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Antimicrobial Peptide Modification of Biomaterials Using Supramolecular Additives

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ABSTRACT: Biomaterials based on non-active polymers functionalized with antimicrobial agents by covalent modification or mixing are currently regarded as high potential solutions to prevent biomaterial associated infections that are major causes of biomedical device failure. Herewith a strategy is proposed in which antimicrobial materials are prepared by simply mixing-and-matching of ureido-pyrimidinone (UPy) based supramolecular polymers with antimicrobial peptides (AMPs) modified with the same UPy-moiety. The N-terminus of the AMPs was coupled in solution to an UPy-carboxylic acid synthon resulting in formation of a new amidic bond. The UPy-functionalization of the AMPs did not affect their secondary structure, as proved by circular dichroism spectroscopy. The antimicrobial activity of the UPy-AMPs in solution was also retained. In addition, the

incorporation of UPy-AMPs into an UPy-polymer was stable and the final material was biocompatible. The addition of 4 mol % of UPy-AMPs in the UPy-polymer material protected against colonization by *Escherichia coli*, and methicillin-sensitive and -resistant strains of *Staphylococcus aureus*. This modular approach enables a stable but dynamic incorporation of the antimicrobial agents, allowing at the same time for the possibility to change the nature of the polymer, as well as the use of AMPs with different activity spectra. © 2018 The Authors. Journal of Polymer Science Part A: Polymer Chemistry Published by Wiley Periodicals, Inc. J. Polym. Sci., Part A: Polym. Chem. **2018**, *56*, 1926–1934

KEYWORDS: antimicrobial peptides; biomaterial; biomaterial associated infections; supramolecular additive

INTRODUCTION Preventing biomaterial associated infections represents one of the main challenges for a new generation of biomaterials which can significantly improve the quality of human life.¹ The growing development of bacterial resistance to common antibiotics is pushing the academic and industrial research toward alternative solutions.² One strategy to achieve this goal is the use of devices made with materials that are able to kill bacteria or to inhibit microbial growth on their surface or in the surrounding environment. In this respect, antimicrobial polymers are receiving increasing interest because of their high tunability in terms of physico-chemical properties, efficacy, resistance, and prolonged life time.³ These polymers either possess inherent antimicrobial activity or are made antimicrobial through

incorporation of organic or inorganic active agents. Examples of antimicrobial polymers belonging to the first class are chitosan,⁴ poly-L-lysine,⁵ quaternary ammonium polymers (e.g., cationic amphiphilic polycarbonates⁶), polyguanidines,⁷ polyethylenimine,⁸ and halogen containing polymers (e.g., N-halamine).⁹ Polymers possessing negligible or no antimicrobial activity can be modified to induce biocidal activity by addition of active antimicrobial agents. These can either be incorporated by simple mixing or chemically bound to the polymer.¹⁰ Silver and copper nanoparticles and titanium zinc oxides are widely used as antimicrobial additives as they exhibit a broad spectrum of antimicrobial activity and are usually blended in the polymers.^{10–12} Organic antimicrobial agents can be either incorporated or chemically bound to

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polymers. Non-covalent incorporation of antibiotics such as triclosan or chlorhexidine is mainly used to prepare releasing systems.^{13–15} Ampicillin and gentamicin have also been chemically bound to several polymers.¹⁰

Recent studies are focusing on the incorporation of antimicrobial peptides (AMPs) into polymeric materials through covalent or physical incorporation.^{16–19} AMPs are currently the most promising antimicrobial agents, mainly because of their broad spectrum of action, high selectivity toward bacterial cells compared to mammalian cells, and low risk to promote bacterial resistance.^{20–23}

Three peptide sequences, SYM11KK (KKFPWWPFKK),²⁴ L₉K₆ (LKLLKLLKLLKLL),^{25–27} and Lasioglossin III (LASIO III; VNWKILGKIHKVVK),^{19,28,29} have been selected based on their broad range activity against both Gram-positive and Gram-negative bacteria and their activity *in vivo*. Moreover, SYM11KK and LASIO III have shown selectivity between mammalian cells and bacterial cells. The SYM11KK is a symmetric Lys-substituted analog of tritriptin, a peptide belonging to a class of evolutionary conserved AMPs present in all mammals namely the *cathelicidin* family. This peptide showed a higher cell selectivity index in solution as compared to tritriptin, that in turn proved to be active also after covalent immobilization onto TentaGel S resin.³⁰ The L₉K₆ peptide is an α -helical lytic peptide derived from *Magainin*, a class of natural AMPs.²⁷ LASIO III has very recently drawn the attention of scientists working on the development of antimicrobial coatings for medical devices as it is very active, even at physiologically relevant salt concentration, and is very selective. A LASIO III N-terminus cysteine modified variant was successfully used as antimicrobial coating on a commercial silicone catheter.¹⁹

Importantly, we have shown that bioactivity can be easily incorporated in supramolecular elastomeric materials via supramolecular interaction.³¹ We have shown that cell adhesion can be induced,^{31,32} and anti-fouling activity can be introduced.^{33–35} As a base polymeric material, our golden standard and well characterized, ureido-pyrimidinone (UPy)-functionalized polycaprolactone (PCL_{2k}-diUPy) is applied for the incorporation of the UPy-peptides (Fig. 1). In previous research we have shown that this PCL_{2k}-diUPy forms well-defined nanofibrous structures at the surface.^{36,37} Intercalation of UPy-peptides into these fibers has shown to lead to enhanced cell adhesion.³⁷

In this context, our research aims to develop a new class of biodegradable antimicrobial materials based on supramolecular interactions, for cardiovascular, renal, and urological applications. A modular approach based on supramolecular building blocks enables to easily prepare instructive, biodegradable scaffolds by simply mixing polymers, and peptides functionalized with UPy-moieties.³¹ Using this strategy, we propose it is possible to incorporate UPy-modified AMP additives into UPy-based polymers to prepare supramolecular anti-infective biomaterials. Here, we show the synthesis of these UPy-AMPs, the *in vitro* antimicrobial activity and biocompatibility of these supramolecular antimicrobial biomaterials.

EXPERIMENTAL

Synthesis of UPy-Modified Antimicrobial Peptides

Materials and Instrumentation

9-Fluorenylmethoxycarbonyl-(Fmoc-)Sieber amide resin (Iris Biotech) Fmoc-protected amino acids, Oxyma Pure and *o*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium-hexafluorophosphate (HATU) were purchased from Merck. All other chemicals for synthesis were purchased from Sigma-Aldrich and used without purification. The UPy-COOH synthon was synthesized as previously described.³⁸

Analytical reversed phase high pressure liquid chromatography–mass spectrometry was performed on a system consisting of the following components: Shimadzu SCL-10A VP system controller, Shimadzu LC-10AD VP liquid chromatography pumps (with an Alltima C18 3u (50 mm × 2.1 mm) reversed phase column and gradients of water–acetonitrile supplemented with 0.1% (v/v) formic acid), a Shimadzu DGU 20A3 prominence degasser, a Thermo Finnigan surveyor auto sampler, a Thermo Finnigan surveyor PDA detector, and a Thermo Scientific LCQ Fleet. Preparative reversed phase high pressure liquid chromatography (prep-RP-HPLC) was performed on a system consisting of the following components: Shimadzu LC-8A preparative liquid chromatography pumps with an Alltima C18 5u (125 × 20 mm) preparative reversed phase column and gradients of water–acetonitrile, supplemented with 0.1% (v/v) trifluoroacetic acid (TFA), a Shimadzu CBM-20A prominence communications bus module and Shimadzu DGU 20A3 prominence degasser, Thermo Finnigan Surveyor PDA detector, Finnigan LCQ Deca XP and Thermo Finnigan surveyor auto sampler.

Synthesis of Antimicrobial Peptides

The AMPs (Supporting Information Table S1) were synthesized in Dimethylformamide (DMF) by manual Fmoc-based solid phase peptide synthesis. The acid labile Sieber resin was used as solid support (0.61 mmol/g, 200 μ mol scale) and Oxyma Pure® (3 eq), in combination with *N,N'*-diisopropylcarbodiimide (DIPCDI; 3 eq), was used as coupling system. Fmoc deprotection of the resin and of each amino acid was achieved using a 20% (v/v) solution of piperidine in DMF. Kaiser test was performed to assess each coupling was successful. For some amino acids a second coupling was performed. To help the swelling of the resin during difficult couplings, Triton-X100 was added to the coupling mixture (40 μ L in 4 mL). Mini-cleavage and liquid chromatography mass spectrometry (LC/MS) were performed every 5 amino acids to check the synthesis. After successful synthesis of the whole peptide, the protected AMPs were cleaved from the resin using 1% (v/v) TFA in Dichloromethane (4 mL × 20, 2 min each). The solutions were collected in water, after which the organic phases were extracted and dried at the rotavapor.

In Solution Coupling of the AMPs with UPy-COOH

The coupling of the protected antimicrobial peptides to the UPy-synthon (Supporting Information Fig. S1) was

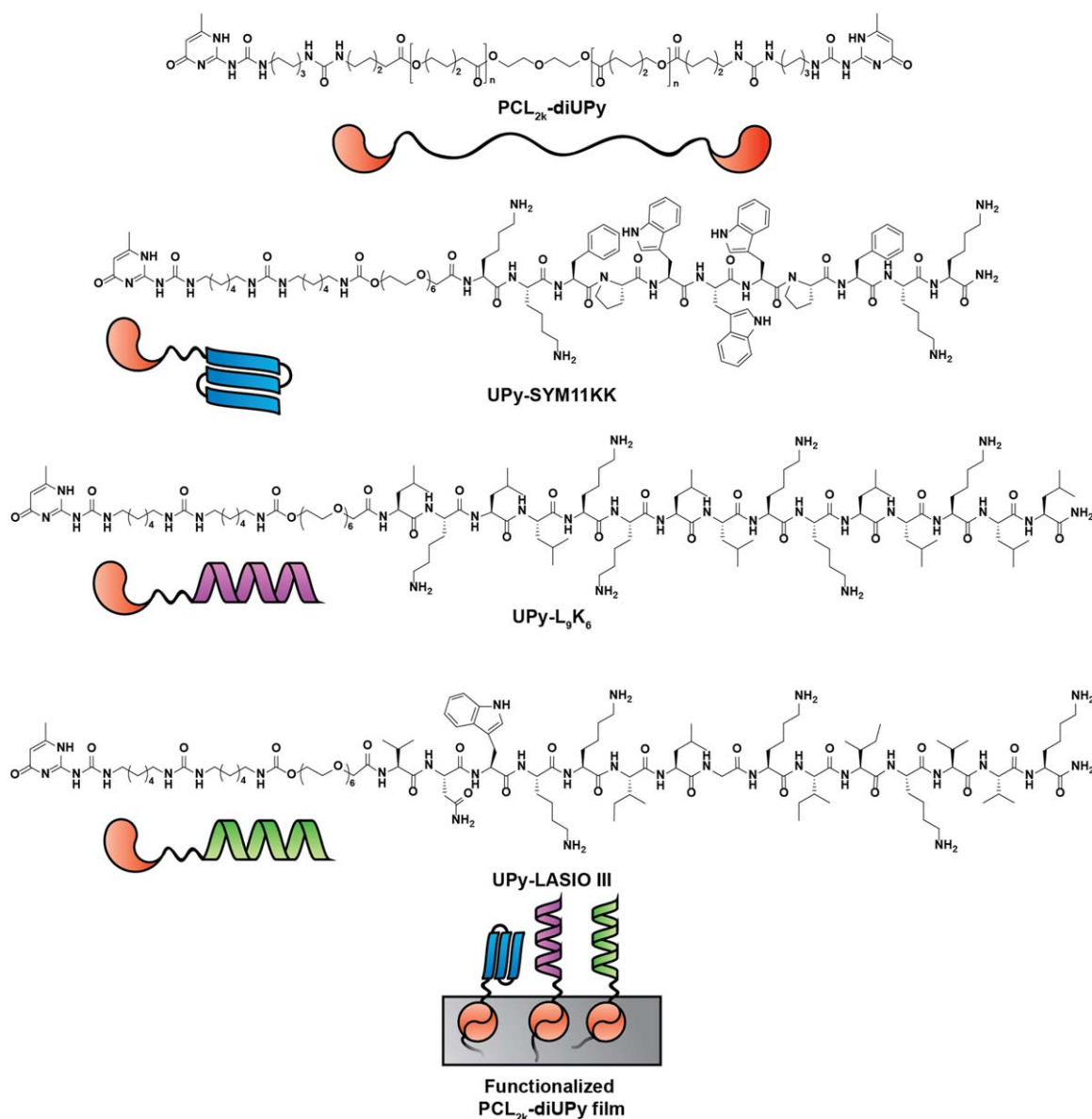


FIGURE 1 Schematic representations and molecular structures of the UPy-polymer and UPy-functionalized antimicrobial peptides SYM11KK, L₉K₆ and LASIO III. [Color figure can be viewed at wileyonlinelibrary.com]

performed in solution. First the UPy-COOH (1.3 eq) was pre-activated for 30 min with HATU (1.1 eq) and *N,N*-diisopropylethylamine (3 eq). Then a solution of the protected peptide (150 $\mu\text{mol}/10\text{ mL}$ dimethylacetamide) was added and the reaction mixture was stirred for 3 h at room temperature. Successful coupling was confirmed by LC/MS. The crude of the reaction was then concentrated at the rotavapor and precipitated in cold diethylether/hexane (8/2, 100 mL). The solid was recovered by centrifugation (3000 rpm/10 min) and dissolved in water/acetonitrile (1/4) to be freeze dried. The pellet was then dissolved in TFA/triisopropylsilane/water (95/2.5/2.5) for deprotection (2.5 h) at room temperature. TFA was evaporated under N₂ flow, the product was precipitated using cold diethylether/hexane (8/2, 100 mL),

centrifuged (3000 rpm/10 min), dissolved in water/acetonitrile (4/1), and freeze dried. The deprotected UPy-AMP was finally purified *via* preparative HPLC.

Circular Dichroism (CD) Spectroscopy

The secondary structure of the UPy-AMPs in the presence and absence of sodium dodecyl sulphate (SDS; 30 mM; Sigma-Aldrich) in water was investigated using a J-815 spectropolarimeter (Jasco, Inc.). The UPy-AMPs concentration for CD analysis was fixed at 50 μM in water. The spectra were recorded from 190 to 250 nm at 25 °C, using a 0.01 cm path length quartz cuvette. The bandwidth was fixed at 3 nm and the spectra were obtained by average over three scans.

Polymer Film Preparation

Films were prepared by drop-casting 50 μL of a 5 w/v % of PCL_{2k}-diUPy solution in hexafluoroisopropanol (HFIP; Fluorochem) with 0, 0.5, 1, 2, and 4 mol % AMPs or UPy-AMPs on glass coverslips of 14 or 15 mm \varnothing (VWR). The samples were allowed to dry overnight to afford homogeneously distributed transparent films.

Surface Hydrophobicity

To assess the effect of AMP and UPy-AMP incorporation, and subsequent water annealing of the samples on the surface hydrophobicity, water contact angles were determined of PCL_{2k}-diUPy films containing 0, 0.5, 1, 2, and 4 mol % UPy-AMP. Samples were left dry, or incubated with MilliQ for 24 h, subsequently water was aspirated and dried for 19 h at 37 °C *in vacuo*. A 4 μL droplet of MilliQ was dropped on the samples, and 5 sec after placement water contact angles were determined with Contact angle system OCA and SCA 202 v4.1.13 build 1020 software (Dataphysics Instruments). The experiment was performed on two different areas of each sample, with three or five replicates per condition.

Leakage of UPy-AMPs and AMPs from PCL_{2k}-diUPy Films

The stability of the incorporation of the UPy-AMPs and unmodified AMPs is investigated through a leakage experiment. Films containing 0 or 4 mol % of UPy-AMPs or AMPs were prepared as previously discussed. The samples were incubated with water at 37 °C for 24 h. The solutions were enriched with 0.1% (v/v) formic acid (Sigma-Aldrich) and analyzed using LC/MS. A calibration curve was drawn by fitting data resulting from the analysis of standard samples in water (enriched with 0.1% (v/v) of formic acid) at different concentrations (0.1, 0.05, 0.01, 0.001, 0.0001 mg/mL). The surface area of the corresponding peak (in the total ion count) was calculated with the ICIS algorithm. All the experiments and measurements were performed in triplicate.

Antimicrobial Activity

Minimal Inhibitory Concentration (MIC_{RPMI}) and 99.9% Lethal Concentration (LC99.9)

The antimicrobial activity in solution of the UPy-AMPs and unmodified AMPs against *Escherichia coli* (*E. coli*; ATCC 8937) as a representative Gram-negative bacterial species, and Methicillin-Sensitive (MSSA; RN 4220³⁹) and -Resistant strains of *Staphylococcus aureus* (MRSA; AMC 201^{40,41}) as representatives of Gram-positive bacterial species was evaluated through MIC and LC99.9 assays. Overnight cultures in Tryptic soy broth (TSB) were diluted 50-fold in fresh TSB and cultured for 2 h at 37 °C. Bacteria were washed twice with 10 mM phosphate buffer, pH 7.0, with 1% (v/v) TSB (PT). The optical density was measured at 620 nm (OD_{620}) and the concentration of the bacteria was adjusted to 1×10^7 colony forming unit (CFU)/mL in PT, based on an established relationship between the number of CFU and the OD_{620} . Different solutions of AMPs and UPy-AMPs (final concentrations of 30–0.06 μM) were prepared by twofold dilution in Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich, w/L-glutamine, w/o sodium bicarbonate) in

a low protein binding 96 wells/plate (Costar Corning) and were incubated at 37 °C with bacteria (final concentration of 1×10^6 CFU/mL). The minimal concentration inhibiting the bacterial growth in RPMI (MIC_{RPMI}) was determined after 24 h of incubation as the lowest concentration (in μM) at which there was no visible bacterial growth. After plating 10 μL aliquots of the undiluted solutions on blood agar plates and 37 °C overnight incubation, the lowest concentration of AMP (in μM) that killed at least 99.9% of the inoculum (LC99.9) after 24 h was determined by colony counting. All incubations were performed in duplicate.

Test for Antimicrobial Activity of Plastics

Films of PCL_{2k}-diUPy with 0, 0.5, 1, 2, or 4 mol % UPy-AMPs were prepared, as previously described, on glass coverslips of 15 mm \varnothing , after which the films were sterilized by irradiation with UV light for 30 min. Test for antimicrobial activity of plastics (JIS Z 2801) was performed to evaluate the antimicrobial activity of the functionalized polymer films against same bacterial strains as described before. Bacterial cultures were prepared as previously described. Fifty microliters of the bacteria suspension were pipetted on the functionalized polymer films and covered with sterilized parafilm disks (13 mm \varnothing). The samples were incubated at 37 °C for 24 h, then 2 mL of phosphate buffer saline (PBS) were added to each sample followed by sonication for 5 min and vortexing for 15 sec. This procedure does not affect bacteria viability.⁴² After 10-fold serial dilution in PBS, 10 μL aliquots of the undiluted and diluted suspensions were pipetted on blood agar plates. The plates were incubated overnight at 37 °C and then survival was determined by colony counting. Experiments were performed in triplicates.

Toxicity for Eukaryotic Cells

Cell Culture

Human kidney 2 cells (HK-2; ATCC) were cultured in Dulbecco's Modified Eagle Medium with high glucose and pyruvate (Life Technologies), supplemented with 10% (v/v) fetal bovine serum (Lonza) and 1% (v/v) penicillin and streptomycin (Life Technologies) under standard culturing conditions at 37 °C and 5% CO₂. PCL_{2k}-diUPy films were fabricated, as described before, with 0 and 4 mol % AMPs or UPy-AMPs added, on a 14 mm \varnothing glass coverslips. All coverslips were mounted in custom holders to prevent possible film detachment from the glass surfaces during cell culture. The holders consist out of a 12-well Transwell insert (Corning) without membrane and a custom ring which can click on the insert. The constructs were subjected to UV-sterilization for 15 min after which cells were seeded at a density of 15×10^3 cells/cm² on the coverslips. Toxicity was assessed by mitochondrial activity, adhering cell number, and cell viability which were determined after 24 h and 7 days of culture.

Mitochondrial Activity

To evaluate mitochondrial activity, culture medium was enriched with 44 μM of Resazurin (Sigma-Aldrich) and incubated on the samples for 3 h at 37 °C. Per sample two

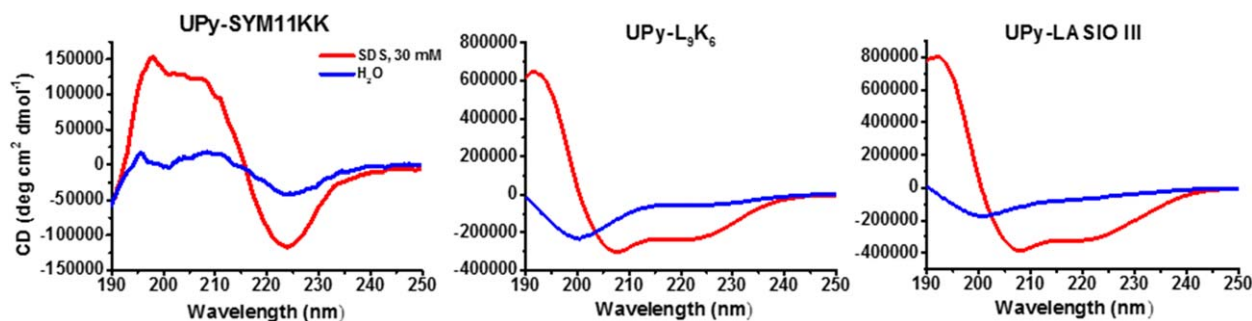


FIGURE 2 Folding of the UPy-antimicrobial peptides. CD spectra of UPy-AMPs (50 μ M) in water (H_2O ; blue line) or 30 mM sodium dodecyl sulfate (SDS; red line) at 25 $^\circ\text{C}$. [Color figure can be viewed at wileyonlinelibrary.com]

aliquots of 200 μL resazurin enriched medium were transferred to a black 96-well plate (Thermo Fisher Scientific) and fluorescence was measured ($\lambda_{\text{ex}} = 550$, $\lambda_{\text{em}} = 584$ nm) with a SynergyTM HT plate reader and Gen5TM software (Bio-Tek Instruments, Inc.). Resulting fluorescence was corrected for background fluorescence and was normalized to PCL_{2k}-diUPy. Experiments were performed with 5 replicates per condition.

Cell Staining and Counting

Following this non-invasive assay, cells were washed with PBS and fixated with 3.7% (v/v) formaldehyde solution (Merck) + 0.5% (v/v) Triton X-100 v/v (Merck) in PBS for 15 min at room temperature (RT). Samples were subsequently washed with PBS and stained for the actin cytoskeleton and nucleus with phalloidin-Atto 488 (Sigma-Aldrich, dilution 1:300) in 0.05% Triton X-100 (v/v) in PBS for 45 min at RT, during the last 10 min 4'-6 diamidino-2-phenylindole (DAPI; 0.1 $\mu\text{g}/\text{mL}$; Sigma-Aldrich) was added. Samples were finally washed with PBS and mounted with mowiol (Sigma-Aldrich). Images were acquired in a quincunx pattern to estimate cell distribution over the whole sample, with the use of a Zeiss Axiovert 200M microscope and AxioVision software (Zeiss). Nuclei were counted with the help of ImageJ 1.48v (National Institutes of Health, USA).

Cell Viability

Cell viability was investigated with a life/dead assay. Samples were washed with PBS and incubated with 10 μM propidium iodide (Thermo Fisher Scientific) and 10 μM Calcein AM (Sigma-Aldrich) or Cell TrackerTM Green CMFDA (Thermo Fisher Scientific) in serum free medium for 20 min at 37 $^\circ\text{C}$. Afterwards the medium replaced with completed culture medium. Cells were imaged with Zeiss Axiovert 200M microscope and AxioVision software. Experiments were performed with 3–5 replicates per condition.

Statistical Analysis

Statistical analysis on samples was performed with the help of Prism 5 (GraphPad Software, Inc.). Sample size $n \geq 5$ were subjected to Kolmogorov–Smirnov test as to assess normality. All samples showed normal distribution. Subsequently a one-way ANOVA was performed with Dunnett post-test

comparing PCL_{2k}-diUPy versus all other conditions. Significance was assumed when $\alpha < 0.05$.

RESULTS AND DISCUSSION

Synthesis of UPy-Modified Antimicrobial Peptides

To stably functionalize the supramolecular biomaterials with AMPs, the selected peptide sequences were modified with an UPy-unit at the N-terminus via an UPy-carboxylic acid synthon (Supporting Information Fig. S1).³⁸ Due to the amphipathic nature of the cationic AMPs, the coupling of the peptides on the resin to the carboxylic group of the UPy-synthon proved to be quite challenging, and the conditions previously developed in our group failed to afford the desired product. To overcome this problem, the peptides were manually synthesized on an acid labile Sieber Amide resin. The mild conditions used to cleave the peptide chains from this resin allow to recover fully protected peptides that were subsequently reacted in solution with the UPy-COOH synthon to afford the desired UPy-AMPs (Fig. 1) with yields ranging from 50% to 60% [Supporting Information Fig. S2(A–C)].

Analysis of UPy-AMPs Secondary Structure Using CD Spectroscopy

It is well known that a correct folding of AMPs when in proximity of the bacterial membrane, is crucial to their antimicrobial activity. Therefore, the secondary structure of our UPy-modified peptides was investigated using CD spectroscopy in water and in SDS (30 mM),⁴³ as this mimics negatively charged membranes. CD analysis of UPy-AMPs showed that the UPy-functionalization of the peptide chain does not affect the correct folding of the peptide, as the results are in agreement with those previously published for the unmodified peptides. Both UPy-L₉K₆ and UPy-LASIO III retained a α -helical^{25,28} structure and UPy-SYM11KK adopted a β -sheet structure²⁴ (Fig. 2).

Antimicrobial Activity

To assess whether the UPy-functionalization influences the antimicrobial activity of the AMPs, both the UPy-AMPs and the corresponding unmodified AMPs were tested *in vitro* against *E. coli*, methicillin-sensitive (MSSA) and methicillin-resistant (MRSA) strains of *S. aureus*. Intriguingly, the UPy-functionalization of the three selected antimicrobial peptides did not cause their inactivation and the antimicrobial activity

TABLE 1 MIC_{RPMI}^a and LC99.9^b of AMPs and UPy-Functionalized AMPs

Peptide	<i>E. coli</i>		MSSA		MRSA	
	MIC _{RPMI} ^a	LC99.9 ^b	MIC _{RPMI} ^a	LC99.9 ^b	MIC _{RPMI} ^a	LC99.9 ^b
SYM11KK	15	15	15	30	30	30
UPy-SYM11KK	30	30	15	15–30	30	30
L ₉ K ₆	3.7	3.7	1.9	1.9–3.7	3.7	3.7
UPy-L ₉ K ₆	1.9–3.7	1.9–3.7	3.7	3.7	7.5	7.5
LASIO III	1.9–3.7	1.9–3.7	1.9	3.7	3.7	3.7
UPy-LASIO III	7.5	7.5	3.7–7.5	7.5	7.5	7.5

^a Defined as the lowest concentration of peptide (μM) at which there is no visible bacterial growth after 24 h.

^b Defined as the lowest concentration of peptide (μM) that killed 99.9% of an inoculum of 1×10^6 CFU/mL in 24 h. All incubations were performed in duplicate.

of the peptides was overall retained for both the concentration of AMP inhibiting the bacterial growth, that is MIC_{RPMI}, and the LC99.9 (Table 1). In particular, the UPy-AMPs are either equally active compared to the corresponding unmodified peptide, or slightly less active.

In the case of the L₉K₆ peptide, the UPy-modified peptide is slightly more active toward *E. coli* compared to the unmodified AMP. However, in general a twofold increase in both the MIC_{RPMI} and LC99.9 occurs upon functionalization of the AMP with the UPy-moiety. The MIC_{RPMI} and LC99.9 values are bacterial species and strain independent. All three UPy-peptides are equally active against Gram-positive and Gram-negative bacteria, which indicates that the UPy-functionalization does not have any effect on the tested activity spectrum of the selected peptides.

Leakage of UPy-AMPs and AMPs from Polymer Films

The stability of the UPy-AMPs and unmodified AMPs incorporation in films of PCL_{2k}-diUPy was investigated through leakage experiments. PCL_{2k}-diUPy films containing 4 mol % of peptides were incubated with water at 37 °C for 24 h.

Surface hydrophobicity strongly increased for L₉K₆, UPy-L₉K₆, LASIO III and UPy-LASIO III, and slightly decreased for SYM11KK and UPy-SYM11KK after water annealing, as shown with water contact angle measurements [Fig. 3(A) and Supporting Information Fig. S3]. The concentration of potential released peptide in the water was determined using LC/MS. All the UPy-modified AMPs are stably incorporated in the films whereas the unmodified LASIO III and L₉K₆ peptides are released from the samples and up to 3% of peptide originally incorporated in the PCL_{2k}-diUPy films is detected after the 24 h incubation [Fig. 3(B)]. Unmodified SYM11KK and UPy-SYM11KK both did not show release from the films. The change in surface hydrophobicity for the bioactive films after annealing, displaying no release of the peptides, might indicate a rearrangement of the peptides at the surface upon water annealing [Fig. 3(A)].

Antimicrobial Activity of Films

Polymer films with different concentrations of UPy-AMPs were prepared and tested *in vitro* against three bacterial strains. The UPy-SYM11KK peptide was not active against all

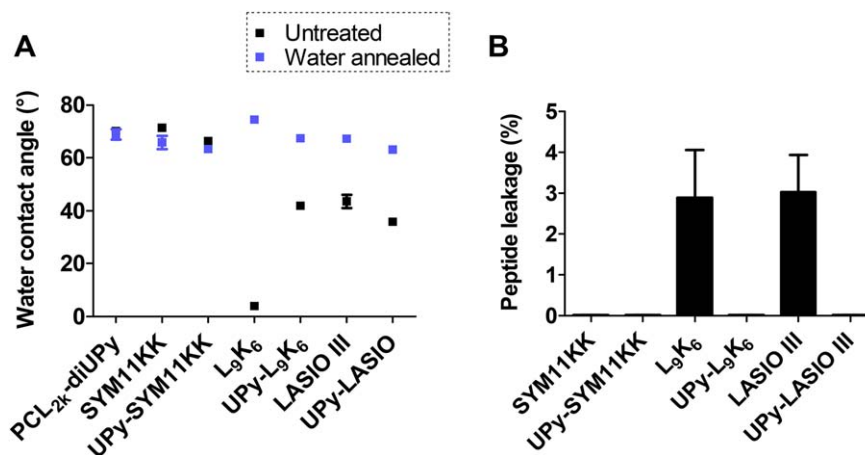


FIGURE 3 Peptide leakage from polymer films. PCL_{2k}-diUPy films containing 4 mol % of UPy-AMPs or AMPs in PCL_{2k}-diUPy which were left dry or have been incubated with MilliQ water for 24 h at 37 °C. (A) Depicting changes in surface hydrophobicity. (B) Percentage of peptide in the water compared to initial amount present in the film. Error bars represent standard deviation of mean for $n = 3$. [Color figure can be viewed at wileyonlinelibrary.com]

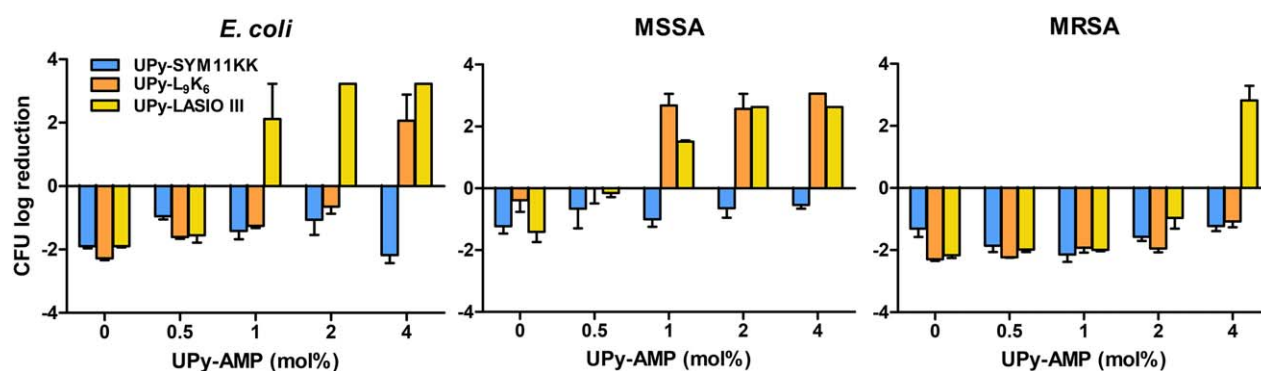


FIGURE 4 Antimicrobial activity of UPy-AMP functionalized PCL_{2k}-diUPy films. Log reduction in colony forming units (CFU) of *E. coli*, MSSA and MRSA inoculated on PCL_{2k}-diUPy films containing 0, 0.5, 1, 2, or 4 mol % of UPy-AMP for 24 h. Error bars represent standard deviation of mean for $n = 3$. [Color figure can be viewed at wileyonlinelibrary.com]

the investigated bacterial strains at any of the concentrations that were screened (Fig. 4). In agreement, the SYM11KK peptide and its UPy-analog showed the lowest antimicrobial activity among the tested peptides in solution (Table 1). Moreover, findings by other research groups show that surface immobilization can decrease AMP activity, such as for tritrypticin.^{30,44,45} Taken together this indicates that the local concentration of exposed UPy-SYM11KK at the biomaterial surface is below active concentrations which prevent bacterial growth on the material.

UPy-L₉K₆ and UPy-LASIO III peptides retained activity against both *E. coli* and MSSA upon incorporation in PCL_{2k}-diUPy films (Fig. 4). Both these UPy-peptides showed a concentration-dependent activity against the two bacterial strains tested. In particular, the samples containing 4 mol % of the two UPy-peptides killed the complete MSSA inoculum. Regarding the antimicrobial activity against *E. coli*, the UPy-LASIO III peptide is more active compared to the UPy-L₉K₆, showing complete killing of the unoculum at a concentration of 2 mol %, whereas even the 4 mol % of UPy-L₉K₆ was able to induce a threefold reduction of the number of CFU against *E. coli*. Both UPy-L₉K₆ and UPy-LASIO III showed similar potency against MRSA in solution, however after incorporation into polymer films only UPy-LASIO III was capable to kill the majority of inoculated MRSA (Fig. 4). Although MSSA could already be affected when 1 mol % of UPy-LASIO III was incorporated the MRSA required 4 mol % to be affected by the same UPy-peptide. The observed increased resistance of MRSA compared to MSSA corresponds with resistances found for UPy-AMPs in solution (Table 1).

Overall, these results show that it is possible to use the AMP additives in combination with UPy-based material to successfully make supramolecular biomaterials with antimicrobial properties.

Eukaryotic Cell Cytotoxicity

It remains unknown whether AMP incorporation into polymer films results in similar toxicity for eukaryotic cells as in

solution. Therefore, HK-2 cells were cultured on PCL_{2k}-diUPy films containing 4 mol % (UPy)-AMPs. After 24 h and 7 days, toxicity was assessed through investigation of cell morphology, metabolic activity, adherent cell numbers, and cell viability.

Despite that unmodified SYM11KK and LASIO III containing films showed comparable cell morphology [Fig. 5(A)] and viability (Supporting Information Fig. S4) with pristine films, a small reduction in metabolic activity was observed for both, compared to pristine PCL_{2k}-diUPy films [Fig. 5(B)]. Cells cultured on UPy-SYM11KK and UPy-LASIO III showed comparable cell morphology, spreading [Fig. 5(A)], metabolic activity [Fig. 5(B)], adherence [Fig. 5(C)], and viability (Supporting Information Fig. S4) as compared to pristine material. Seven-day culture revealed that possible initial effects were overcome as all read-outs for cytotoxicity were similar among SYM11KK, LASIO III, UPy-SYM11KK, and UPy-LASIO III compared to pristine PCL_{2k}-diUPy [Supporting Information Figs. S4 and S5(A-C)]. This is in line with previous research which showed no decrease in viability of cells in contact with AMP functionalized biomaterials.^{19,44}

Incorporation of both L₉K₆ and UPy-L₉K₆ lead to a drastic change in morphology, which appeared to be less spread [Fig. 5(A)], indicative of a stressed phenotype. Moreover, a significant reduction in cell metabolic activity [Fig. 5(B)] and adherence [Fig. 5(B)], and reduced viability (Supporting Information Fig. S4) was observed compared to pristine PCL_{2k}-diUPy on L₉K₆ as well as UPy-L₉K₆ containing films after 24 h of culturing. No cells were present on L₉K₆ containing films after 7 days of culture [Supporting Information Fig. S5(A,C)], accounting for no metabolic activity [Supporting Information Fig. S5(B)]. However, on UPy-L₉K₆ films slow ingrowth from the sides was observed, and intriguingly albeit lower cell numbers metabolic activity was similar to unmodified PCL_{2k}-diUPy films.

The effects of L₉K₆ and LASIO III containing films on cells are likely related to the release profile of these peptides. Following these release profiles we assume a concentration for both peptides up to 3.0 μ M during culture for the first 24 h.

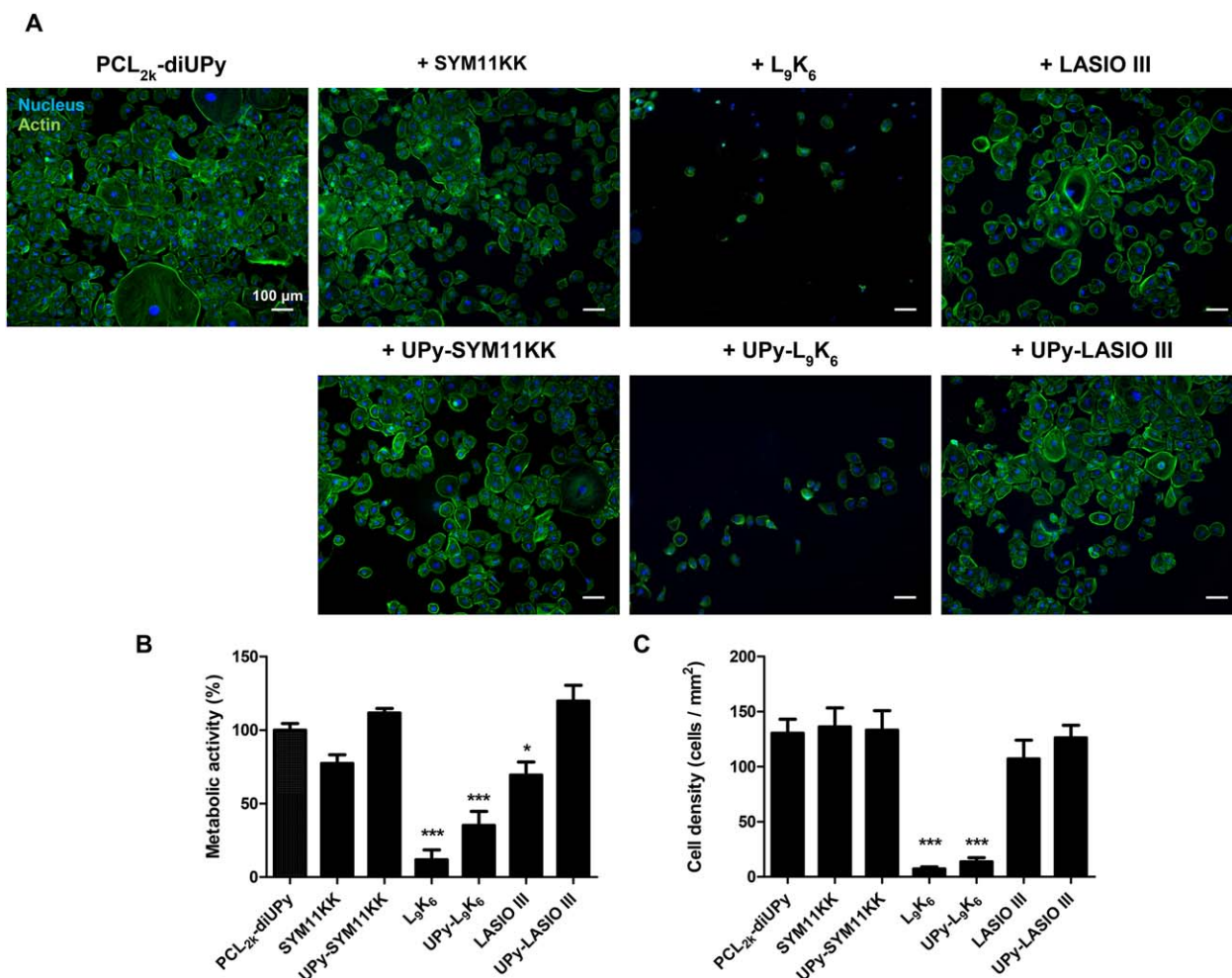


FIGURE 5 Cytotoxic activity of UPy-functionalized and unmodified AMPs on eukaryotic cells. (A) Human kidney-2 cells (HK-2) cultured on PCL_{2k}-diUPy films containing 0 or 4 mol % of UPy-functionalized and unmodified AMPs for 24 h. Cells were stained for the nucleus (blue) and actin cytoskeleton (green). (B) Metabolic activity was determined through resazurin conversion. Results were normalized to PCL_{2k}-diUPy. (C) The number of adhered HK-2 was determined at five different locations per substrate through nuclei counting. *n* = 5 per condition, mean ± SEM are depicted. **p* < 0.05 and ****p* < 0.001 compared to PCL_{2k}-diUPy. [Color figure can be viewed at wileyonlinelibrary.com]

L₉K₆ is known to be toxic for eukaryotic cells with an IC₅₀ of 0.6–1.3 μM, well above the released concentration.²⁷ The smaller effect of LASIO III on cells is due to its lower toxicity (IC₅₀ 4.5–15 μM).²⁸ The peptide UPy-modification prevents peptide release, thereby reaching non-toxic concentrations in the culture medium. We hypothesize that the declining toxicity seen on UPy-L₉K₆ is due to either degradation or surface rearrangement. In future work the toxicity of UPy-L₉K₆ containing biomaterials can be reduced by incorporating d-Lysine into the peptide sequence, which has been shown to increase antibiotic effects and decrease eukaryotic cell cytotoxicity.^{25,26}

CONCLUSIONS

Development of a multi-functional antimicrobial supramolecular polymeric material is shown to be able to induce cell adhesion, and to have antimicrobial properties. This

approach might be the first step toward the reduction of implant rejection due to infection via antimicrobial supramolecular elastomeric materials.

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