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Zwitterionic Peptide Enhances Protein-Resistant Performance of Hyaluronic Acid-Modified Surfaces

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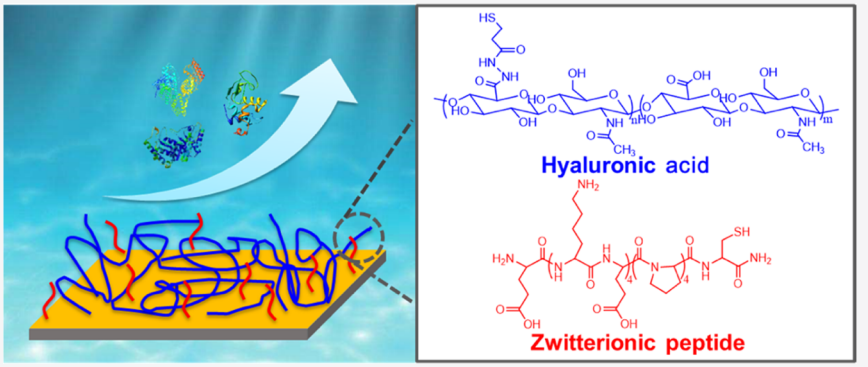

ABSTRACT: A convenient and efficient approach for the surface modification of antifouling materials is highly desirable in numerous applications like affinity-based biosensors. Herein, we fabricated a hybrid antifouling coating on Au surfaces, with thiolated hyaluronic acid (HA) being chemically adsorbed to Au surfaces by the "graft to" approach, followed by a self-assembly of a smaller zwitterionic peptide named p-EK to obtain HA/p-EK-modified surfaces. The real-time sensorgrams of surface plasmon resonance biosensor manifested the successful modification of HA and p-EK on Au surfaces, indicating that there were some bare Au substrates on the HA-modified surfaces for peptide binding. The obtained HA/p-EK surfaces exhibited high hydrophilicity with a water contact angle of 9°. Quartz crystal microbalance and surface plasmon resonance experiments verified that further grafting the zwitterionic p-EK peptide on HA-modified surfaces could enhance the antifouling performance by one time. The improved protein resistance could be mainly contributed by the modification of the zwitterionic peptide that shields the exposed Au substrates from interacting with protein foulings. This strategy by grafting a smaller zwitterionic peptide might provide a novel way to achieve an enhanced protein-resistant performance of the macromolecular coating obtained by the "graft to" surface modification approach.

INTRODUCTION

Surface plasmon resonance (SPR), as a highly sensitive analytical technique, could be employed to obtain the interaction information between molecules from solution with surface-immobilized receptors (e.g., antibody, aptamer, and enzyme) by monitoring the changes of refractive index within 200−300 nm on a metal surface.[1,2] Because of the advantages of label-free and real-time detection, SPR is widely used in a variety of fields, such as theranostics,[3,4] pharmaceutics,[5,6] food safety,[7,8] and environmental monitoring.[1,9] However, surface fouling by nonspecific protein adsorption from complex media (e.g., human serum) could greatly shield the SPR response caused by specific binding of targets, resulting in a reduction of accuracy for target detection.[10,11] Therefore, fabrication of antifouling coatings against nonspecific adsorption is highly demanded to improve the accuracy and sensitivity of target detection by SPR biosensors.[12]

Over the decades, there were numerous surface modification methods being developed for antifouling coatings,[13−15] and one of most commonly used is the "graft from" approach, which makes a polymer grow from the surface by an immobilized initiator.[16,17] Though it could have a good control on thickness and obtain a high graft density for polymers, it is difficult to handle and achieve large-scale fabrication as the synthetic process is always complicated and the reaction condition is extremely harsh (e.g., inert atmosphere).[18,19] Another is the "graft to" approach, which directly grafts polymers on surfaces by physical or chemical adsorption.[20,21] It is much more facile and easier to realize practical applications, but the inherent excluded-volume effects

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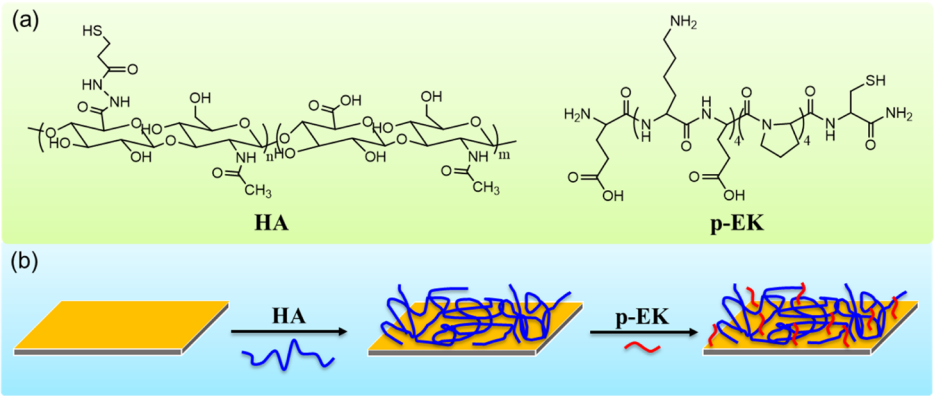
Scheme 1. (a) Molecular Structure of Thiolated HA and p-EK Peptide; (b) Fabrication Process of Thiolated HA and Zwitterionic p-EK-Modified Surface



of polymers would result in a low surface graft density,[22,23] which might affect the antifouling property of the coating.[24] Several strategies have been developed to improve the performance of the coatings fabricated by the "graft to" approach.[25−27] For instance, Emilsson et al. reported a new "graft to" method for poly(ethylene glycol) (PEG), which was performed under a high salt condition to shrink the coil conformation of PEG, thus acquiring a high surface graft density and achieving excellent performance against nonspecific protein adsorption.[27] However, this strategy is limited to few materials like PEG that are sensitive to salts or temperature. Therefore, a universal and facile way to enhance the protein-resistant property of surface coatings by the "graft to" approach is more desirable.

As materials with much low molecular weight (i.e., several hundred to thousand daltons) would have little or even no excluded-volume effect,[28] small molecules like short peptides might be able to fill up the exposed substrates after the surfaces are modified with macromolecules by the "graft to" method so as to improve the protein-resistant performance. To certify this suppose, we first chose thiolated hyaluronic acid (HA), a commercial hydrophilic macromolecule, to act as an antifouling model and grafted it to Au surfaces by forming Au−S bonds in a one-step "graft to" way.[29] As peptides with a zwitterionic structure show capabilities to reduce nonspecific protein adsorption and those composed with glutamic acid (E) and lysine (K) have been demonstrated to exhibit better performance than others,[30,31] a zwitterionic peptide with a sequence of CPPPPEKEKEKEKE (p-EK) was further assembled to the HA-modified Au surfaces, forming a hybrid layer of HA and p-EK-modified Au surfaces (Scheme 1). We used SPR to real-time monitor the surface modification process of HA and p-EK and then measured the wettability and roughness of modified surfaces by a water contact angle instrument and an atomic force microscope, respectively. Finally, quartz crystal microbalance (QCM) as well as SPR were employed to evaluate the antifouling property of modified surfaces toward proteins from a single solution [i.e., bovine serum albumin (BSA) and lysozyme] and complex fluids (i.e., 10% human serum).

MATERIALS AND METHODS

Materials. Thiolated HA (MW = 240 kDa, PDI = 1.9−2.0, the degree of thiolation is between 20 and 25%) was purchased from ESI·BIO Co., Ltd. (California, USA). Zwitterionic p-EK peptide (CPPPPEKEKEKEKE, purity 95%, MW = 1666.9 g/mol) was synthesized by Genscript Biological Co., Ltd. (Nanjing, China). BSA was obtained from Aladdin Reagent Co., Ltd. (Shanghai, China). Lysozyme was purchased from Fluke International Corporation (WA, USA). Phosphate-buffered saline (PBS, containing 10 mM phosphate, 138 mM sodium chloride, and 2.7 mM potassium chloride, pH 7.4) was prepared with ultrapure water. Normal human serum was obtained from Academy of Military Medical Sciences (Beijing, China) and diluted with PBS buffer by 10 times volume to obtain a 10% human serum solution, in which the concentration of protein was ∼5.24 mg/mL.[32]

Fabrication of HA- and HA/p-EK-Modified Au Surfaces. Before experiment, we employed the RCA method to clean the gold chips of the SPR and QCM biosensors. Specifically, the chips were immersed in a mixture solution of water/hydrogen peroxide/ammonium hydroxide (v/v/v = 5:1:1) under 75 °C for 15 min. Then the chips were washed with anhydrous alcohol and ultrapure water for three times, followed by drying the surface with high-purity nitrogen. Afterward, the chips were put into an ultraviolet ozone cleaner and cleaned for 60 min, washed again with anhydrous ethanol and ultrapure water for three times, and blow-dried with high-purity nitrogen before being stored in a refrigerator under 4 °C.

For QCM biosensor chips, we modified the Au surface in an offline way. The cleaned Au chips were first immersed in 1 mg/mL of HA solution for 12 h and then taken out to wash with pure water for three times and blow-dried with nitrogen; thus, the thiolated HA-modified Au surface (Au-HA) was obtained. We further used PBS buffer to prepare p-EK solution (with a concentration of 0.2 mg/mL) and incubated the Au-HA surface in the solution overnight. Finally, the surface was cleaned with ultrapure water for three times and dried with high-purity nitrogen to obtain a HA and p-EK-modified Au surface (Au-HA/p-EK).

Based on the characteristics of less sample consumption and real-time monitoring, we employed the online method to modify HA and p-EK on the SPR Au chips. First, PBS (10 mM pH 7.4) was used as a running buffer to flow over the surface at a rate of 30 μL/min. After a signal baseline was established, 1 mg/mL HA solution was injected and flowed over the Au surface at a rate of 10 μL/min for 30 min. Subsequently, PBS buffer was employed again and flowed at a rate of 30 μL/min to establish a new baseline, finally obtaining the Au-HA surfaces. The Au-HA/p-EK surfaces could be obtained by further injecting the p-EK solution over the Au-HA surfaces with a flow rate of 10 μL/min for 30 min and then rinsing the surface with PBS at a rate of 30 μL/min until reaching a new steady baseline.

Contact Angle Measurement. The water contact angle of bare Au, Au-HA, and Au-HA/p-EK surfaces was measured by a contact angle meter OCA15EC (DataPhysics Instruments, Germany). A 1 μL droplet of water was first added to the sample surface; after it reached the contact equilibrium on the surface, we collected the three-phase interface images by a CCD camera. The contact angle value could be obtained by selecting the baseline and numerical curve fitting of the droplet profile at the three-phase interface. The average contact angle

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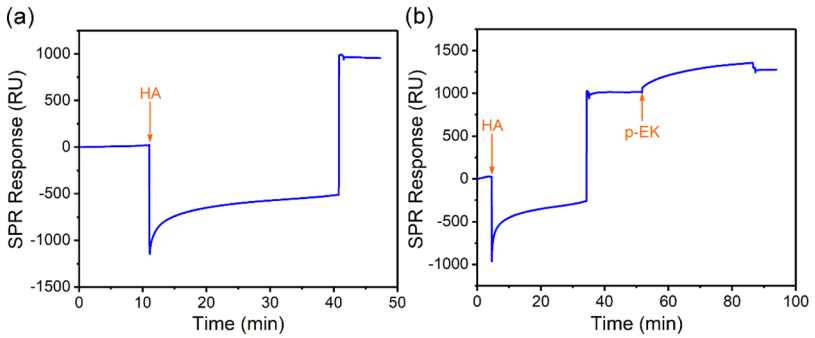

Figure 1. SPR sensorgram showing the modification progress of (a) thiolated HA on Au surface and (b) thiolated HA together with zwitterionic p-EK peptide on Au surface.

was calculated by measuring the values on four different positions for each sample.

Atomic Force Microscopy. The surface morphology of the substrates before and after the p-EK peptide modification was measured by a Nanosurf Flex atomic force microscope (Nanosurf Company, Switzerland) in a tapping mode. Commercial silicon probes (Tap300Al-G, BudgetSensors, Bulgaria) with a spring constant of 40 N/m were used to perform the experiments. The obtained surface roughness was averaged by measuring the images on three different places for each sample.

QCM Measurement. For determining the surface's protein-resistant performance, after mounting the modified chips into the QCM sensors, PBS was chosen to establish a signal baseline with a flow rate of 50 μL/min, and then 1 mg/mL of BSA solution was injected to flow over the surface at a rate of 25 μL/min for 10 min, followed by PBS washing with a flow rate of 50 μL/min to establish a new signal baseline. The adsorption of proteins on surfaces would induce the increase of the crystal's total mass, which is reflected by the decrease of resonance frequency. As a result, the change of resonance frequency (ΔF) could reflect the efficacy of antifouling coatings on surfaces.[33,34]

SPR Measurement. A signal baseline was established by the PBS buffer with a flow rate of 30 μL/min, and then the protein solution with a rate of 10 μL/min was injected into the fluid cell. After a period of 10 min, the sensor surface was rinsed with PBS buffer again to establish a new baseline. For the Biacore T200 SPR biosensor, a shift in the SPR signal of 1 RU corresponds to a surface coverage of 0.1 ng/cm^2. Based on this, we could calculate the amount of nonspecific protein adsorption on surfaces from the SPR angle shift before and after protein adsorption.

RESULTS AND DISCUSSION

Surface Modification of HA and HA/p-EK. As shown in Scheme 1, thiolated HA was chosen as the main coating layer and grafted onto Au chips by Au−S bonds, thereby obtaining the HA-modified Au surfaces (Au-HA). Considering the low surface graft density of macromolecules induced by this "graft to" method, we further employed a zwitterionic peptide (p-EK), which has a much lower molecular weight than HA, to self-assemble to Au-HA surfaces so as to improve the surface antifouling performance against nonspecific protein adsorption.

Figure 1 shows the surface modification process of HA and p-EK on Au surfaces by SPR real-time monitoring. As the SPR signal is relevant to the surface refractive index, which changes with the adsorption of molecules on surfaces or the bulk refractive index of solution, the refractive index of the HA solution lower than that of the PBS buffer caused a sharp decrease of the SPR signal upon the initial stage of flow of the HA solution over Au surfaces. Then, the signal started to increase as HA adsorbed on the surfaces. As shown in Figure 1a, the SPR signal increased significantly for the first 5 min during HA modification; then, the signal raised slowly with time, which suggested that HA presents a fast adsorption behavior on Au surfaces; as HA continued to flow over the surfaces, the already modified HA on Au surfaces might produce certain steric hindrance effect for the free HA in solution coming toward the surfaces, which is not beneficial for the further binding of HA to surfaces; thus, the SPR signal changed a little for the later modification stage. When exchanged to PBS washing again, the increase of refractive index on the surface caused the SPR signal to raise rapidly, which then turned to be stable. Further modification of the p-EK peptide on the Au-HA surfaces is shown in Figure 1b. As the p-EK peptide flowed over the Au-HA surfaces, the SPR signal went up gently. This might be because of the incomplete coverage of HA on the Au surface that exposed certain Au substrates to bind with the peptide. Moreover, the p-EK peptide is easier to adsorb on surfaces than the remaining free HA in solution as the former has a lower molecular weight and would lead to less steric hindrance effect. Consequently, the p-EK peptide could successfully be assembled to HA-modified surfaces and form a complex layer of HA/p-EK on Au surfaces (Au-HA/p-EK).

Characterization of HA and HA/p-EK Coatings. The wettability of surfaces before and after modification is shown in Figure 2; when the Au surfaces were modified with HA, the

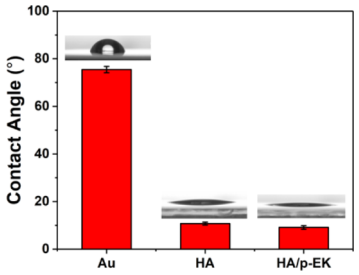

Figure 2. Water contact angle of Au, Au-HA, and Au-HA/p-EK surfaces.

water contact angle dropped from 75 to 11°, demonstrating the strong hydrophilicity of HA. After the graft of the p-EK peptide onto Au-HA surfaces, the water contact angle decreased to 9°, indicating that the zwitterionic p-EK peptide further improved the surface hydrophilicity.

We further used atomic force microscopy to measure the surface topography of Au, Au-HA, and Au-HA/p-EK. As shown in Figure 3a, the Au surface exhibited uniformly distributed nanoscale asperities and had a root-mean-square

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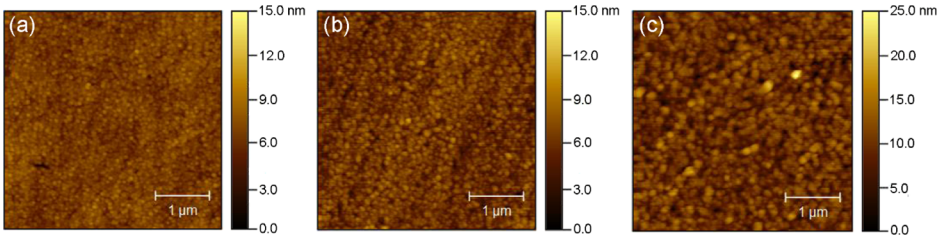

Figure 3. Surface morphology of (a) Au, (b) Au-HA, and (c) Au-HA/p-EK substrates.

roughness of 0.8 nm. After being modified with HA (Figure 3b), the surfaces became a little rougher (with a root-mean-square roughness of 1.1 nm). Further grafting the surfaces with the p-EK peptide made the asperities much larger and increased the roughness to 2.2 nm (Figure 3c), suggesting that the p-EK peptide might fail to form an ordered conformation on the Au-HA surface.

Nonspecific Protein Adsorption on HA- and HA/p-EK-Modified Surfaces. To evaluate the protein-resistant capability, we first employed QCM to measure the nonspecific protein adsorption on the modified surfaces. BSA was chosen as a model protein, and then 1 mg/mL of BSA solution was injected to the fluid cell and flowed over the surfaces; finally, the shift of resonance frequency during protein adsorption was recorded. As shown in Figure 4, BSA adsorbed faster initially

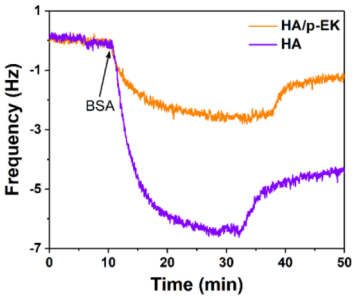

Figure 4. QCM sensorgram of 1 mg/mL of BSA solution on the HA-modified Au surface and HA/p-EK-modified Au surface.

on HA-modified surfaces compared to HA/p-EK-modified surfaces and induced a bigger change of resonance frequency. To be specific, for nonspecific BSA adsorption, the resonance frequency decreased utmost by 7 Hz on the HA-modified surface, whereas by 2.5 Hz on the HA/p-EK-modified surfaces, demonstrating that the zwitterionic p-EK peptide could effectively reduce nonspecific BSA adsorption on surfaces. With continuous PBS washing, some proteins that do not adsorb stably would be desorbed from the surfaces, leading the signal of resonance frequency to increase, which then tended to be steady. Finally, the signal shift of resonance frequency because of BSA fouling on Au-HA surfaces and Au-HA/p-EK surfaces was 4 and 1.5 Hz, respectively. Therefore, further graft of zwitterionic p-EK peptide could significantly improve the protein-resistant capability of HA-modified surfaces.

To verify the results of QCM measurements, SPR was further used to systematically investigate the protein-resistant performance of the modified surfaces. Before experiment, the concentration of HA and p-EK peptide solutions for online surface modification was optimized, as shown in Figure 5. Figure 5a shows that for HA solution, the SPR signal increased from 200 RU to 1000 RU when the concentration increased from 0.1 to 1 mg/mL. Further increasing the concentration to 2 mg/mL made the SPR signal no longer change and showed a signal similar to that for 1 mg/mL HA solution. The results illustrated that the adsorption amount of HA increased when the concentration increased from 0.1 to 1 mg/mL and reached a saturated level (corresponds to an adsorption amount of about 100 ng/cm^2) at a concentration of 1 mg/mL; so, we chose a concentration of 1 mg/mL of HA solution to be used in the following SPR experiments. Figure 5b shows the SPR signal response for different concentrations of p-EK peptide solutions flowing over HA-modified surfaces. As the molecular weight of HA is 120 times higher than that of the p-EK peptide, the molar concentration of even 0.01 mg/mL for the p-EK peptide solution is higher than that of 1 mg/mL HA solution. As seen in Figure 5b, the SPR signal only increased to 75 RU when the concentration of the p-EK peptide solution increased from 0.01 to 0.4 mg/mL. The SPR signal response because of the p-EK peptide adsorption from the different concentrations of peptide solutions did not change so obviously as that for HA, which might be because of the low-molecular-weight of the peptide as well as the repulsion of HA on surfaces toward the adsorption of free peptide from p-EK solutions. Besides, the SPR signal did not change much as the concentration of the peptide solution increased from 0.2 to

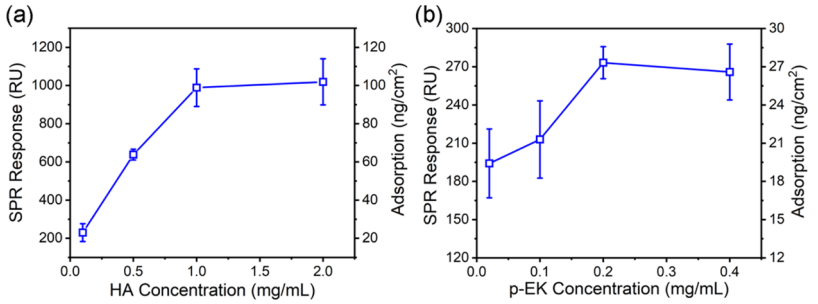

Figure 5. (a) SPR response and the corresponding adsorption of HA solution with different concentrations on the Au surface, (b) SPR response and the corresponding adsorption of zwitterionic p-EK peptide solution with different concentrations on the HA-modified surface.

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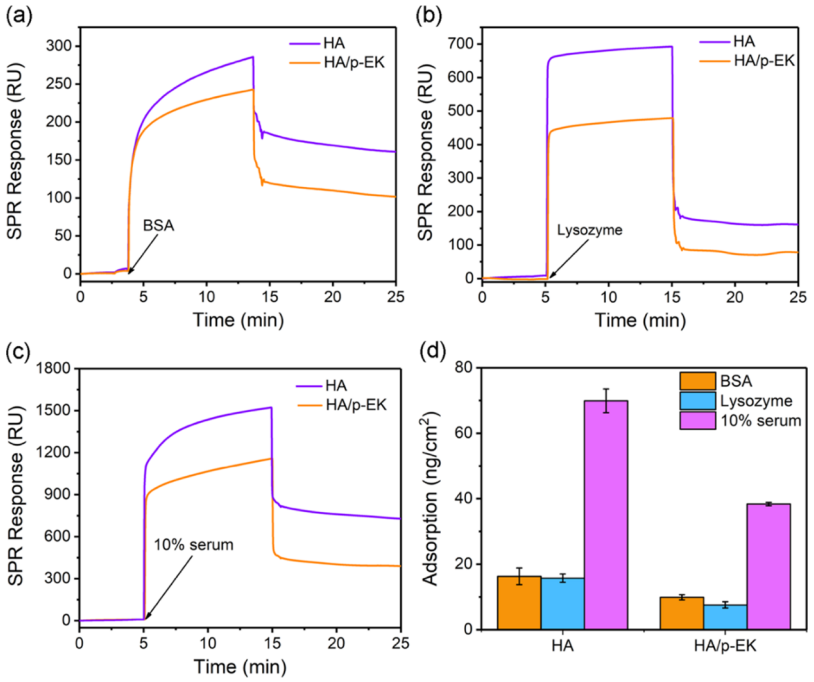

Figure 6. SPR sensorgrams of (a) 1 mg/mL of BSA, (b) 1 mg/mL of lysozyme, and (c) 10% human serum on HA- and HA/p-EK-modified Au surfaces, (d) nonspecific adsorption amount of protein on HA- and HA/p-EK-modified Au surfaces.

0.4 mg/mL. Consequently, the p-EK peptide solution with a concentration of 0.2 mg/mL was selected to be used in the following SPR experiments.

For the protein adsorption measurement by SPR, we first chose BSA and lysozyme as single protein solutions to be tested, which presents as positively charged and negatively charged, respectively, in PBS buffer (pH = 7.4), and the corresponding SPR sensorgrams are shown in Figure 6. As seen in Figure 6a, owing to the higher refractive index of BSA solution than th PBS buffer, the SPR signal initially increased with a sharp rate. Then, the adsorption of BSA on the surfaces made the SPR signal continuously increase but more slowly. Compared to HA/p-EK-modified surfaces, the SPR signal response to BSA adsorption on HA-modified surfaces was much larger, with a maximum value of nearly 280 RU, which means the amount of nonspecific BSA adsorption on HA-modified surfaces is higher than that on HA/p-EK-modified surfaces. When exposed to PBS again, the SPR signal dropped suddenly as the surface refractive index decreased. With continuous PBS rinsing, the weakly adsorbed protein would be washed away from the modified surfaces; so, the SPR signal went down slowly and became stable before reaching the adsorption−dissociation equilibrium. As a result, the SPR signal responses caused by BSA adsorption on HA- and HA/p-EK-modified surfaces are 163 and 99 RU, respectively. Therefore, HA combined with the p-EK peptide presents a better antifouling performance against BSA than HA alone, which is consistent with the result of QCM. Figure 6b shows the SPR sensorgram for lysozyme flowing over the two modified surfaces; similar to that in Figure 6a, the SPR signal increased sharply at the initial stage because of the increased surface refractive index by the lysozyme solution. Because of the smaller size of the lysozyme than BSA, the SPR signal went up even more slowly as the lysozyme adsorbed toward the surface as compared to BSA, as shown in Figure 6a. Afterward, the SPR signal dropped down and gradually became steady under continuous PBS washing. Ultimately, the SPR signal response toward lysozyme adsorption on HA/p-EK-modified surfaces was half of that on HA-modified surfaces, that is, 76 and 158 RU, respectively. Therefore, similar to the result of BSA adsorption, the HA/p-EK-modified surfaces exhibited a much better protein-resistant property toward lysozyme than the surfaces modified with HA alone.

Furthermore, we selected the commonly used 10% human serum as a complex protein medium and tested its fouling level on the two modified surfaces. As shown in Figure 6c, on account of the various proteins and their high content in human blood serum, the SPR signal caused by the increase of the surface refractive index from 10% human serum is much higher than that from the BSA or lysozyme solution. With the protein from human serum adsorbing on the surface, the SPR signal continuously increased. Then the SPR signal tended to be steady with PBS rinsing the surface. Finally, the SPR responses caused by the protein adsorption from 10% human serum toward the Au-HA/p-EK surfaces and Au-HA surfaces were 383 and 767 RU, respectively. Based on the conversion between the SPR signal and the mass amount of substance adsorbed on the surfaces (1 RU = 0.1 ng/cm^2), we obtained the adsorption amount of protein from all the three solutions on the modified surfaces, as shown in Figure 6d. It was seen that the amount of BSA, lysozyme, and protein from 10% human serum on HA-modified surfaces of 16.3, 15.8, and 70 ng/cm^2, respectively, presents certain resistance toward protein adsorption.[29] Nevertheless, further grafting zwitterionic p-EK peptide on Au-HA-modified surfaces improved the antifouling performance by one time, irrespective of being exposed to single protein solution or complex protein medium, and the amount of nonspecific adsorption from the single protein solution could reduce to 10 ng/cm^2, reaching a low fouling level.[35] In summary, the zwitterionic p-EK peptide could assist HA for realizing the enhanced protein-resistant property.

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CONCLUSIONS

A facile and efficient approach for surface modification of antifouling coatings was developed by a mixture of thiolated HA and a zwitterionic peptide. Macromolecular thiolated HA was grafted onto a gold surface by a one-step "graft to" approach. By virtue of less "excluded volume" effect of relatively smaller molecules, a zwitterionic p-EK peptide was employed to occupy the remaining bare Au substrates. SPR results showed that both thiolated HA and zwitterionic p-EK could be successfully modified on Au surfaces in a stepwise manner. The obtained Au-HA/p-EK surfaces were extremely hydrophilic and exhibited superior protein-resistant performance than the surfaces modified with HA alone. The much better antifouling capability could be attributed to the peptide's zwitterionic structure and its ability to prevent the remaining Au substrates from being exposed to foulings like protein. The graft of a zwitterionic peptide to HA-modified surfaces might be applicable to other macromolecule-modified surfaces by the "graft to" approach and provides an efficient way to improve the protein-resistant performance of the surface coating.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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