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Toward Hemocompatible Self-assembling Antimicrobial Nanofibers: Understanding the Synergistic Effect of Supramolecular Structure and PEGylation on Hemocompatibility

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Abstract

A significant challenge associated with systemic delivery of cationic antimicrobial peptides and polymers lies in their limited hemocompatibility toward vast numbers of circulating red blood cells (RBCs). Supramolecular assembly of cationic peptides and polymers can be an effective strategy to develop an array of antimicrobial nanomaterials with tunable material structures, stability and thus optimized bioactivity to overcome some of the existing challenges associated with conventional antimicrobials. In this work, we will demonstrate the supramolecular design of self-assembling antimicrobial nanofibers (SAANs) which have tunable supramolecular nanostructures, stability, internal molecular packing and surface chemistry through self-assembly of *de novo* designed cationic peptides and peptide-PEG conjugates. The interaction of the SAANs with human RBCs was evaluated in a stringent biological assay (beyond a traditional hemolysis assay) where both hemolytic and eryptotic activity were examined to establish a fundamental understanding on the correlation between material structure and hemocompatibility. It was found that although the SAANs showed moderate hemolytic activities, their abilities to induce eryptosis vary significantly and are much more sensitive to the internal molecular packing, supramolecular nanostructure and stability of the nanofiber. Improved hemocompatibility requires PEGylation on stable supramolecular nanofibers composed of highly organized β -sheet structure while PEG conjugation on weakly packed nanofibers composed of partially denatured β -sheets did not show improvement. The current study reveals the fundamental mechanism involved in the selective hemocompatibility improvement of the SAANs upon PEG conjugation. The structure-activity relationship developed in this study will provide important guidance for the future design of a broader family of peptide and polymer-based assemblies with optimized antimicrobial activity and hemocompatibility.

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Graphical Abstract

In this work we will demonstrate the supramolecular assembly of antimicrobial peptides and the effect of PEGylation and nanostructure on biocompatibility with human red blood cells.

Introduction

The ever-growing global issue of bacterial resistance to commonly used small molecule antibiotics reveals an urgent need for the development of novel antimicrobial materials to combat a variety of infectious diseases found in both civilian hospitals and military facilities.¹ As alternatives to conventional small molecule antibiotics, antimicrobial peptides (AMPs) and synthetic cationic polymers hold great promise to overcome bacterial resistance due to their direct action on bacterial cell membranes which are less prone to mutation to avoid bactericides.^{2–11} As a complementary strategy to single chain AMP and polymer-based antimicrobial design, supramolecular assembly of peptides and polymers are of considerable interests for the construction of functional antimicrobial nanomaterials.^{11–20} These materials offer several advantages including (1) versatile chemical functionality that can be built in the assembly from different building blocks (2) greater control over materials stability, structure and thus bioactivity and (3) increased modularity through independently designed subunits for ultimate structural and activity optimization. Recently, several cationic synthetic polymers and peptides have been designed and used as the molecular building blocks to form supramolecular assemblies with tunable, nanostructure-dependent antimicrobial activity.^{12, 15, 18, 21, 22}

A significant challenge associated with systemic delivery of AMPs and cationic polymers lies in their potential cytotoxicity toward human primary cells and control over hemocompatibility toward vast numbers of circulating red blood cells (RBCs) due to their cationic and amphiphilic nature.^{23–26} The evaluation of hemocompatibility has been an important part of materials characterization to validate their potential as systemic delivery vehicles. Hemolysis has been commonly used for the evaluation of hemocompatibility, which is easy to perform and can provide quick feedback on materials capability to disrupt RBCs. However, hemolysis analysis may not be delicate enough to reflect the full spectrum of erythrocyte injuries caused by undesired material-RBC interactions. For example, eryptosis, referred to as programmed death of erythrocytes does not usually break cell

membrane to the degree of causing hemolysis and thus cannot be detected by hemolytic activity analysis.²⁷ Increased numbers of erythrocytes that undergo eryptosis can cause some physiologic derangement or serious health problems,²⁸ such as anemia, microcirculation dysfunction, and thrombogenic activation. Several serious diseases, such as heart failure-associated anemia,²⁹ chronic renal failure,³⁰ hemolytic uremic syndrome,³¹ Wilson's disease,³² and diabetes³³ were found to associate with excessive injured erythrocytes. Therefore, it is important to detect the early process of erythrocyte damage before hemolysis occurs in order to fully establish the credibility of antimicrobial materials as highly hemocompatible systemic delivery vehicles.

We have recently reported a self-assembling antimicrobial nanofiber (SAAN) based on the supramolecular assembly of *de novo* designed Multi-Domain Peptides (MDPs).²¹ MDPs, with a general sequence of $K_x(QL)_yK_z$, can be modularly designed to have tunable intermolecular interactions among the building units to generate nanofibers with intrinsic antimicrobial activity. SAANs are basically vehicle-free AMP delivery systems where MDPs serve dual roles as both antimicrobials and molecular building blocks to program and direct the assembly. In this work, we will thoroughly evaluate their interaction with HRBCs in a stringent biological assay where both hemolytic and eryptotic activity were examined and used to identify critical structural features governing hemocompatibility. Attaching polyethylene glycol (PEG) on synthetic polymers has been demonstrated as an effective strategy to improve the hemocompatibility of antimicrobial materials through the well-known stealth effect of PEG to minimize non-specific interactions of biomaterials with various proteins, cells and lipids in the biological environment.^{34–37} PEGylated SAANs were generated through the self-assembly of MDP-PEG conjugates which were synthesized by attaching PEG (MW=750 Da) at the N-terminus of MDPs. Based on our preliminary results, the eryptotic activity was found to vary significantly among designed SAANs (Table 1) and is much more sensitive to the internal molecular packing, supramolecular nanostructure and stability of the nanofiber than their hemolytic activity. Based on the eryptotic activity assay report, we also observed a differential effect of PEGylation on the hemocompatibility of SAANs. Improved hemocompatibility requires PEGylation on stable supramolecular nanofibers composed of highly organized β -sheet structure while PEG conjugation on weakly packed nanofibers composed of partially denatured β -sheets did not show improvement. Our study thus helps to reveal the fundamental mechanism involved in the selective hemocompatibility improvement of SAANs upon PEG conjugation. The structure-activity relationship developed through this study will provide important guidance for future design of a broader family of peptide and polymer-based assemblies with optimized antimicrobial activity and hemocompatibility.

Experimental section

Peptide synthesis and purification

All peptides were synthesized on a PS3 peptide synthesizer using standard Fmoc-solid phase peptide synthesis. 20% (V/V) piperidine in DMF was used to deprotect Fmoc groups. HCTU and DIPEA were used as amino acid coupling reagents in a molar ratio of 1:1:2.5 (amino acid: HCTU: DIPEA). Fmoc protected amino acids were added in 5 equivalents to the resin.

The N-terminus was acetylated in the presence of 50 equivalents of acetic anhydride and 6 equivalents of DIPEA in DMF. For PEGylated peptides, the N-terminus was reacted with the carboxyl terminated PEG750 in the presence HCTU/DIPEA. The coupling reaction was performed at room temperature for overnight and repeated once. PEG conjugation reaction was confirmed by the Kaiser test. All peptides were cleaved from the resin in a mixture of TFA/Tris /water (95/2.5/2.5 by volume) for 3 hrs. TFA solution was collected and the resin was rinsed twice with neat TFA. After evaporation of the combined TFA solutions, the residual peptide solution was triturated with chilled diethyl ether. Resulting precipitate was centrifuged, washed three times with chilled diethyl ether and dried overnight for HPLC purification. Purification was carried out with a 3% linear gradient using 0.05% TFA containing water and acetonitrile. The mass of each peptide was confirmed by MALDI-TOF mass spectrometry (Applied BioSystems Voyager-DE Pro.) using α -cyano-4-hydroxycinnamic acid as the matrix.

Critical assembly concentration (CAC) determination

Fluorescence measurements were performed at 25 C in 20 mM Tris buffer, pH 7.5 on a Varian Cary Eclipse fluorimeter with excitation wavelength at 280 nm and emission wavelengths between 295 and 500 nm. The bandwidths of the excitation and emission monochromators were either 5 nm or 10 nm. Multiple small increments (1–2 μ L) of a concentrated peptide stock solution (1 mM) were injected in the buffer solution while stirring. As the concentration of the peptide increases in solution, a deviation from linear fluorescence increase is observed suggesting peptide self-assembly and the burial of the tryptophan residues. The data was analyzed with OriginLab v 8.0 with the CAC value estimated at the crossing point.

Hemolytic activity measurement

All human subjects involved in the study were approved through written informed consent by the Medical Ethics Committee of the Second Affiliated Hospital affiliated with the Third Medical University. All experiments were performed in compliance with the relevant laws and institutional guidelines. Human RBCs were incubated with MDPs at different concentrations at 5, 10, 20 and 40 μ M for 3 hrs. Erythrocytes incubated with deionized water and Ringer solution were used as positive (+) and negative (-) controls, respectively. After Incubation, mixtures were centrifuged at $10,016 \times g$ for 3 min and 100 μ L supernatant samples were collected to measure the absorbance at 570 nm with a reference at 655 nm on a Varioskan Flash Multimode Reader (Thermo Fisher, USA). All experiments were conducted in triplicates. The percentage of hemolysis was calculated as:

$$\text{Hemolysis (\%)} = [(A_{\text{sample}} - A_{\text{negative control}}) / (A_{\text{positive control}} - A_{\text{negative control}})] \times 100$$

Measurement of phosphatidylserine (PS) exposure

Human RBCs were incubated with MDPs at different concentrations at 5, 10, 20 and 40 μ M for 24 hrs. Erythrocytes were washed with Ringer solution and resuspended in HEPES buffer (10 mM) containing 5 mM CaCl_2 and 5 μ L annexin-V-fluorescein. After co-incubation at 20 °C for 15 min, the forward scattering (FSC) and Annexin-V-FLUOS fluorescence

intensities were measured by FACS Calibur (BD, USA). The percentage of annexin-V positive RBCs was analyzed using Flowjo software (Treestar, USA). Fluorescence images were obtained by confocal microscopy (LSM 780, Carl Zeiss, Germany).

Erythrocyte membrane localization

To determine the localization of MDPs on HRBCs, 20 μ L of fluorescein-labeled MDP was mixed with 180 μ L erythrocytes solution and incubated at 37 °C. After co-incubation for 2 hrs, erythrocytes were washed with Ringer solution and resuspended in Ringer solution for confocal imaging (LSM 780, Carl Zeiss, Germany).

Results and discussion

Table 1 showed the primary sequences of the two peptide isomers used for the construction of SAANs. Both peptides consist of six repeating units of glutamine (Q) and leucine (L) to drive the assembly into β -sheet nanofibers while the end lysine (K) domains serve to modulate the morphology and stability of the supramolecular nanofiber. Tryptophan (W) was incorporated in the end domain playing dual roles to allow accurate UV quantification of peptide concentration and tune the molecular packing of MDPs within the nanofiber. As demonstrated in our previous work, the site of W is critical to the molecular secondary structure, fiber morphology and antimicrobial activity of SAANs.²¹ 3W62 exhibited a well-defined β -sheet structure while its isomer W362 formed mixed α -helices and β -sheets. The increased β -sheet content is due to the additional hydrophilic and hydrophobic unit, in this case KW included in the central domain to strengthen the intermolecular interactions among the building blocks. In this work, we focus on two new molecular building blocks, P-W362 and P-3W62 by attaching PEG (MW=750 Da) at the N-terminus of W362 and 3W62 in an effort to generate highly hemocompatible PEGylated SAANs for a wide range of biomedical engineering applications.

All four peptides spontaneously self-assembled into nanofibers in aqueous solution as characterized and confirmed by fluorescence microscopy, Circular Dichroism (CD) spectroscopy, Transmission Electron Microscopy (TEM) and small angle x-ray scattering (SAXS). Critical assembly concentration (CAC) was determined by measuring the intrinsic fluorescence emitted by tryptophan as a function of peptide concentration. At the CAC, fluorescence quenching occurs leading to a deviation of the linear trend of fluorescence increase with concentration. As shown in Figure 1, the CAC values are in close proximity with respect to one another suggesting comparable thermodynamic stability.

The four peptides, however, differ dramatically in their molecular secondary structure and packing within supramolecular nanofibers. As described above, W362 showed a mixture of α -helix and β -sheet as a result of the balance of attractive interaction among the (QL) repeating units and the electrostatic repulsion among the lysine residues. 3W62 showed a more defined β -sheet structure due to the additional hydrophilic-hydrophobic repeating unit “KW” to increase the driving force. PEG conjugation was found to slightly weaken β -sheet packing and both peptides showed signs of increased helical content as characterized by the enhancement of peak intensity at 208 nm (Figure 2).

Denaturation may occur at the end of the peptides due to entropic repulsion exerted by polymer chains. The effect of PEG conjugation on the supramolecular nanostructure was characterized by TEM. As reported previously, both peptides self-assembled into short nanofibers.²¹ For P-W362, short nanofibers co-exist with substantial amounts of random near spherical aggregates (Figure 3a). The formation of spheres is presumably due to structural denaturation of W362 upon PEG conjugation leading to reorganization of the building block to form non-specific aggregates. On the other hand, 3W62 did not seem to be much affected in terms of their ability to assemble (Figure 3b). Thorough examination of TEM sample revealed homogeneous nanofibers with negligible numbers of spherical-like aggregates. The effect of PEG conjugation on molecular packing and supramolecular stability was investigated through temperature-dependent CD which provided accurate information on the dynamic secondary structure of MDPs upon heating. Transition temperature (T_m) can be used as a quick assessment tool to qualitatively evaluate the nanofiber stability of various SAANs formulation. Figure SI–2 showed the results of melting curves of 3W62 and P-3W62 by monitoring the ellipticity at 205 nm, a characteristic wavelength for β - α structural transition as a function of temperature.³⁸ Both peptides displayed T_m close to one another suggesting comparable thermal stability and stable β -sheet structure at physiological temperature. Folding was found to be reversible upon cooling which again validates the design principle governing the assembly of MDPs into the most thermodynamically stable supramolecular β -sheet nanofiber. Unfortunately, T_m cannot be accurately determined for W362 and P-W362 based on the melting curves which lack a distinct structural transition profile presumably due to the low β -sheet content in the initial state.

The nanostructure of self-assembled peptides was quantified in more details using SAXS. Normalized data obtained in solutions at concentrations of 3–6 mg/ml are given in Figure 4. The data all show well-defined scattering pattern with a substantial continuous upturn at low Q indicating elongated structures. This is confirmed by the pair distribution function obtained by inverse Fourier transform (IFT), which shows clear asymmetric shapes (Figure SI–3) The data were further quantified using a theoretical model describing solid sheets of dimensions $a < b < c$ where a refers to the height, b refers to the width and c is the length and analyzed on an absolute scale as described in the previous publication.³⁹ W362 formed nanofibers with $a = 3.9$ nm, $b = 5.7$ nm and $c > 30$ nm. 3W62 formed nanostructures of similar dimension with $a = 3.7$ nm, $b = 5.9$ nm $c > 34$ nm. The length of the nanofibers cannot be resolved within the Q -range of the instrument. Although, the data indicate a polydispersity in length, c , we may get a measure of the maximum dimension of the filaments from IFT, yielding a similar value, $c = 78$ and 73 nm, for W362 and 3W62 respectively. Upon PEGylation, significant reduction in intensity was observed for W362 while the scattering intensity did not change much for 3W62. Taking into account the increase in the molecular weight of PEG-peptide conjugate (partially counteracted by the decrease in the overall electron intensity), the reduced intensity suggests a partial disintegration of the nanofiber for W362 which is consistent with the TEM results where smaller fragments were observed. The scattering profile of self-assembled PEGylated peptides was well fitted using a previously described model³⁹ for Gaussian polymer chains attached on the side of the nanofiber along with a contribution from free oligomers. The

analysis yields a nanostructure with $a = 4.4$ nm, $b = 6.0$ nm and $c > 40$ nm and $a = 3.9$ nm, $b = 5.6$ nm and $c > 33$ nm for P-W362 and P-3W62 respectively. In both cases we obtain similar radius of gyration (R_g) of the attached chains at 0.7 nm which is slightly less than the calculated size of about 1 nm for free PEG.

Notably, attaching PEG on W362 and 3W62 did not compromise the antimicrobial activity of the peptides given identical or even reduced MIC values (SI, Table 1). The outer layer of bacterial cell membrane contains excessive negative charges compared to mammalian cells and RBCs. In current work, PEG (MW=750 Da) was used to conjugate at the N-terminus of the peptides. Based on SAXS results, the radius of gyration of PEG is ~ 0.7 nm upon conjugation. It is possible that PEG chain may not completely shield the flanking lysine residues from interacting with the negative lipid membrane to induce membrane disruption and cell death. In future, we will focus on the antimicrobial activity of peptide-PEG conjugates consisting of PEG with different molecular weight (2000 Da and 5000 Da) and different architecture. Through a combination of biophysical study, i.e. SAXS and small angle neutron scattering (SANS) studies with biological evaluation, we will gain deeper mechanistic insights into the effect of SAANs' nanostructure and polymer conformation on their biological activity.

The study of hemocompatibility has been extremely important and indispensable to evaluate the potential of antimicrobial materials to be practically used in a blood-contacting environment. Currently, evaluation of the hemocompatibility of antimicrobials mainly relies on hemolysis analysis, which provides limited information on the effect of materials on erythrocyte injuries. Eryptosis, referred to as programmed death of erythrocytes occurs prior to hemolysis as a result of undesired materials-RBC interaction.⁴⁰ Eryptosis does not usually break cellular membrane integrity to lead to hemolysis. However, excessive eryptotic cells in circulation would induce erythrocytopenia by macrophage engulfment, even cause microcirculation dysfunction and thrombogenesis by adherence to the vascular wall and interaction with blood platelets.⁴¹ In RBCs, the parameters associated with eryptosis can be used to measure erythrocytes injury that cannot be detected by hemolytic activity analysis and thus may represent more sensitive and predictive parameters of toxicity compared with hemolysis. Eryptosis has been used as a metric to evaluate the effects of drugs,⁴² heavy metal ions,⁴³ fungi and bacterial toxins^{44, 45} on cellular structure and function of erythrocytes. However, it has rarely been investigated and used as an active index to assess the hemocompatibility of antimicrobial materials. In this work, we will thoroughly evaluate the hemocompatibility of different SAANs in terms of both hemolytic and eryptotic activity to understand the synergistic effect of PEGylation and supramolecular structure on the hemocompatibility of SAANs. It should also be pointed out that current study is performed not solely for the purpose of evaluating the hemocompatibility of this particular type of SAANs. It will provide invaluable mechanistic insights into the assembly of various other SAANs family with tunable physicochemical properties, antimicrobial activity and hemocompatibility, and could generalize such design principle to guide future design of a broader family of peptide and polymer-based antimicrobial assemblies.

First, the hemolytic activity of SAANs was evaluated according to the established literature protocol⁴⁶ by co-incubation of HRBCs with peptides at varying concentrations. Hemoglobin

released upon hemolysis was measured absorbance at 570 nm for quantitative comparison of the hemolytic activity of different SAANs. As shown in Figure 5, non-PEGylated peptides, W362 and 3W62 showed dose-dependent hemolytic activity with 10–20% of hemolysis occurring at 40 μ M for both peptides.

Hemolysis percentage significantly decreased to ~ 2% upon PEGylation due to the stealth effect of PEG to shield cationic antimicrobials from disrupting the negatively charged cell membrane of RBCs. Based on the hemolytic activity assay reports, it is reasonable to consider the antimicrobial nanofibers formed by P-W362 and P-3W62 to be highly hemocompatible with negligible hemolysis caused by the two peptides. However, evaluation of the eryptotic activity yielded dramatically different diagnostic results in terms of the hemocompatibility of all four peptides. Eryptosis can be characterized by various physicochemical parameters and processes including phosphatidylserine (PS) externalization, cell morphology change, cytosolic biochemical indices and phagocytosis.⁴⁷ In particular, PS translocation acts as a key mark in eryptosis. The externalization of PS can be quickly and reliably detected by fluorescein-labeled annexin V conjugates, which we used as an index to evaluate the effect of SAANs on eryptosis. As shown in Figure SI 4–7, the number of annexin V positive erythrocytes increased in a dose-dependent manner although to varying degree for different SAANs quantified by flow cytometry. Figure 6 showed the percentage of annexin V positive erythrocytes as analyzed by flow cytometry and Figure 7 showed the corresponding confocal images for all four peptides at 40 μ M.

3W62 showed the highest percentage of PS-displaying erythrocytes at ~89%, followed by P-W362 and W362 at ~26% and P-3W62 at ~6%. As shown in Figure SI 8–11 and Figure 7, confocal images confirmed the trend of peptide ability to induce PS externalization. Although the hemolytic activity of PEG-3W62 and PEG-W362 were comparable showing hemolysis rate at ~ 2%, their eryptotic activity is dramatically different. 26% of erythrocytes were found to undergo PS externalization induced by P-W362, while only 6% were found with P-3W62 treated cells. From the materials design point of view, we speculate that the ability of SAANs to induce eryptosis is largely dictated by a combined effect of supramolecular structure, stability and surface chemistry. Although all four peptides are capable of forming nanofibers, the molecular packing and supramolecular cohesion in the nanofiber varied dramatically as shown by CD and TEM (Figure 2 and 3). 3W62, due to the additional hydrophobic-hydrophilic repeating unit, was found to form nanofibers with stronger supramolecular cohesion than that of W362. Improved molecular packing led to a more compact and defined charge interface where a large number of lysine residues were accumulated. These supramolecular charge clusters may cause RBC membrane disruption, cell aggregation and therefore eryptosis (Figure 7B). The molecular structure and packing was compromised in nanofibers formed by W362 due to a shift of energetic balance toward electrostatic repulsion. The nanofiber is composed of partially folded β -sheets with less ordered lysine domains. Decreased interfacial charge ordering and density will alleviate the non-specific electrostatic interaction of SAANs with RBCs and greatly reduce their eryptotic activity. It is worth noting that such difference in hemocompatibility cannot be detected in hemolytic activity assay which usually requires a high degree of cell membrane disruption to cause hemolysis. Eryptosis represents a more sensitive and delicate assay methods to

provide a full spectrum of materials-RBC interaction and predict the fate of injured erythrocytes in blood flow.

It is interesting and also surprising to see the differential effect of surface PEG conjugation on the eryptotic activity of SAANs. Attaching PEG on nanofibers with weak supramolecular cohesion formed by W362 did not improve the eryptotic activity while PEG attachment on nanofibers with strong supramolecular cohesion formed by 3W62 led to significant reduction of eryptosis. The results seem to suggest PEG attachment failed to shield and protect the “weak” nanofibers from exposure to RBCs. We speculate that on one hand, PEGylation can indeed shield the charged domain on W362 nanofibers from interacting with RBC cell membranes. However, on the other hand, PEG attachment on the “weak” nanofiber may further destabilize the molecular packing of individual peptide chains affording them to be more flexible and able to dissociate and intercalate into RBCs leading to enhanced eryptotic activity. The eryptotic activity of P-W362 is then dictated by the balance of reduced supramolecular stability caused by PEGylation and the universal stealth effect of PEG. In contrast, P-3W62 retained a strong β -sheet secondary structure and supramolecular stability upon PEGylation of 3W62 (Figure 2b, 3b and 4). SAAN consisting of P-3W62 basically features a shielded cationic nanofiber where individual building units are held tightly within the nanofiber to prevent from undesired physical contacts and interactions of with RBCs for possible hemolysis.

To further understand the different eryptotic activity exhibited by SAANs, potential mechanisms in eryptosis induced by SAANs were explored by measuring cytosolic Ca^{2+} and Reaction Oxygen Species (ROS). The increase of intracellular ROS induces eryptosis through opening cationic channels. The activation of Ca^{2+} -sensitive potassium channels by oxidative stress increases cytosolic Ca^{2+} concentration which will further affect the skeleton flexibility and stability, intracellular ion balance leading to PS externalization and eryptosis.⁴⁸ Figure 8a showed the level of intracellular ROS quantified by flow cytometry upon incubation of HRBCs with various SAANs at different concentrations. Substantially higher amounts of ROS production was observed for HRBCs treated with 3W62 which showed the most potent eryptotic activity. The level of cytosolic Ca^{2+} is consistently higher for 3W62 than that of HRBCs treated with other peptides (Figure 8b). P-3W62 with the minimum eryptotic activity exhibited consistently low level of both ROS and cytosolic Ca^{2+} across the entire concentration range.

These findings indicate that both oxidative stress and Ca^{2+} entry play a pivotal roles in eryptosis induced by SAANs. The specific biochemical routes and detailed biological mechanisms of SAANs induced ROS and Ca^{2+} flux will be the primary focus of future study and will be reported separately. More quantitative thermodynamic and kinetic analysis will also be performed through a combination of CD, DSC and small angle x-ray/neutron scattering to provide more structural insights into the differential effect of PEGylation of SAANs on RBC's structure and function. However, regardless of the mechanistic origin of SAANs' interaction with RBCs, based on current studies, optimized hemocompatibility can only be achieved by introducing PEG on stable supramolecular assemblies to inhibit both hemolysis and eryptosis. Optimized SAAN formulation based on P-3W62 showed exquisite hemocompatibility in terms of extremely low levels of hemoglobin release as an indicator of

hemolysis, and low levels of Ca^{2+} , ROS and PS externalization characteristic of eryptotic activity.

Conclusions

We have developed a highly hemocompatible antimicrobial nanomaterial based on the supramolecular assembly of *de novo* designed cationic peptides. The hemocompatibility of SAANs was thoroughly investigated in a comprehensive assay where both hemolysis and eryptosis were considered to be important parameters to establish an accurate structure-activity relationship for future materials design and optimization. PEGylation was found to selectively improve the hemocompatibility of SAANs and optimization was achieved by introducing PEG on stably packed supramolecular assemblies to inhibit both hemolysis and eryptosis while retaining good antimicrobial activity. Importantly, we believe the fundamental structure-activity relationship may not be limited to current SAANs platform and may potentially be applied to a broader range of antimicrobial nanomaterials. Additionally, the ability to fabricate highly hemocompatible and versatile SAANs and use them to modify RBCs without compromising their structure and function could open a new avenue in cell surface engineering to meet various needs in biological and biomedical engineering applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. CAC determination through fluorescence measurement of peptides as a function of concentration in Tris buffer (20 mM, pH 7.4) (a) W362, (b) 3W62, (c) P-W362, (d) P-3W62.

Figure 2. CD spectra showing the different secondary structures adopted by each peptide. **(a)** W362 and 3W62, **(b)** P-W362 and P-3W62. Peptides were dissolved in Tris buffer (20 mM, pH 7.4) at 100 μ M.

Figure 3. TEM images of supramolecular nanostructures formed (a) P-W362 (b) P-3W62. Peptides were dissolved in Tris buffer (20 mM, pH 7.4) at 100 μ M. Scale bar: 100nm.

Figure 4. SAXS results and fitting (BM29, ESRF, Grenoble) of self-assembled nonPEGylated and PEGylated MDPs (a) W362 and P-W362 (b) 3W62 and P-3W62. Peptide solution was prepared in Tris buffer (pH=7.4, 20 mM) with a final peptide concentration at ~ 3–6 mg/ml.

Figure 5. Hemolysis induced by SAANs. Peptides were incubated with RBCs for 3 hrs. Hemoglobin release was used to evaluate hemolytic activity of peptides at concentration of 5 μM, 10 μM, 20 μM, 40 μM.

Figure 6.

(a) Percentage of annexin V positive erythrocytes upon incubation of HRBCs with peptides at 40 μ M for 24 hrs as determined by flow cytometry. (A) W362 (B) 3W62 (C) P-W362 (D) P-3W62.

Figure 7. Confocal images of HRBCs as a result of PS externalization upon incubation with peptides at 40 μ M. (A) W362 (B) 3W62 (C) P-W362 (D) P-3W62. Top panel: bright field images. Bottom panel: fluorescence images.

Figure 8.

Figure 8a. (a) Intracellular ROS concentrations and (b) Cytosolic Ca^{2+} concentration after exposure of HRBCs to different peptides at 40 μM.

Table 1

Chemical composition of MDPs used in the study

Peptide	N-terminus	Peptide Sequences*	C-terminus
W362	CH ₃ CO–	W KKKQLQLQLQLQL KK	CONH ₂
3W62	CH ₃ CO–	K KKWQLQLQLQLQL KK	CONH ₂
P-W362	PEG750–	W KKKQLQLQLQLQL KK	CONH ₂
P-3W62	PEG750–	K KKWQLQLQLQLQL KK	CONH ₂

* Alternating hydrophilic-hydrophobic repeating units are highlighted in bold. W362 contains six repeating units and its constitutional isomer 3W62 contains seven.