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Bacterium-Mimicking Nanoparticle Surface Functionalization with Targeting Motifs

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Abstract

In recent years, surface modification of nanocarriers with targeting motifs has been explored to modulate delivery of various diagnostic, sensing and therapeutic molecular cargos to desired sites of interest in *in vitro* bioengineering platforms and *in vivo* pathologic tissue. However, most surface functionalization approaches are often plagued by complex chemical modifications and effortful purifications. To resolve such challenges, this study demonstrates a unique method to immobilize antibodies that can act as targeting motifs on the surfaces of nanocarriers, inspired by a process that bacteria use for immobilization of the host's antibodies. We hypothesized that alkylated *Staphylococcus aureus* protein A (SpA) would self-assemble with micelles and subsequently induce stable coupling of antibodies to the micelles. We examined this hypothesis by using poly(2-hydroxyethyl-co-octadecyl aspartamide) (PHEA-g-C₁₈) as a model polymer to form micelles. The self-assembly between micelles and alkylated SpA became more thermodynamically favorable by increasing the degree of substitution of octadecyl chains to PHEA-g-C₁₈, due to a positive entropy change. Lastly, the simple mixing of SpA-PA-coupled micelles with antibodies resulted in the micelles coated by antibodies, as confirmed with a fluorescence resonance energy transfer (FRET) assay. The micelles coated by antibodies to VCAM-1 or integrin α_v displayed higher binding affinity to a substrate coated by VCAM-1 and integrin $\alpha_v\beta_3$, respectively, than other controls, as evaluated with surface plasmon resonance (SPR) spectroscopy and a circulation-simulating flow chamber. We envisage this bacterium-inspired protein immobilization approach will be useful to improving the quality of targeted delivery of nanoparticles, and can be extended to modify the surface of a wide array of nanocarriers.

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Introduction

The intermolecular assembly of polymers has been previously used to form a wide array of different nanoparticles, including micelles and vesicles. By adding a targeting motif to the nanoparticle surface, these polymeric nanoparticles can be used in a range of *in vitro* and *in vivo* applications, such as separation and sensing devices,^{1, 2} imaging of diseased tissue,³⁻⁵ and targeted delivery of drug molecules.^{6, 7} Amphiphilic molecules constituted with hydrophobic and hydrophilic segments are being commonly used as a building block of the self-assembled nanoparticles, because of the association between hydrophobic segments in aqueous media.⁸ The size and the morphology of resulting nanoparticles are significantly dependent on the molecular weight and the packing parameter of amphiphilic molecules.^{9, 10}

These nanoparticle surfaces are often functionalized with varied bioactive molecules that can specifically bind with surfaces of interest, in order to deliver molecular cargos to a target site and subsequently elevate their desired performance. It is common to covalently conjugate targeting biomolecules to nanoparticles before or after self-assembly.¹¹ Alternatively, the nanoparticle surface and the targeting ligand are connected by the noncovalent interaction between biotin and avidin or streptavidin to each pair.¹¹ These processes, however, require multi-step chemical modifications and effortful purifications, which inadvertently lead to a low production yield and a large production cost. Additionally, chemical conjugation of targeting biomolecules to amphiphilic molecules prior to nanoparticle assembly can lead to reduced biomolecular activity as well as localization into the nanoparticle core.

Apart from these conventional methods, living organisms offer a simple way to present a wide array of biomolecules on its surface. For example, the bacterium *Staphylococcus aureus* expresses protein A (SpA), a surface protein that can bind with the heavy chain in the Fc-region of immunoglobulins.¹² SpA associates with the membrane of *Staphylococcus aureus* and inhibits opsonophagocytosis by binding with the host antibody, so it aids the survival of the bacterium.¹³ Inspired by this process, we hypothesized that insertion of alkylated SpA into the surface layer of a self-assembled nanoparticle will allow us to readily engineer the nanoparticle surface with various antibodies of interest that can function as targeting motifs (Scheme 1)¹⁴. We examined this hypothesis by using SpA modified with palmitic acid (SpA-PA) and directing self-association between SpA-PA and a micelle of poly(2-hydroxyethyl aspartamide) substituted with octadecyl chains, termed PHEA-g-C₁₈. The micelle coupled with SpA-PA was coated by antibodies to vascular cell adhesion molecules-1 (anti-VCAM-1) or those to integrin $\alpha_v\beta_3$ (anti-integrin α_v) via simple mixing of the pre-made micelle with antibodies. The biological association between the SpA-PA of the micelle and the antibody was monitored by analyzing the fluorescence resonance energy transfer (FRET) between the SpA-PA and the antibody, each of which was labeled with FRET donor and acceptor, respectively. The targetability of the resulting micelles toward a substrate coated with VCAM-1 or integrin $\alpha_v\beta_3$ was evaluated with surface plasmon resonance (SPR) spectroscopy and a blood vessel-mimicking flow chamber. Taken together, this study will greatly serve to simplify nanoparticle surface functionalization via a bio-inspired modular self-assembly process. We envisage this technique can potentially elevate the performance level of a wide array of nanoparticles.

Results and Discussion

Preparations and characterizations of PHEA-g-C₁₈ micelles

First, PHEA-g-C₁₈ was synthesized through nucleophilic substitution of designated amounts of octadecylamine and excessive amount of ethanolamine to poly(succinimide) (PSI) (Figure 1a), as characterized with the ¹H NMR spectra (Figure S1). According to integrals of NMR peaks at 0.85 to 0.95 ppm and 4.3 to 4.7 ppm, the degree of substitution for octadecyl chains (*DS*_{C18}) to the polymer, defined as the mole percentage of succinimide units substituted with octadecyl chains, was varied from 2.4 to 6.4 and 14.8 mol % (Table 1). The peak at 0.85 to 0.95 ppm in the NMR spectrum represents protons of methyl groups at the ends of the substituted octadecyl chains, and the peak at 4.3 to 4.7 ppm represents protons on the polyaspartamide backbone (Figure S1). For convenience, PHEA-g-C₁₈ at *DS*_{C18} of 2.4, 6.4, and 14.8 mol % were termed as DS-2.4, DS-6.4, and DS-14.8, respectively.

Addition of PHEA-g-C₁₈ into phosphate buffer saline (PBS) and subsequent sonication created polymeric micelles via *in situ* self-assembly. Increasing *DS*_{C18} of PHEA-g-C₁₈ dramatically decreased the corresponding critical micelle concentration (CMC), which was marked by the significant increase in the emission intensity ratio (*I*₃/*I*₁) between 385 (*I*₃) and 373 (*I*₁) nm of pyrene added in the aqueous micelle suspension¹⁵ (Table 1 & Figure S2). The *z*-average hydrodynamic radii of the micelles, measured via dynamic light scattering (DLS), became larger by increasing *DS*_{C18} of PHEA-g-C₁₈ (Table 1). Micrographs acquired with the transmission electron microscope (TEM) confirmed the spherical morphology of PHEA-g-C₁₈ micelles (Figure 1b). The zeta-potential of micelles, in the range of −10 to −12 mV, were independent of *DS*_{C18} according to the statistical analysis (Figure 1c).

Surface modification of PHEA-g-C₁₈ micelles with palmitic acid-conjugated *Staphylococcus aureus* protein A (SpA-PA)

Chemical reaction of palmitic acid *N*-hydroxysuccinimide ester with amine groups of SpA resulted in the SpA conjugated with palmitic acid, termed SpA-PA (Figure 2a-1). Simple mixing of the SpA-PA with PHEA-g-C₁₈ micelles resulted in self-association between two components (Figure 2a-2), as monitored with the SpA-PA labeled with fluorescein (Scheme S1). In contrast, the unmodified SpA minimally associated with PHEA-g-C₁₈ micelles (data not shown). Interestingly, the percentage of SpA-PA associated with the polymeric micelles increased from 9 (± 0.5 %) to 38 % (± 6 %) by increasing *DS*_{C18} of PHEA-g-C₁₈ from 2.4 to 14.8 %, as quantified with the fluorescence intensity from the micelle following centrifugation-based purification (Figure 2b).

The underlying mechanism by which SpA-PA associated with the PHEA-g-C₁₈ micelles was examined by quantifying changes in the Gibbs free energy (*G*), enthalpy (*H*), and entropy (*S*) of this self-assembly process. *G* calculated using Eq. (1) became a more positive value at a given temperature (*T*) as *DS*_{C18} decreased from 14.8 to 2.4 mol % (Figure 2c).

$$\Delta G = -RT \ln K_{eq}$$

$$\text{where } K_{eq} = \frac{[\text{Micelle-coupled SpA-PA}]}{[\text{Free SpA-PA}][\text{Micelles}]} \quad (1)$$

R is the gas constant, [Micelle-coupled SpA-PA] is the concentration of SpA-PA associated with micelles, [Free SpA-PA] is the concentration of excess SpA-PA, and [Micelles] is the concentration of PHEA-g-C₁₈ micelles. H calculated from the slope of Van't Hoff plot (Figure 2d based on Eq. (2)) was 11.4 kJ/mol for the DS-14.8 micelles coupled with SpA-PA, while H for DS-2.4 micelles was -6.3 kJ/mol (Figure 2e). In addition, S calculated from the intercept of Van't Hoff plot presented a larger, positive number for the association of SpA-PA with DS-14.8 micelles than that with DS-2.4 micelles (Figure 2e).

$$\ln K_{eq} = \left(\frac{-\Delta H}{R} \right) \left(\frac{1}{T} \right) + \frac{\Delta S}{R} \quad (2)$$

These analytical results suggest that the association of SpA-PA with DS-2.4 micelles was thermodynamically unfavorable, as characterized with the negative S and the positive G of the process. In contrast, increasing DS_{C18} led to thermodynamically favorable association between SpA-PA and DS-14.8 micelles as confirmed with the positive S and the negative G . The positive S of the assembly of SpA-PA with DS-14.8 micelles likely indicated the larger release of ions and water molecules caused by the more active insertion SpA-PA into DS-14.8 micelle surface than that into DS-2.4 micelles.⁴ Additionally, the association of DS-2.4 micelles with SpA-PA, an entropy-penalized exothermic process, is referred to as the “non-classical” hydrophobic effect.^{16–18}

FRET analysis of the assembly of antibody-coated PHEA-g-C₁₈ micelles

The PHEA-g-C₁₈ micelle coated by anti-VCAM-1 and anti-integrin α_v was created by simply mixing the SpA-PA-coupled DS-2.4 or DS-14.8 micelles with antibodies at 4 °C for 60 minutes. The FRET assay was conducted to confirm the binding between the antibody and SpA-PA on the micelle surface (Figure 3a). Interestingly, mixing of micelles coated by the fluorescein (i.e., FRET donor)-conjugated SpA-PA with the rhodamine (i.e., FRET acceptor)-conjugated anti-VCAM-1 resulted in a significant decrease of the fluorescein emission intensity maximized at the wavelength of 520 nm and an increase of the rhodamine emission intensity maximized at the wavelength of 580 nm (Figure 3b). These results confirmed the specific association between SpA-PA and antibodies. The degree of FRET (D_{FRET}) between the SpA-PA and the antibody was quantified with the emission intensity of fluorescein in the absence ($I_{fluorescein,0}$) and presence ($I_{fluorescein}$) of rhodamine-labeled antibody following Eq. (3):¹⁹

$$D_{FRET} = 1 - \frac{I_{fluorescein}}{I_{fluorescein,0}} \quad (3)$$

Interestingly, D_{FRET} was higher in the mixture of antibodies with DS-14.8 micelles than with DS-2.4 micelles, which indicates the larger number of antibodies was bound to the SpA-PA coupled on the DS-14.8 micelle surface. Additionally, D_{FRET} with DS-14.8 micelles remained at approximately 0.9 throughout the incubation in PBS for 60 min, while D_{FRET} with DS-2.4 micelles significantly decreased (Figure 3c). The reduced D_{FRET} indicated the active separation of antibodies from the surface of SpA-PA-coupled DS-2.4

micelles and the dissociation of the complex of antibody and SpA-PA from micelles. The binding stability of SpA-PA-coupled DS-14.8 micelles with antibodies was further studied by incubating them in blood mimicking, serum-supplemented media at 37 °C for 60 minutes and analyzing D_{FRET} between the RITC-labeled DS-14.8 micelle and the FITC-labeled antibody (Figure S3). D_{FRET} with DS-14.8 micelles remained at approximately 0.5 for 60 minutes. Additionally, D_{FRET} was minimally changed by adding an excess antibodies to the vascular endothelial growth factor (VEGF) to the suspension of SpA-PA-micelles coupled with the anti-VCAM-1 (Figure 3d). As this experiment was conducted in the blood mimicking, serum-supplemented media, this result implicated that the anti-VCAM-1 bound to the SpA-PA-micelle would minimally dissociate in a blood stream carrying various types of antibodies.

Kinetic analysis of the adhesion of antibody-coated PHEA-g-C₁₈ micelles onto a target substrate

The targeting capability of antibody-coated PHEA-g-C₁₈ micelles was evaluated with surface plasmon resonance (SPR) spectroscopy. VCAM-1 and integrin $\alpha_v\beta_3$ were chosen as the target proteins. It is well known that inflamed endothelium has higher levels of VCAM-1 and integrin $\alpha_v\beta_3$ expression than healthy endothelium.^{20, 21} The targeting efficiency of the antibody-coated SpA-PA-micelles was evaluated by measuring the binding response unit (RU) of the micelle with a model cell membrane, which was built by conjugating VCAM-1 or integrin $\alpha_v\beta_3$ to a monolayer of 11-mercaptopundecanoic acid (MUA) on the SPR chip (Figure 4a). Note that the RU value represents the mass density of the micelles on the model cell membrane,²² due to the association and dissociation of micelles.

Interestingly, during the association period, the sensorgram displayed a steeper increase of RU values with the antibody-coated DS-14.8 micelles (solid lines in Figure 4b) than with the antibody-free micelles (dashed lines in Figure 4b). RU after injections of micelles (RU_{disso}) was 6-fold higher with DS-14.8 micelles coated by anti-VCAM-1 than with micelles free of antibodies (Figure 4d). Likewise, RU_{disso} was 2-fold higher with DS-14.8 micelles coated by anti-integrin α_v than with micelles free of antibodies (Figure 4d). In contrast, DS-2.4 micelles coated by antibodies exhibited a relatively smaller increase in RU values than DS-14.8 micelles (Figure 4c & 4d), because of the smaller loading of antibodies characterized with FRET analysis.

DS-14.8 micelles free of SpA-PA were mixed with anti-VCAM-1. RU_{disso} of the control was almost 2-fold smaller than that of DS-14.8 micelles coated by antibodies using SpA-PA as the linker (Figure S4a). Additionally, RU_{disso} of SpA-PA coupled DS-14.8 micelles coated by anti-VCAM-1 was comparable to that of DS-14.8 micelles chemically linked with antibodies, and also higher than free anti-VCAM-1 (Figure S4b). It is noteworthy that the affinity of binding between the anti-VCAM-1 and the VCAM-1-coated substrate is higher than that between the anti-integrin α_v and the integrin α_v coated substrate (Figure 4b & 4c). Therefore, the difference of binding affinity between anti-VCAM-1-coated micelles and the anti-integrin α_v coated micelles towards their target sites are mainly attributed to the difference of intrinsic interactions between the antibody and antigens on the substrate (Figure S5).

Finally, the targeted adhesion of DS-14.8 micelles coated by anti-integrin α_v was evaluated by injecting fluorescently labeled micelles into a flow chamber that presented a substrate coated with integrin $\alpha_v\beta_3$ (Figure 5a).²³ To simulate *in vivo* circulation, the average bulk flow velocity of the media was set as 1 mm/s. The DS-14.8 micelles coated by antibodies adhered to the substrate more actively than ones without antibodies, as confirmed with a significantly larger fluorescence area (Figure 5b & 5c). In contrast, no significant difference was found between antibody-coated micelles and antibody-free ones for a control substrate free of integrin $\alpha_v\beta_3$ (results not shown).

On a separate note, these micelles and SpA-PA-coupled micelles did not negatively impact cellular metabolic activity as evaluated with the MTT reagent. Endothelial cells incubated with 0.1 mg/ml of different micelles retained similar levels of metabolic activities of cells which were not exposed to micelles (Figure S6).

Overall, this study demonstrates that SpA-PA can act as a linker to bridge antibodies of interest to PHEA-g- C_{18} micelle surfaces. It is suggested that SpA-PA binds with the Fc domain of the antibody without affecting specific interaction between antibodies and target proteins on surfaces. This simple nanoparticle surface modification process driven by thermodynamically self-assembly should be distinguished from previous ones conducted through covalent conjugation of SpA or protein G to the nanoparticle surface.^{24, 25} We envisage that this antibody-immobilization strategy would be useful to functionalize a wide array of nanoparticles assembled via self-assembly between lipids and amphiphilic copolymers.

Experimental Section

Materials were purchased from Sigma-Aldrich (USA) and used without further purification unless otherwise specified.

Synthesis of poly(2-hydroxyethyl-co-octadecyl aspartamide) (PHEA-g- C_{18})

Poly(succinimide) (PSI) was synthesized by thermal condensation of *L*-aspartic acid suspended in sulfolane at 170°C under nitrogen atmosphere for 14 hours with the phosphoric acid (Fisher Scientific) as a catalyst.²⁶ PSI was precipitated in excess methanol and successively washed with deionized water until the pH of the suspension reached neutral. The precipitate was dried by lyophilization. The molecular weight of PSI was determined by gel permeation chromatography (Breeze 2 GPC, Waters), with Styragel® HT column (Waters). *N,N*-dimethylformamide (DMF) containing 20 mM LiBr was used as the eluent, with the elution rate of 1 ml min⁻¹. Polystyrene standards were used for calibration. $M_n = 13,600$ g mol⁻¹ with PDI = 1.4.

149 mg purified PSI was dissolved in 8 ml *N,N*-dimethylformamide (DMF, Fisher Scientific), and octadecylamine (6, 22, and 44 mg for DS-2.4, DS-6.4, and DS-14.8, respectively) was added to the reaction mixture. The reaction was stirred at 70 °C for 24 hours. Next, excess amount of ethanolamine (272, 261, 247 μ L for DS-2.4, DS-6.4, and DS-14.8, respectively) was added and the reactant was further stirred at 45 °C overnight. Following completion of the chemical reactions, the products were dialyzed (MWCO 3500

Da, Fisher Scientific) extensively in deionized water at room temperature for two days and then lyophilized to yield dry powders.

For control experiments, PHEA-g-C₁₈ were chemically conjugated with the anti-VCAM-1 (R&D Systems) using the p-maleimidophenyl isocyanate (PMPI, Thermo Scientific). 4 ml of DS-14.8 polymer in water (0.5 mg/ml) was adjusted to pH 8.5 and then mixed with 3.66 μ L of PMPI in dimethyl sulfoxide (DMSO, 0.45 mg/ml). After being incubated at room temperature for 3 hours, the solution was purified with a centrifugal filter (Amicon® Ultra 3K device, Millipore Corporation). The PMPI-modified DS-14.8 was suspended in PBS at a concentration of 0.1 mg/ml and mixed with designed antibodies for 2 hours at room temperature.

Characterizations of PHEA-g-C₁₈ polymers

¹H NMR (Varian Unity 500 MHz) was applied to analyze the polymer dissolved in DMSO-*d*₆ (Cambridge Isotope Laboratories, Inc.). The integrals of characteristic peaks were used to quantify the degree of substitution of octadecyl chains (*DS*_{C₁₈}) in each sample by Eq. (4).

$$DS_{C_{18}}(\text{mol } \%) = \frac{\text{The integral of the peak in } 0.85 - 0.95 \text{ ppm}/3}{\text{The integral of the peak in } 4.3 - 4.7 \text{ ppm}} \times 100\% \quad (4)$$

Measurement of the critical micelle concentration (CMC)

Pyrene molecules (Acros) were suspended in the polymer solution at a concentration of 10⁻⁴ mg/ml. The fluorescent spectra of the suspensions with varying polymer concentrations were collected using a FluoroMax®-4 spectrometer (HORIBA Jobin Yvon). The excitation wavelength was set at 330 nm and the resulting emission between 350 and 450 nm was collected; the slit widths for excitation and emission were both set as 1 nm. The CMC was determined by the polymer concentration at the point where the emission intensity ratio (*I*₃/*I*₁) between the third vibronic peak at 385 nm (*I*₃) and the first vibronic peak at 373 nm (*I*₁) was significantly increased.¹⁵

Preparation of PHEA-g-C₁₈ micelles

The PHEA-g-C₁₈ polymer was suspended in PBS at a concentration of 0.1 mg/ml and sonicated briefly to make a homogeneous colloidal solution. PHEA-g-C₁₈ micelles formed spontaneously after the sonication.

Morphological and size analysis of PHEA-g-C₁₈ micelles

The micelle suspension was dropped onto a 300-mesh copper grid coated with carbon and formvar (SPI Supplies), and the sample was stained with 2 % (w/v) phosphotungstic acid solution (adjusted to pH 7.4 with NaOH). Then the morphology of self-assembled PHEA-g-C₁₈ micelles was observed using TEM (JEOL 2100 with LaB₆ emitter) operating at an accelerating voltage of 120 keV. Separately, the diameter of PHEA-g-C₁₈ micelles was measured using a Malvern Zetasizer Nano ZS instrument (Malvern, 4 mW He-Ne laser operating at a wavelength of 633 nm) with 173° backscattering. The hydrodynamic radius was determined using Cumulant analysis.²⁷ All measurements were carried out in triplicate

and performed at 25°C. The statistical significance between each two conditions was evaluated using an unpaired, two-tailed Student's t-test in Microsoft Excel. Differences were considered statistically significant when $p < 0.05$.

Synthesis of *Staphylococcus aureus* protein A (SpA) coupled with palmitic acid (SpA-PA)—The chemical conjugation of palmitic acid to SpA has been reported previously.²⁸ In short, SpA was suspended at 1 mg/ml in PBS containing 0.3 % sodium deoxycholate (DOC), 0.1 % sodium bicarbonate, and 0.1 % sodium azide. *N*-hydroxysuccinimide ester of palmitic acid (NHS-PA) was dissolved in ethanol at 10 mg/ml and added to the protein solution to yield a final concentration of 0.1 mg/ml NHS-PA. This mixture was incubated at room temperature for 24 h with constant mixing, and then purified with a centrifugal filter (Amicon® Ultra 3K device). The product was adjusted to 1 mg/ml in PBS with 0.1 % DOC.

Coupling of PHEA-g-C₁₈ micelles with SpA-PA and association percentage of SpA-PA—500 µl of PHEA-g-C₁₈ solution (0.1 mg/ml PBS) and 10 µL of SpA-PA labeled with fluorescein (details in the supporting information) were mixed and sonicated briefly. Then the mixture was incubated at a designated temperature (277, 294, or 310 K; 294 K if not specified) for 10 min, and purified with a centrifugal filter (Amicon® Ultra 100K device, Millipore Corporation). The concentrate was added with PBS equal to the weight spun off during the ultracentrifugation to make the final sample.

For studying the association percentage of SpA-PA, part of the sample was taken out as the control before the ultracentrifugal purification. Both the control and the final sample were measured with the fluorescence emission intensity at 535 (± 20) nm at the excitation of 485 (± 20) nm, using a microplate reader (Infinite 200Pro, Tecan Group Ltd.). The association percentage of SpA-PA, defined as the ratio of the amount of SpA-PA associated with the micelle to the amount of SpA-PA added in prior the assembly process, was calculated from the ratio of fluorescence of the final sample to the control.

FRET analysis for the antibody-micelle association—For FRET analysis to confirm the association between the anti-VCAM-1 and the SpA-PA-coupled micelles, 250 µl of the micelle coupled with fluorescein-SpA-PA was mixed with 12 µL anti-VCAM-1 labeled with rhodamine (details in the supporting information) or mixed with 12 µL PBS as the control. Micelle samples mixed with antibodies and those without were measured with the fluorescence emission intensity in the range of 500 to 700 nm at the excitation of 490 nm using FluoroMax®-4 spectrometer (HORIBA Jobin Yvon).

For experiments to evaluate the binding stability between anti-VCAM-1 and SpA-PA coupled micelles, the micelles were incubated in PBS supplemented with 80 wt% human serum (PAA Laboratories) for 60 minutes. For the experiment to evaluate whether the other antibodies can replace the anti-VCAM-1, 18 µg of the antibody to vascular endothelial growth factors (VEGF, R&D Systems) into the anti-VCAM-1 coated micelles suspended in the 80 wt% serum-supplemented media.

Measurements of the association/dissociation rates of micelles bound to target proteins using surface plasmon resonance (SPR) spectroscopy—A gold sensor chip (GE Healthcare, USA) was modified to present VCAM-1 or integrin $\alpha_v\beta_3$ on the MUA layer (Figure 4a). Details on surface modification of the chip to set up a model cell membrane were described in the supporting information. SpA-PA-coupled micelles coated with or without antibodies at a concentration of 0.1 mg/ml PBS were injected into the flow cell to examine the association and dissociation rates of the micelles with the gold sensor chip modified with the target protein on MUA. The media flow rate was kept constant at 5.0 $\mu\text{L}/\text{min}$. RU_{disso} was defined as the RU reading at the third minute after the sample injection stopped. Micelles chemically conjugated with anti-VCAM-1 were also examined for control experiments.

Analysis of the micelle adhesion onto a protein-coated substrate in a flow chamber—Integrin $\alpha_v\beta_3$ was coated onto the bottom of a 35 mm petri-dish, which was then assembled with the gasket (GlycoTech) and exposed the coated substrate to a flow at an average velocity of 1 mm/s. DS-14.8 polymers were labeled with rhodamine (details in the supporting information), in order to locate DS-14.8 micelles bound to the substrate with a confocal microscope. The suspension of PHEA-g- C_{18} micelles labeled with rhodamine was injected into the chamber via a syringe pump (KDS 100 Infusion Pump, KD Scientific), and the micelles were circulated for 10 min followed by an injection of PBS to remove unbounded micelles. The adhesion of micelles to the substrate was examined by capturing fluorescence from the substrate using a confocal microscope (Leica SP2). Finally, the fluorescence was quantified with ImageJ (NIH). Five different areas of the substrate were analyzed with at least three different samples per condition.

Conclusions

In conclusion, this study successfully demonstrates that alkylated SpA (SpA-PA) acts as a linker to immobilize antibodies of interest on pre-made PHEA-g- C_{18} micelle surfaces. The entropy-driven self-association between SpA-PA and micelles was tuned by increasing the number of alkyl chains ($\text{DS}_{\text{C}_{18}}$) conjugated to PHEA-g- C_{18} . Thus, micelles assembled by PHEA-g- C_{18} with a higher $\text{DS}_{\text{C}_{18}}$ resulted in a larger degree of self-association with SpA-PA and subsequently stable immobilization of antibodies on micelle surfaces, as confirmed with FRET analysis. The micelles functionalized with antibodies to VCAM-1 or integrin α_v via this sequential, biological coupling displayed the enhanced adhesion to target substrates compared with micelles free of antibodies. In sum, this simple yet elaborate bio-inspired protein immobilization would be broadly useful to functionalize nanoparticles with a wide array of proteins of interest. Ultimately, the results of this study will greatly serve to improve the quality of nanoparticles used in various applications including sensing, diagnosis, and clinical treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Assembly of nano-sized micelles using PHEA-g-C₁₈ with controlled DS_{C18}
 (a) The reaction scheme for synthesizing PHEA-g-C₁₈. (b) TEM micrographs of DS-2.4 (b-1), DS-6.4 (b-2), and DS-14.8 micelles (b-3). Scale bar is 200 nm. DS-2.4, DS-6.4, and DS-14.8 represent PHEA-g-C₁₈ at DS_{C18} of 2.4, 6.4, and 14.8 mol %, respectively. (c) The zeta-potential of the micelles with varied DS_{C18} in PBS. The difference of the zeta-potential values between conditions was not statistically significant ($p^* > 0.05$).

Figure 2. Assembly of PHEA-g-C₁₈ micelles coupled with SpA-PA

(a) (a-1) Reaction scheme for conjugating palmitic acid to SpA. **(a-2)** Hydrophobic interaction-induced self-assembly between SpA-PA and PHEA-g-C₁₈. **(b)** Fluorescence analysis to evaluate effects of DS_{C18} of PHEA-g-C₁₈ micelles on the percentage of SpA-PA associated with micelles suspended in PBS. For this analysis, SpA-PA was labeled with fluorescein. **(c)** G underlying the self-assembly between SpA-PA with DS-2.4 (filled triangles) or DS-14.8 (open squares) in PBS. G was calculated from K_{eq} using Eq. (1), and K_{eq} was quantified from the association percentage of SpA-PA presented in **(b)** using Eq. (1). **(d)** Van't Hoff plot for self-assembly between SpA-PA and DS-2.4 (filled triangles) and DS-14.8 (open squares) micelles in PBS. **(e)** S (open bars) and H (filled bars) underlying the self-assembly process of SpA-PA with DS-2.4 and DS-14.8 micelles in PBS. S and H were calculated from **(d)** and Eq. (2).

Figure 3. FRET analysis of the biological coupling of antibody to PHEA-g-C₁₈
 (a) Schematic description of the FRET assay designed to characterize the binding between the fluorescein-conjugated SpA-PA and the rhodamine-labeled antibody on the micelle surface. (b) Fluorescence emission spectra of SpA-coupled micelles mixed with anti-VCAM-1 right after mixing. (b-1) Emission spectra of pure SpA-PA-coupled DS-2.4 micelle suspension (dashed line) and the mixture of SpA-PA-coupled DS-2.4 micelles and antibodies (solid line). (b-2) Emission spectra of pure SpA-PA-coupled DS-14.8 micelle suspension (dashed line) and the mixture of SpA-PA-coupled DS-14.8 micelles and antibodies (solid line). (c) The degree of FRET (D_{FRET}) for the mixture of antibodies with SpA-PA-coupled DS-2.4 micelles (filled bars) and the mixture of antibodies with SpA-PA-coupled DS-14.8 micelles (open bars) right after mixing (0 min) and after incubation in PBS for 60 min, respectively. (d) D_{FRET} for the complex of anti-VCAM-1 with SpA-PA-coupled DS-14.8 micelles right after mixing with the anti-VEGF (0 min) and after incubation with anti-VEGF in the serum-supplemented media for 60 min, respectively.

Figure 4. SPR analyses of PHEA-g-C₁₈ micelles coated with or without anti-VCAM-1 or anti-integrin α_v

(a) Schematic SPR configuration for studying the binding between antibody-coated PHEA-g-C₁₈ micelles and the MUA substrate coupled with target proteins. (b) (b-1) SPR sensorgrams for binding of DS-14.8 micelles coated with anti-VCAM-1 (solid line) and without antibodies (dashed line) to the substrate covered with VCAM-1. (b-2) SPR sensorgrams for binding of DS-14.8 micelles coated with anti-integrin α_v (solid line) and without antibodies (dashed line) to the substrate covered with integrin α_vβ₃. (c) (c-1) SPR sensorgrams for binding of DS-2.4 micelles coated with anti-VCAM-1 (solid line) and without antibodies (dashed line) to the substrate covered with VCAM-1. (c-2) SPR sensorgrams for binding of DS-2.4 micelles coated with anti-integrin α_v (solid line) and without antibodies (dashed line) to the substrate covered with integrin α_vβ₃. In (b) and (c), micelles were coupled with SpA-PA. (d) RU after the injection of micelles (RU_{disso}) for DS-14.8 micelles and DS-2.4 micelles coated with antibodies (filled bars) and without antibodies (open bars).

Figure 5. *In vitro* analysis of the targeted adhesion of SpA-PA-coupled DS-14.8 micelles in a flow chamber

(a) Schematic of the flow chamber. (b) Confocal micrographs of integrin $\alpha_v\beta_3$ -coated substrate in the flow chamber following exposure to micelles coated by anti-integrin α_v (**Image 1**) and those free of antibodies (**Image 2**). Micelles tested were labeled with rhodamine. (c) Quantitative analysis of the number of DS-14.8 micelles adhered to integrin $\alpha_v\beta_3$ -coated substrate. The differences of the fluorescence area percentages between the two conditions were statistically significant ($p^* < 0.05$). Each value and error bar in the plot represents the mean and standard deviation from at least four different areas of the substrate in three different samples per condition.

Scheme 1.

Schematic description of a strategy to immobilize antibodies on the surface of PHEA-g-C₁₈ micelle using the alkylated *Staphylococcus aureus* protein A (SpA), termed SpA-PA.

Table 1

Analysis of the chemical structure and the critical micelle concentration (CMC) of PHEA-g-C₁₈, and the hydrodynamic radius of PHEA-g-C₁₈ micelles.

Sample	DS_{C18} (mol %) ^a	CMC (mg/ml) ^b	Hydrodynamic radius of micelles (nm) ^c
DS-2.4	2.4	2.0×10^{-2}	103
DS-6.4	6.4	1.0×10^{-2}	116
DS-14.8	14.8	0.8×10^{-2}	138

^aDetermined based on ¹H NMR spectra of the polymer

^bDetermined based on fluorescent emission spectra of pyrene in PBS

^cDetermined with a dynamic light scattering unit