used without further purification. Cell lab ware, cell culture plates and flasks were sterile, tissue culture treated, and purchased from Techno Plastic Products (TPP), Switzerland. Cell culture supplements were all from Invitrogen, Thermo Fisher Scientific, Switzerland, if not specified differently.

Bi-functional peptide design An antimicrobial peptide (AMP) (RRWKIVVIRWRR)^[26] was conjugated (GSGSGGS)^[27] to a cellulose-binding peptide (CBP) (WHWTYYW).^[25] The C-terminal end of the bi-functional protein AMP-CBP was modified with the short amino acid sequence GKKKK to increase the solubility in an aqueous environment. For spectrophotometric detection and quantification, the bi-functional peptide was furthermore labelled with an amidated (5,6)-FAM moiety. Following the same concept with small modifications, an RGD-moiety was linked to the C-terminal end of CBP, without a FAM moiety. Custom designed peptides were purchased from CASLO (Lyngby, Denmark). Peptide stock solutions were prepared by dissolving lyophilised peptides in Milli-Q water. Stock solutions were stored at -20°C until further use. Working solutions were prepared by further diluting the peptide in Tris-buffered saline (TBS) at pH 7.5.

Table 1 Peptide sequences

Peptide	Amino acid sequence	Mw [g·mol ⁻¹]
AMP-CBP-FAM	RRWKIVVIRWRR-GSGSGGS-WHWTYYW-GKKKK-(5,6-FAM)-NH ₂	4263.38
CBP-RGD	KKKGG-WHWTYYW-GSGSGG-RGD-SP	2555.26

Bacterial cellulose BC-films were produced as previously reported.^[10c] In brief, wild type *Acetobacter xylinum* (ATCC-700178, LGC Standards, Germany) was cultured at 26.5 °C. After 3 to 4 days, a cellulose layer was formed at the liquid-air interface and harvested. BC was incubated in 1M NaOH for 80 minutes at 80°C, followed by extensive washing in MilliQ water. Purified BC was then dehydrated overnight at room temperature and autoclaved prior to use.

Electrospinning and membrane characterisation Polymer solutions for electrospinning were prepared at a total concentration of 15 % w/v (0.6 g polymer in 4 mL solvent) in a mixture of N,N-dimethylformamide (DMF) and acetone at a ratio of 2 units DMF and 1 unit acetone. Blend solutions of Polyurethane (PUR, Elastollan C95A55, Mw: 85,790 Da, BASF, Germany) and Cellulose Acetate (CA, Mw: 37,000 Da, 40% acetylated) were directly added to the solvent at different weight to weight ratios: pure CA, pure PUR, 5:95 CA: PUR or 10:90 CA: PUR, respectively. Polymer solutions were processed on an electrospinning device developed in house and previously reported.^[48] In short, two high voltage sources of opposite charges (0 to +30kV or 0 to -30 kV, aip Wild AG, Switzerland) maintained the electrical field. An infusion pump (KD scientific, USA) provided a constant polymer flow. Fibres were collected on a planar stainless steel plate. Process parameters for all conditions were an electrical bias of +9 kV and -2kV, a flow rate of 10 $\mu\text{L} \cdot \text{min}^{-1}$ through a needle of 0.8 mm diameter (Unimed S.A., Switzerland) and a needle to collector distance of 15 cm. Spinning time was 120 minutes. Fibre morphology was characterised by scanning electron microscopy (SEM) on a Hitachi S-4800 (Hitachi High-Technologies, Canada). For image acquisition, acceleration voltage was set to 2 kV, at a current flow of 10 μA . Prior to imaging, samples were gold sputter coated (7 nm, EM ACE600, Leica Microsystems, Switzerland). Mean fibre diameters were calculated based on 100 individual fibres on 5 images at random locations on two individual electrospun membranes (total of n = 200), and with n = 150 on one BC film (Image J freeware, <http://rsbweb.nih.gov/ij/>).

Cellulose deacetylation was performed according to the Zemplén Deacetylation as previously reported.^[49] Pure sodium was dissolved in methanol (25 mM), followed by immersing as spun cellulose acetate/polyurethane (CAPU) membranes for 3 hours. Membranes were washed in H₂O and air dried. Deacetylated membranes are henceforth called CPU membranes.

Surface chemical composition was analysed by X-ray photospectroscopy (XPS) on a PHI 5000 VersaProbe II instrument (USA) with a monochromatic AlK α X-ray source with energy resolution at 0.125 eV/step and 29.35 eV. Spectra were calibrated to Carbon 1s at 285 eV to correct for charge effects and analysed with CasaXPS software (Casa Software Ltd, United Kingdom). Potential core-sheath fibre formation was monitored by continuous vanishing of the N1s peak, being selective for PUR, at higher CA concentrations as previously reported.^[14b] Deconvolution of the high resolution C1s signal was performed by fitting C-C and C-H at 284.92 ± 0.12 eV, C-O at 286.41 ± 0.13 eV, O-C-O at 287.63 ± 0.28 , and O-C=O at 289.00 ± 0.14 eV (mean and SD of all measurements). O-C=O content was calculated based on n=5 measurements on CPU membranes and n=7 measurements on CAPU membranes. Results are presented as mean $\pm$ SD. Water in air contact angles were measured on a drop shape analyzer (Krüss GmbH, Germany). For each condition, two different membranes with n=3 water droplets were analysed. Contact angles were automatically calculated by the equipment software implementing the Laplace-Young fit (<https://www.kruss-scientific.com/services/education-theory/glossary/young-laplace-fit/>).

Development and characterisation of peptide functionalised membranes Peptide immobilisation was achieved by incubating BC films in peptide solutions at various concentrations (6, 1, 0.6, 0.06 or 0.006 mg·mL⁻¹) in 1x TBS at pH = 7.5 for specific durations (1, 3, 6, or 24 hours). Films or membranes were placed in 2 mL Eppendorf tubes and incubated with the peptide solution on an orbital shaker at room temperature. Samples were washed 3 times in 1x TBS and placed in culture well plates for spectrophotometric quantification based on the FAM-label of

the peptide on a BioTek's Synergy™ Mx Microplate Reader. (Detailed information in Supporting Information and **Figure S3**.) Based on apparent peptide concentration in this preliminary test with $n = 4$ to 6 membranes, four different conditions were chosen and used to functionalise BC films as well as CPU membranes for biological assays: $6 \text{ mg} \cdot \text{mL}^{-1}$ for 6 hours, $1 \text{ mg} \cdot \text{mL}^{-1}$ for 6 hours, $0.6 \text{ mg} \cdot \text{mL}^{-1}$ for 1 hour, and $0.06 \text{ mg} \cdot \text{mL}^{-1}$ for 3 hours. For each condition, at least $n = 37$ membranes were analysed. CBP-RGD was immobilised at $0.6 \text{ mg} \cdot \text{mL}^{-1}$ for 6 hours. CBP-RGD was not fluorescently labelled and no detailed quantification was carried out.

Antibacterial activity The antibacterial activity of AMP-CBP-FAM against two important wound pathogens, *Staphylococcus aureus* (ATCC 6538, Manassas, USA) and *Pseudomonas aeruginosa* (ATCC 43390, Manassas, USA) was first assessed in a susceptibility test to determine the minimal inhibitory concentration (MIC), followed by a regrowth assay of each bacterial strain cultured individually on functionalised membranes (8 mm in diameter). To determine the MIC, bacterial growth was recorded spectrophotometrically at 595 nm over 18 hours after the addition of AMP-CBP-FAM at different concentrations (0.13 to $64 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$). For the regrowth assay, bacteria were cultured in 30% Tryptic Soy Broth (TSB) supplemented with 0.25% glucose. Bacteria from pre-cultures were diluted to a concentration of OD 0.05 (*S. aureus*) or OD 0.005 (*P. aeruginosa*) at 595 nm and seeded in a volume of $20 \text{ } \mu\text{L}$ on the respective membranes (corresponding to an inoculum of $\sim 5 \cdot 10^6$ bacterial cells). For both strains, bacteria were incubated for 3 hours at 37°C . The films were rinsed with 0.9% NaCl and placed in a new well plate where $800 \text{ } \mu\text{L}$ fresh medium was added. After further 2 hours of incubation, $200 \text{ } \mu\text{L}$ of the supernatant containing regrown bacteria were pipetted in triplicates into a 96-well plate. For the following 12 hours ODs were recorded at 595 nm every 30 minutes in a BioTek's Synergy™ Mx Microplate Reader at 37°C . Growth curves were fitted with a sigmoidal Gompertz function,^[50] based on which the time for bacterial cells to proliferate to a defined OD of 0.1 was calculated. This time correlated directly with the initial viable cell counts, namely, the more

viable cells in the wells, the shorter the lag phase is and the faster a specific OD value can be reached.^[51] Based on this regrowth time, colony forming units (CFU) were quantified by use of a standard growth curve of *S. aureus* or *P. aeruginosa*, respectively and reported relative to bacterial growth on pristine BC or CPU membranes. For each bacterial strain at least n=14 functionalised membranes per condition were analysed in N=3 individual experiments.

In vitro cytocompatibility test in mammalian cell culture All experiments were carried out with normal human dermal fibroblasts (NHDF, female, 47y, caucasian, skin/temple, PromoCell, C-29910, Germany) up to passage 12. NHDF were expanded in Dulbecco's Modified Eagle's Medium (DMEM), high glucose, supplemented with 10% v/v foetal calf serum, 1% glutamine, and 1% penicillin, streptomycin, neomycin (PSN). All experiments were accomplished under aseptic conditions; NHDF culture was maintained in a humidified incubator at 37°C with 5% CO₂. Cell culture on BC films or CPU membranes was performed as reported previously.^[52] Briefly, membranes of 6 mm in diameter were fixed with insect pins (Entosphynx, Czech Republic) in poly(dimethylsiloxane) (PDMS, Sylgard 184, Distrelec, Switzerland) coated cell culture well plates. Cytocompatibility of AMP-CBP-FAM was assessed two-fold: i) in indirect contact by culturing NHDF on TCPS in AMP-CBP-FAM supplemented media (Supplementary Information) and ii) in direct contact by culturing NHDF on AMP-CBP-functionalised membranes. Relevant cell seeding densities were evaluated in preliminary tests. On AMP-CBP-FAM functionalised membranes, NHDF were seeded at a density of 100'000 cells per BC film (~200'000 cells·cm⁻²). The cell density was reduced to 20'000 cells per sample on electrospun CPU membranes (~40'000 cells·cm⁻²). Cell metabolic activity was assessed with alamarBlue on day one and day three according to the manufacturer's instructions. In brief, cells were incubated with a 10% v/v alamarBlue in phenol-red free DMEM solution for 2 hours. Subsequently, the reacted alamarBlue solution was transferred to a 96-well plate, fluorescence of three technical replicates was measured on a Mithras² Plate reader (Berthold Technologies,

Germany) at $\lambda_{\text{ex}}=540$ nm and $\lambda_{\text{em}}=580$ nm. Spectrometric values were background corrected (fluorescence intensity of alamarBlue on membranes without cells) and normalised to day 1 of cells cultured on the respective control group pristine BC, or pristine CPU. All experiments were accomplished in N=3 individual experiments with n=3 replicates in each group.

RGD-functionalised BC membranes were incubated in 1% bovine serum albumin (BSA) for 1 hour to passivate the substrate and reduce protein adsorption. NHDF were then seeded at a density of 20'000 cells per membrane in serum free DMEM and let to adhere for 24 hours. A second batch was supplemented with serum after these 24 hours and cultured for an additional day. Cell metabolic activity was assessed on day 1 and day 2 as described above, in N=3 individual experiments with n=3 replicates each. For immunofluorescence staining, cells were washed in PBS, fixed in 4% v/v formaldehyde in PBS, and permeabilised in 0.1% v/v Triton-X 100. Actin was stained with Alexa-546® conjugated phalloidin (Life Technologies, Thermo Fisher Scientific, Switzerland, $\sim 0.03 \mu\text{M}$ in PBS), nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Life Technologies, Thermo Fisher Scientific, Switzerland; $1 \mu\text{g}\cdot\text{mL}^{-1}$ in PBS). Images were acquired on a Confocal Laser Scanning Microscopy (CLSM, LSM780, Carl Zeiss AG, Switzerland). Cell spreading on RGD-functionalised membranes was quantified based on fluorescent images. The cell body area, positively stained with phalloidin, was quantified automatically with ImageJ and presented relative to the cell number (based on automatic nuclei counts). Results are mean values of 8 images per condition.

Statistical Analysis All numerical results are reported as mean $\pm$ standard deviation (SD). For the relatively small sample number in the here reported assays, normal distribution of the residuals could not be assumed and non-parametric tests were applied. Sample size and data processing is reported for each experiment under the respective paragraph. Statistical significance

among more than two groups was assessed in a Kruskal-Wallis test, followed by pairwise comparison in a Mann-Whitney test. Differences among the means were accepted as significant for $P < 0.05$. Statistical analysis was performed with GraphPad.

Supporting Information

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

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