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Development of nonfouling polypeptides with uniform alternating charges by polycondensation of the covalently bonded dimer of glutamic acid and lysine

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In this work, nonfouling polypeptides with homogenous alternating charges were synthesized by polycondensation of the covalently bonded dimer of glutamic acid (E) and lysine (K) (EK dimer) with benzyloxycarbonyl (*Z*)-protected side chains. This facile method successfully solved the uniformity problem of nonfouling peptides caused by the copolymerization of two different monomers and enabled the incorporation of various terminal functional groups for future applications. The molecular weights (MWs) of the nonfouling peptides can be easily controlled by the ratio of the terminal group, lipoic acid, to the EK dimer. The nonfouling peptides can form self-assembling monolayers (SAMs) on a gold surface through two terminal thiol groups, which were characterized by attenuated total reflection Fourier transform infrared (ATR-FTIR), X-ray photoelectron spectroscopy (XPS) and ellipsometry (ELL). The resistance to nonspecific protein adsorption, cell attachment and bacterial adhesion of these nonfouling peptide SAMs and the *in vitro* cytotoxicity and haemolytic activity of these peptides were also evaluated. The results show that the lowest relative protein adsorptions of antibody (anti-IgG) and fibrinogen (Fg) on the SAMs are 5.1 ± 1.6% and 7.3 ± 1.8%, respectively, determined by enzyme-linked immunosorbent assay (ELISA), where the protein adsorption on a tissue culture polystyrene (TCPS) surface was set to 100%. Almost no obvious cell attachment and bacterial adhesion were observed, and no cytotoxicity and no haemolytic activity *in vitro* were detected. With the advantages of biocompatibility, biodegradability and the abundance of moieties for ligand immobilization, these nonfouling peptides developed by the facile method can be used in a wide range of biomedical applications.

Introduction

With the accelerated investigation of the nonfouling mechanisms in recent years, polyzwitterionic materials, such as poly(2-methacryloyloxyethyl phosphorylcholine) (pMPC),[1–3] poly(sulfobetaine) (pSB),[4,5] and poly(carboxybetaine) (pCB),[6–8] have been recognized as effective nonfouling materials. They are superior to poly(ethylene glycol) (PEG) in the fields of resistance to nonspecific protein adsorption, endurance in an oxidating environment, multi-functionalization,[6,9–13] *etc.* All of these polyzwitterionic materials show good biocompability through excellent resistance to nonspecific protein adsorption and weak interactions with cell membranes.[4,14–17] However, they are not in the most ideal format for bio-applications due to a lack of biodegradability through natural metabolism. They might disrupt normal cell metabolism and eventually cause host or untargeted cell death in gene or nano medicine therapy since their inherent lack of non-biodegradability will lead to the accumulation of the polymers in cells. Therefore, it is necessary to develop biodegradable alternatives to the classical polyzwitterionic nonfouling materials.

For this purpose, biodegradable and biocompatible natural amino acid-based nonfouling peptides, composed of homogenous alternating negative glutamic acid (E) and positive lysine (K) residues, were proposed and investigated since the peptide could possess similar charge distribution features to the zwtterionic polymer, uniformity and neutrality.[12] Recently, the Jiang group[18] also demonstrated that an alternating EK oligopeptide with a four-proline linker and a cysteine residue can form a self-assembling ordered structure on a gold surface. The results clearly show that these peptide coated surfaces efficiently resist nonspecific protein adsorption. Moreover, there

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are plenty of functional groups on the uniformly charged peptides for immobilizing biorecognition molecules in future biomedical applications. All results indicate that this is a new group of promising nonfouling materials. However, to the best of our knowledge, all high quality nonfouling peptides with exact EK-repeating sequences have to use a peptide synthesizer, which is not suitable for mass production. Thus, copolymerization of two monomers containing E and K through *N*-carboxyanhydride (NCA) ring opening polymerization has been developed.[19] However, it is rather difficult to obtain uniform EK repeating sequences due to both the different reactivity of each monomer and the difficulty in controlling the purity of the monomer in each batch, although this problem could be partially solved through different ratios of E to K NCA monomers.

In this work, we designed and synthesized a covalently bonded EK dimer to achieve uniformity, which is the key feature to generate nonfouling properties, of charge alternating nonfouling peptides through polycondensation. Three nonfouling peptides with different degrees of polymerization were successfully prepared through controlling the ratio of the terminal group, lipoic acid, to the EK dimer. The lipoic acid capped EK-repeating polypeptides can form self-assembling monolayers (SAMs) on a gold surface through two thiol groups. The chemical and physical properties of these peptide SAMs were systematically characterized to further verify the formation of SAMs. The functionalities of the SAMs, including resistance to protein adsorption, cell attachment and bacterial adhesion, were investigated. The relationships between the resistance and the peptide MWs were discussed. Moreover, the biocompatibility of these peptides, including *in vitro* cytotoxicity and haemolytic activity of these EK-repeating polypeptides were also examined. All results suggest that these fully biodegradable and biocompatible polypeptides will play important roles in biomedical applications, especially for nano drug carriers.

Experimental

Materials

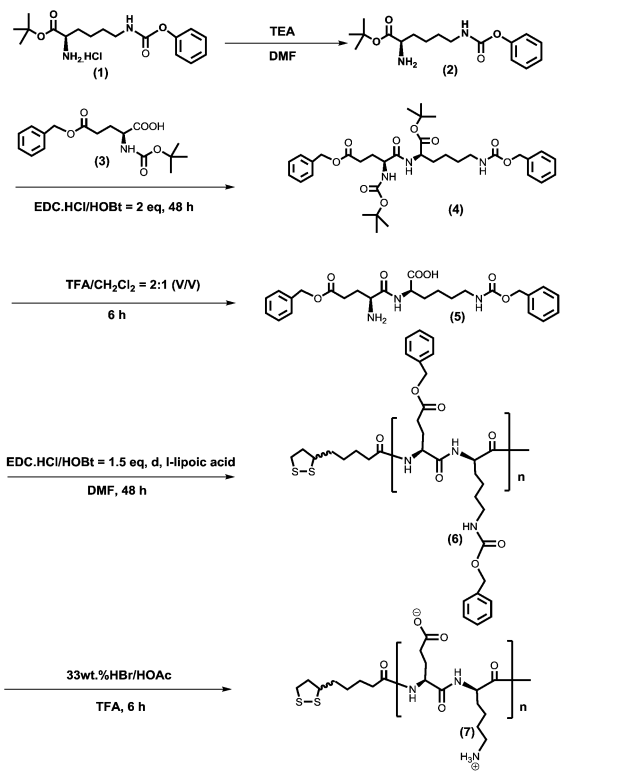
N^{ε}-benzyloxycarbonyl-α-*tert*-butyl-L-lysine hydrochloride (H-Lys(*Z*)-O^{t}Bu·HCl, 99%) and δ-benzyl-*N*-*tert*-butoxycarbonyl-L-glutamic acid (Boc-Glu(*Z*)–OH, 98%) were purchased from Shanghai Hanhong Chemical Co., Ltd. RPMI 1640 medium, trypsin 0.25% and fetal bovine serum (FBS) were purchased from Sijiqing Biological Engineering Materials Co., Ltd. Horseradish peroxidise (HRP)-conjugated goat anti-human IgG (anti-IgG/HRP), horseradish peroxidase (HRP)-conjugated goat anti-fibrinogen (anti-Fg/HRP) and Fg were purchased from Beijing Bosheng Bio-technology Co., Ltd. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl, 98.5%), 2-hydroxybenzotriazole (HOBt, 99%), O-phenylenediamine (OPD, 98%), 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT), fluorescein diacetate (FDA), trifluoroacetic acid (TFA), triethylamine (TEA), and anhydrous dimethylformamide (DMF) were purchased from Aladdin Reagent Co., Ltd. 33 wt% HBr/HOAc solution was purchased from Sigma-Aldrich Co. LLC. Human umbilical vein endothelial cells (HUVECs) were purchased from China Center for Type Culture Collection. All other chemicals were of reagent grade and were used without further purification.

Synthesis route

The synthesis route is summarized in Scheme 1.

Synthesis of N^{α}-(δ-benzyl-*N*-*tert*-butoxycarbonyl-L-glumatyl)-N^{ε}-benzyloxycarbonyl-α-*tert*-butyl-L-lysine (4)

A typical procedure is described as follows: H-Lys(*Z*)-O^{t}Bu·HCl (**1**, 5.87 g, 15.74 mmol) was dissolved in 50 mL anhydrous DMF and 3 mL TEA (21.52 mmol) in 10 mL DMF was added dropwise. After 30 min, the mixture was filtered. Boc-Glu(*Z*)–OH (**3**, 5.06 g, 15 mmol), EDC·HCl (5.75 g, 30 mmol) and HOBt (4.05 g, 30 mmol) were added into the filtrate and then the mixture was reacted for 48 h at room temperature. After complete removal of DMF, the residue was dissolved in dichloromethane and then washed with 10% aqueous citric acid solution, saturated aqueous $NaHCO_3$ solution, water, and saturated brine, and dried over anhydrous $MgSO_4$. The organic solution was concentrated by a rotary evaporator under reduced pressure, and the residue was purified by column chromatography (silica gel, dichloromethane/ethyl acetate = 3 : 1, v/v) to obtain compound **4** (8.40 g, 85.4%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.23–1.45 22H, 1.49–1.71 2H, 1.71–1.83 1H, 1.85–1.98 1H, 2.36–2.44 2H, 2.92–3.02 2H, 3.95–4.03 2H, 4.96–5.05 2H, 5.05–5.11 2H, 6.90–6.97 0.89H, 7.20–7.27 0.88H, 7.28–7.40 10H, 8.05–8.11 0.95H.



Scheme 1 Synthesis route of the homogenous EK polypeptide.

Synthesis of N^{α}-(δ-benzyl-L-glumatyl)-N^{ε}-benzyloxycarbonyl -L-lysine (5)

4 (4.15 g, 6.33 mmol) in 18 mL TFA/CH_2Cl_2 (v/v = 2 : 1) solution was added to a 50 mL round bottom flask. The mixture was stirred for 6 h at room temperature, followed by removal of the solvent by a rotary evaporator under reduced pressure and the residue was dissolved in ethyl acetate again. TEA was added to adjust the pH to 7.0–8.0. Then the mixture was dried and washed with water several times to obtain compound **5** (2.66 g, 84.2%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.19–1.31 2H, 1.32–1.43 2H, 1.49–1.62 1H, 1.64–1.82 2H, 1.82–1.95 1H, 2.41–2.48 2H, 2.90–3.00 2H, 3.36–3.46 1H, 4.01–4.12 1H, 4.94–5.02 2H, 5.02–5.13 2H, 7.23–7.29 0.86H, 7.30–7.40 10H, 8.16–8.28 1H.

Synthesis of lipoic acid capped poly(N^{α}-(δ-benzyl-L-glumatyl)-N^{ε}-benzyloxycarbonyl-L-lysine) (6)

The EK dimer (**5**) (0.2 g, 0.4 mmol), EDC·HCl (0.115 g, 0.6 mmol), HOBt (0.081 g, 0.6 mmol) and different amounts of D,L-lipoic acid (based on the molar ratio of D,L-lipoic acid to the EK dimer (**5**)) were added into 2 mL anhydrous DMF. After complete removal of oxygen and humidity from the reaction system by bubbling filtrated N_2 for about 30 min, the resulting mixture was kept sealed for reaction at room temperature for 48 h. The mixture was poured into excess ethyl ether to precipitate a polymer (**6**). The polymer (**6**) was kept in oil for the next reaction without purification.

Synthesis of lipoic acid capped poly (L-glumatyl-L-lysine) (7)

The *Z* moieties on polymer **6** were removed by a mixed solution of 33 wt% HBr/HOAc and TFA (v/v = 1 : 1) for about 6 h. The EK polypeptide (**7**) was precipitated by ethyl ether, followed by neutralization to pH 7.0 by saturated $NaHCO_3$ solution, dialyzed against deionised (DI) water and lyophilized to obtain a white powder. Full cleavage was confirmed by the disappearance of peaks at 7.20–7.40 and 4.90–5.20 in ^{1}HNMR. A typical yield of the EK polypeptide from the EK dimer is about 45.0%. ^{1}H NMR (D_2O, 400 MHz): δ 1.20–1.50 2H, 1.51–1.59 2H, 1.65–2.06 4H, 2.08–2.36 2H, 2.90–2.94 2H, 3.95–4.23 2H.

Gel permeation chromatography (GPC) analysis

The MW and MW distribution of the polypeptides were determined by Shimadzu gel permeation chromatography equipped with a Waters Ultrahydrogel™ 120 and a Waters Ultrahydrogel™ linear column. The mobile phase was phosphate buffered saline (PBS) (NaCl 150 mM, pH 7.4) with a flow rate of 0.5 mL min^{-1} at 45 °C. Poly(ethylene oxide) was used as a standard.

ATR-FTIR for film structure and ELL for film thickness

ATR-FTIR was conducted on a Fourier transform infrared spectrometer (Bruker Vector 22) and film thickness measurements were conducted on a spectroscopic ellipsometer (J. A. Woollam M-2000D). The bare gold coated chips were used as controls.

XPS measurements[18]

XPS was conducted on an X-probe spectrometer (VG Escalab Mark II) equipped with a monochromatic Mg Kα X-ray source ($h\nu = 1253.6$ eV), a hemi-spherical analyzer, and a multichannel detector. The bare gold coated chips were used as controls.

Protein adsorption assay

Gold chips were immersed in the potassium dichromate solution overnight, followed by rinsing five times with DI water and ethanol, and drying by filtered air. Then pre-treated gold chips were immersed in 2 mg mL^{-1} polypeptide solution for 24 h followed by rinsing with sterile PBS. Fg adsorption on the polypeptide-based SAMs and TCPS control were quantified using the anti-Fg/HRP adsorption.

The samples were first incubated with Fg (10 μg mL^{-1}) for 20 min, followed by rinsing with sterile PBS to remove all free proteins. Then the chips were incubated with anti-Fg/HRP (1 μg mL^{-1}) for another 20 min and rinsed five times with sterile PBS. The tested chips were transferred to a 24-well plate and incubated with 1 mL *o*-phenylenediamine (OPD) (1 mg mL^{-1}) in citrate phosphate buffer (0.1 M, pH 5.0) containing 0.03% hydrogen peroxide. The enzyme activity was quenched by adding an equal volume of 2 N H_2SO_4 after 8 minutes of incubation. The tangerine colour was measured by a UV spectrophotometer at 492 nm. The antibody adsorption was directly measured from the adsorbed anti-IgG/HRP.

Bacterial adhesion assay

Both Gram positive *Staphylococcus aureus* (G^+) and Gram negative *E.coli* (G^-) bacteria were used to investigate the resistance to bacterial adhesion. The *Staphylococcus aureus* seeds were first incubated in the culture medium at 37 °C, 200 rpm for 24 h. Then the *Staphylococcus aureus* was separated by centrifugation and re-suspended in 30 mL of sterile PBS. After rinsing twice with PBS, the bacterial density was adjusted to 1×10^6 cells mL^{-1} ($OD_{670} = 0.015$). Prior to testing, the samples and TCPS controls were immersed in alcohol/water solution (v/v = 3 : 1) for 5 min, followed by sterile PBS for 1 min. Then they were incubated with 1×10^6 cells mL^{-1} bacterial suspension for 1–2 h, washed with sterile PBS and then stained with Live/Dead® BackLight bacterial viability kits. The images were recorded by a CCD camera on a Nikon Eclipse Ti series microscope with a 20× lens. The operation procedure for *E.coli* was the same as that for *Staphylococcus aureu*.

Cell attachment assay

The samples and TCPS controls were transferred to a 24-well plate and each well was loaded with 1 mL HUVECs (4×10^5 cells mL^{-1}) suspension in RPMI 1640 culture medium plus 5% fetal bovine serum (FBS) and incubated for 1 day at 37 °C in 5% CO_2. After the medium was removed, each well was loaded with 1 mL of 5 μg mL^{-1} FDA solution and incubated for 5 min. After rinsing twice with PBS, each well was loaded with 1 mL RPMI 1640 and the images were directly recorded by a CCD camera on a Nikon Eclipse Ti series microscope with a 20× lens.

MTT assay

A typical procedure for the MTT assay is as follows: a 5 mg mL^{-1} MTT stock solution was diluted in RPMI 1640 medium to make a 5 μg mL^{-1} working solution. HUVECs were seeded in a 96-well tissue culture plate at a density of 10 000 cells per well and were cultured in RPMI 1640 medium with 10% FBS. After 1 day of incubation, the medium was removed and the cells were washed with RPMI 1640 medium twice. Then the cells were incubated in 100 μL of RPMI 1640 medium with polypeptides at various concentrations at 37 °C for 24 h, and the metabolic activity was determined by using an MTT assay. After the medium was removed, 100 μL MTT working solution was transferred into each well and the samples were incubated at 37 °C for 4 h. Then, the medium was removed and 100 μL DMSO was added and incubated for 10 min. The absorbance at 570 nm was recorded using a 96-well plate reader SpectraMaxM2, Molecular Devices, Sunnyvale, CA. Cell viability was expressed as the percentage of absorbance of treated cells with various polypeptide concentrations compared to the absorbance of cells that were not treated. Each measurement had three replicate wells.

Haemolytic activity assay

The haemolytic activity assay was performed based on a previous study.[20] Red blood cells (RBCs) were collected by centrifugation of whole blood in sterile PBS at 1500 rpm for 10 min. The RBCs were further washed three times using sterile PBS. After the supernatant had been removed following the last wash, the cells were resuspended in PBS to get a 2% w/v RBC suspension. The tested samples were also prepared in sterile PBS. 150 μL of the sample solution and 150 μL of the 2% w/v RBC solution were added to the centrifuge tube to make a sample final concentration of 5 mg mL^{-1} and incubated for 4 h at 37 °C. Then the mixture was centrifuged and the supernatant was transferred to a 96-well plate. The relative adsorption of the supernatants was measured on a microplate reader at 575 nm. Complete haemolysis was attained using water as the positive control and PBS as the negative control. The haemolytic activity was defined as follows: haemolytic activity% = [(sample absorbance − negative control)/(positive control − negative control)] × 100%.

Results and discussion

Synthesis of the EK polypeptide

In this work, the target EK polypeptide (**7**) with uniform alternating charges was achieved by following the synthesis route in Scheme 1. The ^{1}H NMR spectrum of the EK polypeptide (**7**) is shown in Fig. 1.

Commercially available H-Lys(*Z*)-O^{t}Bu·HCl (**1**) and Boc-Glu(*Z*)–OH (**3**) were coupled using EDC·HCl/HOBt to obtain the fully protected EK dimer (**4**), followed by cleavage of Boc (*tert*-butoxycarbonyl) by using TFA/CH_2Cl_2 (v/v = 2 : 1) solution to get the EK dimer (**5**) with *Z*-protected side chains, which serves as a basic block for the successful synthesis of the EK polypeptide. Complete removal of the protection group Boc was confirmed by disappearance of δ 1.23–1.45 18H in ^{1}H NMR. The EK dimer

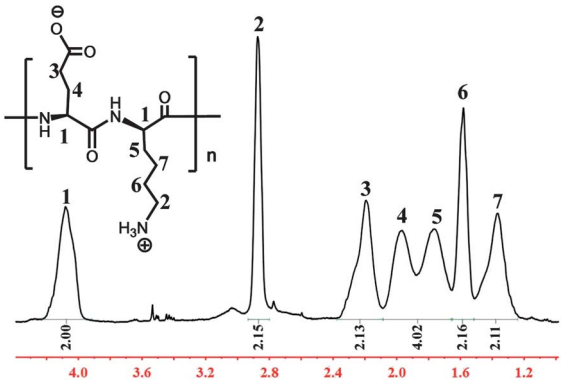


Fig. 1 Nuclear magnetic resonance (^{1}H NMR) spectrum of the EK polypeptide.

(**5**) was condensed by EDC·HCl/HOBt and D,L-lipoic acid serves as the end-group to adjust the MW. The MWs of the synthesized EK polypeptides are 2.8 kDa (PDI = 1.13), 5.4 kDa (PDI = 1.31), and 12.0 kDa (PDI = 1.90) (Table 1). The difference between the feeding molar ratios of the EK dimer (**5**) to lipoic acid and the MWs of the synthesized EK polypeptides might be caused by the side reaction of minor impurities in the EK dimer (**5**). The low PDI of the 2.8 kDa EK polypeptide is achieved by dialysis.

The properties of SAMs characterized by ATR-FTIR, XPS and ELL

In order to verify the formation of the polypeptide SAMs on the gold coated surface, the polypeptide SAMs were investigated by ATR-FTIR, XPS and ELL. From the FTIR spectra (Fig. 2), two typical secondary amide bands, defined as band I ($\nu_{C=O}$, 1600–1750 cm^{-1}) and band II (δ_{N-H}, 1500–1560 cm^{-1}) can be seen. The peaks over 3500 cm^{-1} might mainly come from the water molecules adsorbed on the zwitterionic polypeptide SAMs[21] since water molecules could be strongly bound with the polypeptide through both ionic hydration and hydrogen bond formation. This strong hydration capability leads to the excellent resistance of these zwitterionic polypeptides to protein adsorption, bacterial adhesion and cell attachment.

XPS survey spectra of the EK polypeptide coated gold surfaces and bare gold surface were also measured. By comparing these four survey spectra in Fig. 3, the emergence of N 1s, representing the characteristic element peak of polypeptides, together with the obvious intensity increases of C 1s and O 1s indicate the formation of the EK polypeptide SAMs. Furthermore, detailed scans of carbon, nitrogen and oxygen were also obtained to further investigate the relative element

Table 1 The feed molar ratio of the EK dimer (**5**) to lipoic acid, MW (M_w) and polydispersity index (PDI) of the target EK polypeptide (**7**)

n(EK dimer)/*n*(lipoic acid)	MW (M_w)	PDI	Yield (%)
20 : 1	2.8 kDa	1.13	44.2
40 : 1	5.4 kDa	1.31	47.6
200 : 1	12.0 kDa	1.90	51.4

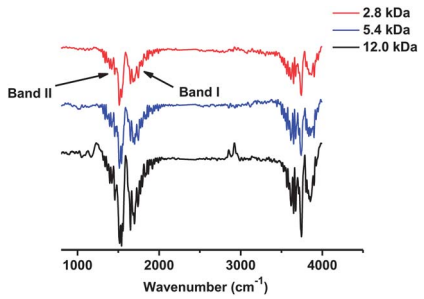


Fig. 2 ATR-FTIR spectroscopy of EK polypeptide SAMs at different MWs. The main characteristic peaks of the secondary amide in band I ($\nu_{C=O}$, 1600–1750 cm^{-1}) and band II (δ_{N-H}, 1500–1560 cm^{-1}) can be seen from the spectra, indicating the formation of the EK polypeptide SAMs on the gold surface.

contents of the SAMs (Table 2). The XPS-measured carbon and nitrogen contents were slightly lower than their theoretical values while the oxygen content was much higher than its theoretical value. The deviation of the oxygen content from its theoretical value is about 24.8%, which is similar to the observation by S. F. Chen and Y. C. Chung, *et al.*[1,22,23] According to their previous results, the high content of oxygen mainly came from the hydration water on the zwitterionic polymer, which will also lead to lower contents of carbon and nitrogen in the XPS assay than the theoretical ones. Moreover, such an observation also agrees with the peaks over 3500 cm^{-1} in the FTIR spectra. All this information suggests that the zwitterionic polypeptide SAMs should obviously contain water due to all samples being exposed to an ambient environment.

Film thickness (or packing density) is an essential parameter which influences the nonspecific protein adsorption of the SAMs. In Fig. 4, when the MWs increase from 2.8 kDa to 12.0 kDa, the corresponding thicknesses of the SAMs are 2.45 ± 0.02 nm, 3.12 ± 0.02 nm and 3.82 ± 0.03 nm, respectively. It is clear that the thickness does not linearly increase with the MW. As the MW increases, the increase rate of the SAM thickness drops. It is worth mentioning that the thickness of the 2.8 kDa EK polypeptide SAMs (2.45 ± 0.02 nm) is rather reasonable. The thickness of the 2.8 kDa EK polypeptide SAMs should be about 3.3 nm or 7.7 nm, for an α-helix format or an all-extending state, respectively. The measured thickness is shorter than the α-helix format, which indicates that the conformation of the 2.8 kDa EK polypeptide on the gold surface is not in the α-helix format. It is believed that the conformation of these polypeptides might be close to randomly coiled since the diameter of the random coil increases more slowly than the increase rate of the MW. Also, the small random coils could pack more densely than large ones, which leads to fewer surface defects for the 2.8 kDa EK polypeptide SAMs.[24] The nonspecific protein adsorption could be reduced consequently since defects are the key reason for protein adsorption on the zwitterionic polymer coated surface. Thus, the lowest nonspecific protein adsorption is achieved by the SAMs formed by the 2.8 kDa EK polypeptide as shown in Fig. 5.

Table 2 Element contents of the polypeptide SAMs determined by XPS (M_w = 2.8 kDa)[a]

Element	Theoretical value (%)	Measured value (%)	Relative deviation (%)
C	70.8	67.3	−5.0
N	12.5	11.9	−4.9
O	16.7	20.8	24.8

[a] The composition of the substrate is not included and the relative element content values are calculated as follows: element% = specific element fitting area/(C + N + O) fitting area × 100%.

Nonspecific protein adsorption assay

The resistance of the polypeptide SAMs to nonspecific protein adsorption was evaluated from the amounts of conjugated HRP on the surface. The adsorption of anti-IgG/HRP was used as the antibody adsorption, while the specific adsorption of anti-Fg/HRP on the physically adsorbed Fg was used to represent the adsorption of Fg. The relative adsorption values were obtained from the comparison between the protein adsorption on the samples and on the TCPS. As shown in Fig. 5, with the increase of the MW from 2.8 kDa to 12.0 kDa, the relative nonspecific protein adsorption for the antibody and Fg increases from 5.1 ± 1.6% and 7.3 ± 1.8% to 11.6 ± 5.6% and 15.2 ± 3.2%, respectively. Moreover, the relative adsorption amounts of Fg were higher than the antibody on these three EK polypeptide SAMs. It is believed that these phenomena are mainly caused by the

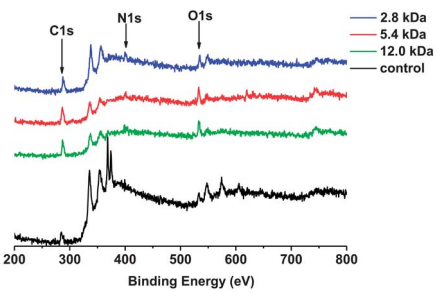


Fig. 3 XPS survey spectra of the samples and bare gold surface. The emergence of N 1s and obvious intensity increase of C 1s and O 1s indicate the formation of the EK polypeptide SAMs on the gold surface.

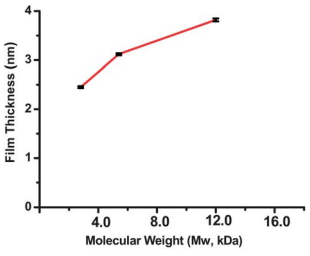


Fig. 4 Film thicknesses of polypeptide SAMs at different MWs. When the MWs increase from 2.8 kDa to 12.0 kDa, the film thicknesses increase from 2.45 ± 0.02 nm to 3.82 ± 0.03 nm. The gradient of the film thickness *versus* MW decreases when the MW increases. The results are shown as means ± standard deviations (SD) (n = 3).

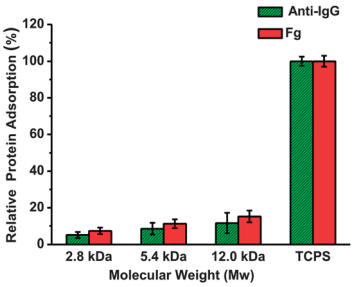


Fig. 5 Relative nonspecific antibody and Fg adsorption on polypeptide SAMs formed by different MW EK polypeptides, where the nonspecific protein adsorption of the TCPS surface was set at 100%. The relative nonspecific protein adsorptions of the antibody and Fg increase from $5.1 \pm 1.6\%$ and $7.3 \pm 1.8\%$ to $11.6 \pm 5.6\%$ and $15.2 \pm 3.2\%$, when the MW of the EK polypeptides increases from 2.8 kDa to 12.0 kDa. The results are means $\pm$ standard deviations (SD) ($n = 3$).

higher tendency of Fg to denature due to its flexible structure.[12] Compared to the pMPC or PEG coated surface, it is believed that the main reason for the high protein adsorption on the EK polypeptide SAMs is the defects on the EK polypeptide SAMs.

There are two factors which might contribute to the increase of the protein adsorption as the MW increase. The large size of high MW polypeptide might cause big cavities among the immobilized polypeptide chains, where proteins can adsorb. The second possible factor is protein adsorption through multiple contact points between protein molecules and the EK polypeptide chains. Long-chain polypeptides are flexible and have more contact points available with protein molecules, which might cause nonspecific protein adsorption.[25] On the contrary, these two issues can be avoided in the SAMs formed by the short EK polypeptide. In short, the SAMs formed by the short EK polypeptide could result in better resistance through a dense monolayer structure and strong hydration of zwitterionic molecular structures.

Cell attachment and bacterial adhesion assay

In general, it is believed that the protein resistance of surfaces could also exhibit resistance to cell attachment and bacterial adhesion. Here, HUVECs, derived from the endothelium of veins from the umbilical cord, were use to test the resistance to cell attachment. In this experiment, FDA is chosen to stain HUVECs to observe cell attachment and cytoplasmic spreading. Thus, we examined cell attachment on various SAM surfaces with different MWs after 24 h incubation (images are not shown). The representative morphology of cells on the EK polypeptide SAM and its control surface (TCPS) are presented in Fig. 6. The results show no HUVEC attachment on the SAMs of various EK polypeptides, whereas the HUVECs grow normally in the same culture well. Furthermore, HUVECs display good attachment and spreading conditions on the TCPS surface with an additional 5 mg mL^{-1} of polypeptide ($M_w = 2.8$ kDa) in the culture medium (Fig. 7), indicating there is good compatibility between the polypeptide and the cells.

Microbial adhesion onto surfaces and the subsequent formation of a biofilm are critical issues for many biomedical

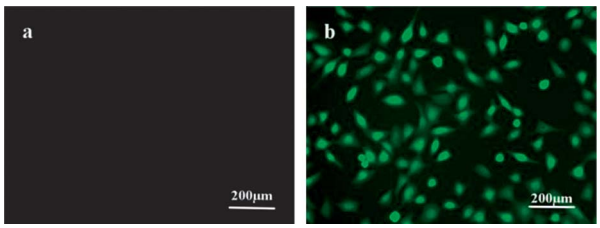


Fig. 6 Cell images of HUVECs on (a) representative EK polypeptide SAM ($M_w = 2.8$ kDa) surface and (b) TCPS surface. Cells were stained with FDA. No cell attachment was detected on the SAM surfaces and a large amount of cells were found on the TCPS surface.

and engineering applications.[26] *Staphylococcus aureus* and *E. coli* are commonly used model bacteria in scientific research. *Staphylococcus aureus* can cause skin infections, endocarditis, osteomyelitis and pneumonia. *E. coli* can cause urinary tract infections or other infections. Thus, microbial adhesion assays were performed to further investigate the fouling resistance of the SAMs to Gram positive (G^+) *Staphylococcus aureus* and Gram negative (G^-) *E. coli.* The results show that there was no obvious attached *Staphylococcus aureus* and *E. coli* (data not shown) observed on the polypeptide SAM surfaces, while a large amount of attached *Staphylococcus aureus* was present on the TCPS surface (Fig. 8). In short, all results indicate that the polypeptide SAM surface showed good resistance to nonspecific protein adsorption, cell attachment and bacterial adhesion.

MTT and haemolytic activity assay

In order to demonstrate the good biocompatibility of the EK polypeptides, *in vitro* cytotoxicity and haemolytic activity of the EK polypeptides were investigated. The EK polypeptides show no toxicity up to 5 mg mL^{-1} concentration after 24 h incubation. The cell viabilities vary between 87.4% and 107.6% when the polypeptide concentrations vary within 0.01–5 mg mL^{-1} (Fig. 9). Even when the concentration is up to 5 mg mL^{-1}, the corresponding cell viability can be maintained at $87.4 \pm 4.5\%$ of the normal cell value, indicating no cytotoxicity *in vitro*. Moreover, the cell morphology in the 5 mg mL^{-1} ($M_w = 2.8$ kDa) EK polypeptide solution shows no apparent difference to the control solution.

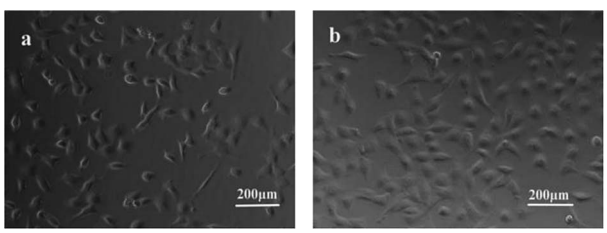


Fig. 7 Representative morphology of cells cultured in (a) RPMI 1640 growth media and (b) with an additional 5 mg mL^{-1} EK polypeptide ($M_w = 2.8$ kDa). Almost no apparent differences between the sample and its control were found even though the polypeptide feed concentration is up to 5 mg mL^{-1}, indicating no cytotoxicity *in vitro* for the polypeptides.

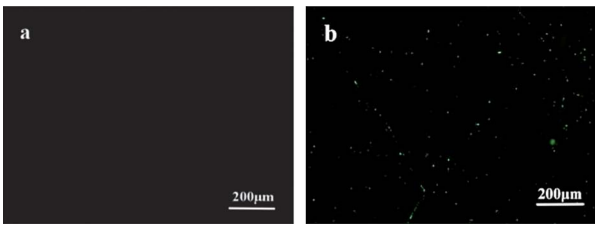


Fig. 8 Bacterial adhesion images of Gram positive (G^+) *Staphylococcus aureus* on (a) the representative polypeptide SAMs (M_w = 2.8 kDa) and (b) the TCPS surface. No obvious adhered bacteria were detected on the SAMs and a large amount of bacteria were found on the TCPS surface.

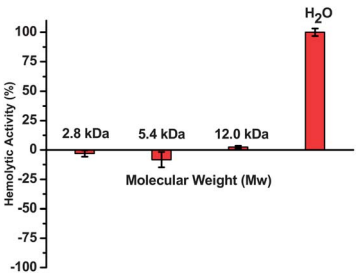


Fig. 10 Haemolytic activities for the polypeptides with various MWs at a concentration of 5 mg mL^{-1}. For samples with MWs of 2.8 kDa, 5.4 kDa and 12.0 kDa, no haemolytic activity was detected. The results are shown as means ± standard deviations (SD) (n = 3).

Furthermore, the haemolytic activity assay (Fig. 10) also indicates the low interaction between EK polypeptides and the cell membrane. It was found that the light absorbance of the supernatants after removal of red blood cells with 2.8 kDa, 5.4 kDa and 12.0 kDa EK polypeptides was very low. The haemolytic activity of the 2.8 kDa and 5.4 kDa EK polypeptides was even slightly lower than that of the negative control (PBS solution). Such a phenomenon might be due to the protection imparted by the EK polypeptides to red blood cells. The highest haemolytic activity is observed for 12.0 kDa EK polypeptides, for which the haemolytic activity is just 2.4 ± 1.1%. Such a low haemolytic activity agrees with the cytotoxicity results and also suggests that the low cytotoxicity should be attributed to the low interactions of the EK polypeptides with the protein and cell membrane. In short, all the EK polypeptides exhibit very good biocompatibility and are very good candidates for biodegradable nonfouling materials.

Conclusions

In this work, nonfouling polypeptides with uniform alternating charges were prepared by the polycondensation of a covalently bonded EK dimer using facile EDC·HCl/HOBt chemistry. The results show that the EK polypeptides can have excellent resistance to nonspecific protein adsorption and biocompatibility the same as other nonfouling zwitterionic polymers. Furthermore, they also have the inherent biodegradability of natural polypeptides. The new synthesis route through EK dimer polycondensation solves the nonuniformity problem in the NCA ring opening polymerization of mixed E and K monomers. Moreover, good control of the MW of EK polypeptides is achieved by this new method. The results indicate that a rather short polypeptide (2.8 kDa) is sufficient to resist nonspecific protein adsorption. It is expected that this kind of polypeptide could be used as an alternative to other synthetic nonfouling materials, such as PEG, to avoid non-biodegradability in biomedical applications, especially for nano drug carriers.

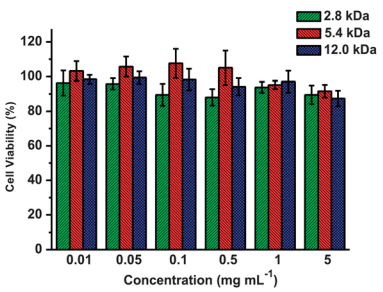


Fig. 9 Cell viabilities of the different MW polypeptides (M_w = 2.8 kDa, 5.4 kDa and 12.0 kDa) at various concentrations. When the polypeptide concentrations vary within 0.01–5 mg mL^{-1}, the cell viabilities vary between 87.4% and 107.6%, indicating no cytotoxicity *in vitro* for the polypeptides. The results are shown as means ± standard deviations (SD) (n = 3).

Acknowledgements

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