Reduced nonspecific adsorption on covalently immobilized protein surfaces using poly(ethylene oxide) containing blocking agents

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Abstract

In a number of applications, e.g. DNA/protein micro-array technology, enzyme-linked immunosorbent assay (ELISA) technology or surface plasmon resonance (SPR) technology, the covalent coupling of proteins to surfaces is required. Following the covalent coupling of proteins, the remaining reactive groups should be blocked in order to avoid covalent binding of the analyte to the reactive surface. To this end, preferably blocking agents containing groups that avoid nonspecific adsorption should be used. These blocking agents are typically ethanolamine and cysteine for protein coupling via amino groups and thiol groups, respectively. This report presents novel blocking agents containing poly(ethylene oxide) (PEO) groups. These blocking agents show enhanced qualities to avoid nonspecific adsorption and can therefore have advantages in versatile protein-surface technologies.
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1. Introduction

The adsorption of proteins on solid surfaces is influenced by the physical and chemical characteristics of the real sample (blood, urine, waste water,...). Changes in pH, ionic strength or particular additives in the sample can cause desorption of the adsorbed proteins. These problems can be overcome using covalent coupling procedures. Currently, there is a growing interest in the covalent coupling of proteins, e.g. antibodies, to solid substrates. The use of covalent coupling procedures in DNA or protein micro-array technology and in enzyme-linked immunosorbent assay (ELISA) is increasing because of the higher robustness of this approach. For immunosensor applications the affinity biosensor interface consists of antibodies, which are preferably covalently attached onto the transducer surface. In most cases, the surface should be activated before the attachment of proteins [1,2]. After covalent coupling, the remaining reactive groups should be blocked in order to avoid covalent binding of the analyte to the surface. To achieve specific recognition, the analyte should bind to the bioreceptors via specific interactions and not to the surface itself. Therefore, the blocking agents should have groups which are excellent in preventing nonspecific adsorption such as hydroxyl or poly(ethylene oxide) groups [3–9]. Nonspecific signals due to interferences constitute a major problem in diagnostic applications, where an analyte at low concentrations must be detected in the presence of a much larger concentration of nonspecific molecules. In order to couple proteins by their amino groups, typically the 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide and *N*-hydroxysuccinimide procedure is used to activate a carboxylic group on the surface [1]. Subsequently, the remaining activated carboxylic groups are in general blocked with ethanolamine (Fig. 1) [1,8]. For covalent coupling to the thiol groups of proteins (e.g. reduced antibody), a similar activation is used in combination with an extra crosslinker 2-(2-pyridinyldithio)ethaneamine hydrochloride (PDEA) [8]. The unreacted groups are then usually blocked with cysteine (Fig. 2).

In this contribution, we report on the replacement of ethanolamine and cysteine by triethylene glycol monoamine: $H_2N-(CH_2-CH_2-O)_3-H$ and triethylene glycol monothiol: $HS-(CH_2-CH_2-O)_3-H$, respectively. The ability to couple triethylene glycol monoamine to
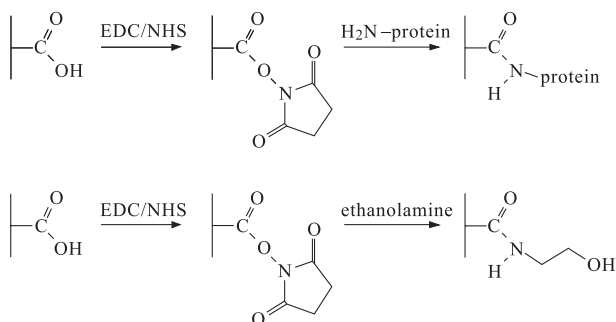

Fig. 1. The covalent coupling of the amino groups of proteins to a carboxylic-terminated surface and the blocking of unreacted *N*-hydroxysuccinimide esters via ethanolamine.
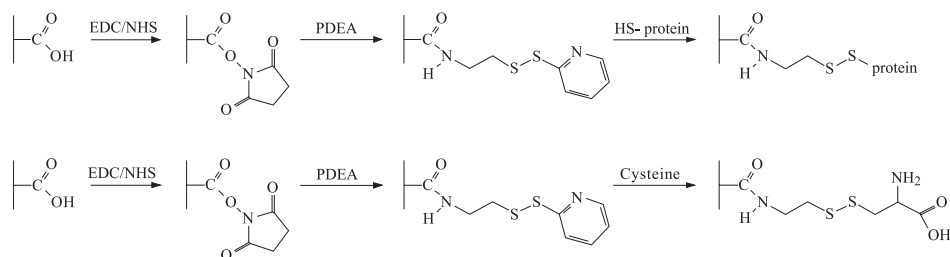
Fig. 2. The covalent coupling of the thiol groups of proteins to a carboxylic-terminated surface and the blocking of unreacted groups via cysteine.

activated carboxylic groups and its excellent qualities against nonspecific adsorption were described by Chapman et al. [9]. However, the procedure used by Chapman et al. is only useful for amino coupling procedures and is based on the use of a combination of organic solvents. This approach can be used to realize surfaces with good qualities against nonspecific adsorption but not as a blocking procedure after covalent coupling of proteins via their amino groups. As a matter of fact, proteins will denaturate in organic solvents and will loose their ability to recognize their complement [10]. In order to test the poly(ethylene oxide) containing blocking agents, we have used self-assembled monolayers (SAMs) of thiols on gold. These SAMs are known for their stable bond to gold and for their reproducible behavior [11]. They may be used in applications such as electroanalytical chemistry [12,13], molecular electronics [14], corrosion research [15,16] and biomaterial research [17–19]. In our approach, we deposited 16-mercapto-1-hexadecanoic acid (HS-$(CH_2)_{15}$-COOH) on a gold surface, which generates a carboxylic-terminated surface. This surface is subsequently used to evaluate the enhanced qualities of the poly(ethylene oxide) containing blocking agents with and without antibody coupling to the surface.

2. Materials and methods

All materials and reagents were used as commercially received. Anti-human transferrin (anti-HT) was supplied by Diamed Eurogen (Belgium). Human IgG reagent (IgG), human transferrin (HT) and cysteine were obtained from Sigma. 16-Mercapto-1-hexadecanoic acid (16-MHA) (>90%) was purchased from Aldrich. Ultrapure ethanol was purchased from Riedel-DeHaën. 1-Ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), ethanolamine and 2-(2-pyridinyldithio)ethaneamine hydrochloride (PDEA) were purchased from Biacore. To characterize the adsorption of proteins on SAM coated gold slides, we used the Biacore® 2000 Surface Plasmon Resonance (SPR) system provided with a SIA-kit® (bare gold substrates) [20,21]. The deposition of the SAM and the working procedures for the SPR system have been described in Frederix et al. [22]. The immobilization degree of proteins in a Biacore SPR system is reported as refractive units (RU). One RU corresponds to a shift in the resonance angle of approximately 0.1 millidegrees [20]. The poly(ethylene oxide) (PEO) containing blocking

agents are synthesized based on reported methods [23–25]. Triethylene glycol monoamine is currently available at Molecular Biosciences.

3. Results and discussion

In a first test, the carboxylic groups of the SAMs of 16-MHA on gold were activated with EDC-NHS, immediately followed by a covalent coupling of the PEO containing blocking agents. The resulting surface was subsequently evaluated for its ability for protein resistance without antibody coupling. This surface should be regarded as the worst possible situation to test nonspecific adsorption, since antibodies will also prevent nonspecific adsorption. To evaluate the nonspecific adsorption, IgG (100 ng/ml–100 μg/ml) was used because it is present in the blood in high concentrations and its adsorption is thoroughly documented [26–28]. In a later stage, we performed a real immunosensing experiment where we covalently immobilized anti-HT and recognized HT and a nonspecific analyte, e.g. IgG. The buffers and activation of the carboxylic groups and the immobilization of antibodies were similar to those described by Frederix et al. [22] Ethanolamine or triethylene glycol monoamine (1 M in water) was reacted for 40 min. The blocking agent used after a coupling with the thiol groups of proteins requires an extra activation step (Fig. 2). To this end PDEA (127 mM in 0.1 M borate buffer pH 8.5) was added to the activated surface for 10 min, followed by a 40-min reaction with the thiol containing blocking agents cysteine or triethylene glycol monothiol (167 mM in 0.1 M formate buffer with 1 M NaCl pH 4.3).

The adsorption characteristics of different concentrations of IgG on the amino group containing blocking agents are shown in Fig. 3. It is very clear that the adsorption is much
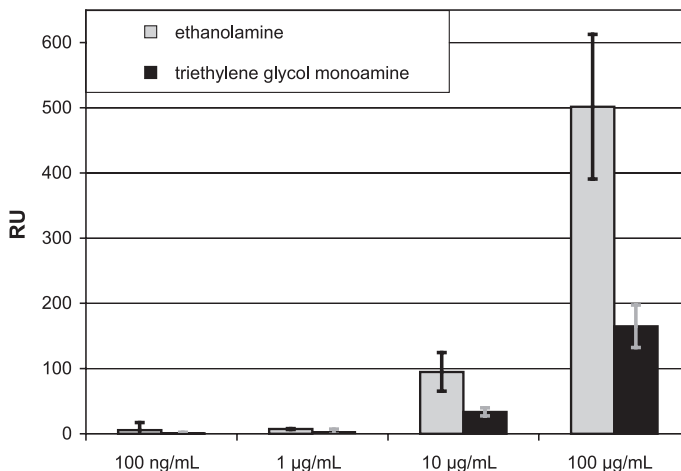

Fig. 3. The adsorption of different concentrations of IgG on ethanolamine and triethylene glycol monoamine blocked carboxylic surfaces.

smaller on monoamine triethylene glycol compared to ethanolamine blocked surface (results are averaged over two injections on two lines on two different samples). To calculate the surface coverage, the obtained RU values can be converted to ng/cm^2. For example the adsorption of the highest concentration (100 μg/ml IgG) on the ethanolamine blocked surface gave a response of ∼ 500 RU, which yields a monolayer coverage between 8% and 38% depending on the orientation of the antibodies [22]. The same concentration adsorbed on the surface treated with the PEO containing blocking agent resulted in 3% to 13% coverage, which is significantly less than the ethanolamine blocked surface.

A similar approach was followed for the covalent coupling of the thiol group containing blocking agents. The carboxylic groups were activated via EDC/NHS and PDEA and subsequently blocked with cysteine or triethylene glycol monothiol. Again the nonspecific adsorption is significantly lower using the PEO containing blocking agent (Fig. 4). For the highest concentration of IgG (100 μg/ml), the monolayer coverage is between 4% and 18% on a cysteine blocked surface compared to only between 0.5% and 2% for the triethylene glycol monothiol blocked surface.

For real immunosensing or biosensor applications, the nonspecific adsorption will be lower since the surface is already partially blocked by the immobilized antibody. In order to access the influence of the PEO groups on the recognition of the analyte, we covalently coupled anti-HT and evaluated the affinity binding of HT and of a nonspecific analyte (high concentrations of IgG). Fig. 5 demonstrates that the recognition is slightly larger on the surface with immobilized anti-HT subsequently blocked
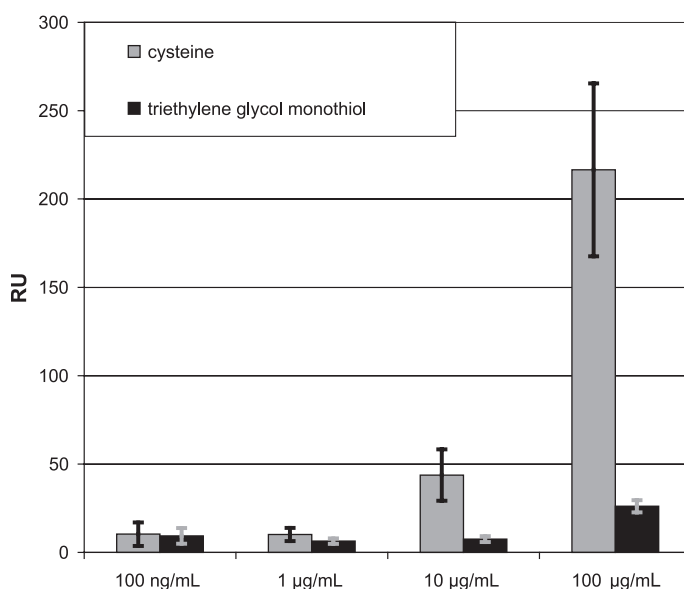

Fig. 4. The adsorption of different concentrations of IgG on cysteine and triethylene glycol monothiol blocked carboxylic surfaces.
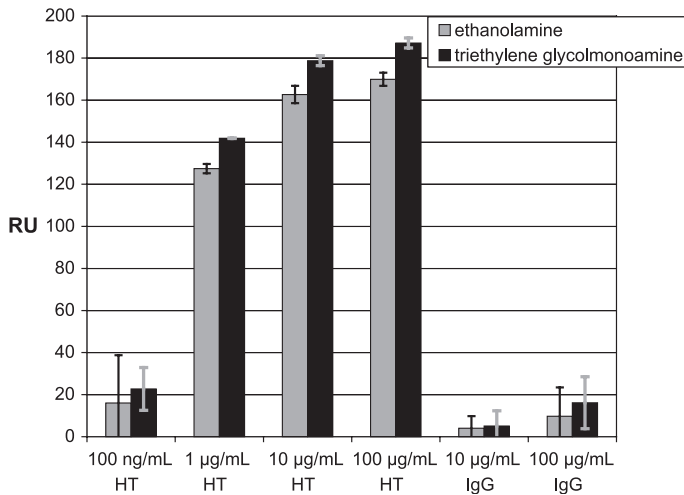
Fig. 5. The recognition of HT by the covalently immobilized anti-HT. After the immobilization of anti-HT, the unreacted groups are blocked with monoamino triethylene glycol or ethanolamine.

with triethylene glycol monoamine, although a similar amount of antibody was immobilized on the triethylene glycol monoamine blocked surface and the ethanolamine blocked surface. The nonspecific adsorption is negligible and therefore not significantly different for the two chosen chemistries. This is probably due to the high anti-HT coverage on both surfaces. The procedure used was optimized to achieve a high anti-HT immobilization [22]. The anti-HT immobilization calculated from the SPR signal is 270 ± 7 ng/cm^2, which is close to a monolayer coverage. The difference in nonspecific adsorption is expected to be more pronounced if fewer antibodies are immobilized which is useful for kinetic calculations and determinations of affinity constants. We can therefore conclude that by using the PEO containing blocking agents, the recognition is definitely not negatively influenced and that the nonspecific adsorption can be reduced.

4. Conclusion

In this report, we have shown the enhanced qualities of PEO containing blocking agents for covalently bound protein coupling procedures using either the amino groups or the thiol groups of the proteins or biomolecules. In both cases, the nonspecific adsorption is much smaller on the PEO containing blocking agents compared to the classically used blocking agents. The recognition of the antigen is not negatively influenced and even slightly higher using the novel blocking agents described in this report. This approach could therefore be a possible solution for nonspecific adsorption interference problems in ELISA tests, micro-array technology, biosensors or the widely used surface plasmon resonance technology.

Acknowledgements

References

[1] Cass T, Ligler FS. Immobilized biomolecules in analysis: a practical approach. New York: Oxford Univ. Press; 1998.

[2] Hermanson GT, Mallia AK. Immobilized affinity ligand techniques. London: Academic Press; 1992.

[3] Kingshott P, Griesser HJ. Surfaces that resist bioadhesion. Curr Opin Solid State Mater Sci 1999;4:403–12.

[4] Prime KL, Whitesides GM. Self-assembled organic monolayers-model systems for studying adsorption of proteins at surfaces. Science 1991;252:1164–7.

[5] Ostuni E, Yan L, Whitesides GM. The interaction of proteins and cells with self-assembled monolayers of alkanethiolates on gold and silver. Colloids Surf, B Biointerfaces 1999;15:3–30.

[6] Wang RLC, Kreuzer HJ, Grunze M. Molecular conformation and solvation of oligo(ethylene glycol)-terminated self-assembled monolayers and their resistance to protein adsorption. J Phys Chem, B 1997; 101:9767–73.

[7] Lindblad M, Lestelius M, Johansson A, Tengvall P, Thomsen P. Cell and soft tissue interactions with methyl- and hydroxyl-terminated alkane thiols on gold surfaces. Biomaterials 1997;18(15):1059–68.

[8] Cooper MA. Optical biosensors in drug discovery. Nat Rev, Drug Discov 2002;1:515–28.

[9] Chapman RG, Ostuni E, Yan L, Whitesides GM. Preparation of mixed self-assembled monolayers (SAMs) that resist adsorption of proteins using the reaction of amines with a SAM that presents interchain carboxylic anhydride groups. Langmuir 2000;16:6927–36.

[10] Coligan JE, Dunn BM, Ploegh HL, Speicher DW, Wingfield PT. Current protocols in protein science. USA: Wiley; 1995.

[11] Ulman A. An introduction to ultrathin organic films. San Diego, USA: Academic Press; 1991.

[12] Mandler D, Turyan I. Applications of self-assembled monolayers in electroanalytical chemistry. Electroanalysis 1996;8(3):207–13.

[13] Bard AJ, Abruña HD, Chidsey CE, Faulkner LR, Feldberg SW, Itaya K, et al. The electrode-electrolyte interface—a status-report. J Phys Chem 1993;97:7147–73.

[14] Matsui H, Porrata P, Douberly GE. Protein tubule immobilization on self-assembled monolayers on Au substrates. Nano Lett 2001;1(9):461–4.

[15] Jennings GK, Munro JC, Laibinis PE. Effects of film crystallinity on the protective properties of self-assembled monolayers of alkanethiols on copper. Adv Mater 1999;11(12):1000–3.

[16] Laibinis PE, Whitesides GM. Self-assembled monolayers of *N*-alkanethiolates on copper are barrier films that protect the metal against oxidation by air. J Am Chem Soc 1992;114:9022–8.

[17] Castner DG, Ratner BD. Biomedical surface science: foundations to frontiers. Surf Sci 2002;500:28–60.

[18] D'Souza SF. Immobilization and stabilization of biomaterials for biosensor applications. Appl Biochem Biotechnol 2001;96:225–38.

[19] Kasemo B. Biological surface science. Surf Sci 2002;500:656–77.

[20] Stenberg E, Persson B, Roos H, Urbaniczky C. Quantitative determination of surface concentration of protein with surface plasmon resonance using radiolabeled proteins. J Colloid Interface Sci 1991;143(2): 513–26.

[21] Jönsson U, Fägerstam L, Ivarsson B, Johnsson B, Karlsson R, Lundh K, et al. Real-time biospecific interaction analysis using surface-plasmon resonance and a sensor chip technology. BioTechniques 1991; 11(5):620–7.

[22] Frederix F, Bonroy K, Laureyn W, Reekmans G, Campitelli A, Dehaen W, et al. Enhanced performance of an affinity biosensor interface based on mixed self-assembled monolayers of thiols on gold. Langmuir 2003;19(10):4351–7.

[23] Roberts C, Chen CS, Mrksich M, Martichonok V, Ingber DE, Whitesides GM. Using mixed self-assembled monolayers presenting RGD and (EG)(3)OH groups to characterize long-term attachment of bovine capillary endothelial cells to surfaces. J Am Chem Soc 1998;120(26):6548–55.

[24] Pale-Grosdemange C, Simon ES, Prime KL, Whitesides GM. Formation of self-assembled monolayers by chemisorption of derivates of oligo(ethylene glycol) of structure $HS(CH_2)_{11}(OCH_2CH_2)Meta$-OH on gold. J Am Chem Soc 1991;113(1):12–20.

[25] Williams LM, Evans SD, Flynn TM, Marsh A, Knowles PF, Bushby RJ, et al. Kinetics of the unrolling of small unilamellar phospholipid vesicles onto self-assembled monolayers. Langmuir 1997;13:751–7.

[26] Ortega-Vinuesa JL, Tengvall P, Wälivaara B, Lundström I. Stagnant versus dynamic conditions: a comparative adsorption study of blood proteins. Biomaterials 1998;19:251–62.

[27] Jin G, Tengvall P, Lundström I, Arwin H. A biosensor concept based on imaging ellipsometry for visualization of biomolecular interactions. Anal Biochem 1995;232:69–72.

[28] Silin V, Weetall H, Vanderah DJ. SPR studies of the nonspecific adsorption kinetics of human IgG and BSA on gold surfaces modified by self-assembled monolayers (SAMs). J Colloid Interface Sci 1997;185:94–103.