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Coupling of Antibodies to β-Cyclodextrin-Coated Gold Surfaces via an Intermediate Adamantyl-Modified Carboxymethylated Dextran Layer

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Abstract

β-cyclodextrin-coated sensors chips were obtained by grafting amino-β-cyclodextrin or amino-polymers of β-cyclodextrin (poly-β-CD-NH_2) to functionalized gold surfaces. An additional carboxymethylated dextran bearing adamantyl groups (Ad-Dex-COOH) was then immobilized onto the surface by formation of inclusion complexes between β-cyclodextrin cavities and adamantyl groups. Such multilayered structures were stable in aqueous media. However, the initial β-cyclodextrin-coated surface could be recovered by using sodium dodecylsulfate solutions (SDS). After activation with *N*-hydoxysuccinimide, dextran-coated sensor chips were used to bind antibodies. The immunoreactivity of the resulting biosensors was examined. Moreover, conditions leading to the complete regeneration of the initial surface were investigated. Throughout this study, interfacial adsorption and desorption phenomena were followed in real time by an optical technique, Surface Plasmon Resonance (SPR).

Introduction

For the last decades, cyclodextrin bonded chromatography supports have been used to separate enantiomers and other isomers [1]. More recently, it was shown that β-cyclodextrin (β-CD) coated surfaces could be utilized to build multilayered structures by formation of inclusion complexes [2]. It is e.g. possible to immobilize poly(ethylene glycol)s bearing adamantyl groups onto gold surfaces covered by polymers of β-cyclodextrin (poly-β-CD). Resulting multilayers are stable in aqueous media. Moreover, the initial β-CD-coated surface can be recovered after exposure to organic solvents or sodium dodecylsulfate (SDS).

In this study, similar systems are used to bind antibodies to gold surfaces and elaborate optical biosensors. First, aminated polymers of β-CD (poly-β-CD-NH_2) or amino-β-cyclodextrin (β-CD-NH_2) are grafted to the gold film. Then a carboxymethylated dextran layer bearing adamantyl groups (Ad-Dex-COOH) is bound to the β-CD-coated surface by formation of inclusion complexes. Finally, antibodies (rabbit IgG) are coupled to the dextran layer and the immunoreactivity of resulting biosensors is evaluated. Interfacial modifications are studied by Surface Plasmon Resonance (SPR). This optical method is sensible to refractive index modifications at the interface. Thus, it is possible to follow in real time the binding of additional layers to the surface as well as their desorption in the presence of suitable reagents. Moreover, surface concentration Γ (g cm^{-2}) for additional layers can be calculated from experimental resonance curves [3, 4]:

$$\Gamma = \frac{(n - n_{\mathrm{liq}}) \cdot h}{dn/dc} \qquad (1)$$

with n: refractive index of the layer; n_{liq}: refractive index of the liquid in the flow cell; h: thickness of the layer (determined by fitting resonance curves); dn/dc: refractive index increment.

Experimental

Materials

β-CD-NH_2 was prepared according to the procedure described by Melton *et al.* [5]. Copolymers of β-cyclodextrin and epichlorohydrin [6] were modified by amine groups by a similar method, except for the reduction step where triphenylphosphine was used to reduce azide functions [2]. The modification ratio was determined with 2,4,6-trinitrobenzene sulfonic acid (75 NH_2 per mole of poly-β-CD) [7].

Ad-Dex-COOH with low COOH contents (up to 1 COOH/5 glucose units) were prepared by addition of chloroacetic acid to a basic aqueous dextran solution (M = 40,000 g mol^{-1}) (45 min at 50 °C). Then the carboxymethylated dextran was modified by adamantyl groups by esterification with 1-adamantanecarbonyl chloride [2].

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Ad-Dex-COOH with one COOH function per glucose unit was obtained by reaction of adamantyl-modified dextrans with succinic anhydride in DMF (overnight at 70 °C).

Grafting of β-CD-NH_2 and poly-β-CD-NH_2 to gold surfaces

The grafting of β-CD-NH_2 and poly-β-CD-NH_2 to gold surfaces was performed as described previously [2]. Briefly, gold-coated (50 nm) glass chips were functionalized with 11-mercaptoundecanoic acid (HS-$(CH_2)_{10}$-COOH). After activation of COOH functions with N-hydroxysuccinimide (NHS) [8], amino-β-CD (10 g L^{-1}) or poly-β-CD bearing amine groups (1 g L^{-1}) was grafted to the surface. Then, residual NHS ester groups were blocked by reaction with ethanolamine.

Blocking of residual amine groups of poly-β-CD-NH_2 after immobilization

In some cases, residual amine groups of poly-β-CD-NH_2-coated surfaces were blocked before reaction with Ad-Dex-COOH. In the first method, the sensor chip was exposed for 1 h to acetic anhydride (4.2 mL) in 1-methyl-2-pyrrolidinone (2.4 mL) and borate buffer pH = 8 (0.11 mL). In another method a fresh sulfo succinimidyl acetate solution (40 μmol L^{-1}) in pH = 7.4 PBS was used to block amine groups (2-fold 30 min).

Binding of antibodies to β-CD-coated surfaces via Ad-Dex-COOH layers

Ad-Dex-COOH (5 g L^{-1} in water) was bound to the β-cyclodextrin-coated surface by formation of inclusion complexes. After rinsing with NaCl (1 mol L^{-1}) and activation by NHS [8], rabbit IgG was coupled to the surface. After reaction of residual NHS ester groups with ethanolamine (0.5 mol L^{-1}; pH = 7.8), the immunoreactivity of resulting biosensors was evaluated by passing anti-rabbit IgG in the flow cell. Sample concentrations were 0.2 g L^{-1} for rabbit IgG and 0.05 g L^{-1} for anti-rabbit IgG, in pH 7 PBS (20 mmol L^{-1} phosphate buffer with 0.15 mol L^{-1} NaCl).

Surface plasmon resonance method

As shown on Figure 1a, the gold-coated glass chip was placed against a glass prism. Solutions were injected into the flow cell by means of a peristaltic pump. A *p*-polarized laser beam (intensity I_0) was directed to the prism and reflected onto a photodiode (intensity I). The reflectivity R = I/I_0 was measured as a function of the incidence angle (resonance curves, Figure 1b) or was monitored as a function of time (Figure 1c). By fitting resonance curves before and after circulation of samples in the flow cell, it was possible to calculate surface concentrations for polymers or for biomolecules bound to the surface (equation 1) [4].

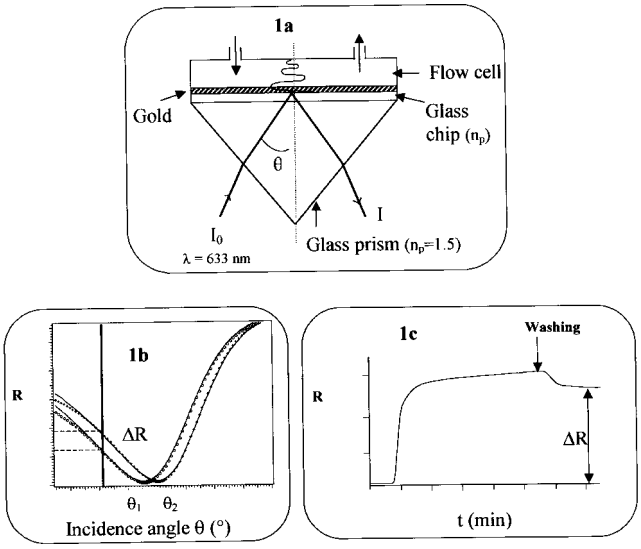

Figure 1. (a) A schematic representation of the experimental setup. The prism (refractive index 1.515) is brought in optical contact with the gold-coated glass slide using an index matching oil. The laser beam (λ = 633 nm) is totally reflected at the multilayered interface. (b): A schematic curve showing changes in reflectivity (R = I/I_0) versus incidence angle. The minimum resonance angle shifts from θ_1 towards θ_2 when an additional layer is deposited on the surface. (c) The variation of reflectivity versus time at a given incidence angle is used for monitoring the adsorption (or desorption) process of molecules onto (or from) the surface.

Results and discussion

Binding of adamantyl-modified dextrans to β-CD-coated surfaces

In an earlier study [2], it was shown that additional layers could be bound to β-CD-coated surfaces by passing adamantyl-modified poly(ethylene glycol)s in the flow cell of the SPR instrument. In this study, the formation of inclusion complexes between immobilized β-CD cavities and various adamantyl-modified dextrans and adamantyl-modified carboxymethylated dextrans was investigated. Surface concentrations Γ_{dex} calculated for dextran layers, using dn/dc = 0.15 mL g^{-1} [3], are reported in Table I. In the case of Ad-Dex-COOH layers, these values were determined after washing with NaCl, because partial desorption of the polymer occurred during this step. This demonstrates that some dextran chains were probably bound by electrostatic interactions, since inclusion complexes are stable in the presence of saline solutions [9].

Surface concentrations for dextran polymers are of the same order as values reported for modified poly(ethylene glycol)s [2]. However, when the adamantyl content is kept constant (7%), it appears (Table I) that surface concentrations determined for Ad-Dex-COOH are lower than for Ad-Dex. Thus, it can be concluded that the formation of inclusion complexes between Ad-Dex-COOH and poly-β-CD surfaces is hindered by COOH groups, in spite of the presence of amine functions on poly-β-CD-NH_2 chains. Moreover, for a given COOH content (12%), surface concentrations slightly increase with the number of adamantyl groups per dextran chain, illustrating the influence of this parameter on the stability of immobilized dextran layers.

Table 1. Surface concentrations (Γ_{dex}) for dextran layers bound to poly-β-CD-NH_2-coated gold surfaces, versus concentration and composition of Ad-Dex and Ad-Dex-COOH polymers

Polymer	C (g L^{-1})	% Ad[a]	% COOH[a]	Γ_{dex} (g cm^{-2})
Ad-Dex	0.05	7	0	$1.1\ 10^{-7}$
	10	7	0	$1.9\ 10^{-7}$
Ad-Dex-COOH	5	0.9	7	$1.5\ 10^{-7}$
		2.1	12	$0.7\ 10^{-7}$
		4.5	12	$1.1\ 10^{-7}$
		7	100	$0.5\ 10^{-7}$

[a] Number of adamantyl (Ad) or COOH groups per 100 glucose units.

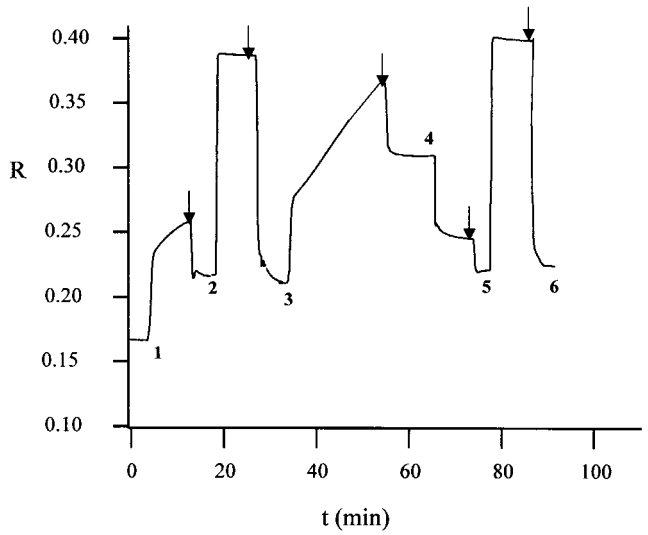

Figure 2. Variations of reflectivity as a function of time for [MUA/poly-CD-NH_2/Ad-Dex-COOH]-coated gold surfaces without blocking of residual amine groups. Step **1**: binding of rabbit IgG (0.2 g L^{-1}) to the activated Ad-Dex-COOH layer; step **2**: reaction of residual NHS-ester groups with ethanolamine; step **3**: binding of anti-rabbit IgG (0.05 g L^{-1}); step **4**: partial desorption of anti-rabbit IgG by HCl 0.1 M; step **5**: regeneration attempts with SDS 1%. Arrows indicate washing steps.

As described for modified poly(ethylene glycol)s [2], additional Ad-Dex and Ad-Dex-COOH dextran layers could be completely removed from the surface by using 1% SDS solutions (in water or acid glycine buffers), demonstrating again that the initial poly-β-CD-NH_2 coated surface could be regenerated under these conditions.

Coupling of antibodies to Ad-Dex-COOH layers immobilized onto poly-β-CD-NH_2 surfaces

After activation of COOH functions by NHS, rabbit IgG was coupled to the reactive dextran layer (step 1 on Figure 2). After reaction of residual NHS ester groups with ethanolamine (step 2 on Figure 2), it appeared that rabbit IgG was bound to the surface since the reflectivity level was higher at 3 than at 1. Moreover, as illustrated on Figure 2 (step 3), the binding of anti rabbit IgG to resulting biosensors was efficient (reflectivity level at 4 higher than at 3).

As shown in Table II, the amounts of antibody bound to Ad-Dex-COOH-coated sensor chips increased with the number of COOH functions on polymer chains (Γ_{IgG} calculated with dn/dc = 0.183 [3]). Values obtained for Ad-Dex-COOH polymers with 100 COOH per chain, were e.g. higher than for Ad-Dex-COOH with 12 carboxylic groups per chain, in spite of a lower surface concentration for the dextran layer.

Table 2. Surface concentrations for IgG (mole cm^{-2}) and Ad-Dex-COOH (g cm^{-2}) as a function of the number of COOH groups per 100 glucose units

% COOH in Ad-Dex-COOH	Γ_{dex} (g cm^{-2})	Γ_{IgG} (mole cm^{-2})
12	$1.1\ 10^{-7}$	$1.6\ 10^{-13}$
100	$0.5\ 10^{-7}$	$2.3\ 10^{-13}$

These values were about 100-fold lower than maximum binding capacities obtained with dextran hydrogels by Stenberg *et al.* [3]. However the hydrogel structure provides much more numerous sites for covalent binding of biomolecules than a thin dextran layer.

Regeneration of poly-β-CD-NH_2 -coated surfaces after coupling of biomolecules

As shown in the first part of this study, Ad-Dex and Ad-Dex-COOH layers could be removed from the surface by using SDS solutions. However, after binding of IgG and anti-IgG to Ad-Dex-COOH-coated sensor chips and desorption of the anti-IgG layer by HCl (step 4 on Figure 2), the circulation of SDS solutions into the flow cell was fully ineffective, since reflectivity levels at 6 and at 5 were exactly the same (step 5 on Figure 2). The lack of regeneration of initial poly-β-CD-NH_2-coated sensing layers after use can be explained as follows. Some of the reactive NHS-ester functions formed during activation by NHS can react with residual NH_2 groups of the poly-β-CD-NH_2 layer, resulting in covalent linkages between the Ad-Dex-COOH polymer and the immobilized poly-β-CD-NH_2. Thus desorption of the dextran layer is no more possible after the activation step. This assumption was confirmed experimentally in the case of naked Ad-Dex-COOH sensor chips (data not shown).

For this reason, attempts were made to block residual NH_2 functions after binding of poly-β-CD-NH_2 to the gold surface. By using acetic anhydride before the immobilization of Ad-Dex-COOH and activation by NHS, only 60–65% of additional layers (Ad-Dex-COOH and IgG) were removed from the surface by SDS solutions. It can be concluded that NH_2 groups were still present on the surface after the blocking step. Typical sensorgrams observed after blocking of amine groups by sulfosuccinimidyl acetate are shown on Figure 3. In this case, regeneration of the initial surface by SDS solutions is rather efficient (about 80%) since the reflectivity level at 13 is nearly the same as at 4 (before the immobilization of Ad-Dex-COOH). Experimental conditions could be probably further improved to obtain a complete regeneration of the initial surface (reaction time, concentration of reagents). However, blocking procedures being time consuming, a shorter method where the blocking step could be avoided was evaluated.

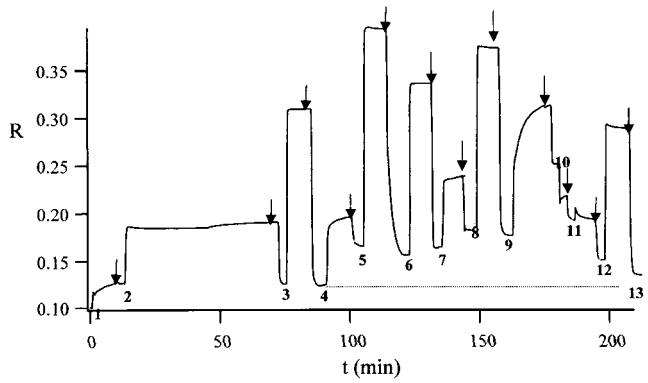

Figure 3. Blocking of residual NH_2 functions of poly-CD-NH_2 by sulfosuccinimidyl acetate, subsequent immobilization of IgG, anti-rabbit IgG and regeneration. Step **1**: grafting of poly-CD-NH_2; step **2**: blocking of NH_2 functions by sulfosuccinimidyl acetate; step **3**: reaction of residual reactive NHS-ester groups with ethanolamine; step **4**: binding of Ad-Dex-COOH; step **5**: NaCl 1 mol L^{-1}; step **6**: activation of COOH groups by NHS; step **7**: binding of rabbit IgG (0.2 g L^{-1}); step **8**: reaction of residual reactive NHS-ester groups with ethanolamine; step **9**: binding of anti-rabbit IgG (0.05 g L^{-1}); step **10**: partial desorption of anti-rabbit IgG by HCl 0.1 M; steps **11** and **12**: regeneration with SDS 1% in water and acid glycine buffer, respectively. Arrows indicate washing steps.

Coupling of antibodies to Ad-Dex-COOH layers immobilized onto monomeric β-CD-NH_2 surfaces

In order to avoid side reactions generated by residual amine functions, poly-β-CD-NH_2 was replaced by monoamino-β-cyclodextrin. The binding of Ad-Dex-COOH and rabbit IgG to the surface was performed as described for poly-β-CD-NH_2 surfaces. Sensorgrams obtained after exposure to Ad-Dex-COOH, IgG, anti-IgG and regeneration solutions were similar to those shown on Figure 3. However, in the case of surfaces coated with monomeric cyclodextrin, the blocking step (step 2) was suppressed. In both cases (poly-β-CD-NH_2 and CD-NH_2-coated sensor chips), the reaction of residual NHS-ester groups with ethanolamine induced a slight desorption of the Ad-Dex-COOH layer (step 8 on Figure 3). On the other hand, in the case of monoamino-β-cyclodextrin, the regeneration by SDS solutions was fully efficient since reflectivity levels at 13 and at 4 were exactly the same (data not shown). This result confirms that free amine groups on poly-β-CD-NH_2 chains were really responsible for the lack of regeneration of poly-β-CD-NH_2 -coated surfaces.

Conclusion

It is possible to elaborate optical biosensors by an easy procedure using reactive dextran layers bound to β-CD-coated gold surfaces by formation of inclusion complexes. After grafting of antibodies, the resulting multilayered structures were stable in aqueous buffers and their biological activity was demonstrated. When amino-β-CD was used to anchor additional layers, the biosensors obtained by this method could be regenerated after utilization and used for other experiments.

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