Investigation of nonfouling polypeptides of poly(glutamic acid) with lysine side chains synthesized by EDC·HCl/HOBt chemistry

Qinghua Yang\textsuperscript{a,b}, Wenchen Li\textsuperscript{c}, Longgang Wang\textsuperscript{a}, Guangzi Wang\textsuperscript{a}, Zhen Wang\textsuperscript{a}, Lingyun Liu\textsuperscript{c} and Shengfu Chen\textsuperscript{a*}

\textsuperscript{a}Key Laboratory of Biomass Chemical Engineering of Ministry of Education, Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, P.R. China; \textsuperscript{b}School of Medical Engineering, Hefei University of Technology, Hefei 230009, P.R. China; \textsuperscript{c}Department of Chemical and Biomolecular Engineering, University of Akron, Akron, OH 44325, USA

(Received 30 April 2014; accepted 1 July 2014)

Nonfouling polypeptides with homogenous alternating charges draw peoples’ attentions for their potential capability in biodegradation. Homogenous glutamic acid (E) and lysine (K) polypeptides were proposed and synthesized before. In this work, a new polypeptide formed by poly(glutamic acid) with lysine side chains (poly(E)-K) was synthesized by facile EDC·HCl/HOBt chemistry and investigated. Results show that these polypeptides also have good nonspecific protein resistance determined by enzyme-linked immunosorbent assay. The lowest nonspecific adsorption of the model proteins, anti-IgG and fibrinogen (Fg), on the self-assembling monolayers (SAMs) surface of poly(E)-K was only 3.3 ± 1.8 and 4.4 ± 1.6%, respectively, when protein adsorption on tissue culture polystyrene surface was set as 100%. And, the relative nonspecific protein adsorption increases when the polypeptide molecular weight increases due to the repression of low density polymer brushes. Moreover, almost no obvious cytotoxicity and hemolytic activity in vitro were detected. This work suggests that polypeptides with various formats of homogenous balanced charges could achieve excellent nonspecific protein resistance, which might be the intrinsic reason for the coexistence of high concentration serum proteins in blood.

Keywords: polypeptides; nonfouling; biocompatibility; SAMs; poly(E)-K

1. Introduction

Polypeptide-based nonfouling materials could be biocompatible alternatives of polyvinyl-based ones due to their potential in natural structure-originated properties, such as biodegradability, compatibility and nontoxicity of themselves, and their degradable products. Recently, polypeptides, with homogenous alternating glutamic acid (E) and lysine (K) (poly(EK)), were synthesized and showed high nonfouling property, which proved that the zwitterionic structure mimic at nanometer scale could be an efficient way to obtain resistance to nonspecific protein adsorption.[1–3] Moreover, a facile method was successfully developed to solve the uniformity problem of nonfouling peptides caused by copolymerization of EK dimers in large quantities for potential biomedical applications.[2]
On the other hand, the methacrylate-based materials containing amino acids were proved to be effective antifouling materials. Liu et al. developed poly(serine methacrylate), poly(lysine methacrylamide) and poly(ornithine methacrylamide) for fouling resistance.[5] Ishii also developed copolymers of N-methacryloyl-L-histidine and n-butyl methacrylate to reduce surface biofouling and found the 50% content of the histidine monomer one as the most excellent candidate.[6] Shiraishi synthesized several poly(methacrylate) copolymer-based microspheres, among which poly(O-methacryloyl-L-serine-co-methyl methacrylate) (poly(SerMA-co-MMA)) showed the most effective suppression of protein adsorption.[7] More interestingly, a series of lysine-containing polymers developed by Chen H group [6–9] showed both fouling resistance and fibrinolytic activity originated from the synergetic effect of the ε-amino group and the carboxyl group, while additional 2-hydroxyethyl methacrylate (HEMA) or poly(ethylene glycol) (PEG) methacrylate was introduced to control the density of lysine in these copolymers. Klok also developed an oligo(ethylene glycol) (OEG)-containing surface with lysine brushes by ring-opening polymerization (ROP) of lysine N-carboxyanhydride (NCA).[10]

Here, we proposed a new structure, poly(glutamic acid) with lysine side chains to investigate the fouling resistance. This polypeptide has structure similar to lysine-based polymethacrylamide developed by Chen H group [6–9], which could show extra fibrinolytic activity besides the fouling resistance observed in poly(EK). The detailed synthesis route is shown in Scheme 1. DL-lipoic acid was applied to adjust molecular weight (MW) by attaching it to the N-terminal of the polypeptides. The lipoic acid-capped poly(E)-K can form SAMs on gold surface through two thiol groups. The polymer was verified by nuclear magnetic resonance (1H NMR) and gel permeation chromatography (GPC). Chemical and physical properties of the SAMs were systematically studied by attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS), and Ellipsometer (ELL). The relationships between protein resistance, SAM thickness, and polypeptide MW were also discussed. The biocompatibility including in vitro cytotoxicity and hemolytic activity of polypeptides was also investigated. All results indicated that these new polypeptides with lysine side chains are good biocompatible nonfouling materials through reducing nonspecific interactions with protein molecules and cell membranes.

2. Materials and methods

2.1. Materials

α-Benzyl-Nε-benzyloxycarbonyl-L-lysine hydrochloride (H-Lys(Z)-OBzl.HCl, 99%) and α-tert-butyl-Nα-(tert-butoxycarbonyl)-L-glumatic acid (Boc-Glu-OtBu, 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 peroxidase (HRP)-conjugated goat anti-human IgG (anti-IgG/HRP), HRP-conjugated anti-fibrinogen (anti-Fg/HRP), and Fg were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC.HCl, 98.5%), 2-hydroxybenzotriazolehydrate (HOBT, 99%), O-phenylenediamine (OPD, 98%), trifluoroacetic acid (TFA), N,N-dimethylformamide (DMF), dimethylsulfoxide (DMSO), and triethylamine (TEA) were purchased from Aladdin Reagent Co., Ltd. Thirty-three weight percent HBr/HOAc solution was purchased from Sigma-Aldrich Co. LLC. All other chemicals were of reagent grade. All chemicals were used without further purification.
2.2. Synthesis route

The synthesis route is summarized in Scheme 1.

Scheme 1. Synthesis route of the poly(E)-K polypeptide.
2.2.1. Synthesis of \( \delta \)-(a-benzyl-N\(^\varepsilon\)-benzyloxycarbonyl-L-lysyl)-a-tert-butyl-N\(^\alpha\)-tert-butoxycarbonyl-L-glutamic acid (4)

To a solution of H-Lys(Z)-OBzl·HCl (1, 6.70 g, 11.78 mmol) in 30 mL anhydrous DMF, about 2 mL TEA (14.50 mmol) was added and kept reacting for 20 min under rigorous agitation followed by filtration to remove the precipitation. Then, BOC-Glu-OtBu (3, 3.56 g, 11.78 mmol), EDC.HCl (4.52 g, 23.56 mmol), and HOBt (3.18 g, 23.56 mmol) were added to the filtrate under \( \text{N}_2 \) bubbling for about 30 min. The reaction was quenched with 10\% aqueous citric acid solution after 48 h at room temperature, and the organic layer was washed with saturated aqueous NaHCO\(_3\) solution, water, saturated brine, and dried over anhydrous MgSO\(_4\). The solvent was removed by a rotary evaporator under reduced pressure, and the residue was purified by a column chromatography (silica gel, ethyl acetate/dichloromethane = 1:3, V/V) to obtain the compound (4) (6.92 g, 89.5\%). \(^1\)HNMR (DMSO-\( d\_6 \), 400 MHz): \( \delta \) 1.21–1.45 22H, 1.49–1.69 2H, 1.70–1.96 2H, 2.35–2.46 2H, 2.92–3.03 2H, 3.94–4.10 2H, 4.95–5.12 4H, 6.96–7.01 0.93H, 7.20–7.26 0.93H, 7.29–7.42 10H, 8.05–8.11 0.98H.

2.2.2. Synthesis of \( \delta \)-(a-benzyl-N\(^\varepsilon\)-benzyloxycarbonyl-L-lysyl)-L-glutamic acid (5)

To a 30 mL TFA/\( \text{CH}_2\text{Cl}_2 \) (V/V = 2:1) solution, 4 (8.0 g, 12.20 mmol) was added. The mixture was stirred for 6 h at 0 \( ^\circ \)C, followed by removal of the solvent by a rotary evaporator under reduced pressure to get an oil residue. The residue was dissolved in ethyl acetate and TEA was added to make a neutral solution. The organic solvent was removed by rotary evaporator and the residue was suspended in water under agitation for several times and the mixture was filtrated to get the compound (5) (4.60 g, 75.7\%). \(^1\)HNMR (DMSO-\( d\_6 \), 400 MHz): \( \delta \) 1.19–1.41 4H, 1.53–1.72 2H, 1.75–1.93 2H, 2.21–2.37 2H, 2.87–2.98 2H, 3.25–3.31 1H, 4.14–4.24 1H, 4.94–5.03 2H, 5.05–5.13 2H, 7.22–7.26 0.80H, 7.27–7.39 10H, 7.45–7.85 2H, 8.65–8.74 0.96H.

2.2.3. Synthesis of poly (\( \delta \)-(a-benzyl-N\(^\varepsilon\)-benzyloxycarbonyl-L-lysyl)-L-glutamic acid) (6)

To a solution of dimer (5) (0.2 g, 0.4 mmol) in DMF (2 mL), EDC.HCl (0.12 g, 0.6 mmol) and HOBt (0.08 g, 0.6 mmol) were added. After bubbling with filtrated \( \text{N}_2 \) for about 30 min, the resulting mixture was kept for reaction at room temperature for 48 h. After completion of the reaction, the mixture was poured into excessive ethyl ether to isolate the polymer (6). The polymer (6) was kept in oil for next reaction without purification.

2.2.4. Synthesis of poly(N\(^\delta\)-L-lysyl-L-glumatic acid) (7)

Deprotection of Z moiety was achieved by a mixed solution of 33wt.% HBr/HOAc and TFA(V/V = 1:1).\(^{[11]}\) The product was precipitated by ethyl ether, neutralized to pH 7.0 by saturated NaHCO\(_3\) solution, dialyzed against deionized (DI) water, and lyophilized to obtain a white powder (7). Full cleavage was confirmed by total disappearance of peaks at \( \delta \) 7.22–7.39 and 4.94–5.13 in \(^1\)HNMR spectroscopy. From the (E)-K dimer, the average yield for the polypeptide is about 40\%. \(^1\)HNMR (D\(_2\)O, 400 MHz): \( \delta \) 1.20–1.40 2H, 1.42–1.78 4H, 1.80–2.10 2H, 2.10–2.40 2H, 2.80–2.90 2H, 3.92–4.32 2H.
2.3. **GPC assay**

The MW and polydispersity index (PDI) of polypeptides were determined by a Waters Ultrahydrogel™ 120 column 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 at 40 °C. Poly(ethylene oxide) with different MWs were used as standards.

2.4. **ATR-FTIR for film structure and ELL for film thickness**

Gold-coated chips were rinsed with ethanol and DI water, dried by filtered air, and placed in a UV cleaner for 20 min. Then, chips were incubated with a PBS solution (pH 7.4) of 3 mg/mL polypeptide for 24 h. The chips were then rinsed several times by PBS, followed by drying with filtered air to make clean chips for measurement. ATR-FTIR was used to verify the formation of the SAMs on chips by detecting the characteristic groups of the polypeptides. Thickness of the film was measured by a Spectroscopic Ellipsometer (J. A. Woollam M-2000D). For each sample, five separate spots were measured and bare gold-coated chips were used as references.

2.5. **XPS measurement**

The pretreatment for the chips are the same as that for FTIR and ELL. XPS was conducted on an X-Probe Spectrometer (VG ESCALAB MARK II) equipped with a monochromatic Mg Kα X-ray source (hv=1253.6 eV), a hemi-spherical analyzer, and a multichannel detector. For each SAM, three separate spots were examined. The bare gold-coated chips were used as references.

2.6. **Protein adsorption assay**

Nonspecific protein adsorption of the polypeptide SAMs on gold surfaces and tissue culture polystyrene (TCPS) control were determined by anti-IgG/HRP and Fg adsorption. The samples were first incubated with anti-IgG/HRP (1 µg/mL) or Fg (10 µg/mL) for 20 min, and rinsed with PBS to remove all free proteins. For Fg adsorption, the samples were further incubated with anti-Fg/HRP (10 µg/mL) for another 10 min. After that, all tested samples and TCPS controls were placed in a 24-well plate and incubated with 1 mL O-phenylenediamine (OPD) (1 mg/mL) in citrate phosphate buffer (0.1 M, pH 5.0) containing 0.03% hydrogen peroxide. Enzyme activity was quenched by adding an equal volume of 2N H2SO4 after 6–8 min incubation. The tangerine color was measured by a UV spectrophotometer at 492 nm.

2.7. **MTT assay**

A typical procedure for the MTT assay is described as follows: MTT was dissolved in PBS (pH 7.4) to obtain a 5 µg/mL MTT working solution. HUVECs were seeded in a 96-well tissue culture plate at a density of 10,000 cells per well and cultured in a RPMI 1640 medium with 10% FBS. After one day incubation, the medium was removed and the cells were washed by RPMI 1640 medium twice. Then, polypeptides were added at various concentrations of 0.01, 0.05, 0.1, 0.5, 1, and 5 mg/mL, followed by additional 1 day incubation. Then, for each concentration, MTT solutions were loaded for three wells. The MTT solution was removed after 4h incubation, then 100 µL DMSO was
added to each well followed by 10 min shaking to get a complete dissolving of the MTT reduction product in cells. The cell viability was determined by measuring the absorbance of each sample at 492 nm on a Microplate Reader.

2.8. Hemolytic assay [13–15]

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 by sterile PBS. After the supernatant was removed following the last wash, the cells were resuspended in PBS to get a 2% w/v RBC suspension. The tested polypeptide samples were also prepared in sterile PBS. One hundred and fifty microliters of the sample solution and 150 μL of the 2% w/v RBCs solution were added to a centrifuge tube to make a sample with final concentration of 5 mg/mL 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 supernatants was measured on a Microplate Reader at 575 nm. Complete hemolysis was attained using water as the positive control, and PBS was used as the negative control. The hemolytic activity is defined as follows: Hemolytic activity% = [(sample absorbance − negative control)/(positive control − negative control)] × 100%.

3. Results and discussions

3.1. Synthesis of the polypeptides

In this work, the proposed polypeptides (poly(E)-K) were synthesized by Scheme 1. Commercially available H-Lys(Z)-OBzl.HCl and BOC-Glu-OtBu were coupled by facile EDC·HCl/HOBt chemistry, followed by removal of BOC (tert-butoxycarbonyl) in TFA. Weak organic base TEA was used to remove TFA to get a Z-protected dimer (5), which serves as a key unit for successful polymerization. Three peaks (m/z) at 456.3, 500.3, and 538.1 (from left to right) are found in ESI-MS spectroscopy (see details in ESI, Figure S1), which are assigned to ([M – CO2] + H)+, [M + H]+, and [M + K]+, where M represents the dimer (5). And, the 1HNMR spectroscopy (Figure S2) also indicates that the dimer (5) was successfully synthesized for polymerization in the next step. The dimer (5) was then condensed by EDC·HCl/HOBt chemistry to get a Z-protected polypeptide (6), and DL-lipoic acid was used as the end group to adjust the MW of polypeptide. The final product (7) was achieved by total cleavage of the protection moiety of Z, which can be confirmed by the disappearance of peaks at δ 7.27–7.39 and 4.94–5.13 in 1HNMR spectroscopy. Three poly(E)-K polypeptides at the MW of 3.5 kDa (PDI = 1.08), 5.6 kDa (PDI = 1.44), and 13.7 kDa (PDI = 1.83) were obtained (Table 1) and their 1HNMR spectroscopy is shown in Figure S3. The difference between the theoretical MW determined by the feeding molar ratio of dimer (5) to lipoic acid

Table 1. The feeding molar ratio of dimer (5) to lipoic acid, weight-average MW and PDI of the target polypeptides.

| n(Dimer)/n(Lipoic acid) | MW (kDa) | PDI   |
|------------------------|----------|-------|
| 20:1                   | 3.5      | 1.08  |
| 40:1                   | 5.6      | 1.44  |
| 200:1                  | 13.7     | 1.83  |
and the deviation of MW of the synthesized poly(E)-K polypeptides from theoretical values might be caused by side reactions of minor impurities in dimer (5). The very low PDI of 3.5 kDa poly(E)-K polypeptide is possibly caused by its low polymerization degree and the dialysis during preparation in which some of short poly(E)-K peptides could be removed to narrow MW distribution.

### 3.2. SAM properties characterized by ATR-FTIR, XPS, and ELL

Chemical and physical properties of the polypeptide SAMs were systematically characterized by ATR-FTIR, XPS, and ELL. In Figure 1, the main characteristic peaks of the secondary amide, band I ($\sigma_{C=O}$, 1600–1750 cm$^{-1}$), band II ($\delta_{N-H}$, 1500–1650 cm$^{-1}$) can be found, indicating the existence of polypeptide SAMs on the gold surfaces. The detailed spectra of the band I (carbonyl region) clearly show two peaks for amide in lower wavenumber region and carboxyl groups in lysine side chains in higher wavenumber region, respectively. Survey and detailed scans of XPS spectra were obtained to provide further evidences for the existence of the SAMs on gold surface and to investigate relative element contents of the polypeptide SAMs. By comparing the sample and control spectra in Figure 2, both the newly emerging peak of N1s, serving as the characteristic element peak of polypeptides, and the obvious intensity increase of C1s and O1s indicate the polypeptide SAM formation.

In order to quantify the relative element contents of the SAMs, detailed scans of carbon, nitrogen, and oxygen were acquired. The detected carbon and nitrogen contents

![Figure 1. ATR-FTIR spectroscopy of polypeptide SAMs.](image)

Notes: Band I ($\sigma_{C=O}$, 1600–1750 cm$^{-1}$), band II ($\delta_{N-H}$, 1500–1600 cm$^{-1}$), the main characteristic peaks of the secondary amide can be seen, indicating the existence of polypeptide SAMs on gold surfaces. The insert picture shows the details in band I range.
are 63.6 ± 2.4 and 10.2 ± 1.3% (Table 2), respectively, which are slightly lower than their theoretical values of 70.8 and 12.5%. However, oxygen content (26.7 ± 1.6%) increases significantly from its theoretical value (16.7%) with a large relative deviation of 59.9 ± 9.6%, which had also been observed by Chen and Chung et al. [16,17]. According to their results,[16,18] the large amount of the hydration water of the zwitterions of the polypeptides could increase the content of oxygen and lower the contents of carbon and nitrogen, found in XPS measurements comparing the theoretical value.

Film thicknesses are determined by ELL. In Figure 3, when the MWs of tested samples are 3.5, 5.6 and 13.7 kDa, the corresponding thicknesses of the SAMs are 2.8 ± 0.1, 3.4 ± 0.2, and 3.8 ± 0.6 nm, respectively. As the MW increases, the slope factor slightly decreases, indicating that the thickness of the SAMs does not linearly increase with the MWs. The possible reason for this phenomenon is that longer polymers have more flexibility and are in a ‘collapsed’ state, thus leading to a slower thickness increase.

3.3. Nonspecific protein adsorption assay

As a fundamental property of the nonfouling materials, the nonspecific protein adsorption of the polypeptide SAMs was evaluated by enzyme-linked immunosorbent assay.

Table 2. Element contents of polypeptide SAMs detected by XPS (MW = 3.5 kDa).

| Element | Theoretical value (%) | Measured value (%) | Relative deviation (%) |
|---------|-----------------------|-------------------|------------------------|
| C       | 70.8                  | 63.6 ± 2.4        | 10.2 ± 3.4             |
| N       | 12.5                  | 10.2 ± 0.8        | 18.4 ± 6.4             |
| O       | 16.7                  | 26.7 ± 1.6        | 59.9 ± 9.6             |

Notes: The compositions of the substrate are not considered here and the relative elemental content value is calculated as follows: element% = specific element fitting area/(C + N + O) fitting area × 100%.
using HRP-conjugated anti-IgG and Fg as model proteins. The relative adsorption of Fg was determined by a modified sandwich method. As shown in Figure 4, when MWs increase from 3.5 to 13.7 kDa, the relative nonspecific protein adsorption for anti-IgG and Fg increases from 3.3 ± 1.8, 4.4 ± 1.6 to 11.6 ± 2.8, 15.9 ± 5.6%, respectively. And for all samples tested, Fg has a higher relative adsorption than anti-IgG. It

Figure 3. Film thickness of the polypeptide SAMs vs. MW. Notes: When the MWs of the polypeptides are 3.5, 5.6, and 13.7 kDa, the corresponding thicknesses of the SAMs are 2.8 ± 0.1, 3.4 ± 0.2, and 3.8 ± 0.6 nm, respectively. As the MWs increase, the slope factor slightly decreases, indicating that the thickness of the SAMs has no linear relationship with the MW. The results are means ± standard deviation (SD) (n = 3).

Figure 4. Relative nonspecific protein adsorption of polypeptide SAM surfaces with different MWs, where the adsorption of TCPS surface was set as 100%. Notes: When the MW increases from 3.5 to 13.7 kDa, the relative nonspecific protein adsorption for anti-IgG and Fg increases from 3.3 ± 1.8, 4.4 ± 1.6 to 11.6 ± 2.8, 15.9 ± 5.6%, respectively. And for all samples tested, the polypeptide SAMs show good nonspecific protein resistance properties. The results are means ± standard deviation (SD) (n = 3).
is believed that this phenomenon is mainly caused by the higher tendency of Fg in denaturation due to the flexible structure of Fg.[19]

The reason for the increased relative nonspecific protein adsorption could be ascribed to the cavity increase when the polypeptide MWs increase. According to Currie[20] and Halperin’s results[21], when the particle size is smaller than the space among polymer brushes, the particles can diffuse into the brushes and repress and reorganize polymer brushes, which lead to protein contact with the surface of the substrate and adsorb on it. As for our case, when the MWs increase, cavities among polypeptide chains may become larger due to the low surface packing density of the polymer judged from slow increase of polymer film thickness, which leads to more protein adsorption on the SAM surface, as Currie[20] and Halperin[21] observed. Another possible reason might be the synergetic effect of multiple weak interactions between protein molecule and longer polypeptide chain.[22] Long chain polymer is more flexible and has more contact sites available for protein adsorption, whereas short chain polymer may form a more compact layer with fewer contact sites; thus, lead to lower protein adsorption. It should be noted that all of our samples with various MWs show relatively high efficiency to reduce both anti-IgG and Fg adsorption, even if their PDI is not in a very narrow range, indicating that this kind of polypeptides are highly fouling resistant and the uniformity of MW is not very critical. Comparing the resistant property of the polypeptide SAMs with the OEG SAMs reported by Whitesides et al.[23,24] the protein adsorption on the 3.5KDa polypeptide SAMs, the lowest one among three tested samples, is slightly higher than on the OEG SAMs. However, it is a reasonable results since the complex structure of poly(glutamic acid) with lysine side chains hinders the high density SAMs formation. Last, based on our preliminary results (data not shown), these polypeptides show selective plasminogen-binding capability, which is a key factor for fibrinolytic activity of lysine-based fouling-resistant materials.[6–9] All suggest that these polypeptides might be ideal candidates for blood-contact coatings.

![Figure 5](image)

**Figure 5.** Cell viability of the polypeptides under different polypeptide concentrations. Notes: From the histogram, we can find that when the polypeptide concentrations increase from 0.01 to 5 mg/mL, the cell viabilities vary from 120.9 ± 5.0% to 85.6 ± 3.2%, indicating no cytotoxicity *in vitro* for the polypeptides. The results are means ± standard deviation (SD) (n = 3).
3.4. MTT and hemolytic activity assay

MTT and hemolytic activity assay were conducted by following the procedures described in 2.7 and 2.8, respectively. When the polypeptide concentration increases from 0.01 to 5 mg/mL, the cell viability gradually decreases from 120.9 ± 5.0 to 85.6 ± 3.2%, indicating no obvious cytotoxicity in vitro for the polypeptides (Figure 5). It is believed that the decreased cell viability is mainly caused by the high concentration of the hydrophobic lipoic acid terminal group of the polypeptide, which might increase the interaction with the cell membrane of HUVECs and interfere their growth. However, this interference is not very obvious even when the concentration of the polypeptide reaches to 5 mg/mL.

Furthermore, the hemolytic activity assay result (Figure 6) indicates low interactions between the polypeptides and cell membrane at 5 mg/mL polypeptides. It was found that the light absorbance of the supernatants was very low after the removal of RBCs incubated with 3.5, 5.6, and 13.7 kDa polypeptide. For three tested samples, the adsorption was even lower than the negative control (PBS solution). Such phenomenon might come from the protection to RBCs by polypeptides. The highest hemolytic activity was observed for the 3.5 kDa polypeptide, of which hemolytic activity was just −14.2 ± 13.3%. Such low hemolytic activity agrees with the cytotoxicity results and also suggests that the low cytotoxicity should be attributed to low interactions of the polypeptides with protein and cell membrane. In short, all the polypeptides exhibit very good biocompatibility and are excellent candidates for fouling-resistant materials.

4. Conclusions

In this work, a kind of fouling-resistant polypeptides was synthesized by facile EDC-HCl/HOBt chemistry. Successful synthesis of the polypeptides was verified by 1HNMR and GPC. The chemical and physical properties of the polypeptide SAMs were systematically evaluated by ATR-FTIR, XPS, and ELL. The existence of the
SAMs on the gold surface, the relative element contents of the SAMs, and the thickness of the SAMs were determined. Furthermore, results from nonspecific protein adsorption resistance assay show that this kind of material has good nonspecific protein resistance. In MTT and hemolytic assay, even if the feeding concentration is up to 5 mg/mL, no obvious cytotoxicity or hemolytic activity for the polypeptides in vitro was detected. The design of a homogenous charge distribution and the facile EDC·HCl/HOBt synthesis chemistry provides a clear guide to explore other fouling-resistant homologies. As a candidate of biofunctional materials, these fouling-resistant polypeptides will be investigated in our successive works.

**Funding**

The authors appreciate financial support from the National Nature Science Foundation of China [grant number 21174127]; the PhD Programs Foundation of Ministry of Education of China [grant number 20110101110034]; the Zhejiang Provincial Natural Science Foundation of China [grant number LZ13E030001]; and the department education of Zhejiang province [grant number Z200804487].

**Supplemental data**

Supplemental data for this article can be accessed http://dx.doi.org/10.1080/09205063.2014.941262.

**References**

[1] Nowinski AK, Sun F, White AD, Keefe AJ, Jiang SY. Sequence, structure, and function of peptide self-assembled monolayers. J. Am. Chem. Soc. 2012;134:6000–6005.

[2] Yang QH, Wang LG, Lin WF, Ma GL, Yuan J, Chen SF. Development of nonfouling polypeptides with uniform alternating charges by polycondensation of the covalently bonded dimer of glutamic acid and lysine. J. Mater. Chem. B. 2014;2:577–584.

[3] Nowinski AK, White AD, Keefe AJ, Jiang SY. Biologically inspired stealth peptide-capped gold nanoparticles. Langmuir. 2014;30:1864–1870.

[4] Liu QS, Singh A, Liu LY. Amino acid-based zwitterionic poly(serine methacrylate) as an antifouling material. Biomacromolecules. 2013;14:226–231.

[5] Liu QS, Li WC, Singh A, Cheng G, Liu LY. Two amino acid-based superlow fouling polymers: poly(lysine methacrylamide) and poly(ornithine methacrylamide). Acta Biomater. 2014;10:2956–2964.

[6] Ishii T, Wada A, Tsuzuki S, Casolaro M, Ito Y. Copolymers including L-histidine and hydrophobic moiety for preparation of nonbiofouling surface. Biomacromolecules. 2007;8:3340–3344.

[7] Shiraiishi K, Ohnishi T, Sugiyama K. Preparation of poly(methyl methacrylate) microspheres modified with amino acid moieties. Macromol. Chem. Phys. 1998;199:2023–2028.

[8] Li D, Chen H, Wang SS, Wu ZQ, Brash JL. Lysine-poly(2-hydroxyethyl methacrylate) modified polyurethane surface with high lysine density and fibrinolytic activity. Acta Biomater. 2011;7:954–958.

[9] Li D, Chen H, McClung WG, Brash JL. Lysine-PEG-modified polyurethane as a fibrinolytic surface: effect of PEG chain length on protein interactions, platelet interactions and clot lysis. Acta Biomater. 2009;5:1864–1871.

[10] Wang J, Gibson MI, Barbey R, Xiao SJ, Klok H-A. Nonfouling polypeptide brushes via surface initiated polymerization of N-oligo(ethylene glycol)succinate-L-lysine N-carboxyanhydride. Macromol. Rapid. Commun. 2009;30:845–850.

[11] Klok H-A, Rodríguez-Hernández J. Dendritic-graft polypeptides. Macromolecules. 2002;35:8718–8723.

[12] Chen SF, Cao ZQ, Jiang SY. Ultra-low fouling peptide surfaces derived from natural amino acids. Biomaterials. 2009;30:5892–5896.
[13] Wang LG, Wang Z, Ma GL, Lin WF, Chen SF. Reducing the cytotoxicity of poly(amide-amine) dendrimers by modification of a single layer of carboxybetaine. Langmuir. 2013;29:8914–8921.

[14] Gouda N, Miyata K, Christie RJ, Suma T, Kishimura A, Fukushima S, Nomoto T, Liu X, Nishiyama N, Kataoka K. Silica nanogelling of environment-responsive PEGylated polyplexes for enhanced stability and intracellular delivery of siRNA. Biomaterials. 2013;34:562–570.

[15] Gao WW, Langer R, Farokhzad OC. Poly(ethylene glycol) with observable shedding. Angew. Chem. Int. Ed. 2010;49:6567–6571.

[16] Chen SF, Zheng J, Li LY, Jiang SY. Strong resistance of phosphorylcholine self-assembled monolayers to protein adsorption: insights into nonfouling properties of zwitterionic materials. J. Am. Chem. Soc. 2005;127:14473–14478.

[17] Chung YC, Chiu YH, Wu YW, Tao YT. Self-assembled biomimetic monolayers using phospholipid-containing disulfides. Biomaterials. 2005;26:2313–2324.

[18] Chen SF, Li LY, Zhao C, Zheng J. Surface hydration: principles and applications toward low-fouling/nonfouling biomaterials. Polymer. 2010;51:5283–5293.

[19] Chen SF, Jiang SY. A new avenue to nonfouling materials. Adv. Mater. 2008;20:335–338.

[20] Currie EPK, Norde W, Cohen Stuart MA. Tethered polymer chains: surface chemistry and their impact on colloidal and surface properties. Adv. Colloid Interface Sci. 2003;100:205–265.

[21] Halperin A. Polymer brushes that resist adsorption of model proteins: design parameters. Langmuir. 1999;15:2525–2533.

[22] Wu J, Wang Z, Lin WF, Chen SF. Investigation of the interaction between poly(ethylene glycol) and protein molecules using low field nuclear magnetic resonance. Acta Biomater. 2013;9:6414–6420.

[23] Kane RS, Deschatelets P, Whitesides GM. Kosmotropes form the basis of protein-resistant surfaces. Langmuir. 2003;19:2388–2391.

[24] Prime KL, Whitesides GM. Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide): a model system using self-assembled monolayers. J. Am. Chem. Soc. 1993;115:10714–10721.