J. Biochem. 2008;144(6)781–788
doi:10.1093/jb/mvn134

Modification of Antimicrobial Peptide with Low Molar Mass Poly(ethylene glycol)

Genghui Zhang[1,2], Baozhong Han[1,2], Xiaoyan Lin[1,2], Xin Wu[1,2] and Husheng Yan[1,2,*]

[1]Key Laboratory of Functional Polymer Materials, Ministry of Education; and [2]Institute of Polymer Chemistry, Nankai University, Tianjin 300071, P. R. China

Received July 16, 2008; accepted September 23, 2008; published online October 9, 2008

PEGylation of peptide drugs prolongs their circulating lifetimes in plasma. However, PEGylation can produce a decrease in the *in vitro* bioactivity. Longer poly(ethylene glycol) (PEG) chains are favourable for circulating lifetimes but unfavourable for *in vitro* bioactivities. In order to circumvent the conflicting effects of PEG length, a hydrophobic peptide, using an antimicrobial peptide as a model, was PEGylated with short PEG chains. The PEGylated peptides self-assembled in aqueous solution into micelles with PEG shell and peptide core. In these micelles, the core peptides were protected by the shell, thus reducing proteolytic degradation. Meanwhile, most of the *in vitro* antimicrobial activities still remained due to the short PEG chain attached. The stabilities of the PEGylated peptides were much higher than that of the unPEGylated peptides in the presence of chymotrypsin and serum. The antimicrobial activities of the PEGylated peptides in the presence of serum, an *ex vivo* assay, were much higher than that of the unPEGylated peptide.

Key words: antimicrobial peptides, micelles, PEGylation, peptides, self-assembly.

Abbreviations: CD, circular dichroism; CFU, colony form unit; CMC, critical micelle concentration; DBU, 1,8-diazabicyclo [5,4,0] undec-7-ene; DCM, dichloromethane; DIC, diisopropyl carbodiimide; DLS, dynamic laser scattering; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; FDA, Food and Drug Administration; Fmoc, 9-Fluorenylmethyloxycarbonyl; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MIC, minimal inhibitory concentration; mPEG-COOH, monomethoxy poly(ethylene glycol) carboxylic acid; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol); TEM, transmission electron microscopy; TFA, trifluoroacetic acid.

Nowadays plenty of peptides and proteins have been explored for pharmaceutical applications by virtue of their high biological activity and specificity (*1–4*). Especially great progress has been made recently in (i) discovery of novel peptides and proteins, (ii) a better understanding of the mechanism of action *in vivo* and (iii) improvements in expression or synthesis of proteins and peptides that closely resemble fully human proteins and peptides. About one-third of drug candidates in clinical trials today are polypeptides (*4*). Despite of these tremendous advances, the peptide drugs suffer from short circulating lifetime due to proteolytic degradation and rapid kidney clearance (*3–6*). Therefore, the use of high doses is required to maintain therapeutic efficacy which in turn increases the chance for the development of an adverse immune response and also the cost for clinical application. These shortcomings have limited their usefulness as effective drugs.

Various methods have been developed to overcome these problems associated with peptide and protein drugs. These methods include incorporating them into drug-delivery vehicles such as liposomes, substituting D-amino acids for L-amino acids to reduce susceptibility to certain proteases, fusing them to albumin to improve half-life, covalent modification by polymer conjugation to reduce proteolytic degradation, kidney clearance and immunogenicity (*7–10*). The covalent modification with poly(ethylene glycol) (PEG), which is termed PEGylation, is one of the most successful strategies for modification of peptide and protein drugs (*3–7*). PEG presents unique properties such as (i) lack of immunogenicity, antigenicity and toxicity; (ii) high solubility in water and in many organic solvents; (iii) high hydration and flexibility of the chain, which prevents protein adsorption; and (iv) approval by Food and Drug Administration (FDA) for internal use. The attached PEG chains, along with the associated water molecules, act like a shield to protect the modified peptide and protein drugs, thus reducing degradation by proteolytic enzymes, and the antigenic epitopes of the drugs, reducing reticuloendothelial system clearance and recognition by the immune system. PEG conjugation also increases the apparent size of the drugs, thus reducing renal filtration and altering biodistribution. These effects of PEGylation significantly prolong the circulating lifetime of the modified peptide and protein drugs. Higher the number and the molecular weight of PEG chains attached, the better is the foregoing protection for the drugs and the longer is the prolonged circulating lifetime. However, PEGylation can also produce a decrease in the *in vitro* activity of the modified drugs and this decrease is especially notable for peptides and low-molecular proteins. The decrease is positively correlated with the number and the molecular weight of PEG chains attached (*7*, *11*, *12*). Therefore the

*To whom correspondence should be addressed. Tel: +86-22-23501705, Fax: +86-22-23503510, E-mail: yanhs@nankai.edu.cn

effects of the number and the molecular weight of PEG chains on the circulating lifetime and the *in vitro* bioactivity upon PEGylation are conflict. The negative effect of PEGylation on the *in vitro* bioactivity is generally offset in biological systems by the increased circulating lifetime for protein drugs (*12*, *13*). Several PEGylated protein drugs have received market approval and many others are already in advanced clinical investigation (*4*, *7*). However, the benefit of PEGylation due to the circulating lifetime prolonging cannot even offset the loss of the *in vitro* bioactivity for some of PEGylated peptides studied (*14–17*). This *in vitro* bioactivity decrease caused by PEGylation may be ascribed to the following factors: (i) direct attachment of the PEG chains to the receptor-binding (bioactive) sites; (ii) steric hindrance for the binding with the receptor from the PEG chains; and (iii) here we proposed the third factor, the binding free energy loss due to the additional translational and rotational entropy loss upon binding of the PEGylated peptide or protein to the receptor caused by the molecular weight increasing upon PEGylation. This is because both translational and rotational entropies are positively correlated with the molecular mass (*18*). The higher the molecular weight of the PEG chain attached, the greater is the loss of the translational and rotational entropy upon the binding of the PEGylated peptide to the receptor. However, this kind of entropy effect for the bioactivity may be neglectable for high-molecular proteins which recognize small substrates.

To maximize protection while minimizing reduction in bioactivity, we proposed a novel PEGylation strategy for hydrophobic peptide drugs, as schematically shown in Fig. 1. Peptides are PEGylated with short PEG chains and the resulting PEGylated peptides self-assemble in aqueous solution into micelles with PEG shell and peptide core. In these micelles, the core peptides would be well protected by the PEG shell, thus reducing degradation by proteolytic enzymes and recognition by the immune system. The size of the aggregates is much greater than that of the unimers, thus reducing kidney clearance. As a consequence, the circulation lifetime of the PEGylated peptides should be greatly prolonged. Meanwhile, the decrease in the *in vitro* bioactivity caused by the PEGylation would be small because of the small length of the PEG chains attached. Moreover, the aggregates may also act as a depot for sustained release through equilibrium between the aggregates and the unimers. Herein, we use an antimicrobial peptide as a model peptide to investigate its PEGylation, aggregation, stability and bioactivities. Antimicrobial peptides have been considered as potential candidates for the treatment of antibiotic-resistant bacterial infections (*19*, *20*). Like other peptide drugs, antimicrobial peptides consisting of L-amino acids are easily degraded by proteolytic enzymes and inactivated in blood, plasma and serum (*15*, *21–26*), which limit their usefulness.

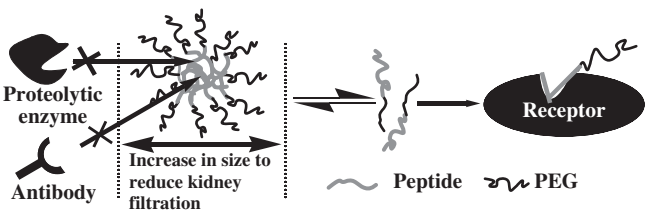

Fig. 1. **Schematical illustration of self-assembly of PEGylated peptides and the shielding effect by PEG shell.**

EXPERIMENTAL PROCEDURES

Peptide Synthesis—GLLALILWIKRKR-NH_2, denoted as MA, was synthesized by solid-phase peptide synthesis with manual Fmoc/tBu strategy on MBHA-rink amide resin. The Fmoc group on the starting resin was removed by treatment with 20% piperidine/dimethylformamide (DMF). The deprotected resin was suspended in a solution of Fmoc-protected amino acid (3 eq.), diisopropyl carbodiimide (DIC; 3 eq.) and *N*-hydroxybenzotriazole (HOBt; 3 eq.) in DMF/dichloromethane (DCM; 1/1 volume ratio) and the mixture was stirred until a negative Kaiser test. A second coupling reaction may be required to achieve quantitative conversion. Before the next coupling reaction, the Fmoc group was removed by treatment with 4% 1,8-diazabicyclo [5,4,0] undec-7-ene (DBU) in DMF/DCM (1/1 volume ratio). After the incorporation of all amino acids, the peptide was cleaved with a mixture of trifluoroacetic acid (TFA)/phenol/H_2O/thioanisole (95/2/2/1 volume ratio). The resulting cleavage solution was precipitated with cold diethyl ether to give crude peptide. The peptide was purified by preparative reversed-phase HPLC. The purity of the peptide was determined by HPLC (Fig. S1 of the Supplementary Data). The mass spectrometry of the peptide was shown in Fig. S2 of the Supplementary Data.

Synthesis of Monomethoxy PEG Carboxylic Acid—Monomethoxy PEG carboxylic acid (mPEG-COOH) was prepared according to a literature method (*27*). A solution of mPEG (0.01 mol, MW 750 or 1100) and potassium hydroxide (0.7 g, 0.0125 mol) in distilled water (15.0 g) was cooled to 0.5°C and stirred for 30 min. To this was added slowly acrylonitrile (0.85 g, 0.016 mol) and the mixture was stirred for 2 h at 0–5°C. Concentrated sulfuric acid (30.0 g) was added slowly and the mixture was heated for 3 h at 95–100°C. After cooling to room temperature, distilled water (200 ml) was added, and the mixture was extracted with DCM (3×30 ml). The extract was dried with anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The conversions were determined by titration with 0.1 M NaOH to be 65.2% and 73.7% for $mPEG_{750}$ and $mPEG_{1100}$, respectively.

Synthesis of PEGylated Peptides—Resin-bound MA prepared as shown above was mixed with mPEG-COOH (4 eq.), DIC (4 eq.) and HOBt (4 eq.) in DCM/dimethyl sulfoxide (DMSO; 4/1 volume ratio) and the mixture was stirred until a negative Kaiser test (about 2 days). The PEGylated peptides were cleaved from the resin, precipitated and purified with the same procedures as for MA described above. The purities of $mPEG_{750}$-MA and $mPEG_{1100}$-MA, respectively, were shown in Fig. 1S of the Supplementary Data. The mass spectrometry profiles of $mPEG_{750}$-MA and $mPEG_{1100}$-MA were shown in Figs S3 and S4 of the Supplementary Data, respectively.

Determination of Antimicrobial Activity—The antimicrobial peptide or its PEGylation derivatives were assayed in nutrient broth (5 g of nutrient broth and 5 g of NaCl per liter medium) under aerobic conditions. Two-fold serial dilutions of the antibiotics were added to 1 ml of the culture medium containing the inocula of the test organism ($\sim 10^6$ CFU) in mid-logarithmic phase of growth. After incubation for 20 h at 37°C, the growth inhibitions were determined by optical density measurements at 600 nm. The antibacterial activity is expressed as minimal inhibitory concentration (MIC). The microorganisms used were Gram-positive bacteria, *Staphylococcus aureus*, *Bacillus subtilis* and Gram-negative bacteria *Escherichia coli*.

Determination of Antimicrobial Activity in the Presence of Serum—The antimicrobial peptide or its PEGylation derivatives were dissolved in the nutrient broth culture medium containing 20% bovine calf serum. The culture medium was incubated for 1 h at 37°C. To this was added the inocula of the test organism (the final concentration of serum in the mixture was retained to 20% by adding a certain amount of serum). The mixture was incubated for 30 h at 37°C. The MIC in the presence of serum was obtained in the same way as shown above.

Determination of Haemolytic Activity—The haemolytic activities of the antimicrobial peptide or its PEGylation derivatives were tested against human erythrocytes. The erythrocytes were suspended in phosphate-buffered saline (PBS). A solution of the sample in PBS was mixed with the same volume of erythrocyte suspension in PBS. The final erythrocyte concentration was 2.5%. The mixture was incubated for 60 min at 37°C with gentle stirring. The suspension was then centrifuged and the absorbance of the supernatant was measured at 414 nm. Controls for zero haemolysis (blank) and 100% haemolysis consisted of erythrocyte suspended in PBS and 1% Triton, respectively.

Degradation in 20% Serum—Five hundred microlitres of calf serum (frozen, Beijing Newprobe Biotechnology Co. Ltd., Beijing, China) and 2 ml of solution (1.0 mg/ml) of the antimicrobial peptide or its PEGylation derivatives were warmed to 37°C and were mixed. A sample (100 μl) was taken immediately after mixing. The mixture was incubated at 37°C and samples were taken during the incubation. Ethanol (200 μl) was added to each of the samples immediately after being taken to precipitate serum proteins. After cooling at 4°C for 10 min, the sample mixture was centrifuged at 10,000 r.p.m. for 2 min. The concentration of the antimicrobial peptide or its PEGylation derivatives in the supernatant was determined by reversed-phase HPLC. The same assay was done as shown above except that the calf serum was replaced with heat-treated (65°C for 20 min) calf serum.

Degradation in Chymotrypsin Solution—The antimicrobial peptide or its PEGylation derivatives solution in HEPES buffer (pH 7.5) was mixed with a chymotrypsin stock solution (100 μg/ml) in 0.1 M HCl. The final concentrations of chymotrypsin and the peptide or its PEGylation derivatives were 3.64 and 330 μg/ml, respectively. A sample was taken immediately from the mixture. The mixture was then incubated at 37°C and samples were taken from the mixture at intervals of 10 min during the incubation. All samples taken were cooled in ice-bath and analysed by reversed-phase HPLC immediately.

α-Helical Contents Determined by Circular Dichroism—The antimicrobial peptide or its PEGylation derivative was dissolved in PBS at a series of concentration (0–30 μM) and the circular dichroism (CD) spectra were recorded using a JASCO-J-715 spectropolarimeter under nitrogen flush in 1-cm path length cells at 25°C. The spectra were recorded from 200 nm to 250 nm scanning and the average of three recordings was taken. The α-helical contents were calculated by: $\alpha\text{-helix}\ (\%) = -([\theta]_{222} + 2340)/30300$, where $[\theta]_{222}$ is the experimentally observed absolute mean residue ellipticity at 222 nm (*28*). Figure S5 in the Supplementary Data shows the α-helical contents of MA, $mPEG_{750}$-MA and $mPEG_{1100}$-MA determined by CD at different concentrations.

Dynamic Laser Scattering Observation for the Micells—The peptide derivates were dissolved in PBS and then the well-assembled sample solution was filtrated by Millipore (pore size <400 nm) before observation. The dynamic laser scattering (DLS) was performed on a laser light scattering spectrometer (BI-200SM, Brookhaven, New York, USA) equipped with a digital correlator (BI-9000AT, Brookhaven, New York, USA) at 514 nm at room temperature.

Determination of Critical Micelle Concentration—The critical micelle concentrations (CMCs) were determined by pyrene solubilization method according to literature procedures (*29*, *30*). An excess of powdered pyrene was added to a series of different concentrations of PEGylated peptides. The mixtures were sonicated for >8 h and then centrifuged at 4,000 r.p.m. for 1 h to sediment excess pyrene. The concentrations of pyrene in the supernatants were detected by UV absorption at 336 nm.

RESULTS AND DISCUSSION

Our strategy is that peptides modified with low molecular weight PEG chains self-assemble in aqueous solution into micelles. As the PEG chains are highly hydrophilic, the peptides should be hydrophobic so that the PEGylated peptides can self-assemble in aqueous solution into expected micelles. We designed a strongly hydrophobic analogue of melittin, a well-studied antimicrobial peptide with 26 amino acid residues, with high antimicrobial activity based on our previous researches (*31*, *32*). The amino acid sequence of this antimicrobial peptide, MA, which is the 12–24 segment residue of melittin with substitution of proline at residue 3 and serine at residue 7, respectively, with leucines. MA was synthesized by solid-phase peptide synthesis with manual Fmoc strategy using MBHA-rink amide resin as the support. MA was PEGylated at the N-terminal amino group (of glycine) with 750 and 1100 PEG by solid-phase method by coupling corresponding mPEG-COOH, mPEG-COOH, to resin-bound MA with side chain protections prepared as described above. Cleaved MA and PEGylated MAs, denoted as $mPEG_{750}$-MA and $mPEG_{1100}$-MA, from the resin were purified by

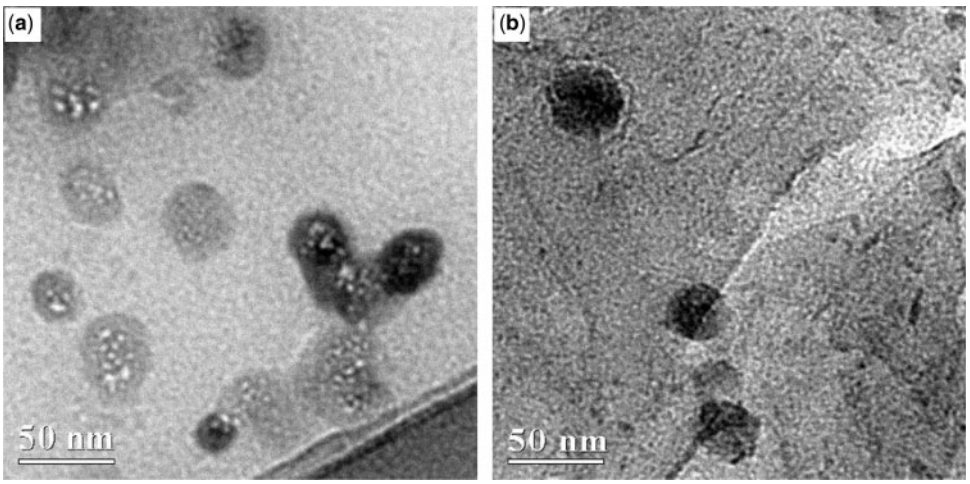

Fig. 2. **TEM images of mPEG$_{750}$-MA (a) and mPEG$_{1100}$-MA (b) aggregates.**

preparative reversed-phase HPLC. The solubility of MA in water was very small due to its hydrophobicity. However, the solubility of the PEGylated peptides was enhanced markedly. Therefore, the water solubility increasing for hydrophobic peptides upon PEGylation is another advantage.

Micellization of the PEGylated peptides in aqueous solution was studied by transmission electron microscopy (TEM), DLS and CMC determination by pyrene solubilization method. Figure 2 shows the TEM images of the aggregates formed from mPEG$_{750}$-MA and mPEG$_{1100}$-MA in aqueous solution. It can be seen that the PEGylated peptides formed aggregates with diameters of 20–40 nm. The aggregation was further confirmed by DLS of the samples in PBS (Fig. 3). The particle size determined by DLS was in accordance with that determined by TEM. The DLS measurement also showed the secondary aggregation of the PEGylated peptides.

CMC is an important parameter for micellization. The most common method to determine CMC is by a fluorescence spectroscopy measurement using pyrene as a hydrophobic probe. CMC values can be usually obtained by monitoring the changes in the ratio of the vibrational band intensity (i.e. I/III ratio) of pyrene (*33*). The PEGylated peptides studied in this article possess tryptophan residues, which have fluorescence and thus can interfere with the pyrene fluorescence. Thereby this fluorescence method is not applicable for the determination of CMC of the PEGylated peptides. Here we used a pyrene solubilization method (*29*, *30*) to determine CMC of the PEGylated peptides in aqueous solution. Pyrene is insoluble in aqueous medium, but it can be entrapped by hydrophobic cores of micelles in aqueous solution. Therefore, the solubility of pyrene should be correlated with the concentration of the micelles. Figure 4 shows the plot of the pyrene solubility against the concentration of mPEG$_{750}$-MA and mPEG$_{1100}$-MA. It can be seen that the pyrene solubility was close to zero at lower concentrations of the PEGylated peptides. When the concentration was higher than certain value, the pyrene solubility increased linearly with the increase in the concentration, indicating that after this concentration value the PEGylated peptide formed micelles and the

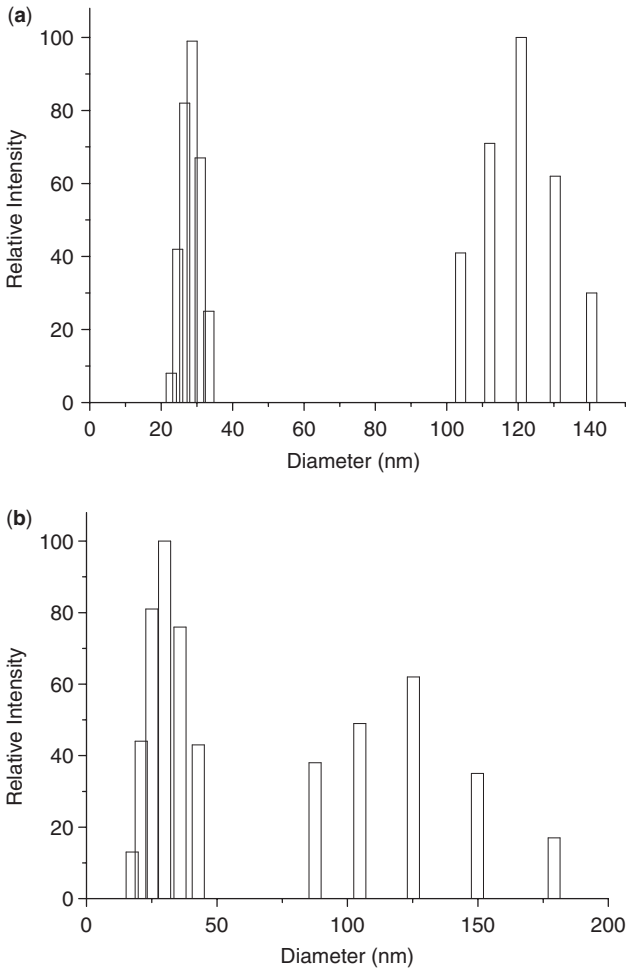

Fig. 3. **Hydrodynamic diameter distribution of aggregates formed from mPEG$_{750}$-MA (a) and mPEG$_{1100}$-MA (b) determined by DLS.**

number or concentration of the micelles increased linearly with the increase in the concentrations of the PEGylated peptide. This intersection concentration value should be CMC. The CMCs were taken as the

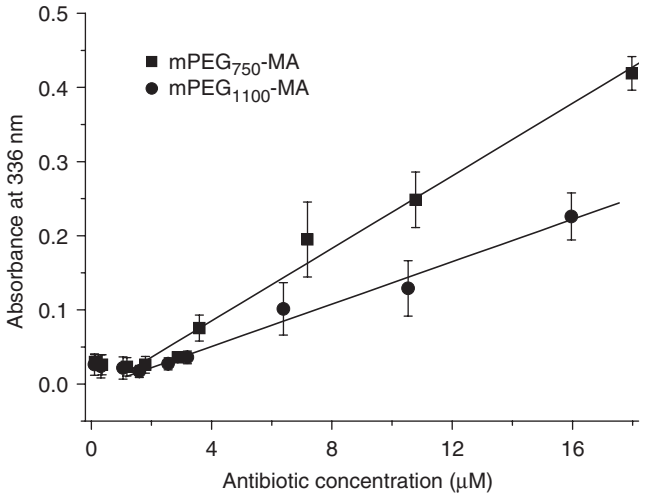

Fig. 4. **Plot of the absorbance of pyrene dissolved against the concentration of the PEGylated peptides.** Results are means with SDs of three trials.

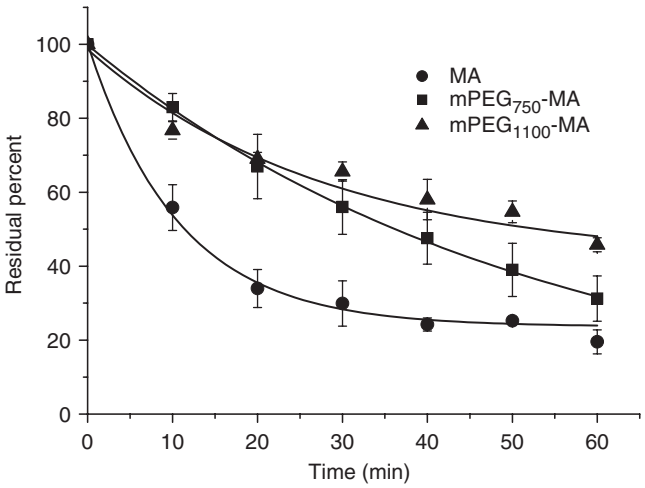

Fig. 5. **Degradation profiles of MA, mPEG$_{750}$-MA and mPEG$_{1100}$-MA incubated with chymotrypsin (3.64 μg/ml) at 37°C and pH 7.5.** Results are means with SDs of three trials.

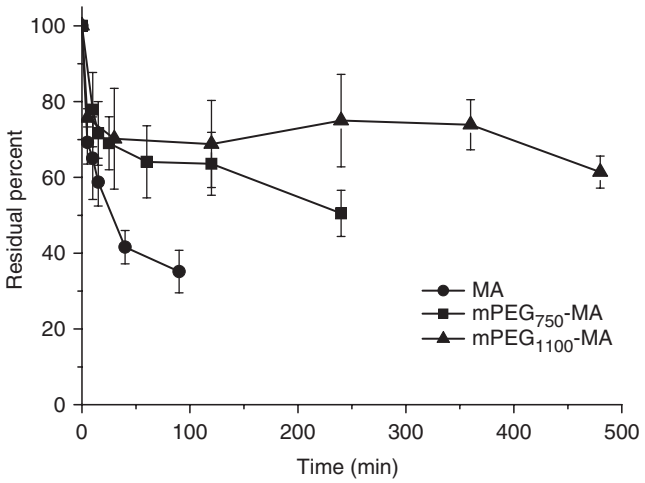

Fig. 6. **Degradation profiles of MA, mPEG$_{750}$-MA and mPEG$_{1100}$-MA incubated in 20% bovine calf serum at 37°C.** Results are means with SDs of three trials.

intersections of the tangents to the curves at the inflections and the values were 1.9 ± 0.3 and 2.3 ± 0.5 μM, respectively, from Fig. 4. The above results indicate that these two PEGylated peptides possess quite low CMC.

Ultraviolet CD measurements showed that both of MA and the PEGylated peptides adopted predominantly random coil conformation with a small proportion of α-helical structures (Fig. S5 of the Supplementary Data). For mPEG$_{750}$-MA and mPEG$_{1100}$-MA, the contents of the α-helical structures decreased rapidly before CMC and then reached a plateau or increased slightly after CMC with the concentration increasing, indicating the peptide in the micelles adopted predominantly random coil conformation.

The proteolytic stabilities of MA and its PEGylated derivatives in the presence of chymotrypsin were investigated, as shown in Fig. 5. It can be seen that MA was rapidly degraded in the presence of chymotrypsin, while the degradation rates of mPEG$_{750}$-MA and mPEG$_{1100}$-MA were much lower. The degradation half-lives for MA, mPEG$_{750}$-MA and mPEG$_{1100}$-MA were calculated based on the data in Fig. 5 by assuming first-order kinetics to be 11.4, 36.0 and 53.4 min, respectively. There were 3.2- and 4.7-fold increases in the half-lives after PEGylation with 750 and 1,100 Da PEGs compared with the unPEGylated peptide.

It has been reported that *in vivo* stability of peptides in blood is modelled well by *in vitro* stability in serum or plasma (*34–36*). Figure 6 shows that the stabilities of the PEGylated peptides were much higher than that of the unPEGylated peptide in the presence of serum. The unexpected rapid concentration decrease of the PEGylated peptides during the first 10 min, as shown in Fig. 6, might be caused by the association of the PEGylated peptides with the serum components. The formed complex was precipitated upon adding ethanol for the precipitation of proteins in the serum and removed in the analysis of the concentrations of the PEGylated peptides (see the EXPERIMENTAL PROCEDURES section). After ~10 min, all the components associating with the PEGylated peptides in the serum might be consumed. It has been reported that defensin and non-defensin cationic antimicrobial peptides strongly bind to C1 complement in serum (*37*). In comparison, the concentrations of the peptide and PEGylated peptides upon incubation in 20% heat-treated serum changed little (Fig. S6 of the Supplementary Data), indicating heat treating may inactivate both the proteolytic enzymes and the components. To our best knowledge, significant prolonging half-lives of peptides can only be obtained by PEGylation with 2 kDa or higher molecular weight PEG, in most cases, 5–50 kDa PEG (*11*, *15–17*, *38*, *39*). Therefore, the much lower degradation rates of mPEG$_{750}$-MA and mPEG$_{1100}$-MA in the presence of chymotrypsin and serum must be ascribed to the PEG shell shielding protection for the peptide core against proteolytic degradation.

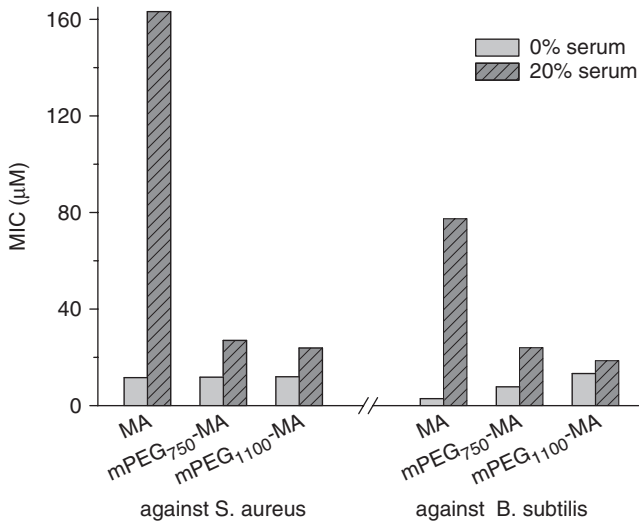

Fig. 7. **Antimicrobial activities of MA, $mPEG_{750}$-MA and $mPEG_{1100}$-MA before and after incubation in 20% bovine calf serum at 37°C for 60 min against Gram-positive bacteria, *S. aureus* and *B. subtilis*.** MIC values are representative of results from three or more separate experiments.

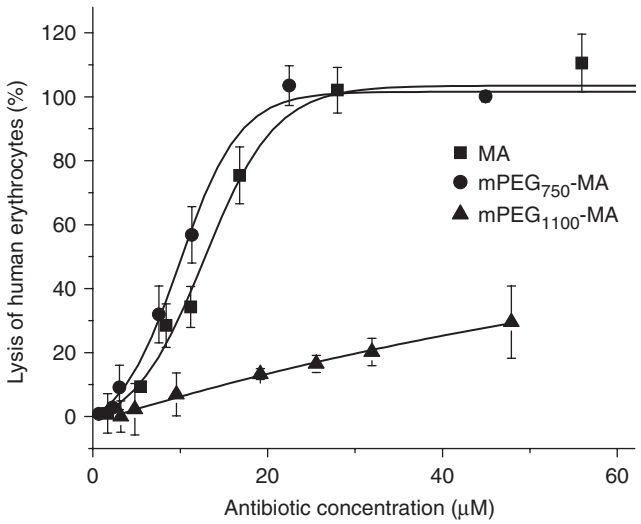

Fig. 8. **Haemolysis of MA, $mPEG_{750}$-MA and $mPEG_{1100}$-MA against human erythrocytes.** Results are means with SDs of three trials.

The antimicrobial activity of the antimicrobial peptide and its PEGylated derivatives against Gram-positive bacteria, *S. aureus*, *B. subtilis* and Gram-negative bacteriium *E. coli* was examined. Due to the short PEG chains, all the bioconjugates held effective antimicrobial activity except for $mPEG_{1100}$-MA against *E. coli*. The antimicrobial activity of MA, $mPEG_{750}$-MA and $mPEG_{1100}$-MA against Gram-positive bacteria, *S. aureus* and *B. subtilis* before and after incubation in 20% serum at 37°C for 60 min was showed in Fig. 7. After incubation in 20% serum, the antimicrobial activity of MA decreased drastically, with MICs, i.e. the concentrations at which 100% inhibition of growth was observed after incubation, increasing from 12 μM to 163 μM against *S. aureus* and 3–77 μM against *B. subtilis*; while the antimicrobial activity of $mPEG_{750}$-MA and $mPEG_{1100}$-MA decreased only slightly, with MICs increasing from 12 μM and 12 μM, respectively, to 27 and 24 μM against *S. aureus*, and 8 and 13 μM, respectively, to 24 and 19 μM against *B. subtilis*. Furthermore, the antimicrobial activity of the PEGylated peptides was much higher than that of the unPEGylated peptide after the incubation in 20% serum, indicating that positive benefit was got upon PEGylation. In comparison, PEGylations of antimicrobial peptides tachyplesin I (*15*) and nisin A (*17*) with 5 kDa PEG chains led to a drastic decrease or even a complete loss of their antimicrobial activities. The antimicrobial activity determined in the presence of serum showed that the net benefit of PEGylation of tachyplesin I is negative (*15*). The extent of decrease of *in vitro* antimicrobial activity of magainin 2 analogue after PEGylation with 5 kDa PEG was not so significant, but there was still four times increase in MIC (*40*).

Some of antimicrobial peptides such as melittin are also cytotoxic to mammalian cells, e.g. erythrocytes. Therefore, only antimicrobial peptides and their derivatives with a high antimicrobial activity and a low cytotoxicity to the healthy eukaryotic cells are of practical interest. The haemolytic activities of MA, $mPEG_{750}$-MA and $mPEG_{1100}$-MA were shown in Fig. 8. It can be seen that MA exhibited strong cytotoxicity to erythrocytes with HC_{50} (concentration required for 50% haemolysis) of 13 μM. An interesting finding is that PEGylation of MA with 750 Da PEG slightly increased its haemolytic activity (HC_{50} 10 μM), while PEGylation with 1,100 Da PEG markedly decreased its haemolytic activity. Interestingly, the order of the relative haemolytic activities of MA, $mPEG_{750}$-MA and $mPEG_{1100}$-MA were in accordance with that of their antimicrobial activities against *E. coli*.

CONCLUSIONS

A hydrophobic antimicrobial peptide, MA, with high antimicrobial activity was designed and synthesized. MA was modified with 750 and 1,100 Da PEG chains, forming PEGylated peptides, $mPEG_{750}$-MA and $mPEG_{1100}$-MA, respectively. The PEGylated peptides self-assembled in aqueous solution into micelles with peptide core and PEG shell. CMC of $mPEG_{750}$-MA and $mPEG_{1100}$-MA in aqueous solution were 1.9 ± 0.3 and 2.3 ± 0.5 μM, respectively. The proteolytic stability of the PEGylated peptides increased significantly due to the shielding protection of the core peptide by the PEG shell. The antimicrobial activities of the PEGylated peptides in the presence of serum, an *ex vivo* assay, were much higher than that of the unPEGylated peptide. In addition, the PEGylation of MA with 1,100 Da mPEG markedly decreased the haemolytic activity.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

FUNDING

National Natural Science Foundation of China (20774048).

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Lee, V.H.L. (1991) *Peptide and Protein Drug Delivery*. Mercel Dekker, New York
2. Marx, V. (2005) Watching peptide drugs grow up. *Chem. Eng. News* **83**, 17–24
3. Lien, S. and Lowman, H.B. (2003) Therapeutic peptides. *Trends Biotechnol.* **21**, 556–562
4. Harris, J.M. and Chess, R.B. (2003) Effect of PEGylation on pharmaceuticals. *Nat. Rev. Drug Discov.* **2**, 214–221
5. Sato, A.K., Viswanathan, M., Kent, R.B., and Wood, C.R. (2006) Therapeutic peptides: technological advances driving peptides into development. *Curr. Opin. Biotechnol.* **17**, 638–642
6. Roberts, M.J., Bentley, M.D., and Harris, J.M. (2002) Chemistry for peptide and protein PEGylation. *Adv. Drug Deliv. Rev.* **54**, 459–476
7. Pasut, G. and Veronese, F.M. (2007) Polymer-drug conjugation, recent achievements and general strategies. *Prog. Polym. Sci.* **32**, 933–961
8. Syed, S., Schuyler, P.D., Kulczycky, M., and Sheffield, W.P. (1997) Potent antithrombin activity and delayed clearance from the circulation characterize recombinant hirudin genetically fused to albumin. *Blood* **89**, 3243–3252
9. Dennis, M.S., Zhang, M., Meng, Y.G., Kadkhodayan, M., Kirchhofer, D., Combs, D., and Damico, L.A. (2002) Albumin binding as a general strategy for improving the pharmacokinetics of proteins. *J. Biol. Chem.* **277**, 35035–35043
10. Meyer, J., Whitcomb, L., and Collins, D. (1994) Efficient encapsulation of proteins within liposomes for slow release in vivo. *Biochem. Biophys. Res. Commun.* **199**, 433–438
11. Esposito, P., Barbero, L., Caccia, P., Caliceti, P., D'Antonio, M., Piquet, G., and Veronese, F.M. (2003) PEGylation of growth hormone-releasing hormone (GRF) analogues. *Adv. Drug Deliv. Rev.* **55**, 1279–1291
12. Bowen, S., Bowen, S., Tare, N., Yamasaki, M., Okabe, M., Horii, I., and Eliason, J.F. (1999) Relationship between molecular mass and duration of activity of polyethylene glycol conjugated granulocyte colony-stimulating factor mutein. *Exp. Hematol.* **27**, 425–432
13. Bailon, P., Palleroni, A., Schaffer, C.A., Spence, C.L., Fung, W.-J., Porter, J.E., Ehrlich, G.K., Pan, W., Xu, Z.-X., Modi, M.W., Farid, A., Berthold, W., and Graves, M. (2001) Rational design of a potent, long-lasting form of interferon: a 40 kDa branched polyethylene glycol-conjugated interferon-alpha 2a for the treatment of hepatitis C. *Bioconjug. Chem.* **12**, 195–202
14. Youn, Y.S., Na, D.H., and Lee, K.C. (2007) High-yield production of biologically active mono-PEGylated salmon calcitonin by site-specific PEGylation. *J. Control. Release* **117**, 371–379
15. Imura, Y., Nishida, M., Ogawa, Y., Takakura, Y., and Matsuzaki, K. (2007) Action mechanism of tachyplesin I and effects of PEGylation. *Biochim. Biophys. Acta* **1768**, 1160–1169
16. Lumb, K.J., DeCarr, L.B., Lucinda, F.M., Mays, M.R., Buckholz, T.M., Fisk, S.E., Pellegrino, C.M., Ortiz, A.A., and Mahle, C.D. (2007) Novel selective neuropeptide Y2 receptor PEGylated peptide agonists reduce food intake and body weight in mice. *J. Med. Chem.* **50**, 2264–2268
17. Guiotto, A., Pozzobon, M., Canevari, M., Manganelli, R., Scarin, M., and Veronese, F.M. (2003) PEGylation of the antimicrobial peptide nisin A: problems and perspectives. *Il Farmaco* **58**, 45–50
18. Mammen, M., Shakhnovich, E.I., Deutch, J.M., and Whitesides, G.M. (1998) Estimating the entropic cost of self-assembly of multiparticle hydrogen-bonded aggregates based on the cyanuric acid-melamine lattice. *J. Org. Chem.* **63**, 3821–3830
19. Zhang, L. and Falla, T.J. (2006) Antimicrobial peptides: therapeutic potential. *Expert Opin. Pharmacother.* **7**, 653–663
20. Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389–395
21. Wade, D., Boman, A., Wahlin, B., Drain, C.M., Andreu, D., Boman, H.G., and Merrifield, R.B. (1990) All-D amino acid-containing channel-forming antibiotic peptides. *Proc. Natl Acad. Sci. USA* **87**, 4761–4765
22. Darveau, R.P., Cunningham, M.D., Seachord, C.L., Cassiano-Clough, L., Cosand, W.L., Blake, J., and Watkins, C.S. (1991) Beta-lactam antibiotics potentiate magainin 2 antimicrobial activity in vitro and in vivo. *Antimicrob. Agents Chemother.* **35**, 1153–1159
23. Oren, Z., Hong, J., and Shai, Y. (1997) A repertoire of novel antibacterial diastereomeric peptides with selective cytolytic activity. *J. Biol. Chem.* **272**, 14643–14649
24. Yeaman, M.R., Gank, K.D., Bayer, A.S., and Brass, E.P. (2002) Synthetic peptides that exert antimicrobial activities in whole blood and blood-derived matrices. *Antimicrob. Agents Chemother.* **46**, 3883–3891
25. Papo, N., Oren, Z., Pag, U., Sahl, H.G., and Shai, Y. (2002) The consequence of sequence alteration of an amphipathic alpha-helical antimicrobial peptide and its diastereomers. *J. Biol. Chem.* **277**, 33913–33921
26. Braunstein, A., Papo, N., and Shai, Y. (2004) In vitro activity and potency of an intravenously injected antimicrobial peptide and its DL amino acid analog in mice infected with bacteria. *Antimicrob. Agents Chemother.* **48**, 3127–3129
27. Yan, H., Li, S., Sun, X., Mi, H., and He, B. (2003) Individual substitution analogs of Mel(12-26), melittin. *FEBS Lett.* **554**, 100–104
28. Sun, X., Chen, S., Li, S., Yan, H., Fan, Y., and Mi, H. (2005) Deletion of two C-terminal Gln residues of 12-26-residue fragment of melittin improves its antimicrobial activity. *Peptides* **26**, 369–375
29. Kastantin, M., Ananthanarayanan, B., Lin, B., Ressl, J., Black, M., and Tirrell, M. (2007) Increase of fluorescence anisotropy upon self-assembly in headgroup-labeled surfactants. *Macromol. Biosci.* **7**, 189–194
30. Powell, M.F., Grey, H., Gaeta, F.C.A., Sette, A., and Colon, S.M. (1992) Peptide stability in drug development: a comparison of peptide reactivity in different biological media. *J. Pharm. Sci.* **81**, 731–735
31. Hoffmann, R., Bulet, P., Urge, L., and Otvos, L. Jr. (1999) Range of activity and metabolic stability of synthetic antibacterial glycopeptides from insects. *Biochim. Biophys. Acta* **1426**, 459–467
32. Fredholt, K., Adrian, C., Just, L., Larsen, D.H., Weng, S., Mossc, B., and Friis, J.G. (2000) Chemical and enzymatic stability as well as transport properties of a Leu-enkephalin analogue and ester prodrugs thereof. *J. Control. Release* **63**, 261–273
33. Panyutich, A.V., Szold, O., Poon, P.H., Tseng, Y., and Ganz, T. (1994) Identification of defensin binding to Cl complement. *FEBS Lett.* **356**, 169–173
34. Kopecky, E.-M., Greinstetter, S., Pabinger, I., Buchacher, A., Römisch, J., and Jungbauer, A. (2006) Effect of oriented or random PEGylation on bioactivity of

a factor VIII inhibitor blocking peptide. *Biotechnol. Bioeng.* **93**, 647–655
35. Pan, C.Q., Li, F., Tom, I., Wang, W., Dumas, M., Froland, W., Yung, S.L., Li, Y., Roczniak, S., Claus, T.H., Wang, Y.J., and Whelan, J.P. (2007) Engineering novel VPAC2 selective agonists with improved stability and glucose lowering activity in vivo. *J. Pharmacol. Exp. Ther.* **320**, 900–906
36. Imura, Y., Nishida, M., Ogawa, Y., Takakura, Y., and Matsuzaki, K. (2004) Effects of PEGylation on the activity of tachyplesin I. *J. Pharm. Soc. Jap*. **124** (Suppl. 4), 117–120
37. Harris, J.M., and Kozlowski, A. (1997) Poly(ethylene glycol) and related polymers monosubstituted with propionic or butanoic acids and functional derivatives thereof for biotechnical applications. US Patent No. 5672662.
38. Chen, Y.J., Yang, J.T., and Martinez, H.M. (1992) Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersiont. *Biochemistry* **11**, 4120–4131
39. Kim, J.-H., Domach, M.M., and Tilton, R.D. (1999) Pyrene micropartitioning and solubilization by sodium dodecyl sulfate complexes with poly(ethylene glycol). *J. Phys. Chem. B* **103**, 10582–10590
40. Imura, Y., Nishida, M., and Matsuzaki, K. (2007) Action mechanism of PEGylated magainin 2 analogue peptide. *Biochim. Biophys. Acta* **1768**, 2578–2585