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Single step biocompatible coating for sulfhydryl coupling of receptors using 2-(pyridinyldithio)ethylcarbamoyl dextran

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Abstract

2-(Pyridinyldithio)ethylcarbamoyl dextran (PDEC dextran) is developed herein as a novel biosensor coating material aimed for direct and facile fabrication of a sulfhydryl-specific capture surface on gold; the site-directed immobilization of a single free cysteine residue presented protein (human albumin serum, HSA) on PDEC dextran coated surfaces without prior activation and subsequent specific binding of mouse monoclonal anti-HSA antibody to the resultant surface were demonstrated using surface plasmon resonance (SPR) assays.

Keywords

Biosensor; SPR; coating; sulfhydryl-specific; coupling

Introduction

The immobilization of molecules at surfaces in a controlled manner is essential for binding and functional assays on solid phases. For proximity-based biosensors, careful control of the interfacial architecture between the transducer and the solution phase are required in order to maximize the signal due to receptor binding, and minimize non-specific binding (NSB). To this end, several strategies for immobilizing biological molecules on biosensor surfaces have been developed and broadly used in the literature. Substrates have been decorated with oligo- and poly-ethylene glycol n-alkanethiolates that form self-assembled monolayer (SAM) coatings on metals.[1-3] These oligo-ethylene glycol containing SAMs are prone to auto-oxidation and, being planar, provide only a limited capacity scaffold to which materials can be attached. The use of a biocompatible hydrogel matrix attached to a gold surface via a SAM for application in surface plasmon resonance (SPR) biosensors has found great utility in analytical biochemistry [4]. In this case an n-alkanethiol SAM with an omega-functionalized hydroxyl group was activated using an epoxy intermediate for attachment of carboxymethylated dextran. However, this procedure requires four steps, the use of organic solvents and aggressive and toxic reagents such as epibromohydrin, bromoacetic acid and strong alkali.

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Attachment of biological molecules via the sulfhydryl groups of available or engineered cysteine residues on a receptor to a suitably sulfhydryl-reactive surface has long been employed in protein chromatography [5]. For biosensing applications, sulfhydryl reactive surfaces are normally generated by converting a carboxylic acid moiety surface to a 2-(pyridinyldithio) ethaneamine (PDEA) surface [6]. However the residual carboxylic acid groups impart a significant negative charge to the polymer, which in turn leads to increased NSB via electrostatic interactions [7]. Direct attachment of polymer coatings to metal surfaces via an electrostatically neutral sulfur is unfortunately compromised by the tendency to oxidation of free sulfhydryl groups [8]. This can occur during purification, storage or deposition, and can also affect the reactivity and ultra-structure of a polymer as sulfhydryl groups are converted into intra-polymer disulfide groups. Such sulfur-mediated cross-linking of a polymer or hydrogel results in a more viscous, gel-like structure that is less suitable for use in biosensors due to concomitant compromised diffusion of receptors and analytes within the hydrogel [9].

In response to these limitations we have developed a novel polymeric material that does not possess free sulfhydryl groups and that can be directly chemisorbed to gold in a single step using aqueous solvents.

Materials and Methods

Materials

Aldrithiol-2, 2-cysteamine hydrochloride, 4-nitrophenyl chloroformate, 4-di(methylamino) pyridine (DMAP), N-methylmorpholine, anhydrous DMSO, anhydrous pyridine, D, L-Dithiothreitol (**DTT**), **2-mercapto pyridine**, phosphate buffered saline (PBS), human serum albumin (HSA) and bovine serum albumin (BSA) were from Sigma-Aldrich. Dextran T70 was purchased from Amersham Biosciences (Little Chalfont, Britain). Mouse monoclonal to human serum albumin (mAb anti-HSA) and mouse monoclonal to bovine serum albumin (mAb anti-BSA) anti-bodies were from Abcam plc (Cambridge, Britain). AffiniPure Rabbit Anti-mouse IgG antibody (Fc (gamma) Fragment Specific) was purchased from Jackson ImmunoResearch Laboratory.

Instrumentation and sensors

Biacore 2000 optical biosensor and Biacore SIA bare gold sensor chips were purchased from Biacore AB (Uppsala, Sweden).

Preparation of 2-(pyridinyldithio)ethaneamine (PDEA)—Aldrithiol-2 (4.41 g, 20 mmol) was dissolved in 20 ml of methanol and 0.8 ml of acetic acid. Into the solution was added dropwise 2-cysteamine hydrochloride (1.14 g, 10 mmol) in 50 ml of methanol over a period of 1 hour. The mixture was stirred at room temperature overnight. The resulting yellow solution was concentrated *in vacuo*. The resulting yellow oil residue was then thoroughly washed by vigorously stirred with 50 ml of diethyl ether. The clear supernatant was decanted off, and the residual yellow solid dissolved in methanol (10 ml). To the solution was added 50 ml of diethyl ether. The resultant precipitates were collected by filtration. This procedure was repeated three times, and the pale white solid was purified by recrystallisation (methanol / diethyl ether) to give a white solid 1.5 grams.

Typical procedure for the preparation of 4-nitrophenyl carbonated dextran—To a solution of dextran T70 (Amersham Biosciences, 1.6 grams, 29.6 mmol hydroxyl) in anhydrous dimethylsulfoxide (18 ml) and anhydrous pyridine (16 ml), were added 4-nitrophenyl chloroformate (Aldrich, 800 mg, 4 mmol) and catalytic amount of 4-di(methylamino) pyridine with gently stirring and externally ice-water cooling. The reaction mixture was stirred at the temperature for 5 hours, and then slowly added into a mixture of

methanol and diethyl ether (1:1) (150 ml) with vigorously stirring. The precipitates formed were collected by filtration, and dispersed again in 150 ml of the same solvent mixture. The precipitates were collected by filtration, and the above procedure was repeated three times. The white solid collected was dried under high vacuum to give 1.57 grams of a white powder.

4-Nitrophenol (11.6 mg) and 4-nitrophenyl carbonated dextran T70 (25.9 mg) were dissolved in d^6 -DMSO (1ml). ^1H NMR (400MHz, d^6 -DMSO): selected δ 6.90 (2H of 4-nitrophenol, d), 7.54 (2H, d), 8.08 (2H of 4-nitrophenol, d), 8.30 (2H, d). Functionality degree: 10.5% (mol glucose unit).

Typical procedure for the preparation of 2-(pyridinyldithio)ethyl carbamoyl dextran (PDEC dextran)—To a solution of 4-nitrophenyl carbonated dextran T70 (1.57 g) in anhydrous dimethylsulfoxide (20 ml) and anhydrous pyridine (6 ml), were added N-methylmorpholine (400 μL) and 2-(pyridinyldithio)ethaneamine (640 mg). The mixture was stirred at 25 °C for 18 hrs, and then it was slowly added into a mixture of methanol and ether (1:1) (150 ml) with vigorously stirring. The precipitates formed were collected by filtration, and washed with the same solvent mixture as described above three times (150 ml each time). The collected white solid was dried under high vacuum to give 1.0 gram of white powder that was stored in a -20 °C freezer until use.

^1H NMR (400MHz, d^6 -DMSO): selected δ 7.21 (1H of pyridinyl, t), 7.42 (1H of carbamoyl N-H, br), 7.75 (1H of pyridinyl, d), 7.81 (1H of pyridinyl, t), 8.43 (1H of pyridinyl, d).

Instrument preparation

Biacore 2000 instrument were cleaned using a 'Desorb' method at 25 °C before the start of the experiment. After docking a maintenance chip, the instrument was consecutively primed with desorb solution 1 (0.5% sodium dodecyl sulphate, SDS), desorb solution 2 (50 mM glycine, pH9.5) and finally degassed MilliQ water.

PEDC dextran deposition onto Biacore SIA bare gold surface—After docking the SIA bare gold chip and priming the instrument with degassed MilliQ water, the gold sensor chip was cleaned by flowing over an aqueous solution of 100 mM NaOH 1% (w/v) Triton $\times$ 100 for 4 minutes. Then a solution of 2 mg/mL (w/v) underivatized dextran T70 in MilliQ water was injected through one of the sensor flowcells as a reference surface and a solution of 2 mg/mL (w/v) PDEC dextran through the rest three flowcells for 7 minutes at a flow rate of 10 $\mu\text{L}/\text{min}$. The signal differences before and after contacting with the polymer solutions were recorded to represent the material loading on the sensor chip surfaces, 1000RU = 1 ng/mm^2 [10].

PEDC dextran coating stability—100 mM NaOH solution was injected to flow over the PDEC dextran coated sensor surface for 1 minute at a flow rate of 10 $\mu\text{L}/\text{min}$. The injection was repeated 20 times followed by 20 times injection of 100 mM NaOH 1% (w/v) Triton $\times$ 100. The signal differences before and after contacting with the alkaline solutions were recorded to represent the material loss from the sensor chip surfaces.

Static deposition of PEDC dextran onto Biacore SIA bare gold surface—Two Biacore SIA bare gold sensor chips were placed in a 40 mm glass Petri dish with the gold coating side uppermost. 200 μL of 100 mM NaOH 1 % Triton in MilliQ water was dropped on one chip, and another 200 μL solution on the other and the dish covered. The chips were then incubated at room temperature for 5 minutes, then washed with MilliQ water 10 times. In the same manner, the chips' gold coated surfaces were contacted with 0.1 % PDEC MilliQ water solution for 16 hours, then cleaned with MilliQ water 10 times, and left to air dry.

Release and quantification of 2-mercapto pyridine from PEDC coated sensor chips—PDEC coated sensor chips were placed in a 40 mm glass Petri dish with the polymer coating side uppermost. 200 μ L of 1 mM D, L-Dithiothreitol (DTT) in MilliQ water was dropped on one chip, and another 200 μ L solution on the other. The chips were then incubated at room temperature for 2 hours, and then the solutions were collected with a pipette. The absorbances of the solutions were recorded on a CARY 1E UV-Visible spectrophotometer (Varian Inc, Palo Alto, USA) at 270 nm. Standard solutions were prepared at 2 μ M, 4 μ M, 6 μ M, 8 μ M and 10 μ M of 2-mercapto pyridine in 1 mM DTT aqueous solution, and their absorbances were recorded on the CARY 1E UV-Visible spectrophotometer at 270 nm.

Human serum albumin (HSA) immobilization—The instrument was primed with PBS buffer before conducting the following biological assays.

A control polymer surface was generated by injecting 50 mM L-cysteine in 100 mM formate buffer pH4.3 into one of the PDEC dextran coated flowcell at a flow rate of 10 μ L/min. The contact time was 5 minutes.

A HSA solution (1mg/mL) in 100 mM borate buffer pH8.5 flowed over both the above control and the active PDEC dextran flowcells for 5 minutes at a flow rate of 10 μ L/min. The signal differences before and after contacting with the protein solution were recorded to represent the immobilization level of the protein on the sensor chip surfaces. The exposure of the protein solution to both control and active surfaces was repeated 5 times.

Anti-HSA antibody interaction with the surface bound HSA

Solutions of 333 nM mAb anti-HSA antibody in PBS pH7.4 (Fig. 3 – i), 10 mM NaCl-HCl solution pH3.0 (Fig. 3 – ii), 10 mM NaCl-HCl solution pH2.0 (Fig. 3 – iii), 333 nM mAb anti-BSA antibody in PBS pH7.4 (Fig. 3 – iv), 333 nM rabbit anti-mouse IgG antibody in PBS pH7.4 (Fig. 3 – v), 10 mM NaCl-HCl solution pH2.0 (Fig. 3 – vi), 333 nM mAb anti-HSA antibody in PBS pH7.4 (Fig. 3 – vii) and again 10 mM NaCl-HCl solution pH2.0 (Fig. 3 – viii) were consecutively flowed over a surface bound with HSA at a flow rate of 10 μ L/min. The exposure time of each above solution to the surface was 5 minutes. The signal differences before and after each solution injection were recorded to represent anti-bodies association to or dissociation from the sensor chip surface.

A BSA control surface was generated in the same way as generating HSA active flowcell described above. The association of mAb anti-HSA antibody to both the HSA active surface and the BSA control surface, and the surfaces regeneration with 10 mM NaCl-HCl solution pH2.0 were repeated 7 times.

Results and Discussion

Preparation of PDEC dextran

To prepare PDEC dextran, dextran T70 (**1**) was firstly activated with 4-nitrophenyl chloroformate in the presence of excessive anhydrous pyridine and a catalytic amount of 4-dimethylamino pyridine (DMAP) at 0 °C for 5 hours. The functionality degree of the resultant 4-nitrophenoxycarbonyl dextran (**2**), which was evaluated with ¹H-NMR using 4-nitrophenol as the internal reference, was 10.5 % molar of glucose unit, i.e. there was one active 4-nitrophenyl carbonate group within 10 glucose units in average. **2** was then allowed to react with 2-(pyridinyldithio)ethane amine (PDEA) in a mixture of anhydrous DMSO and pyridine to produce **3**, PDEC dextran. Good solubility, up to 4% (w/v) in MilliQ water without forming gel over several weeks at room temperature, is considered to be crucial for surface fabrication.

PDEC dextran deposition and its stability

In order to investigate application in biosensing, a gold sensor chip was first cleaned by flowing over an aqueous solution of 100 mM NaOH 1% (w/v) Triton $\times 100$ for 4 min. in a Biacore 2000 SPR biosensor (Biacore AB). Exposure of bare gold surface to 2% (w/v) PDEC dextran solutions resulted in deposition of between 3500 – 4000 RU, which corresponds [10] to 3.5 – 4 ng/mm² of polymer. A reference channel exposed to a negative control: underivatized 2% (w/v) dextran T70, exhibited a signal shift of < 20 RU or 20 pg/mm² (data not shown). This suggests that, at least in part, chemisorption of PDEC dextran was mediated by the coordination of the reactive sulfur moiety to gold.

The chemical stability of the resultant coating sensor surfaces was investigated via repeated exposure to alkaline solutions (Fig. 1, 20 times 100 mM NaOH injections followed by 20 times 100 mM NaOH/1% Triton $\times 100$ injections). Initial exposure of PDEC dextran to NaOH resulted in the loss of ~ 1200 RU or ~ 1.2 ng/mm², most likely due to removal of physisorbed rather than chemisorbed material. However, upon subsequent repeated exposure as described above, only 5 RU, or ~ 5 pg/mm² of material was removed from the surface. PDEC dextran was also shown to be resistant to injected pulses of 1 M NaCl, 10 mM glycine pH 2.0, and 100 mM HCl. The same material stored at -20 °C for one year showed similar levels of immobilization to the freshly prepared material prepared and was subsequently demonstrated to retain activity for receptor capture (vide infra).

Human serum albumin (HSA) immobilization

Having demonstrated facile deposition and subsequent chemical resistance of PDEC dextran we wished to probe the utility of the sulfhydryl reactivity inherent in the polymer for biosensing applications with a pharmacologically relevant receptor. The remaining sulfhydryl reactive functionality, (2-pyridinyl)disulfanyl, in the polymer coating matrix on single SPR chip, which was fabricated in a Petri dish in a static mode, is in the range of 1.0 – 1.5 nmol that was quantified, with a UV-Visible Spectrophotometer at the wavelength of 270 nm, from the concentration of 2-mercaptopyridine released by 1 mM DTT treatment for 2 hours. Human serum albumin (HSA,) was chosen as a model protein as coupling of HSA to aldehyde or carboxyl surfaces is problematic due to the extremely low pI (4.7) of the protein. In addition, amine-coupling results in a random orientation on the surface and the different drug binding sites can be sterically occluded by the linkage to the solid support near that site. HSA is a 66 kDa globular protein that possesses a single sulfhydryl group at residue 34, which should allow a site-specific immobilization. In order to ascertain whether immobilization of HSA to PDEC dextran was mediated via the residue 34 cysteine, and not via simple physisorption, a control polymer surface was generated by pre-treatment with 50 mM L-cysteine in 100 mM formate buffer pH 4.3. This step effectively ‘caps’ the reactive 2-pyridinyldithio groups in PDEC dextran as demonstrated by the detection of 2-mercaptopyridine in the UV adsorbance assay. HSA solutions (1 mg/ml) in 100 mM borate buffer pH 8.5 were then repeatedly exposed for 5 min. to both un-reacted PDEC dextran (Fig. 2, ○) and the control, capped polymer (Fig. 2, ▲). Whilst ~ 2500 RU or ~ 2.5 ng/mm² of protein was coupled to the PDEC dextran surface, less than 150 RU or 0.15 ng/mm² was bound to the control surface. This result suggests that coupling of HSA to the surface was indeed mediated by the reactive 2-pyridinyldithio groups of PDEC dextran. The result further demonstrates that cysteine-capped PDEC dextran is relatively resistant to non-specific physisorption of HSA, despite the introduction of a zwitterionic charge arising from the cysteine residues on the surface.

Anti-HSA interaction with the surface bound HSA

The above result confirms facile deposition and specific coupling of a protein using the novel polymer. However to demonstrate utility in biosensing assays it is important to further establish that the immobilization conditions and interfacial micro-environment do not destroy protein

activity for analyte binding. To probe the remaining activity of PDEC dextran surface bound HSA, 333 nM of a mouse monoclonal anti-HSA IgG antibody (mAb; 150 kDa) in PBS pH 7.4 was passed over a surface that had been coated with 2230 RU of immobilized HSA. This resulted in an equilibrium response for antibody binding of 1750 RU (Fig. 3A-i), and a calculated maximal saturating response (R_{max}) for binding of the bivalent antibody to a monovalent receptor of 2500 RU. When compared to the experimentally determined value for R_{max} of 2230 RU, this gives a protein activity level of 89%. We note however that the binding stoichiometry of antibody : HSA could lie between the range of 1 : 2 to 1 : 1, as not all of the surface immobilized HSA may possess the correct spatial separation and orientation to bind stoichiometrically to antibody. With this caveat, the effective protein activity on the surface is thus predicted to lie within the region of 45–89%. The binding complex was not dissociated by 10 mM NaCl-HCl solution pH 3.0 (Fig. 3A-ii), but was disrupted at pH 2.0, restoring the initial baseline level signal trace (Fig. 3A-iii). To probe the specificity and selectivity of analyte-binding to the active surface, 333 nM mouse monoclonal anti-BSA IgG (Fig. 2A-iv) and 333 nM rabbit anti-mouse antibodies (Fig. 3A-v) in PBS pH 7.4 were applied sequentially across the HSA immobilized surface for 5 min. Neither protein resulted in association of more than 90 RU or 90 pg/mm² of material, confirming that the surfaces were highly bio-specific for analyte.

Regeneration of free HSA was achieved by injection of 10 mM NaCl-HCl solution pH 2.0 (Fig. 3A-vi). Repeated injection of 333 nM of anti-HSA mAb in PBS pH 7.4 resulted in an equilibrium level of 1730 RU (Fig. 3A-vii), within 2% of the initial response (Fig. 3A-i). To further probe assay reproducibility, new assay surfaces were used with HSA (1240 RU) and BSA (2650 RU) challenged by mAb anti-HSA antibody binding and regeneration repeated seven times. For these assays antibody binding generated 1080 ± 25 RU on the HSA surface (Fig. 3B, solid line), and only 58 ± 2 RU on the reference BSA channel (Fig. 3B, dotted line).

Conclusion

In conclusion we have fabricated a novel biosensor coating material that possesses “activated” reactive groups for direct and facile deposition on gold surfaces with subsequent capture of sulfhydryl presenting receptors. This procedure is far simpler than published methods that rely on a multi-step conversion of carboxylated dextran using carbodiimides and cross linking reagents such as 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) [4,8]. In addition, the novel coating overcomes several of the disadvantages reported for disulfide-containing polymers [11] on gold, which exploit short alkyl chain moieties for attachment. Finally, early (one year) stability trials suggest that the activated material is compatible with storage and shipping that is a pre-requisite to commercialization.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Scheme 1.
Preparation of PDEC dextran.

Figure 1. Stability of PDEC dextran surface to alkaline solutions, 20 times 1 minute injections of 100 mM NaOH in MilliQ water followed with 20 times 1 minute injections of 100 mM NaOH and 1% Triton $\times 100$ in MilliQ water.

Figure 2.

The specific immobilization of human serum albumin (HSA) onto PDEC dextran coated surface via active disulfides. Sequential 5 minutes injections of 1 mg/mL HSA solutions in 100 mM borate buffer pH8.5 over the active PDEC dextran surface (○) and the capped surface (▲) which was treated with 5 minutes injection of 50 mM L-cysteine in 100 mM formate buffer pH4.3 beforehand at flow rate 10 μ L/min.

Figure 3.

The specific binding of anti-HSA antibody to HSA surface, regeneration and reproducibility. **A:** Antibodies binding to HSA (2232 RU immobilized) surface and regenerations: **i:** 5 minutes injection of 50 $\mu\text{g/mL}$ anti-HSA in PBS buffer pH7.4; **ii:** 5 minutes injection of 10 mM NaCl-HCl solution pH3.0; **iii:** 5 minutes injection of 10 mM NaCl-HCl solution pH2.0; **iv:** 5 minutes injection of 50 $\mu\text{g/mL}$ anti-BSA in PBS buffer pH7.4; **v:** 5 minutes injection of 50 $\mu\text{g/mL}$ Rabbit anti-Mouse antibody in PBS buffer pH7.4; **vi:** 5 minutes injection of 10 mM NaCl-HCl solution pH2.0; **vii:** 5 minutes injection of 50 $\mu\text{g/mL}$ anti-HSA in PBS buffer pH7.4; **viii:** 5 minutes injection of 10 mM NaCl-HCl solution pH2.0. **B:** Seven times repeats of anti-HSA binding to HSA (1241 RU immobilized, solid line) active surface and BSA (2643 RU immobilized, dotted line) control surface on PDEC dextran coated Biacore sensor chip.