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ARTICLE

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An in vitro evaluation of the biocompatibility of proline-alanine-serine peptides compared with polyethylene glycol and polyglycerol

Qianyu Zhang | Hongjing Chen | Huali Chen

College of Pharmacy, Chongqing Medical University, Chongqing, China

Correspondence
Qianyu Zhang, College of Pharmacy, Chongqing Medical University, No. 1, Yixueyuan Rd, Chongqing 400016, China.
Email: qyzhang@cqmu.edu.cn

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Abstract

PASylation has been recently reported as a feasible alternative to PEGylation, which in essence is using polypeptides constituted of a combination of proline, alanine and serine for the hydrophilic modification of pharmaceuticals. In this work, we focused on the biocompatibility evaluation of two PAS peptides, $(PAS)_8$ and $(PA_3)_7$ as well as the more frequently used polymers polyethylene glycol (PEG) and polyglycerol (PG). It has been verified in this study that $(PAS)_8$ and $(PA_3)_7$ both exhibited low cell toxicity against HUVEC and RAW 264.7 cell lines. They also showed negligible RBC hemolysis and agglutination, which demonstrated adequate hemocompatibility. Their potential interactions with bovine serum albumin have also been investigated, and the results indicated little hydrophobic interactions between the polymers and protein. In conclusion, $(PAS)_8$ and $(PA_3)_7$ as well as PEG and PG all showed considerable compatibility and safety in these studies, suggesting that $(PAS)_8$ and $(PA_3)_7$ could be considered as potential candidates for PEG replacement in future studies.

KEYWORDS
biocompatibility, hemagglutination, hemolysis, PAS peptide, polyethylene glycol, protein-polymer interactions

1 | INTRODUCTION

Modification of hydrophilic polymers on biopharmaceuticals or nanomedicines such as liposomes stabilizes the pharmaceutical formulations, decreases their interactions with blood serum proteins when injected intravenously and potentially elevates the drug delivery efficiency due to the extended circulation time in the system. In this regard, polyethylene glycol (PEG) has long been the polymer of choice. PEGylated drugs or drug carriers such as liposomes have been widely applied in pharmaceutical industry to improve formulation stability and increased in vivo circulation.[1–4] However, the dominant role in drug delivery of PEG has been challenged lately due to its induction of anti-PEG antibody as well as the anaphylactic reactions.[5–7] To overcome this, polypeptides have been proposed as potential replacements as they can be devised with tunable hydrophilicity and in principle display better biocompatibility and biodegradability. Among them, PASylation was designed as an alternative for PEGylation by Skerra et al. and has been recently reported to prolong in vivo half-time and stability of protein drugs such as leptin, interferon, thymosin and even antibody Fab' fragments.[8–17] PAS was named based on the amino acids, which are proline, alanine and serine that compose the polypeptide sequence, and they were arranged in a random coil conformation that resembles the morphology of PEG.[10] The omission of serine from the sequence was feasible in view of the fact that the appropriate arrangement of proline and alanine could also retain the disordered conformation of the peptide sequence while maintaining its hydrophilicity, demonstrating the potential and flexibility of a rich PAS sequence library.[18] PAS peptides demonstrated potential beyond protein modification, as it could also be incorporated into nano-sized drug carriers such as nano-ferrintin, nanoghost (which

Qianyu Zhang and Hongjing Chen contributed equally to this work.

was a poly lactic-co-glycolic acid polymeric core surrounded by PAS-expressing mammalian cell membranes as the coating) and pDNA-loading polyplexes.[19–24] For example, $(PAS)_8$ has shown to be able to shield and protect polyplexes and display similar properties to PEG of comparable length.[24] On the other hand, our lab has also recently demonstrated that similar to PEG, another PAS sequence $(PA_3)_7$ could endow long circulating effect to liposomes and increase their serum stability while lowering the engulfment by macrophages.[25]

Although PAS peptides showed certain potential in drug delivery both in vitro and in vivo, their biocompatibility has not been systematically investigated yet, which should be worthing looking into as they are now potential candidates to replace PEGylation in drug design. Therefore, in this study, the cytotoxicity and hemocompatibility of the two representative PAS peptides, $(PAS)_8$ and $(PA_3)_7$, were probed compared to PEG 2000 (with a comparable molecular weight to the peptides). Polyglycerol (PG) was also included in this study for it is also considered as a hydrophilic polymeric alternative to PEG in pharmaceutical designing and we have shown before that polyglycerol fatty acid esters-incorporated liposomes exhibited prolonged circulation in mice and similar biodistribution to PEGylated liposomes.[4,26–28] Besides the cytotoxicity and hemocompatibility studies, the potential hydrophobic interactions of these polymers with proteins (with bovine serum albumin selected as the model protein) have also been investigated.

2 | MATERIALS AND METHODS

2.1 | Materials, cell lines, and animals

Two PAS peptides $(PAS)_8$ and $(PA_3)_7$ were synthesized according to the standard solid phase peptide synthesis by ChinaPeptides Co. Ltd. (Shanghai, China). Polyethylene glycol with a molecular weight of 2000 Da (PEG 2000) was purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). MTT, polyglycerol-10 (PG-10), 8-aniline-1-naphthalene sulfonic acid (ANS) and bovine serum albumin (BSA) were obtained from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Other chemicals and reagents were of analytical grade.

Murine macrophage cell line RAW 264.7 and human umbilical vein endothelial cell line HUVEC were both obtained from ATCC and maintained in DMEM (Solarbio, Beijing, China) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Israel) at 37°C in a humidified 5% CO_2 atmosphere.

Female BALB/c mice (18–20 g) were purchased from Chongqing Ensiweier Laboratory Animal Co. Ltd. (Chongqing, China). Mice were raised in sterile animal laboratories, and all animal experiments were approved by the Laboratory Animal Welfare and Ethics Committee of Chongqing Medical University.

2.2 | ANS fluorescence assay on BSA binding

The variation in the surface hydrophobicity of BSA due to the presence of different polymers was determined using ANS assay as previously reported.[29,30] Increasing aliquots of different polymers were added into BSA at weight ratios of 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0 (which was denoted as BSA only). The final concentration of BSA was maintained at 50 μg/mL for all samples. ANS was added into each of the samples to reach a final concentration of 80 μM. The excitation wavelength was set at 350 nm and the emission was scanned between 400 and 600 nm (SpectraMax M5, Molecular Devices, USA). The fluorescence intensities were recorded and all assays were conducted under room temperature.

2.3 | Cytotoxicity assay

RAW 264.7 and HUVEC were seeded onto 96-well plates at a density of 1×10^4 cells/well and allowed for attachment overnight. Different polymers were added into the wells and the incubation continued for another 48 h. Cell viabilities were determined by MTT assay according to the manufacturer's instructions.

2.4 | Hemolysis assay

Fresh blood from BALB/c mice was collected, and the red blood cells (RBCs) were obtained after centrifugation and washed with phosphate buffer saline (PBS) for three cycles until the supernatant became colorless. The RBCs suspension (2%, v/v) was then prepared and mixed with polymer solutions at a volume ratio of 1:1 and incubated under 37°C. After 1 and 24 h of incubation, the supernatant was separated after centrifugation and subjected to measurement for hemoglobin release by a microplate reader (SpectraMax M5, Molecular Devices, USA) at 545 nm. Samples incubated with 0.5% sodium dodecyl sulfonate served as the positive control (100% hemolysis) and samples incubated with PBS served as the negative control (0% hemolysis) respectively. The degree of hemolysis was calculated by the following equation:

$$\text{Hemolysis ratio} = (A - A_{0\%})/(A_{100\%} - A_{0\%}).$$

where A, $A_{0\%}$, and $A_{100\%}$ represented the absorbance of samples incubated with liposomes, samples with 0% hemolysis and samples with 100% hemolysis respectively.

2.5 | RBC agglutination assay

Blood from BALB/c mice was collected and RBCs were collected as described in 2.4. The RBCs suspension (1%, v/v) was mixed with polymer solutions at a volume ratio of 1:1 and incubated under 37°C for 1 h in a 96-well U-bottom plate to allow for full sedimentation. At the end of incubation, the plate was visually assessed for agglutination against a white background. PBS served as the negative control.

2.6 | Statistics analysis

All values are represented as mean ± SD. Statistical analysis was performed using two-tailed Student t-test, one-way or two-way ANOVA

(GraphPad Prism, San Diego, CA). Difference was considered as statistically significant at the levels of $^*p < 0.05$ and a p value higher than 0.05 was considered as no significance.

3 | RESULTS

3.1 | ANS fluorescence assay on BSA binding

The wavelength of maximum emission was found to be at 495 nm, which appeared to be consistent in all samples. Results in Figure 1a showed that within 6 h of incubation, the fluorescence of ANS in BSA only along with other groups all gradually decreased over time (with significant differences when comparing the fluorescence at 0 and 6 h of all groups respectively), which might be due to the relaxation and unfolding of BSA in the aqueous solution, conforming to the previous report.[30] Meanwhile, no significant differences can be observed between BSA only and all the other BSA-polymer solutions, indicating the lack of significant hydrophobic interactions between the polymers and BSA, including the two PAS peptides. A similar trend was also obtained when the mass ratios between polymers and BSA decreased from 8 to 0.125 (Figure 1b), with no significance in the ANS fluorescence change, suggesting the absence of detectable hydrophobic interactions under all concentrations. By comparison, PEG 2000 monostearate (C18-PEG 2000) was also included in the study and tested side by side with PEG 2000 (Figure S1). It could be concluded that C18-PEG 2000 showed a concentration-dependent effect on in ANS fluorescence signal as the mass ratio between C18-PEG 2000 and BSA increased from 0.5 to 8 (Figure S1). This was clearly induced by the hydrophobic stearate chain in C18-PEG 2000, which showed a great contrast compared with PEG 2000.

3.2 | Cytotoxicity assay

The toxicity of polymers on HUVEC and RAW 264.7 cells was presented in Figure 2. A highest concentration of 4 mg/mL was set due to the limit of the solubility of $(PAS)_8$ peptide. It can be concluded from Figure 2 that the cytotoxicity of different polymers remained comparatively low after 48 h of incubation with both HUVEC and

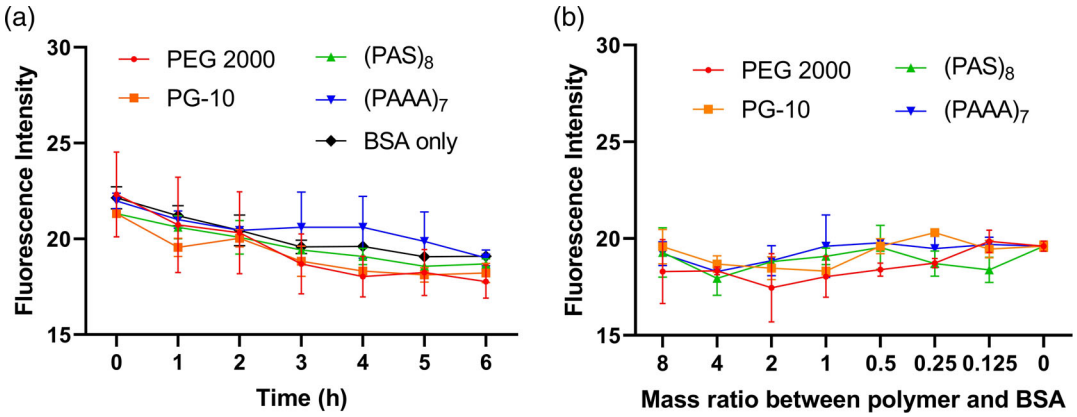

FIGURE 1 8-aniline-1-naphthalene sulfonic acid (ANS) fluorescence change caused by the interactions between different polymers and bovine serum albumin (BSA). (a) Effect of incubation time and polymer type on the ANS fluorescence after incubating polymers with BSA at a mass ratio of 1:1. (b) Effect of the mass ratio between polymers and BSA on ANS fluorescence after incubating the polymers with BSA for 4 h. Values represent mean ± SD with $n = 3$.

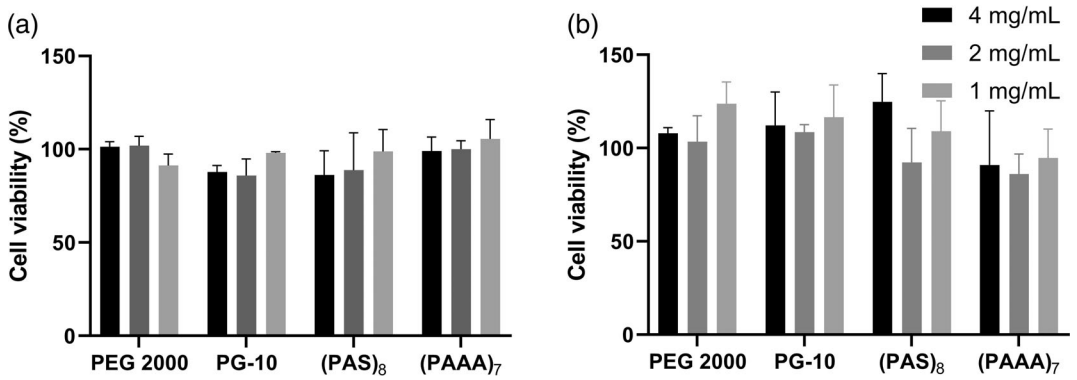

FIGURE 2 Cytotoxicity of polymers of different concentrations on HUVEC (a) and RAW 264.7 (b) after 48 h of incubation. Values represent mean ± SD with $n = 3$.

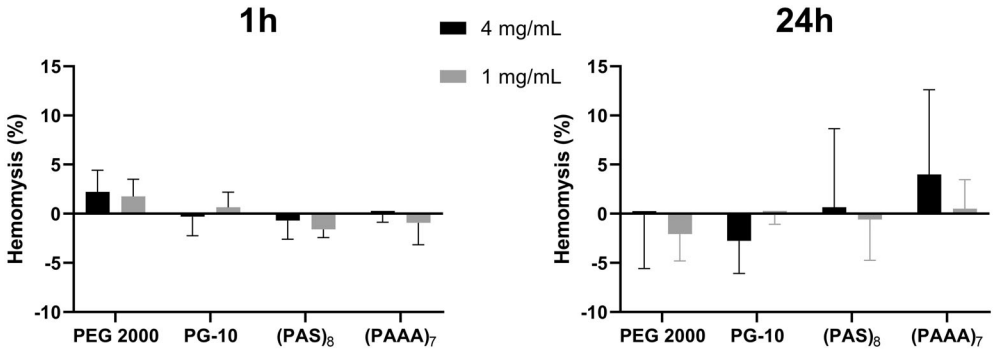

FIGURE 3 Hemolytic assay by incubating 2% mice red blood cells (RBCs) with different concentrations of PEG 2000, PG-10 and PAS peptides for 1 and 24 h. Values represent mean ± SD with $n = 3$.

RAW 264.7 cells with the cell viabilities all above 85% under all concentrations.

3.3 | Blood compatibility

To evaluate the possible effect of the polymers on mice RBCs, both hemolysis and agglutination assays were performed. As can be seen in Figure 3, $(PAS)_8$ and $(PA_3)_7$ both showed negligible hemolysis (below 5%) under both 1 and 4 mg/mL within 24 h of incubation.

The sedimentation of RBC at the bottom of each well as a dot indicated a negative agglutination reaction (with PBS serving as the negative control), and the results showed no significant effect of the PAS peptides on the RBC agglutination formation at a concentration up to 4 mg/mL, similar to PEG 2000 and PG-10 as demonstrated in Figure 4.

4 | DISCUSSION

Compared to the synthesis of polymers, polypeptides can be synthesized in a more well-controlled manner with defined sequences and can even be bio-synthesized.[31–34] Moreover, polypeptides can be degraded into low- or non-toxic metabolites with relative ease due to the omnipresence of protease in vivo.[35] This might indicate relatively low toxicity and immunogenicity, which make them attractive alternatives to polymers such as PEG. PEG has long been a prevalent anti-fouling polymeric material due to its capacity to repel protein adsorption, which exerts profound influence on lowering cell adhesion or even bacteria attachment. Nevertheless, it also produces anti-PEG and induces anaphylactic reaction.[36–38] PAS peptides were designed based on the fact that they potentially possessed PEG-like properties with hydrophilic long chains and random coils.[8,10] Whether this structural resemblance endows PAS peptides with similar protein-repelling characteristics needs further exploration. ANS is a fluorescence probe used in the characterization of protein hydrophobicity, as its fluorescence emission would increase strongly when binding to the hydrophobic pockets in the protein.[30,39] The interactions between

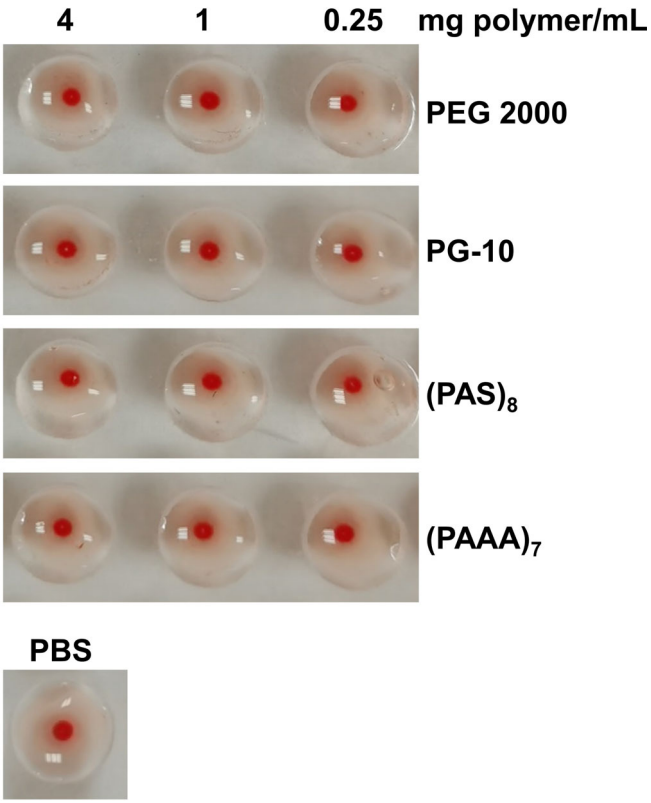

FIGURE 4 Mice red blood cells (RBC) agglutination assay by incubating 1% mice RBC with polymers of different concentrations on a U-shaped bottom plate for an hour. phosphate buffer saline (PBS) served as the negative control.

polymers and proteins induce protein conformation changes or other hydrophobic bindings, which expose or shield the hydrophobic regions and change the intensity of ANS fluorescence. It could be deduced from Figure 1 that $(PAS)_8$ and $(PA_3)_7$ both showed very little effect on the change of conformation and ANS binding site of BSA at all weight ratios tested, same as PEG 2000 and PG-10. The results indicated their potential protein-repelling property, which is critical in designing long-circulating pharmaceuticals as well as biomedical devices.

HUVEC and RAW 264.7 cells were chosen as they could be considered as relatively reliable and simple in vitro cellular models to evaluate the toxicity of the materials on endothelium and immune cells.[40,41] From Figure 2 it could be concluded that the cytotoxicity of different polymers remained comparatively low after 48 h of incubation with both HUVEC and RAW 264.7 cells. In one of our precious works, we have evaluated the cytotoxicity of $(PA_3)_7$-modified liposomes, which exhibited negligible toxicity over several cell lines (with the highest PAS peptide concentration reaching around 0.14 mg/mL).[25] Here we have proved that even at a higher concentration of 4 mg/mL, both PAS peptides still showed low cell toxicity. An upper limit of 4 mg/mL of the polymers was set due to the limitation of $(PAS)_8$ peptide. In fact, other PAS peptides such as $(AAPAAPAPAAPAAPAPAAPA)_n$ and $(ASPAAPAPASPAAPAPSAPA)_n$ all showed much higher solubility in previous studies (up to 100 mg/mL).[18] It would be interesting to investigate the biocompatibility of these PAS peptides with much higher molecular weights and solubility in future studies.

Red blood cells (RBCs) are going to be one of the major cell components to be encountered upon the entrance of the polymers or polymer-mediated pharmaceuticals into the blood stream, and the hemolysis and agglutination assays provide a relatively reproducible and biologically relevant method to help characterize the biocompatibility of tested materials as they can be used to reflect the possible change in RBC integrity, plasticity, adhesiveness and propensity to aggregation during the interactions with polymers.[42] Results from Figures 3, 4 displayed low hemolysis rates and almost no RBC agglutination after incubating polymers with RBCs, preliminarily demonstrating the considerable hemocompatibility of the selected polymers, suggesting their potential safe application if used in vivo.

Overall, here we have proved that $(PAS)_8$ and $(PA_3)_7$ showed considerable protein-repelling properties, low toxicity as well as acceptable hemocompatibility. They are relatively shorter versions of the PAS peptides (with molecular weights around 2000 Da) which should be more applicable in nanomedicine design since they could be synthesized chemically with defined sequences and guaranteed purity, which facilitates the fabrication process. PAS peptides with much longer sequences are usually synthesized using bioengineering technology and more widely applied in protein modification, and their biocompatibility needs further verification as polymers of various molecular weights exhibit different levels of hydrophilicity, solubility and toxicity, hence affecting their biocompatibility.

5 | CONCLUSIONS

In this study, we have investigated the biocompatibility of two PAS peptides, $(PAS)_8$ and $(PA_3)_7$, along with the hydrophilic polymers PEG 2000 and PG-10. Little hydrophobic interactions could be detected between the polymers with BSA using ANS fluorescence assay, which indicated the potential protein-repellent properties of the polymers. Similar to PEG 2000 and PG-10, these two PAS peptides also showed low cytotoxicity on RAW 264.7 and HUVEC cells, and caused negligible levels of hemolysis and agglutination of mice RBCs. In conclusion, we have shown that $(PAS)_8$ and $(PA_3)_7$ presented considerable biocompatibility in these in vitro studies, which warrants them as feasible candidates as the hydrophilic polymers used in the modification of pharmaceuticals.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. No writing assistance was utilized in the production of this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Qianyu Zhang https://orcid.org/0000-0002-0758-2432

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SUPPORTING INFORMATION

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