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Full Paper

Crosslinked platform coatings incorporating bioactive signals for the control of biointerfacial interactions

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Control over biointerfacial interactions on material surfaces is of significant interest in many biomedical applications and extends from the modulation of protein adsorption and cellular responses to the inhibition of bacterial attachment and biofilm formation. Effective control over biointerfaces is best achieved by reducing non-specific interactions on the surface while also displaying specific bioactive signals. We have developed a poly(ethylene glycol) (PEG)-based multifunctional coating that provides effective reduction of protein fouling while enabling covalent immobilisation of peptides in a one or two-step manner. The highly protein resistant properties of the coating, synthesised via the crosslinking of PEG diepoxide and diaminopropane, were confirmed via Europium-labelled fibronectin adsorption and cell attachment assays. The ability to covalently incorporate bioactive signals was demonstrated using the cyclic peptides cRGDfK and cRADfK. L929 cells showed enhanced attachment on the biologically active cRGDfK containing surfaces, whilst the surface remained non-adhesive when the non-biologically active cRADfK peptide was immobilised. The crosslinked PEG-based coating also demonstrated excellent resistance towards *Staphylococcus aureus* attachment in a 48 hour biofilm assay, achieving a >96% reduction compared to the control surface. Additionally, incorporation of the antimicrobial peptide (AMP) melimine during coating formation further significantly decreased biofilm formation (>99%).

1. Introduction

The ability to control the biological response to material surfaces effectively is of interest in a wide range of biomedical applications, such as biosensors, cell culture tools and implantable medical devices. Biological responses that are of particular interest include non-specific interactions such as protein adsorption and biofouling, mammalian cellular responses such as attachment, growth and differentiation and also microbial attachment and biofilm formation.^[1] Many adverse clinical events including infection, inflammation and implant encapsulation, result from the undesired non-specific interactions between the device surface and the biological environment. These adverse interactions can limit the performance of devices and can result in their failure.^[2] Therefore, much of the work that has been carried out in the field of biomaterials has focused on the prevention of these non-specific interactions.^[3] The successful control over these non-specific interactions in turn then provides the opportunity to display bioactive signals on these surfaces that lead to exclusively specific biointerfacial interactions.

The concept of immobilising bioactive signals such as peptides to enable specific biointerfacial interactions on a background coating which otherwise prevents non-specific interactions is considered to have outstanding importance for a range of biomedical applications.^[3, 4] Cell attachment to a

material surface is mediated by proteins and other biomolecules that adsorb onto synthetic surfaces within seconds once the material comes in contact with body fluids or cell culture medium. Therefore non-specific protein adsorption and cell attachment are interrelated. A similar relationship exists between non-specific protein adsorption and the initial phase of microbial attachment.^[5] A range of polymer-based coating strategies have resulted in the effective reduction of non-specific protein/biomolecule adsorption and subsequent events such as cell attachment and microbial attachment.^[6] Particularly successful strategies have included high-density graft polymer coatings made from charge neutral hydrophilic polymers such as poly(ethylene glycol) (PEG), polyacrylamide, poly(2-hydroxypropyl methacrylamide) and poly(2-hydroxypropyl acrylamide), zwitterionic polymers such as poly(2-methacryloyloxyethyl phosphorylcholine) and poly(carboxybetaine methacrylate), and naturally occurring polymers such as dextran.^[7-9] However, crosslinked coatings are also effective in both preventing protein adsorption and attachment of various bacterial strains.^[10]^[11] In both cases, PEG-based coatings have received most of the attention due to its availability, biocompatibility and excellent low-fouling properties.^[12]

Specific bioactive signals that can enhance cell-material interactions when displayed on otherwise low-fouling coatings include in particular integrin-binding peptides or peptide mimetics.^[13] One of the most commonly studied group of bioactive peptides are those that contain the Arg-Gly-Asp (RGD) sequence, which is present in many extracellular matrix (ECM) proteins such as fibronectin, laminin and many collagens.^[14] It has been shown that this sequence is highly effective in promoting cell attachment of a wide range of cell types to a variety of substrate materials. However, linear RGD peptides lack selectivity in regard to the integrins they interact with.^[15] This has led to the emergence of semi-synthetic cyclic peptides incorporating the RGD sequence. These cyclic peptides show improved stability as well as higher affinities for specific integrins.^[14, 16-18] Immobilisation of peptides has also shown effectiveness in regard to combating bacterial colonisation on surfaces. These antimicrobial peptides (AMPs) act by disrupting bacterial cell membranes via direct interaction with

the lipid bilayer structure.^[19] While antibiotics target specific cellular activities and the numbers of antibiotic resistant bacteria are on the rise, the direct targeting of membranes by AMPs makes it very difficult for bacteria to develop complete resistance.^[19] Although there are concerns regarding adverse effects towards mammalian cells, synthetic AMPs such as melimine overcome these issues. Melimine, a synthetic peptide derived from portions of the cationic peptides melittin and protamine, demonstrates broad spectrum antimicrobial capabilities while being non-cytotoxic and non-haemolytic at therapeutic levels.^[20, 21] Additionally, *in vitro* and *in vivo* studies demonstrate that melimine retains its antimicrobial properties even when immobilised onto surfaces, significantly reducing bacterial colonisation and biofilm formation.^[22, 23]

Although both low fouling coatings and bactericidal coatings, including silver releasing surfaces, show promising outcomes in regard to reducing biofilm formation, there is broad agreement that multifunctional coatings that display multiple layers of defence, such as low-fouling characteristics combined with the display of AMPs, will lead to improved outcomes.^[1, 24] To date, various coatings have been prepared that not only provide low-fouling properties but also allow the display of bioactives. For example, Harbers *et al.* have described a novel PEG-based coating that can be applied onto a variety of surfaces in a one-step process while displaying functional groups that allow the immobilisation of peptides.^[25] The described coating was highly resistant to both mammalian and bacterial cell attachment while specific cell attachment could be induced with the immobilisation of the cell adhesive RGD-peptide. Fernandez *et al.* further explored the resistance of these coatings towards various clinical bacterial strains following exposure to bodily fluids such as urine, saliva and blood plasma.^[11] These coatings, known commercially as OptiChem[®], were able to significantly reduce the adhesion of these bacteria while remaining stable in body fluids for over a week. Other coatings focused on forming UV or thermally crosslinked PEG-based copolymer coatings with compositional flexibility on polymeric substrates.^[26] The crosslinked coatings also allowed peptide conjugation via 1,1-carbodiimidazole coupling which provide specific cell-material interactions.

Following from this work Schmitt *et al.* have described crosslinked PEG-containing coatings possessing azlactones which allow coupling of peptides via amide or thioester linkages following coating formation.^[27, 28] These defined surfaces may aid in understanding the influence of chemically defined surfaces in human mesenchymal stem cell behaviour.

These simple crosslinked coatings demonstrate excellent low-fouling properties and the available functional groups allow the immobilisation of bioactives such as peptides following coating formation. However these coatings require the synthesis of precursors, high temperatures (> 100 °C) and a vacuum environment for curing purposes.^[11, 25, 26] These conditions are detrimental for the stability of bioactive molecules such as peptides and therefore prohibit the incorporation of these molecules into the coating in a one-step manner. In contrast, we have investigated a PEG-based low-fouling platform coating applied easily using commercially available precursors that also allows the incorporation of highly specific biologically active molecules in a one-step manner during coating formation. Initially the synthetic cyclic cell adhesion mediator peptide cRGDfK was incorporated to demonstrate the ability of the PEG-based coating to support the covalent incorporation of peptides. Europium-labelled protein adsorption experiments and mammalian cell attachment studies demonstrated the low fouling properties of the coating while cell culture experiments also demonstrated that the specific cell-adhesive function of the cRGDfK was maintained after immobilisation. Separately, the antimicrobial peptide melimine was incorporated into the PEG based coating and subsequent bacterial biofilm assays demonstrated the excellent low-fouling properties of the coating as well as the activity of the AMP.

Overall, the simple and effective coating platform that we have established provides access to a broad range of biologically active surfaces and therefore it is expected that this platform will find applications in a number of biomedical devices where effective control over biointerfacial interactions is required.

2. Experimental Section

2.1. Allylamine plasma treatment

Allylamine plasma coatings were obtained using a custom built reactor according to a previously established procedure.^[29] Briefly, tissue culture polystyrene (TCPS) 48 well plates (Thermo-Scientific, Nunc, Roskilde, Denmark) were placed on the lower rectangular electrode of the plasma reactor in a glass chamber (height = 350 mm, diameter = 170 mm). The distance between the lower and upper electrodes was 150 mm. The allylamine monomer (Sigma-Aldrich, 98 %) was degassed 5 times prior to deposition. The deposition was carried out twice for 25 s with an initial pressure of 0.2 mbar (200 kHz, 20 W).

2.2. PEGDAP coating

Poly(ethylene glycol) diglycidyl ether (PEGDGE) (Sigma-Aldrich, 6,000 Da) and 1,3-diaminopropane (DAP) (Sigma-Aldrich, ≥ 99 %) were dissolved in MilliQ water to produce a 4 mM PEGDGE-based crosslinking solution (PEGDAP) (PEGDGE : DAP molar ratio of 3:1, with excess PEGDGE). The 4 mM stock solution was then diluted to afford solutions of 2 mM, 1 mM, 0.5 mM, 0.05 mM and 0.005 mM of the PEGDAP solution. 175 µL of the stock solution and each dilution thereof were pipetted into 4 wells of a freshly allylamine plasma treated 48 well TCPS plate. The plate was then covered with a lid and placed in a 60 °C shaker oven overnight. After drying of the solution (18 h), the plate was immersed in fresh MilliQ water and washed over 5 hours with 5 changes of fresh MilliQ water. Following washing, the now PEGDAP coated tissue culture polystyrene plate (abbreviated as ^XPEGDAP_{TCPS} where X denotes the concentration of PEGDAP coating solution in mM placed on the ALAPP coated TCPS) was placed in a laminar flow cabinet to dry and stored prior to use.

2.3. XPS Analysis

X-ray photoelectron spectroscopy (XPS) analysis was performed using an AXIS Ultra DLD spectrometer (Kratos Analytical Inc., Manchester, UK) with a monochromated Al K α source at a power of 54 W (4.5 kV $\times$ 12 mA) for survey spectra and 144 W (12 kV $\times$ 12 mA) for high resolution scans. The total pressure in the main vacuum chamber during analysis was typically between 10⁻⁹ and 10⁻⁸ mbar. Survey spectra were acquired at a pass energy of 160 eV. To obtain more detailed information about chemical structure, oxidation states etc., high resolution spectra were recorded from individual peaks at 40 eV pass energy (yielding a typical peak width for polymers of 1.0 eV).

Each specimen was analysed at an emission angle of 0° as measured from the surface normal. Assuming typical values for the electron attenuation length of relevant photoelectrons the XPS analysis depth (from which 95 % of the detected signal originates) ranges between 5 and 10 nm for a flat surface.

Data processing was performed using CasaXPS processing software version 2.3.15 (Casa Software Ltd., Teignmouth, UK). All elements present were identified from survey spectra. The atomic concentrations of the detected elements were calculated using integral peak intensities and the sensitivity factors supplied by the manufacturer.

2.4. Quantification of adsorbed proteins

The labelling of fibronectin with Europium and the subsequent protein adsorption assay were performed as previously described,^[30] albeit fibronectin was used instead of human serum albumin. Briefly, 1 nmol fibronectin from human serum (Sigma-Aldrich) in 100 μ L of 0.1 M bicarbonate buffer

(pH 9.3) was treated with a 15-fold excess of Eu-labelling reagent (Delfia Eu-N1 ITC chelate, Perkin Elmer) at 4 °C for 18 h. Labelled fibronectin was purified via dialysis. Protein adsorption assay was performed with 10 % FBS (fetal bovine serum) in PBS (phosphate buffered saline) supplemented with 0.3 µg/mL Eu-labelled fibronectin (approximately 1/100 of the endogenous fibronectin component of FBS). Wells of 24 well ultra-low attachment (ULA) (Corning Costar, NY, USA) plates, TCPS plates and ²PEGDAP_{TCPS} plates were incubated with supplemented FBS solution (140 µL) at 20 °C for 20 h then washed 5× with PBS and 3× with MilliQ water. Following the final wash, Eu(III)-liberating Enhancement Solution (Perkin Elmer) was added (150 µL) and allowed to incubate with the surfaces for 1 h. Following incubation, 100 µL of the Eu-complex solution was removed from each well surface and transferred to a white 96-well plate for time-resolved fluorescence assay on a PHERAstar instrument with excitation and the emission measured at 337 and 620 nm, respectively, as per Perkin Elmer product instructions. Standard solutions were used to determine concentrations of Eu(III).

2.5. Cyclic-peptide and melimine functionalisation

500 µM solutions of the cyclic-peptides, cyclo(Arg-Gly-Asp-D-Phe-Lys) (cRGDfK) and cyclo(Arg-Ala-Asp-D-Phe-Lys) (cRADfK) (Peptides International, KY, USA) were prepared in fresh MilliQ water. The 500 µM stock solutions were then diluted to give 250, 100, 50, 10, 1 and 0.01 µM solutions. cRGDfK and cRADfK were incorporated into the ²PEGDAP_{TCPS} coatings using a one-step (²PEGDAP_{TCPS}-OS-cRGDfK/cRADfK) and a two-step incubation (²PEGDAP_{TCPS}-TS-cRGDfK/cRADfK) procedure (abbreviated as ^XPEGDAP_{TCPS} where X denotes the concentration of PEGDGE coating solution in mM placed on the ALAPP coated TCPS, OS = one-step, TS = two-step). For ²PEGDAP_{TCPS}-OS-cRGDfK/cRADfK samples, 175 µL of 2 mM PEGDAP solution was added into the wells of a freshly allylamine plasma treated 48 well TCPS plate. Immediately after,

175 μ L of the 500 μ M stock solution and the dilutions of the cRGDfk or cRADfk solutions were added to the wells containing the PEGDAP solution. The plate was then placed into a 37 °C shaker oven for 36 h to allow reaction and drying of the solution. The plate was then washed over 24 h and used in cell attachment studies. For 2 PEGDAP_{TCPS}-TS-cRGDfk/cRADfk samples, 175 μ L of 2 mM PEGDAP solution was added into the wells of a freshly allylamine plasma treated 48 well TCPS plate and placed in a 60 °C shaker oven overnight (18 h). The plate was then washed over 5 hours and allowed to dry. 175 μ L of the 500 μ M cyclic-peptide solutions and the dilutions thereof were placed into the wells of the PEGDAP coated TCPS plate. The plate was then placed in a 37 °C shaker oven for 4 h. Subsequently the solutions in the wells were aspirated and the wells were washed (5 $\times$) with fresh MilliQ water before being used in cell attachment studies.

Melimine (American Peptide Company, CA, USA) – an antimicrobial peptide derived from portions of melittin and protamine,^[21] was separately incorporated into the PEGDAP coatings in a one-step (2 PEGDAP_{TCPS}-OS-Mel) and two-step incubation (2 PEGDAP_{TCPS}-TS-Mel) procedure. A 5 mg/mL solution of melimine was prepared in MilliQ water and incorporated into the PEGDAP coatings in the same manner as cRGDfk and cRADfk. Following washing (24 h) and drying of the coated wells, the bottom sections of the wells were removed using a 12 mm drill punch. The coated cutouts were subsequently used in *Staphylococcus aureus* (*S. aureus*) biofilm assays.

2.6. Cell attachment studies

L929 mouse fibroblasts (cell line ATCC-CCL-1, Rockville, MD) were used to investigate cell attachment and spreading on samples. Cells were cultured in minimum essential medium (MEM, Life Technologies, CA, USA) containing 10 % (v/v) foetal bovine serum (FBS, Sigma-Aldrich) and 1 % (v/v) non-essential amino acids (Life Technologies, CA, USA). Cells were harvested with TrypLE Express (Life Technologies, CA, USA) to produce a single cell suspension. Cells were collected by centrifugation at 300 g for 5 mins at 4 °C and re-suspended in media at 1×10^6 cells/mL of media.

Prior to cell seeding all samples were pre-incubated with a double strength antibiotic-antimycotic solution (Anti-Anti, Life Technologies, CA, USA) in phosphate buffered saline (PBS) for 1 h at room temperature. Cells were seeded into sample ${}^2\text{PEGDAP}_{\text{TCPS}}$, ${}^2\text{PEGDAP}_{\text{TCPS-OS-cRGDfK/cRADfK}}$ and ${}^2\text{PEGDAP}_{\text{TCPS-TS-cRGDfK/cRADfK}}$ wells along with tissue culture polystyrene (TCPS) control surfaces at 2.5×10^4 cells/cm² and incubated at 37 °C, 5 % CO₂ for 24 h. In the case of samples containing cyclic-peptides, FBS containing and serum-free media were used. Samples were photographed under bright-field using an Olympus IX-71 inverted microscope then subsequently prior to any quantification, all of the control and PEGDAP coated surfaces were washed twice with fresh medium (500 µL) to remove any non-adherent cells.

MTS assays can be utilised to determine cell viability based on metabolic activity of cells. Therefore an MTS assay was employed to determine which surfaces allow the least cell attachment relative to the TCPS control by measuring the metabolic activity of any adherent cells. Stock solutions of 4 mM, 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega, WI, USA) in PBS and 3 mM of phenazine methosulfate (PMS, Sigma) in PBS were used to make up a mixture of MTS and PMS reagent. A working assay reagent solution was made up by addition of 2 mL of MTS and 100 µL of PMS stock solutions per 10 mL of complete medium. Media was removed from the sample and control wells and washed with 500 µL of fresh medium; subsequently 365 µL of the assay reagent solution was added to wells then incubated for 3 h at 37 °C, 5 % CO₂.

For colorimetric analysis, plates were read in a BioTek plate reader at wavelengths 490 nm and 655 nm. The difference in readings at the two wavelengths was used to calculate metabolic activity of adherent cells compared to TCPS.

2.7. Cytotoxicity studies

Cytotoxicity assessment of materials was performed according to the International Standard ISO10993-5/12. Tested surfaces were in the 48 well plate format. Untreated TCPS control surfaces, $^2\text{PEGDAP}_{\text{TCPS}}$ and $^4\text{PEGDAP}_{\text{TCPS}}$ surfaces were incubated with 300 μL per well with complete minimum essential medium (MEM, containing 10 % (v/v) FBS and 1 % (v/v) non-essential amino acids for 66 hours in a sealed humidified chamber placed on a rocker (Seoulin Mylab™) at 20 rpm at 37 °C in a 5 % CO_2 incubator.

Media from test wells were removed and serially diluted at 1 in 2 with cell culture medium to afford 50, 25 and 12.5 % dilutions. 100 μL aliquots of neat incubated medium and its dilutions were added in quadruplicate to wells previously seeded with L929 cells (18,000 cells per well, 16 h) in a 96 well plate format. The additional controls added to wells containing pre-seeded cells consisted of medium alone, 5 %, and 10 % (v/v) dimethylsulfoxide (DMSO, Sigma) in medium. The plate was subsequently incubated for 21 h (37 °C, 5 % CO_2). An MTS assay was performed to assess cell viability as previously described for cell adhesion studies.

2.8. *Staphylococcus aureus* biofilm assay and analysis of bacterial adhesion

Bacterial strain *Staphylococcus aureus* (*S. aureus* clinical strain 38) (SA38) was streaked onto chocolate agar (Oxoid, UK) and incubated overnight at 37 °C. A single colony was cultured in 10 mL of tryptic soy broth (TSB) (Oxoid, UK) overnight to log phase at 37 °C. Optical density of the resulting culture was adjusted to OD_{660} of 0.1 (corresponding to 1×10^8 cfu/mL) in TSB media (with 0.25 % w/v glucose). Bacterial numbers were confirmed by a retrospective viable count. Previously prepared TCPS, $^2\text{PEGDAP}_{\text{TCPS}}$ and melimine coated ($^2\text{PEGDAP}_{\text{TCPS}}\text{-OS-Mel}$ and $^2\text{PEGDAP}_{\text{TCPS}}\text{-TS-Mel}$) cutouts were

sterilised with 70 % w/v ethanol for at least 1 hour beforehand. For 48 hours adhesion experiments, samples were placed in 2 mL of 1×10^8 cfu/mL TSB culture containing 0.25 % w/v glucose and incubated at 37 °C with shaking for 24 h, then the media was replaced with fresh TSB (2 mL) and incubated for a further 24 h (total of 48 hours). The samples were then washed with PBS before examination by fluorescent staining.

Samples used in the *S. aureus* biofilm assay were stained with LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Inc., OR, USA) as described in previous studies.^[22, 31-33] Briefly, microscopic observation and image acquisition was performed with Olympus FV1200 Confocal Inverted Microscope. Images obtained from 10 areas on each of duplicate samples for each surface were analysed using ImageJ software. The image analysis results were measured as the average area of cells per field of view and are reported as the average percentage coverage.

2.9. Statistical analysis

A minimum of three experimental repeats ($n \geq 3$, unless otherwise specified) were used in each study and the results are presented as mean $\pm$ standard error. Statistical significance was determined by an independent Student's t-test, and a confidence level of 95 % ($p < 0.05$) was considered to be statistically significant unless otherwise mentioned.

For microbial attachment prior to comparing the groups, equality of variances was tested using Levene's test. Unequal variances were adjusted by transforming the data using square root transformation. Differences between the groups were analysed using linear mixed model ANOVA, which adjusts the correlation due to repeated observations. Post hoc multiple comparisons were performed using Dunnett T3 comparison for the 48 h adhesion experiment.

3. Results and Discussion

3.1. Coating preparation

Plasma polymerisation is a robust and commonly used technique to introduce functional groups on various material surfaces.^[34, 35] While versatile and strongly adherent coatings can be achieved by this method, since custom plasma polymerisation instrumentation is utilised, optimization of the deposition parameters may be required when different designs or equipment are used. In this study, allylamine was used in the plasma treatment of polystyrene surfaces to introduce amine functional groups. Subsequently, an aqueous solution containing poly(ethylene glycol) diglycidyl ether (PEGDGE) and diaminopropane (DAP) (with a PEGDGE:DAP ratio of 3:1) was placed on the allylamine plasma coated (ALAPP) tissue culture polystyrene (TCPS) surfaces. The epoxide groups of the PEGDGE are able to react with the amines present on the allylamine coating as well as the DAP in solution, allowing for covalent surface immobilization and crosslinking to take place between the PEG polymers to ultimately form the PEGDAP coating (**Figure 1**). Each amine functional group can react with up to two epoxide groups,^[36] the PEGDGE : DAP ratio of 3 : 1 allows for crosslinking and also provides unreacted epoxide groups resulting from the excess PEGDGE in the solution (Figure S1). The unreacted epoxide groups are then able to provide conjugation points for amine containing bioactive molecules such as peptides. In this study the PEGDAP coating was carried out on TCPS substrates, however we were able to demonstrate and confirm, via XPS and cell attachment assays, coating of other substrate materials including polypropylene, glass, silicon wafers, poly(dimethyl siloxane) and polyurethane (results not shown).

For a new coating method to be translated into commercial products, it is essential that the precursors are cost effective, easily procurable and that the coating manufacture is facile and amenable for scale-up. For this purpose, we have utilised commercially available precursors in this study which allow the preparation of effective low fouling coatings using a simple technique.

Moreover, coatings prepared by this method also allow direct coupling of amine containing bioactive molecules. High density surface grafting of polymers is a versatile technique for the preparation of low-fouling and/or highly functional coatings with a large range of polymers. However, often catalysts and/or an inert atmosphere (i.e. nitrogen) are required for the preparation of such surfaces.^[37, 38] These required conditions often can be an obstacle in the scale-up and wide application of such coatings. Epoxy-amine chemistry was selected for the PEGDAP coatings since initiators, catalysts or an inert atmosphere is not required.^[36] Its wide application, low cost, and availability in various structures and multiple functional groups^[12] makes PEG an ideal polymer for large scale coating manufacture. For this reason PEG was employed in the preparation of the multifunctional coatings in this study.

Figure 1. Preparation of the PEGDAP coating. A TCPS substrate is treated by allylamine plasma polymer (ALAPP) deposition to provide an amine functionalised surface. The ALAPP interlayer is then exposed to PEGDGE and diaminopropane to produce the crosslinked PEGDAP coating.

3.2. XPS analysis

X-ray photoelectron spectroscopy (XPS) analysis was conducted to determine the effect of the PEGDGE concentration on the amount of PEG immobilised on the allylamine interlayer coating. Following the analysis of the XPS survey spectra and quantification of the atomic concentrations of carbon (C), nitrogen (N) and oxygen (O) there was a clear trend visible. As the PEGDGE concentration was increased, the O atomic concentration (%) also increased (**Table 1**), which was reflected on the elemental ratios, as a significant increase in the O/N ratios is observed (Supplementary Figure S2).

Table 1. Elemental composition of surfaces used in this study as determined by XPS analysis (atomic concentrations in %).

	ALAPP	5 nM	50 nM	500 nM	5 μ M	50 μ M	500 μ M	1 mM	2 mM	4 mM
O (%)	13.6 $\pm$ 0.3	13.2 $\pm$ 0.4	13.9 $\pm$ 0.2	13.3 $\pm$ 0.3	13.9 $\pm$ 0.5	14.3 $\pm$ 0.7	18.8 $\pm$ 0.6	21.3 $\pm$ 1.4	22.4 $\pm$ 0.9	23.8 $\pm$ 0.5
N (%)	13.4 $\pm$ 0.2	12.1 $\pm$ 0.4	12.9 $\pm$ 0.2	12.2 $\pm$ 0.2	12.8 $\pm$ 0.3	10.8 $\pm$ 1.7	8.9 $\pm$ 0.9	7.7 $\pm$ 1.3	6.9 $\pm$ 0.4	6.0 $\pm$ 0.5
C (%)	72.9 $\pm$ 0.4	74.7 $\pm$ 0.6	73.2 $\pm$ 0.1	74.5 $\pm$ 0.2	73.1 $\pm$ 0.5	74.9 $\pm$ 1.0	72.3 $\pm$ 0.8	71.1 $\pm$ 0.5	70.7 $\pm$ 1.37	70.2 $\pm$ 0.3

The increase in the O concentration clearly demonstrates the immobilization of PEG on these surfaces due to the high O content present in the PEG polymer. C1s spectra also confirm the incorporation of PEG in the coating. As seen in **Figure 2**, the shoulder corresponding to the C-O bond increases in intensity as the PEGDGE concentration is increased, relative to the main C-H shoulder, reaching a maximum at the highest concentration used, 4 mM (⁴PEGDAP_{TCPS}). PEG contains C-O bonds in every repeat unit of the polymer hence the increase in C-O intensity in the C1s spectra correlates well with the effect of the increased PEGDGE concentration.

Figure 2. XPS C1s high resolution spectra obtained from analysis at increasing concentrations of PEGDGE. Increase in the C-O shoulder can be observed clearly as the concentration of PEGDGE in coating solutions is increased.

3.3. Cell attachment and protein adsorption

The reduction of non-specific protein adsorption is a desired feature in certain applications including antimicrobial coatings, since this can be a first layer of defence by resisting bacterial attachment.^[11] A study with L929 fibroblasts was conducted to determine the lowest concentration of PEGDGE required to minimise cell attachment to demonstrate the low fouling property of the PEGDAP coatings. L929 fibroblasts were able to significantly adhere and expand onto the treated surfaces up to a PEGDGE concentration of 50 μM , whereby a small number of rounded cells could be observed (Figure 3).

Figure 3. L929 fibroblast cell attachment on PEGDAP surfaces after 24 h. Up to a PEGDGE concentration of 50 μM , all L929 fibroblasts are able to adhere and spread on the surfaces. From 500

μM concentration, cells display a more rounded shape and non-adherent cells begin to form clusters. At 2 and 4 mM concentrations, the number of adherent cells are minimised and all cells remain either non-adherent individuals or form non-adherent cell clusters.

As the PEGDGE concentration was further increased, the number of clumped/rounded non-adherent cells increased significantly until no adherent cells could be seen for $^2\text{PEGDAP}_{\text{TCPS}}$ and $^4\text{PEGDAP}_{\text{TCPS}}$ surfaces (i.e. all cells appeared round and in clumps). An MTS assay was also carried out to quantify these observations (**Figure 4a**) by measuring the metabolic activity of any adherent cells, where a significant reduction in metabolic activity, hence number of adhered cells was observed for $^{0.5}\text{PEGDAP}_{\text{TCPS}}$ surfaces compared to the control TCPS and allylamine surfaces. Further reductions are observed with increasing PEGDGE concentration up to 2 and 4 mM, where a minimum is observed. The results of the cell attachment assay correlate well with the results obtained from XPS analysis; higher PEG content in the coatings reduced the attachment of cells on the surfaces.

The ability of PEG to resist protein adsorption is a significant factor in minimising fouling. The prevention of protein adsorption on surfaces also indicates resistance to the attachment of cells since proteins are not available to be recognized and used by the cells for adherence. To confirm the resistance of protein adsorption by the PEGDAP coating, an assay using Europium-labelled fibronectin was conducted on $^2\text{PEGDAP}_{\text{TCPS}}$, commercial ultra-low attachment (ULA), and control TCPS surfaces.^[30] Test surfaces were incubated in 10 % FBS in PBS supplemented with Eu-labelled fibronectin (c.a. 1 % relative to the endogenous fibronectin present in FBS) for 20 h, washed, and Eu(III) was liberated from the labelled fibronectin. Total fibronectin adsorbed to the test surfaces was calculated based on 620 nm wavelength emission of the liberated Eu(III) from the time-resolved fluorescence measurement. Relative to the TCPS surface (274.1 ng/cm^2), as expected the ULA surface showed a significantly lower fibronectin adsorption (8.4 ng/cm^2), while an even further reduction was observed for $^2\text{PEGDAP}_{\text{TCPS}}$ with greater than 99.8 % lower fibronectin adsorption (0.07 ng/cm^2). This

demonstrates that the $^2\text{PEGDAP}_{\text{TCPS}}$ coating is extremely resistant to protein adherence; where the adsorbed protein is at negligible levels. (Figure 4b).

Figure 4. a) L929 mouse fibroblast attachment after 24 h relative to TCPS in % obtained via MTS assay. b) Total adsorbed fibronectin on test surfaces; TCPS, commercial Ultra-Low Attachment (ULA) surface and $^2\text{PEGDAP}_{\text{TCPS}}$ as determined by incubation in 10 % FBS in PBS, supplemented with Eu-labelled fibronectin, after 20 h. (***) $p < 0.001$)

PEG has been utilised for many applications for the preparation of hydrogels for drug delivery and tissue engineering applications and for increasing circulation of bioactive molecules *in vivo*.^[39-41] All of these applications rely on the resistance of PEG towards non-specific protein adsorption and related biological responses such as immune responses. Similarly the PEGDAP coatings are extremely resistant to non-specific protein adsorption and are able to minimise cell attachment as confirmed by the conducted assays.

3.4. Cytotoxic evaluation of the PEGDAP coating

As a coating designed for implantable medical devices, the PEGDAP coating must not release any toxic compounds in a biological environment. In order to determine if any cytotoxic compounds were leaching from the PEGDAP coatings a cytotoxicity study with L929 cells was conducted. In the cytotoxicity study only surfaces with ²PEGDAP_{TCPS} and ⁴PEGDAP_{TCPS} coatings were used since these coatings showed the best performance in resisting cell attachment. Initially the test surfaces were incubated in medium for 66 h at 37 °C. Subsequently, previously seeded L929 cells were grown in the presence of the PEGDAP incubated media and their dilutions (50, 25 and 12.5 %) for 21 h. L929 cells were also grown in fresh medium, medium incubated with TCPS and 5 and 10 % (v/v) DMSO (in medium) as controls respectively. Following 21 h incubation, an MTS assay was employed to determine cell viability. All cytotoxicity studies were performed according to the International Standard ISO10993-5/12 and cell viability below 70 % was considered cytotoxic.

For TCPS incubated medium and the fresh medium controls there was no reduction in cell viability. Conversely, a significant reduction was observed in the case of 5 and 10 % DMSO in medium (below 60 and 20 %, respectively) as expected (**Figure 5**). There was no reduction in cell viability for any of the PEGDAP incubated surfaces regardless of the concentration of the incubated medium. This result follows many other studies demonstrating the non-toxic properties of PEG-based polymer systems both *in vitro* and *in vivo*.^[42-44] Similarly, in the case of PEGDAP coatings no *in vitro* toxicity was observed.

Figure 5. *In vitro* cell toxicity assessment in PEGDAP incubated media. Only the results for neat incubated solutions of ²PEGDAP_{TCPS} and ⁴PEGDAP_{TCPS} are shown, since even at the highest concentration no reduction in cell viability was observed. Cytotoxicity assessment of materials was performed according to the International Standard ISO10993-5/12 and cell viability below 70 % was considered cytotoxic.

3.5. Functionalisation of PEGDAP coating with cyclic peptides

Coatings to control biointerfacial responses, including prevention of bacterial attachment, are designed to present multiple strategies to achieve this control. PEGDAP coating possesses low-fouling capabilities as demonstrated by the cell attachment and protein adsorption assays, but additionally, the coating was designed to allow facile incorporation of peptides and other amine containing bioactive molecules. PEGDAP coatings were prepared with excess PEGDGE to DAP which would allow for free epoxide functional groups to be available. To demonstrate the facile incorporation of bioactives, functional cRGDfK and non-functional cRADfK peptides were

incorporated as model bioactive compounds into the PEGDAP coatings in a one-step procedure ($^2\text{PEGDAP}_{\text{TCPS-OS-cRGDfK/cRADfK}}$) and a two-step incubation ($^2\text{PEGDAP}_{\text{TCPS-TS-cRGDfK/cRADfK}}$) procedure. With the PEGDAP coating, peptides can be incorporated in a one-step procedure during the crosslinked coating formation. On the other hand the commercial OptiChem® coatings' requirement of high temperature curing only allows for immobilisation of peptides post-coating formation,^[11] similar to the two-step procedure for the PEGDAP coatings where the coating is deposited prior to peptide immobilisation. Cell attachment studies using L929 cells and subsequent analysis via an MTS assay demonstrated the successful incorporation of the cyclic peptides. The attachment of cells onto $^2\text{PEGDAP}_{\text{TCPS-OS-cRGDfK}}$ and $^2\text{PEGDAP}_{\text{TCPS-TS-cRGDfK}}$ coated surfaces could be observed easily while no significant cell attachment was seen on the cRADfK incorporated surfaces (Figure 6). For the TCPS controls, a significant attachment of L929 fibroblasts could be observed even in serum-free conditions. Fibroblasts are known to produce extracellular matrix proteins including collagen and fibronectin,^[45-47] hence even in serum-free conditions the produced proteins would be able to adsorb onto the TCPS surface which in turn allow the cells to attach.

Figure 6. 24 h cell attachment assay on a), b) TCPS surface, c), d) ${}^2\text{PEGDAP}_{\text{TCPs-OS-cRGDfK}}$ e), f) ${}^2\text{PEGDAP}_{\text{TCPs-TS-cRGDfK}}$, g), h) ${}^2\text{PEGDAP}_{\text{TCPs-OS-cRADfK}}$, i), j) ${}^2\text{PEGDAP}_{\text{TCPs-TS-cRADfK}}$. Cell attachment

assays were conducted both in the presence (a, c, e, g, i) and absence (b, d, f, h, j) of FBS (Cyclic-peptide concentration of 500 μ M shown). Attachment and spreading of L929 fibroblasts was observed on all cRGDfK containing surfaces while cells remained non-adherent on cRADfK modified surfaces.

From the MTS assay, a concentration dependant response was observed for 2 PEGDAP_{TCPS}-OS-cRGDfK and 2 PEGDAP_{TCPS}-TS-cRGDfK surfaces in both serum-containing and serum-free media regardless of the incorporation method, whereby with increasing concentration, the number of adherent cells also increased (**Figure 7**). On the other hand, there were a small number of cells attached on the 2 PEGDAP_{TCPS}-OS-cRADfK and 2 PEGDAP_{TCPS}-TS-cRADfK surfaces at all concentrations of the peptide, but this number was not affected by the incorporation method or the presence of serum. Integrins present in mammalian cell membranes interact specifically with RGD,^[48] hence significant L929 attachment was only observed on the cRGDfK incorporated coatings and not the non-biologically active cRADfK counterpart. Based on this, it is possible to see that the cellular interaction with the peptide is specific and that with both the one-step and two-step peptide immobilization methods; the conjugated cRGDfK is biologically active and can be recognized by the cell transmembrane proteins. In the study, both serum-containing and serum-free media were also utilised to demonstrate that the L929 cells were specifically interacting with the conjugated peptide and it was not the result of the proteins present in the FBS containing media.

The facile incorporation of the cRGDfK demonstrates the versatility of the PEGDAP coating to allow for multifunctional properties. In this case cRGDfK was used as a model bioactive to demonstrate the PEGDAP coating's capability for multifunctionality. For this study a range of concentrations were used and the relevant effect was observed. Other bioactive molecules may be effective at lower or higher concentrations than have been observed in this study and may require different incorporation conditions.

Figure 7. 24 h L929 cell attachment assay for $^2\text{PEGDAP}_{\text{TCPS}}\text{-OS-cRGDfK/cRADfK}$ and $^2\text{PEGDAP}_{\text{TCPS}}\text{-TS-cRGDfK/cRADfK}$ surfaces a) with and b) without FBS at increasing concentrations of cyclic peptides, relative to TCPS control surface analysed via MTS ($n \geq 3$, error bars represent standard error of the mean).

3.6. Prevention of biofilm formation

The reduction of non-specific interactions and the provision of low protein fouling surfaces is also a desired feature in antimicrobial coatings due to the fact that such surfaces can act as a first layer of defence by resisting bacterial attachment. The PEGDAP coating is designed as a low protein fouling

platform coating; however this platform also allows the introduction of further layers of defence. The ability to covalently immobilise and display bioactive molecules such as AMPs allows an enhanced resistance towards biofilm formation. Melimine is a synthetic peptide derived from parts of melittin and protamine, which has shown excellent bactericidal effects when immobilized onto surfaces^[21, 22]. Following the demonstration of successful incorporation of cell adhesive peptides into the PEGDAP coating, melimine was incorporated into the PEGDAP coating in either a one or a two-step manner, in the same fashion as cRGDfK and cRADfK. The incorporation of the peptide was confirmed via XPS analysis (See Supplementary Information Table S1 and Figure S3). In order to demonstrate the synergistic effect of the non-adhesive PEGDAP coating and the immobilised melimine, a preliminary biofilm assay using *S. aureus* was conducted on TCPS control, ²PEGDAP_{TCPS}, ²PEGDAP_{TCPS}-OP-Mel and ²PEGDAP_{TCPS}-TS-Mel surfaces. Previous studies^[48-50] using PEG-based surfaces showed reductions over 90 % in the adhesion of *S. aureus* and *S. epidermidis* over 24 hours. Similarly, in the case of ²PEGDAP_{TCPS} coated surfaces, a significant reduction of 96.3 % in *S. aureus* coverage was observed (**Figure 8**). A single strategy against biofilm formation can be effective, as is the case for the ²PEGDAP_{TCPS} coating alone, however, in order to maximise the prevention of biofilm formation, the employment of multifunctional strategies is necessary. As in the case for ²PEGDAP_{TCPS}-OS-Mel surfaces, a further significant reduction in *S. aureus* (99.4 %) attachment is observed when melimine was incorporated in a one-step manner. Even though ²PEGDAP_{TCPS}-TS-Mel also showed low attachment of *S. aureus* (98.8%), there was no statistical difference in comparison to the ²PEGDAP_{TCPS} coating alone. This correlates well with the amount of melimine incorporated into the PEGDAP coating. With the one-step procedure, more peptide can be incorporated together with PEGDGE in one-step as part of the coating. On the other hand with the two-step method, the melimine is introduced after the coating formation, hence the peptide is only conjugated to the available epoxide groups on the surface. As can be seen in Supplementary Information Table S1, the

atomic concentration of nitrogen is significantly higher for the one-step coating ($8.56 \% \pm 0.30$) compared to the two-step coating ($5.71 \% \pm 0.07$), which is also reflected in the O/N elemental ratios for the two coating methods (one-step (O/N = 2.15), two-step (O/N = 3.40)). C1s high resolution spectra also reflects this (Supplementary Figure S3), the C-O shoulder which results from the PEG is more prominent in the two-step procedure, because the presence of more melimine is able to mask the C-O shoulder in the one-step coating.

Although PEG-based polymers possess highly desirable properties such as being low-fouling and bacterial resistant, these polymers also suffer from certain disadvantages such as susceptibility to oxidation and associated degradation.^[38] In order to overcome this disadvantage, other low-fouling polymers such as poly(N-hydroxypropyl acrylamide), poly(N-hydroxypropyl methacrylamide), or zwitterionic polymers could be employed^[7, 38] using the same platform coating approach to prepare multifunctional coatings, enabling sophisticated control over biointerfacial interactions.

Figure 8. Confocal image of live/dead stained *S. aureus* (SA38) on a) TCPS, b) 2 PEGDAP_{TCPS}, c) 2 PEGDAP_{TCPS}-OP-Mel, and d) 2 PEGDAP_{TCPS}-TS-Mel. e) Graph showing the surface coverage of *S. aureus* (SA38) observed on PEGDAP and melimine modified PEGDAP coatings relative to TCPS (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, $n \geq 3$, error bars represent standard error of the mean).

4. Conclusions

We have successfully developed a multifunctional platform coating which provides low protein fouling while at the same time allowing the immobilisation of bioactive molecules in a one or two-step manner. The low fouling properties of the coating were investigated via a Europium-labelled fibronectin adsorption assay as well as an L929 cell attachment assay. In these assays, excellent resistance of the PEGDAP coating in regard to protein adsorption and cell attachment was demonstrated. Furthermore, the cyclic peptides cRGDfK and cRADfK were utilised to demonstrate the ability of the coating to support the covalent immobilisation of bioactive molecules without impairing their biological activity. The specific activity of the incorporated biomolecule, cRGDfK, was confirmed in cell attachment assays when compared to its non-biologically active cRADfK counterpart. In addition, the same peptide immobilisation strategy was used to produce surfaces displaying the antimicrobial peptide melimine on a low fouling background surface. 48 h biofilm assays using a clinical strain of *S. aureus* showed excellent resistance of the PEGDAP platform coating towards bacterial attachment. Moreover, *S. aureus* attachment was further significantly reduced when the antimicrobial peptide melimine was incorporated into the PEGDAP coating in a one-step procedure. Overall, it is expected that the versatile coating platform described here will find applications in biomedical applications where sophisticated control over biointerfacial interactions is required. Advantages of the coating platform include in particular the use of commercially available

precursors, the robust and simple application technique and the facile incorporation of bioactive compounds in one or two steps.

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Supporting Information

Schematic showing crosslinking of PEGDGE and diaminopropane (Figure S1). Elemental ratios of PEGDAP coatings obtained from XPS analysis (Figure S2). Selected XPS elemental composition (Table S1) and high resolution C1s spectra (Figure S3) of ALAPP, $^2\text{PEGDAP}_{\text{TCPS}}$, $^2\text{PEGDAP}_{\text{TCPS-OS-Mel}}$ and $^2\text{PEGDAP}_{\text{TCPS-TS-Mel}}$ highlighting differences in coating composition. This supporting Information is available from the Wiley Online Library.

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PEG-based crosslinked platform coatings described in this study allow for the facile covalent incorporation of bioactive cell adhesive and antimicrobial peptides. The coatings provide low-biofouling properties while maintaining the function of the incorporated peptides. Cell attachment studies confirmed the activity of the cRGDfK peptide while incorporation of the antimicrobial peptide melimine further reduced bacterial colonisation on surfaces.

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Crosslinked platform coatings incorporating bioactive signals for the control of biointerfacial interactions

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