Preparation of a Sensing Membrane for C-Reactive Protein

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Summary: 11,11′-dithiobis(undecanyl succinate) (DS11SACOOH) was synthesized to develop a new surface for immobilization of proteins or antibodies on a gold substrate. DS11SACOOH is a bifunctional linker that has a disulfide group to form SAM (self-assembled monolayer) on a gold substrate and two carboxylic moieties for reaction with biomolecules such as peptide, DNA, etc. After preparation of SAM on a gold-coated quartz cell with DS11SACOOH, the SAM surface was activated with NHS (*N*-hydroxysuccimide). The terminal NHS ester was reacted with anti-CRP (C-reactive protein). The binding behavior of CRP on the surface was monitored by use of SPR (surface plasmon resonance) sensor system.

Keywords: C-reactive protein; immobilization; self-assembled monolayer; sensors; surface plasmon resonance

Introduction

C-reactive protein (CRP) is a clinical maker, widely used in diagnosis and management of various clinical conditions. Especially, CRP has been identified as an independent predictor for cardiovascular diseases such as heart attack and stroke.[1–4] The CRP level is typically less than 2 mg/L for healthy individuals.[5] Therefore, the measurement of the CRP in high sensitivity may provide a powerful method to predict risk of heart attack and stroke. Recently piezoelectric microcantilever[6,7] and a microfluidic network[8] have been known as new detection methods for CRP. Surface plasmon resonance (SPR) that measures molecular interactions in real time without labels is also used for detection of CRP. In SPR-based biosensor systems, the sensing surface is usually made of gold or silver.[9] Biomolecular interaction takes place on this surface and the binding event is transduced into an opto-electric signal. The sensor surface is generally derivatized with suitable immobilization linkers for biding biomolecules to get reproducible and consistent results. Several immobilization methods suitable for SPR immunoassays have been reported. Antibodies have been immobilized by their oxidized glycochains[10] or by their reduced disulphide bonds.[11] Self-assembled layers of modified Protein A and Protein G have also been fabricated for their potential applications in immunosensor systems.[12,13] A silanization chemistry using 3-aminopropyltriethoxysilane has also been used to attach antibodies to the gold surface via a glutaraldehyde linker for sensitive SPR sensing.[14] For a more specific linkage of receptors, use of biotin–avidin complex has been reported.[15] Recognition elements could also be attached to the SPR sensor surface using a gold-binding polypeptide. SPR sensor surfaces were fabricated by attaching fluorescyl groups to the surface, which were used for the binding of anti-fluorescyl antibody.[16] Thin polymer film coating, which provides a hydrophilic surface with

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sufficient amino groups, was also applied to gold to investigate biomolecular interactions with the polymer-coated surface.[17]

In the present study, we synthesized a bifunctional compound, 11,11′-dithiobis (undecanyl succinate) (DS11SACOOH) for SAM (self-assembled monolayer) formation on a gold substrate. The resulting surface was activated with NHS (*N*-hydroxysuccinimide) and reacted with anti-CRP. The interaction between CRP and anti-CRP was investigated by use of surface plasmon resonance (SPR) biosensor system.

Experimental Part

Chemical Reagents

11-mercaptoundecanol, sodium perborate momohydrate, succinic anhydride, *N*-hydroxysuccinimide, and triethylamine were purchased from Aldrich Co. Anti-C-reactive protein (produced in goat), C-reactive protein (from human plasma) and bovine serum albumin (BSA) were obtained from Sigma. All other chemicals were used as received. All buffer and solutions were made form particle free distilled Milli-Q water.

Synthesis of 11,11′-dithiobis(undecanol)[18]

$NaBO_3 \cdot H_2O$ (0.98 g, 9.8 mmol) and 11-mercaptoundecanol (1.00 g, 4.9 mmol) were stirred in MeOH (15 mL) and water (2.5 mL) for 220 min at room temperature. The solvent was evaporated and water (50 mL) added. The suspension was extracted with $CHCl_3$ (100 mL), and the combined extracts were washed with *satd.* Na_2CO_3 (20 mL), dried ($MgSO_4$) and evaporated to yield the product as a white solid which did not require further purification (0.92 g, 92 %). ^{1}H NMR (400 MHz, $CDCl_3$): 3.63 (m, 4H, OCH_2), 2.67 (t, 4H, SCH_2), 1.63 (m, 4H, CH_2), 1.56 (m, 4H, CH_2), 1.39–1.24 (m, 30H, $(CH_2)_7$, OH).

Synthesis of DS11SACOOH

11,11′-dithiobis(undecanol) (0.5 g, 1.23 mmol) and succinic anhydride (0.25 g, 2.46 mmol) were added to two-neck bottom flask under the argon. The mixture was dissolved in dry $CHCl_3$ (20 mL) and the solution was cooled to 0 °C. Triethylamine (0.88 mmol) was added dropwise to the cooled solution. The solution was allowed to reach ambient temperature and then stirred for 3 hr. When conversion was complete, the reaction mixture was partitioned between water (50 mL) and $CHCl_3$ (50 mL) and then the layers were separated. The aqueous layer was further extracted with $CHCl_3$ (50 mL × 3). The combined extracts were washed with water, dried ($MgSO_4$), and evaporated to yield the crude product, which was purified by recrystallization in ethanol (30 mL) to give a white solid. (0.59 g, 78%). ^{1}H NMR (400 MHz, $CDCl_3$): 4.11 (t, 4H, CH_2OCO) 2.71 (m, 8H, $OCCH_2CH_2CO$), 2.67 (t, 4H, SCH_2), 1.63 (m, 4H, CH_2), 1.56 (m, 4H, CH_2), 1.39–1.24 (m, 28H, $(CH_2)_7$).

Preparation of SAM

A gold-coated SPR quartz cell (purchased from K-MAC, 18 mm × 18 mm × 10 mm) was first treated by dipping into a boiling solution of H_2O_2 (35%), NH_3 (25%) and distilled water in a 1:1:5 ratio mixture for 10 min and then rinsed thoroughly with distilled water. The cleaned gold-coated SPR quartz cell was immediately used for thiol linkage by soaking in DS11SACOOH solution (2 mM in ethanol) for 12 hr at room temperature. This was rinsed with ethanol and dried under argon. The terminal carboxylate on the gold-coated SPR quartz cell was reacted with NHS (1 mmol) and *N*-(3-dimethylaminopropyl)- *N*′-ethylcarbodiimide hydrochloride (1 mmol, EDAC) in CH_2Cl_2 (20 mL) for 12 hr. The resulting surface was washed with CH_2Cl_2 and dried with air before use in the SPR set-up.

SPR Measurements

The gold-coated SPR cell of the surface terminal NHS ester was put into the optical path of the SPR setup (K-MAC, model: SPRi LAB). SPR measurements were

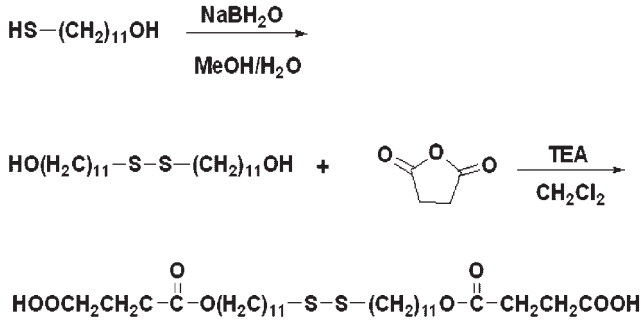

Figure 1.
Synthetic pathway of DS11SACOOH.

performed at 25 °C and PBS (phosphate, pH 7.4) was used for washing before addition of anti-CRP. The flow rate in the experiment kept 20 μL/min for measuring the angle scan and time scan. PBS was introduced into the cell, followed by anti-CRP (10 mg/L in PBS). Scan was recorded after stable response was obtained. Ethylamine (0.7% in water) was eluted for 8 min in the cell to quench the residual NHS ester and then cell was washed with PBS. Non-specific binding was checked after addition of BSA (10 mg/L in PBS). CRP (10 mg/L in PBS) was introduced into the cell for measuring the binding with anti-CRP. After the stable response for the binding of anti-CRP was obtained, the cell was washed with PBS. The schematic diagram for the experimental procedure was shown in Figure 2.

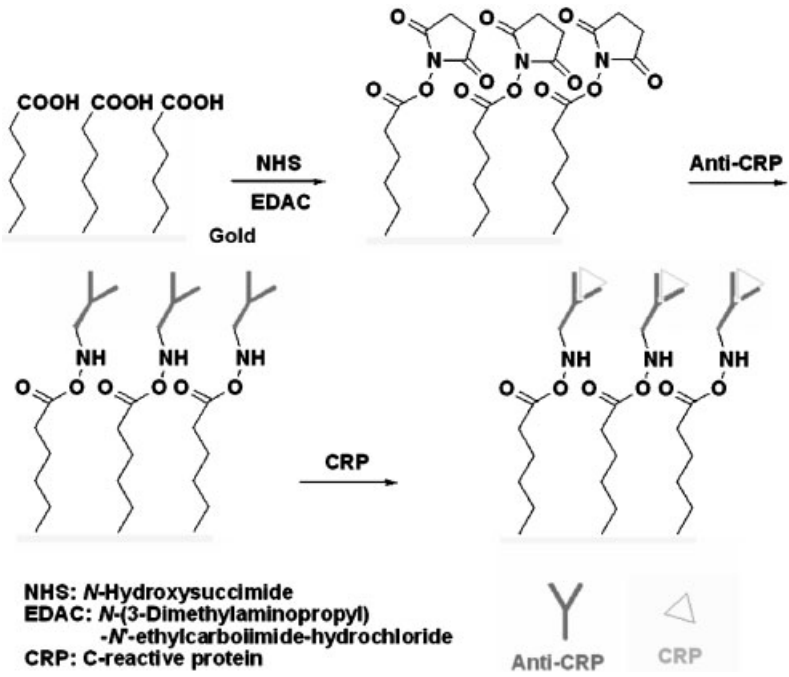

Figure 2.
Immobilization of anti-CRP to the SAM of DS11SACOOH on a gold substrate.

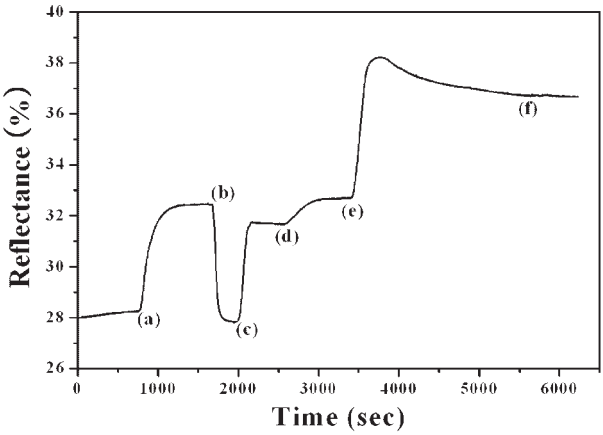

Figure 3.
Shift in SPR intensity for the terminal NHS ester on a gold surface: (a) addition of anti-CRP (10 mg/L in PBS); (b) addition of ethylamine (0.7% in H_2O); (c) PBS flow (pH 7.4); (d) addition of BSA (10 mg/L in PBS); (e) addition of CRP (10 mg/L in PBS); (f) addition of BSA (10 mg/L in PBS).

Results and Discussion

As shown in Figure 1, DS11SACOOH was synthesized by two steps with a high overall yield to develop a new surface to orient proteins or antibodies on a gold substrate. The structure was confirmed by NMR spectroscopy and FT-IR. DS11SACOOH is a bifunctional linker that has a disulfide group to form SAM on a gold substrate and two carboxylic moieties to interact with functional molecules.

Figure 3 shows a typical SPR sensorgram measured at a fixed angle (47.5°). After the SAM of terminal NHS ester on a gold-coated quartz cell presents stable response in PBS, addition of anti-CRP shows a fast increase of the SPR intensity (Figure 3, (a)), which should be a result of the reaction of anti-CRP with terminal NHS ester. Excess aqueous ethylamine was used to quench the residual NHS ester (b) and PBS was eluted (c) until the sensorgram kept constant. Non-specific binding was checked with

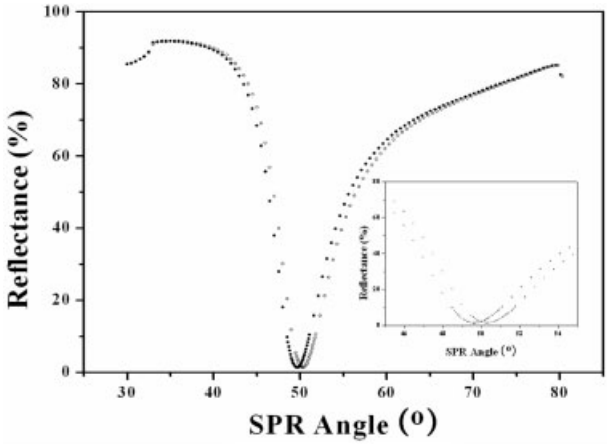

Figure 4.
SPR resonance curve for the terminal NHS ester on a gold surface (Figure 3, (a), closed circle) and after CRP loading on the surface of immobilized anti-CRP (Figure 3, (f), open circle).

BSA (10 mg/L in PBS) (d). A slight increase in SPR intensity was observed, probably due to the non-specific binding. Upon addition of CRP (10 mg/L in PBS), a sharp and dramatic increase of intensity was recorded (e). After stable response curve was obtained through elimination of free CRP with PBS, non-specific binding was checked again by an addition of BSA (f). The result should be the specific interaction between CRP and anti-CRP, which is primarily attributed to uniform anti-CRP coupling on the SAM of terminal NHS ester.

As shown in Figure 4, SPR resonance curves for the terminal NHS ester on a gold surface (Figure 3, (a)) and after CRP loading on the surface of immobilized anti-CRP (Figure 3, (f)) are obtained. The angle shift from SPR resonance curves for (a) and (f) of Figure 3 is 0.65°.

Conclusions

DS11SACOOH was synthesized to develop a new surface to orient proteins or antibodies on a gold substrate. After the SAM preparation on a gold-coated quartz cell using DS11SACOOH, the SAM surface was activated with NHS. The terminal NHS ester was reacted with anti-CRP. The binding behavior of CRP on the surface was monitored by use of SPR. The SPR results show the specific interaction between CRP and anti-CRP. The SAM of terminal NHS ester could be applied to construction of other immunosensors or biochips.

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