Immobilization of a Functionalized Poly(ethylene glycol) onto β-Cyclodextrin-coated Surfaces by Formation of Inclusion Complexes: Application to the Coupling of Proteins

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ABSTRACT: The aim of this study was the immobilization of COOH-modified poly(ethylene glycol) (PEG) layers onto β-cyclodextrin-coated surfaces by formation of inclusion complexes, in view of biosensors applications. To this end, PEGs with one phenyladamantyl and one carboxylic end group (Ad-PEG-COOH) were prepared according to a three-step procedure. After modification of PEG with 4-toluenesulfonylchloride, the reaction of the tosyl intermediate with the alcoholate of 4-(1-adamantyl)-phenol was carried out in tetrahydrofuran to avoid the formation of by-products. Then, it was shown by high performance liquid chromatography that the association between β-cyclodextrin cavities and Ad-PEG-COOH polymers was not hindered by the presence of the COOH group. Last, the Ad-PEG-COOH polymer was immobilized onto β-cyclodextrin-coated gold surfaces by formation of inclusion complexes. The immobilization was performed in water, at room temperature, with a rapid kinetics. After activation of COOH groups with *N*-hydroxysuccinimide, β-lactoglobulin was coupled to the biocompatible PEG layer. Functionalization of the gold surface with β-cyclodextrin cavities, immobilization of Ad-PEG-COOH onto the surface, and coupling of the protein to the reactive PEG layer were followed in real time by surface plasmon resonance imaging system. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 100: 2362–2370, 2006

Key words: β-cyclodextrin; functionalization of polymers; adamantane-modified polyethers; high performance liquid chromatography (HPLC); surface plasmon resonance (SPR)

INTRODUCTION

Cyclodextrins (CD) are cyclic oligosaccharides consisting of 6, 7, or 8 glucopyranose units, which are called α-, β-, and γ-cyclodextrin, respectively. These molecules have the ability of forming inclusion complexes with hydrophobic moieties.[1] In the case of adamantane derivatives, the association is quite strong, so that the β-CD/adamantane system has been used to immobilize polymers bearing adamantane groups onto β-CD-modified interfaces.[2,3] For example, we have previously examined the binding of adamantane-end-capped methoxy-poly(ethylene glycol) (Ad-PEG-OCH_3) to β-CD-coated gold surfaces, by using surface plasmon resonance (SPR) for detection.[3] We showed that the immobilization was carried out under mild conditions, had rapid kinetics, and resulted in the formation of multilayered structures, which were stable in aqueous media. The aim of the present work is to evaluate the suitability of this easy procedure to form biocompatible polymer films, which could be used to immobilize biomolecules onto solid materials in view of biosensors applications.

In the past decades, considerable attention has been focused on COOH-modified surfaces for the preparation of biosensors. Particularly, carboxymethylated dextran hydrogels are developed by Biacore for the binding of proteins onto gold chips, according to a well-established procedure.[4,5] In this article, we report another immobilization method involving the formation of inclusion complexes between a biocompatible poly(ethylene)glycol (PEG) derivative and β-CD-coated gold surfaces, which allows the coupling of biomolecules. To this end, PEGs with OH terminal ends modified by both adamantyl and COOH groups (Ad-PEG-COOH) were synthesized and adsorbed onto β-CD-modified gold surfaces using this procedure. To examine the influence of the COOH function on the formation of inclusion complexes, the association constants of β-CD cavities with Ad-PEG-COOH and Ad-PEG-OCH_3 polymers, respectively, were compared. An affinity high performance liquid chromatography (HPLC) method with UV detection was em-

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ployed.[6] For this reason, polymers bearing a terminal phenyladamantyl group were selected because of their UV-absorption properties.[7] The reaction conditions reported previously for the synthesis of Ad-PEG-OCH_3 from HO-PEG-OCH_3[7] were modified because of the formation of large amounts of by-products when Ad-PEG-OH was prepared from OH-PEG-OH according to this method. The adsorption of Ad-PEG-COOH onto β-CD-modified gold chips and the subsequent binding of a test protein (β-lactoglobulin) to the resulting surfaces were examined using a SPR imaging system. SPR is an optical method, which is sensitive to changes in the refractive index occurring in the vicinity of a metal:dielectric interface.[8] Thereby, this technique allows the monitoring of interfacial modifications in real time resulting from adsorption (or desorption) processes occurring within tens of nanometer of the gold surface.[9]

EXPERIMENTAL

Chemicals

β-Cyclodextrin (β-CD) was obtained from Roquette (Lestrem, France). Poly(ethylene glycol) (OH-PEG-OH; MW = 4600 g/mol), 4-toluenesulfonylchloride, 4-(1-adamantyl)-phenol, sodium hydride, succinic anhydride, sodium borohydride, Raney nickel, 4-dimethylaminopyridine (DMAP), 11-mercaptoundecanoic acid (MUA), *N*-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), and triethylamine were purchased from Aldrich (St Quentin Fallavier, France), while 4-toluenesulfonylchloride was from Fluka (St Quentin Fallavier). β-Lactoglobulin from bovine milk (90%) was acquired from Sigma (St Quentin Fallavier). Protein solutions were prepared in 10 m*M* phosphate buffer containing 2.7 m*M* KCl and 0.137*M* NaCl (PBS). All organic solvents (dichloromethane, diethyl ether, ethanol, pyridine, and tetrahydrofuran (THF)) were obtained from SDS (Aix en Provence, France).

Characterization of modified PEGs by NMR and size exclusion chromatography

^{1}H NMR and heteronuclear multiple bond correlation spectra of the polymers were recorded in DMSO-d_6, on a Bruker AVANCE 300 MHz spectrometer, using the solvent signal as internal reference.

The average molecular masses of the polymers were determined by size exclusion chromatography (SEC) using refractive index and light scattering detectors. Modified PEG samples were analyzed in chloroform on a PL gel column (50, 10^2, 10^3 nm, 5 μm; Polymer laboratories, Shropshire, UK) using polystyrene standards.

Preparation of Ts-PEG-OH from OH-PEG-OH

Tosyl-modified PEG (Ts-PEG-OH) was prepared from OH-PEG-OH under conditions that were similar to those described for the preparation of Ts-PEG-OCH_3 from OH-PEG-OCH_3 (2 h at 0°C, in dichloromethane, with triethylamine as HCl scavenger).[7] In the present study, the reaction was carried out using 2.2 equiv of 4-toluenesulfonylchloride per OH-PEG-OH chain (preliminary dried for 16 h at 40°C under vacuum) to obtain 50% of modification (1 tosyl group per chain) on average. After purification, the Ts-PEG-OH polymer was characterized by ^{1}H NMR and SEC.

^{1}H NMR (DMSO-d_6): δ = 7.77, 7.48 (4H, A_2B_2 dd, J = 8.1 Hz, aryl ring); 4.61(1H, t, 6 Hz, OH), 4.10 (2H, t, J = 3 Hz, C**H$_2$**OTs); 3.50 (broad s, polymer backbone); 2.41 (3H, s, CH_3 of the tosyl group).

Preparation of Ad-PEG-OH from Ts-PEG-OH

Phenyladamantyl-modified PEG was prepared from the reaction of Ts-PEG-OH with the alcoholate of 4-(1-adamantyl)-phenol. Experimental conditions described for the preparation of Ad-PEG-OCH_3 from Ts-PEG-OCH_3[7] were not suitable. So the synthesis of Ad-PEG-OH was carried out as follows. The alcoholate of 4-(1-adamantyl)-phenol was formed by adding 1.12 g (5 mmol) of 4-(1-adamantyl)-phenol to 100 mg (4 mmol) of NaH suspended in 60 mL of THF freshly distilled under a nitrogen atmosphere. The mixture was stirred for 1 h at room temperature. During this time, 5 g (1 mmol) of freshly prepared Ts-PEG-OH were dissolved under nitrogen in 60 mL of THF (gentle heating may be necessary to make the dissolution easier). Thereafter, the polymer solution was added dropwise under nitrogen to the alcoholate of 4-(1-adamantyl)-phenol. The reaction mixture was stirred at 40°C for 48 h under nitrogen. The resulting Ad-PEG-OH polymer was purified by precipitation in diethyl ether followed by recristallization using ethyl alcohol, before being characterized by ^{1}H NMR and SEC.

^{1}H NMR (DMSO-d_6): δ = 7.23, 6.89 (4H, A_2B_2 dd, J = 8.7 Hz, aryl ring); 4.57(1H, t, CH_2O**H**), 4.04 (2H, t, J = 6 Hz, CH_2OAd); 3.50 (broad s, polymer backbone); 2.03, 1.81, 1.71 (15H, broad s, adamantyl protons).

Preparation of Ad-PEG-COOH from Ad-PEG-OH

Ad-PEG-OH (1 g, 0.2 mmol) was dissolved in 13 mL of THF (gentle heating may be necessary). Then, 24.5 mg (0.2 mmol) of DMAP, 27 μL (0.2 mmol) of triethylamine, and 200 mg (2 mmol) of succinic anhydride were added. The reaction mixture was stirred for 48 h at room temperature. After evaporation of THF under reduced pressure and drying, 15 mL of carbon tetrachloride were added to the reaction product to remove

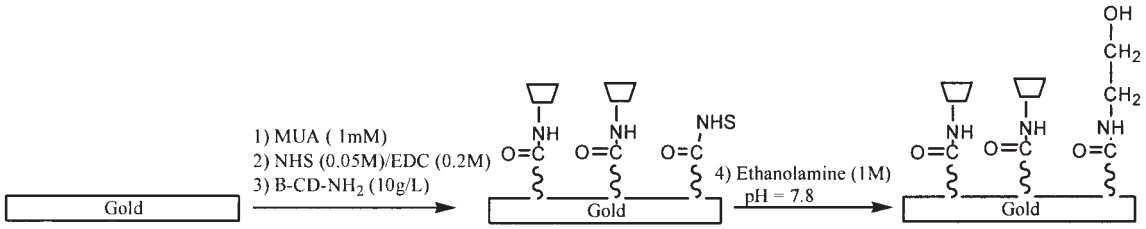

Figure 1 Preparation of the β-CD-modified gold surface.

the excess of succinic anhydride by filtration on a cellulose membrane (0.45 μm). The Ad-PEG-COOH polymer was purified by precipitation in diethyl ether followed by recristallization using ethyl alcohol, before being characterized by ^{1}H NMR.

^{1}H NMR (DMSO-d_6): δ = 7.23, 6.85 (4H, A_2B_2 dd, J = 8.7 Hz, aryl ring); 4.04 (2H, t, J = 6 Hz $\mathbf{CH_2O}CO(CH_2)_2CO_2H$); 4.1 (2H, t, J = 6 Hz, CH_2OAd); 3.50 (broad s, polymer backbone); 2.03, 1.81, 1.71 (15H, broad s, adamantyl protons).

Preparation of β-CD-NH$_2$

β-CD was first modified by 4-toluenesulfonyl chloride in pyridine at 8°C, as described in the literature.[10,11] Then, the mono-tosylate derivative was treated with sodium azide in DMF, as reported by Petter et al.[12] Last, azido-β-CD (0.7 mmol) was reduced in water (40 mL) using a Raney nickel slurry (2 mL) and sodium borohydride (10 mmol). After 24 h at 70°C, the suspension was filtered. The clear filtrate was dialyzed for 8 h against water, using Spectra/por membrane with MW cut-off of 1000 (Fisher Bioblock Scientific, Illkirch, France). Finally, mono-amino-β-cyclodextrin (β-CD-NH$_2$) was lyophilized.

Grafting of β-CD-NH$_2$ onto gold surfaces

As reported in Figure 1, gold-coated glass chips were modified with MUA as described previously.[3] Then, COOH functions were activated into NHS-ester groups by reaction with NHS in the presence of EDC. The activation was carried out in batches for 30 min. The coupling of β-CD-NH$_2$ to the activated surface was performed on the SPR instrument, by passing the β-CD-NH$_2$ solution (10 g/L in water) for 30 min in the flow cell. After rinsing with water, residual NHS-ester groups were blocked with ethanolamine (1 mol/L; pH = 7.8).

Binding of Ad-PEG-COOH to the β-CD-modified gold surface

The Ad-PEG-COOH solution (1 g/L in water) was passed over the β-CD-coated gold surface for 7 min, at a flow rate of 20 μL/min. Then, the chip was rinsed with water. Changes in reflectivity were followed by SPR.

Coupling of β-lactoglobulin to the Ad-PEG-COOH-modified gold surface

Carboxylic groups were activated for 15 min by circulation in the flow cell of the NHS–EDC mixture (0.05 and 0.2 mol/L, respectively). The resulting NHS-ester functions were grafted with the β-lactoglobulin (Fig. 2) by passing the protein solution (0.2 g/L in PBS) in the flow cell of the SPR instrument. Thereafter, the chip was rinsed with water.

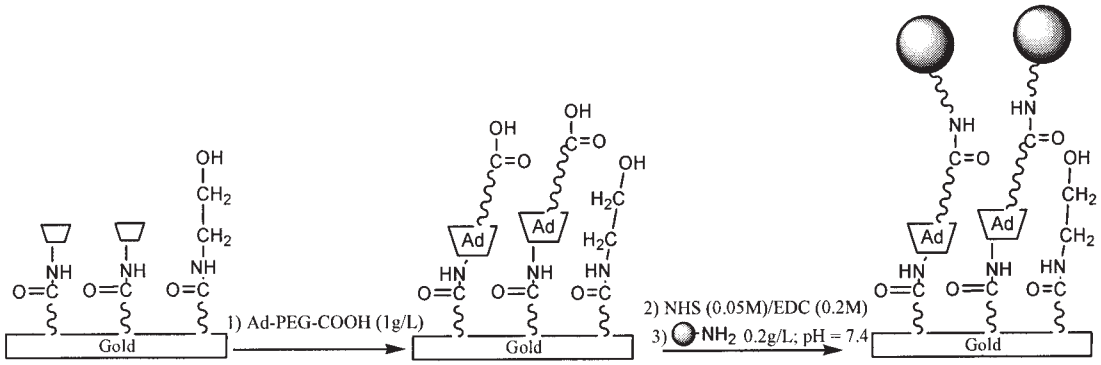

Figure 2 Binding of Ad-PEG-COOH to the modified gold surface (reaction 1), followed by the coupling of the protein (reactions 2 and 3).

HPLC measurements

The determination of association constants between adamantyl-modified PEGs and β-CD was performed using a competitive affinity chromatography method.[13,14] Chromatographic experiments were performed with a modular HPLC system consisting of a LC 9A Shimadzu pump (Kyoto, Japan), a Rheodyne 7125 sampling valve with a 20 μL loop (Berkeley, CA, USA), and a Thermo-Electron Spectra 100 variable-wavelength UV detector (San Jose, CA). Chromatograms were recorded with a Kipp and Zonen (Delft, The Netherlands) chart-recorder (type BD 41). The chromatography column (100 × 4.6 mm ID) contained a diol-silica support modified with a polymer of β-CD (poly β-CD), which was described in an earlier study.[6] For the determination of affinity constants between Ad-PEG-OCH_3 and β-cyclodextrin cavities, the mobile phase was water containing various concentrations of hydroxypropyl β-CD (from 1 to 5 mmol/L). In the case of Ad-PEG-COOH, a 20 mmol/L Tris-HCl buffer (pH = 7.0) was used. All experiments were carried out at 1 mL/min. Diluted polymer solutions (10^{-5} mol/L) were injected onto the poly β-CD-coated column (injection volume: 20 μL). Phenyladamantyl-modified polymers (Ad-PEG-COOH and Ad-PEG-OCH_3) were monitored at 276 nm.

SPR measurements

As described previously,[3,15] the experimental arrangement used for SPR measurements consisted of a *p*-polarized He–Ne laser (intensity I_0) with a wavelength of 633 nm, a glass prism (n = 1.515) covered by a gold-coated glass slide (gold thickness ≈ 45 nm), and a photodiode to measure the intensity I of the reflected light. A flow cell (14 μL) was applied against the sensing surface. Solutions were injected into the system using a peristaltic pump. The experiments were under computer control.

After the injection of the reference medium (either water or PBS) onto the sensor surface and stabilization of the baseline, the solution of interest (polymer or protein) was passed in the flow cell. Thereafter, the surface was rinsed with the reference medium. Data were examined in terms of the response, that is, the change in the reflectivity R (ratio I/I_0) due to the immobilization of polymers or proteins to the surface.

RESULTS AND DISCUSSION

Preparation of PEG derivatives

Preparation of the tosyl intermediate (Ts-PEG-OH)

Various amounts of 4-toluenesulfonylchloride were tested to prepare PEGs bearing approximately one tosyl group per chain. The extent of modification was determined by ^{1}H NMR from signals corresponding to the OH proton at the polymer terminal end (δ = 4.61 ppm; integration I_1) and protons close to the tosyl group (C**H$_2$**OTs, δ = 4.10 ppm; integration I_2) according to the eq. (1):

$$\%\mathrm{Ts} = 100 \times \frac{I_2}{I_2 + 2I_1} \quad (1)$$

When using 2.2 mol of 4-toluenesulfonylchloride per chain of PEG, the expected modification ratio was obtained (from 50 to 58%). For lower amounts of 4-toluenesulfonylchloride (1 and 2 mol/chain), the extents of modification were equal to 22 and 43%, respectively. Moreover, it was demonstrated by SEC that the molecular weights of polymers were similar before and after modification, indicating that the reactions were performed without any chain cleavage.

However, the presence of unmodified PEG and of Ts-PEG-Ts in the Ts-PEG-OH preparation could not be excluded, since analysis by ^{1}H NMR does not make it possible to distinguish these compounds from Ts-PEG-OH. The proportions of unmodified, mono- and disubstituted PEG was determined by HPLC after the reaction with 4-(1-adamantyl)-phenol. This point will be discussed in the next paragraph.

Preparation of Ad-PEG-OH

The nucleophilic displacement of tosyl groups by the alcoholate of 4-(1-adamantyl)-phenol was first carried out according to the procedure described in a previous paper, using dichloromethane as solvent and 2.5 mol of sodium hydride per mole of 4-(1-adamantyl)-phenol.[7]

Figure 3 shows a typical ^{1}H NMR spectrum of polymers obtained under these conditions. As expected, signals corresponding to the tosyl group (δ = 7.48 and 7.77 ppm) were no longer observable, whereas peaks due to the adamantyl moiety could be clearly detected at δ= 1.71, 1.81, and 2.03 ppm. However, this ^{1}H NMR spectrum displayed several anomalies: (i) an unexpected singlet was observed at 5.2 ppm, (ii) signals attributed to aryl protons were splitted in two parts (δ = 6.86 and 6.94 ppm; δ = 7.23 and 7.26 ppm), (iii) values higher than 12 were determined for the ratio [adamantyl protons/C**H$_2$**OAd] instead of 7.5. Since a large excess of reagent was used to prepare Ad-PEG-OH, the last point could be explained by the presence of free 4-(1-adamantyl)-phenol in the reaction product, in spite of an extensive purification step. However, as shown in Figure 3, there was no signal at 6.67 and 7.12 ppm due to aromatic protons of free 4-(1-adamantyl)-phenol. Thus, the anomalous [adamantyl protons/CH_2OAd] ratio measured by ^{1}H NMR was more prob-

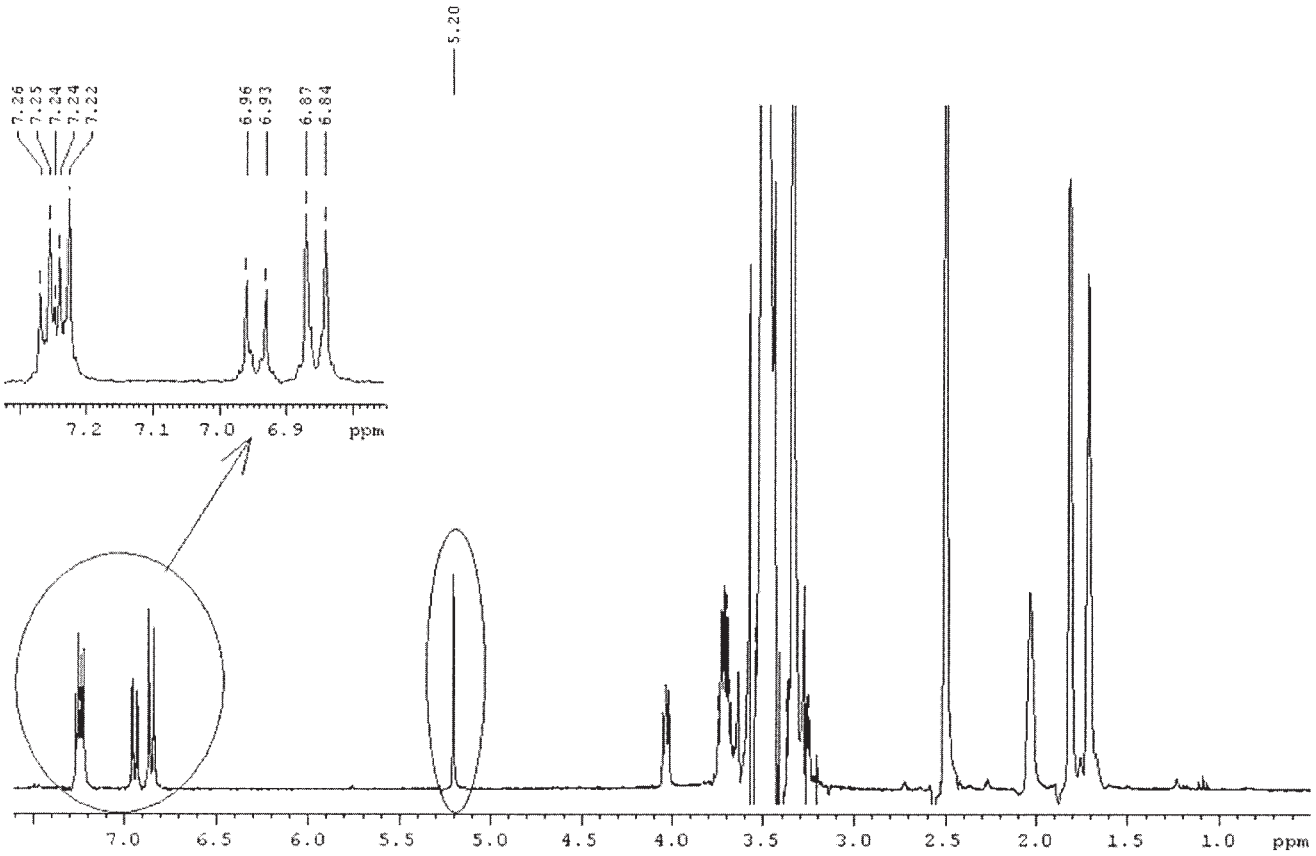

Figure 3 ^{1}H NMR spectrum of the Ad-PEG-OH polymer prepared in dichloromethane.

ably due to the formation of derivatives of PEG bearing two adamantyl end-groups per chain.

In addition to the anomalous ^{1}H NMR spectra, the SEC profiles of Ad-PEG-OH obtained in previous conditions (dichloromethane, excess of NaH) displayed several populations of polymer corresponding to the monomeric, dimeric, and to a less extent, trimeric form of PEG, respectively, (data not shown). These results suggested that side reactions leading to the coupling between polymer chains were taking place.

A possible reaction scheme is proposed in Figure 4 to explain ^{1}H NMR and SEC data. The structure of the resulting by-products (products A and B) and signal assignments are reported in this figure. The presence of the singlet at 5.2 ppm ($H_{c'}$ protons) and of unexpected doublets at 6.94 and 7.26 ppm ($H_{a'}$ and $H_{b'}$ protons) can be elucidated by the formation of the product A, according to a mechanism involving dichloromethane, the alcoholate of 4-(1-adamantyl)-phenol, and the Ts-PEG-O$^-$ form of the polymer (reactions 2 and 4 in Fig. 4). The structure of the by-product A was supported by complementary 2D NMR experiments. On the HBMC spectra, correlation signals corresponding to the coupling of $H_{c'}$ protons with carbon atoms of the polymer backbone, with the quaternary aryl carbon and with carbon atoms bearing $H_{a'}$ and $H_{b'}$ protons could be observed (data not shown). The formation of the derivative of PEG bearing two adamantyl groups per chain (product A) can account for the anomalous value determined for the ratio [adamantyl protons/C**H$_2$**OAd].

Moreover, SEC profiles can be explained by the presence of unreacted sodium hydride after reaction with 4-(1-adamantyl)-phenol. The excess of NaH enables the formation of alcoholate forms of the polymer (Ts-PEG-O$^-$, Ad-PEG-O$^-$). Thus, coupling reactions between PEG chains can occur, resulting in the formation of dimers and trimers of PEG, which were detected by SEC. An example of reaction leading to the formation of a dimer of PEG (product B) is given in Figure 4.

To avoid these side-reactions, Ad-PEG-OH was synthesized according to a similar procedure, using THF instead of dichloromethane. Moreover, in the first step, the amount of sodium hydride was reduced to prepare the alcoholate of 4-(1-adamantyl)-phenol (only 0.9 mol of NaH per mole of reagent). As shown in Figure 5, the ^{1}H NMR spectrum of Ad-PEG-OH obtained in these conditions was as expected (no signal at 5.2 ppm, no splitting of signals attributed to aryl protons, ratio [adamantyl protons/C**H$_2$**OAd] equal to 7.5). In addition, there was a single peak on SEC

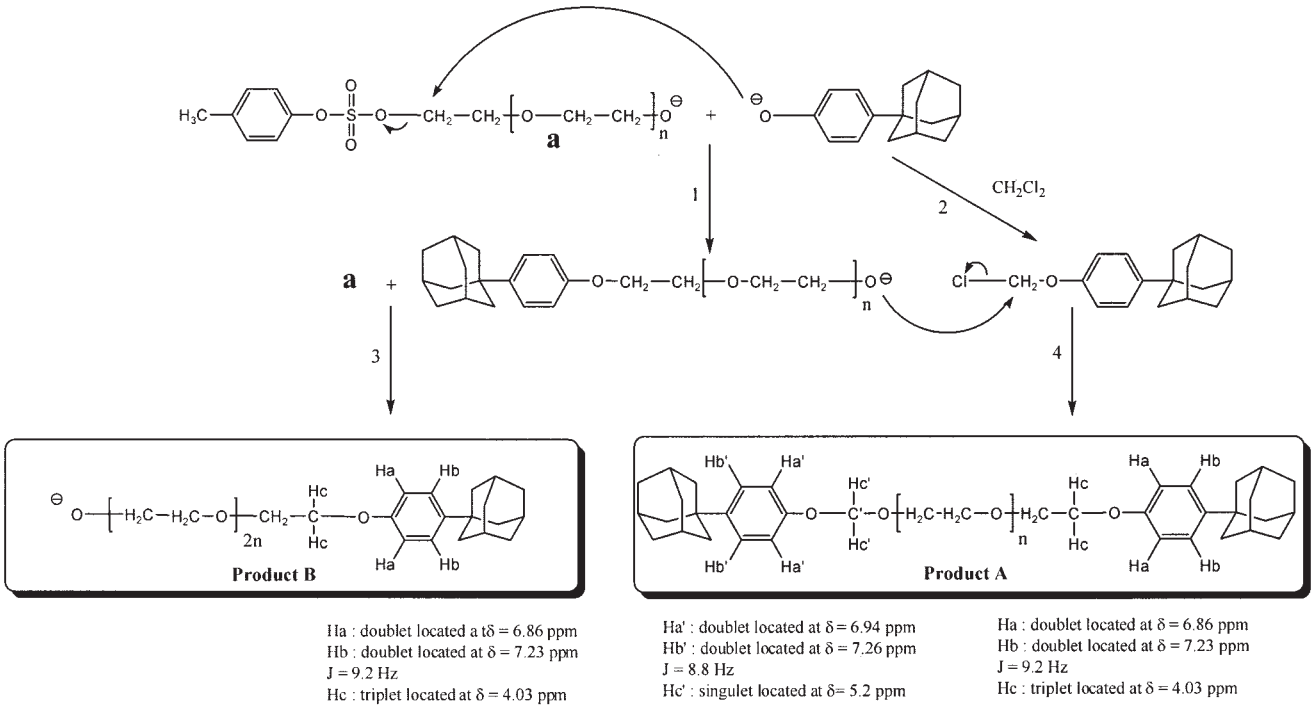

Figure 4 Preparation of Ad-PEG-OH in dichloromethane: mechanisms proposed for the formation of by-products A and B.

chromatograms, corresponding to the monomeric form of Ad-PEG-OH (data not shown).

The proportions of OH-PEG-OH and of Ad-PEG-Ad in the Ad-PEG-OH preparation were determined by HPLC, on a poly β-CD column. The unmodified PEG was eluted at the void volume when using pure water for elution (refractive index detection), whereas the mono- and disubstituted species were eluted with 5 m*M* hydroxypropyl β-CD in the mobile phase (UV detection). It was shown that Ad-PEG-OH prepara-

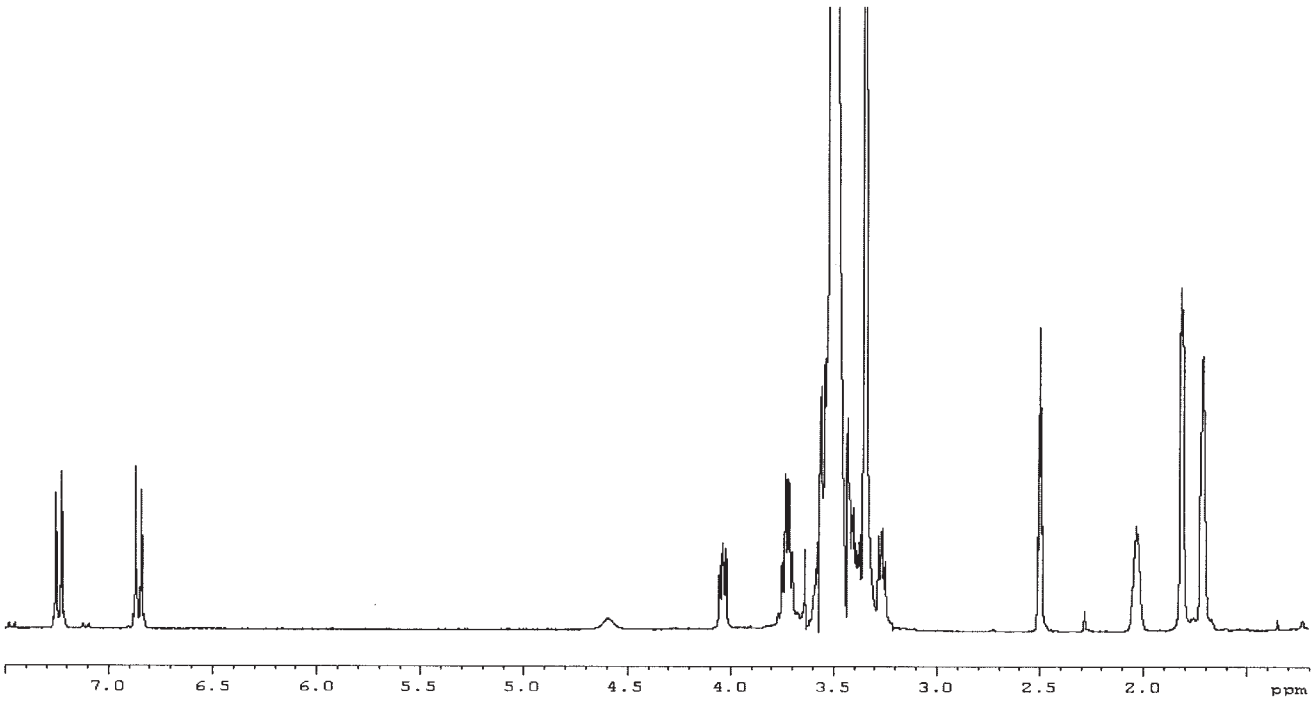

Figure 5 ^{1}H NMR spectrum of the Ad-PEG-OH polymer prepared in THF.

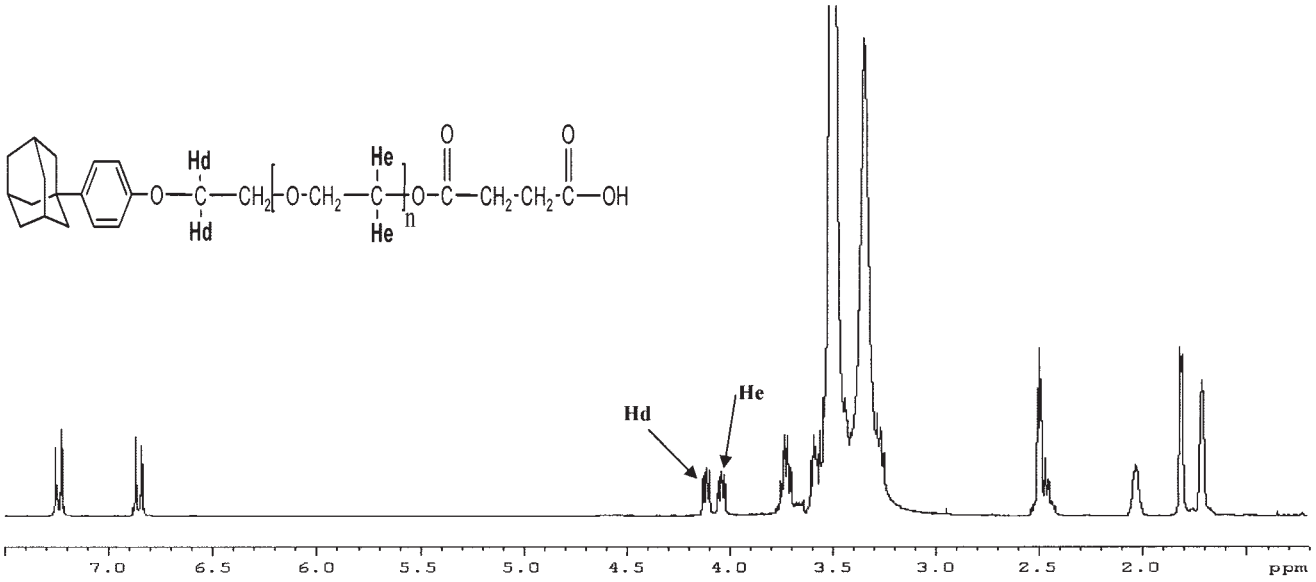

Figure 6 ^{1}H NMR spectrum of the Ad-PEG-COOH polymer.

tions contained small amounts of OH-PEG-OH and Ad-PEG-Ad representing less than 15% of the total. Therefore, they were used without any further purification.

Preparation of Ad-PEG-COOH

Ad-PEG-COOH was prepared by the reaction of Ad-PEG-OH with succinic anhydride after modification of procedures described in the literature.[16–18] The reaction was performed in THF using a 10 molar excess of succinic anhydride. In these conditions, the signal corresponding to the OH group (δ = 4.57 ppm) disappeared whereas peaks due to the protons adjacent to the succinic moiety (H_e) could be detected at 4.04 ppm with the same integration intensity than protons adjacent to the phenyladamantyl group (H_d) at 4.1 ppm (Fig. 6).

Influence of the COOH group on the association constants between adamantyl-modified PEGs and β-CD

The association constants between β-cyclodextrin cavities and adamantyl-modified polymers were determined by competitive affinity chromatography. In this technique, the solute (A) is injected in the affinity column (X), using a mobile phase containing a ligand (L). Interactions between the solute, the stationary phase, and the competitor in the mobile phase are described by two equilibria:

$$A + X \leftrightarrow AX \quad K_{AX} \tag{2}$$

$$A + L \leftrightarrow AL \quad K_{AL} \tag{3}$$

The value of K_{AL} can be easily determined by injecting the solute in the presence of various concentrations of ligand in the eluent.

This method was used to compare the affinity of Ad-PEG-COOH and Ad-PEG-OCH_3 for β-cyclodextrin cavities and to study the influence of the COOH group on the formation of inclusion complexes. To this end, both polymers were analyzed onto a poly β-CD column with a mobile phase containing hydroxypropyl β-CD (HP β-CD). Thus, in the present study, X represents immobilized β-CD cavities and L corresponds to HP β-CD in the eluent. K_{AX} and K_{AL} are the association constants between PEGs and β-cyclodextrin cavities (immobilized and free, respectively).

The model used for the determination of binding constants assumes that (i) only 1:1 complexes are formed between the solute and β-CD cavities, (ii) there is no interaction between the soluble ligand (HP β-CD) and immobilized β-CD cavities, (iii) there is a single type of interaction centers on the support.[6] In these conditions, the retention volume of phenyladamantyl-modified PEGs (V_R) is given by the following equation[14]:

$$\sqrt{\frac{1}{V_R - V_0}} = \sqrt{\frac{1 + [L]K_{AL}}{Q_X K_{AX}}} + [A]\sqrt{\frac{K_{AX}}{Q_X(1 + [L]K_{AL})}} \tag{4}$$

In this relation, V_0 is the void volume of the column, Q_X represents the accessible amount of immobilized β-CD cavities, and [A] is the solute concentration at the peak maximum.

At 0 concentration ([A]→0), a linear relationship is obtained when $1/(V_R\text{-}V_0)$ is plotted versus the ligand concentration in the mobile phase [L] (eq. (5)).

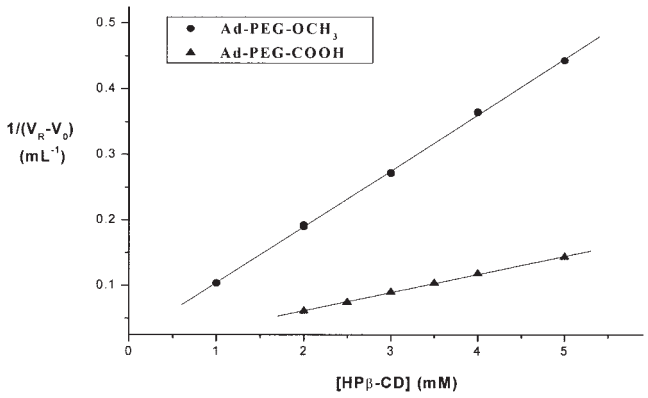

Figure 7 Plot of $1/(V_R\text{-}V_0)$ versus [HP β-CD] for Ad-PEG-OCH_3 and Ad-PEG-COOH (10^{-5} mol/L) injected onto a poly β-CD column (100 × 4.6 mm ID). Mobile phase: water or 20 m*M* Tris-HCl (pH = 7.0) for Ad-PEG-OCH_3 and Ad-PEG-COOH, respectively, containing HP β-CD. Flow rate: 1 mL/min. Injection volume: 20 μL. Detection at 276 nm.

$$\frac{1}{V_R - V_0} = \frac{1}{Q_X K_{AX}} + [L]\frac{K_{AL}}{Q_X K_{AX}} \qquad (5)$$

The association constant K_{AL} can be determined using this equation from the slope to ordinate intercept ratio.

This method was applied to Ad-PEG-COOH and to the reference polymer (Ad-PEG-OCH_3), using concentrations of HP β-CD from 1 to 5 mmol/L. Both polymers were injected at very low concentrations (10^{-5} mol/L). In the case of Ad-PEG-COOH, measurements were performed at pH = 7.0 (20 mmol/L Tris-HCl buffer) to study the influence of the negative charge on the formation of inclusion complexes. As reported in Figure 7, a linear relationship was observed for both polymers as $1/V_R\text{-}V_0$ was plotted versus [HP β-CD], showing that the chromatographic model could be applied. K_{AL} values were determined from the slope to ordinate intercept ratio (eq. (5)) for both the reference polymer and Ad-PEG-COOH. As expected for PEGs under investigation in this study (MW = 5000 g/mol), the formation of inclusion complexes with β-CD cavities was not hindered by the presence of a negative charge at the other end of the PEG chain, since the association constants were nearly the same (5200 and 4500 L/mol (±13%), respectively). Therefore, the immobilization of Ad-PEG-COOH layers onto β-CD-modified surfaces was examined, using SPR as a detection method.

Immobilization of Ad-PEG-COOH onto β-CD-modified gold chips: a SPR study

The procedure reported in a previous work for the functionalization of gold surfaces with β-CD cavities involved a copolymer of β-CD bearing amino groups (poly-β-CD-NH_2). Various polymers have been adsorbed on these interfaces, by formation of inclusion complexes. Particularly, hydrophobically end-capped, but chemically inert, PEG derivatives have been used as model polymers to assess the properties of resulting multilayers in terms of stability and regenerability.[3]

The purpose of the present study was to apply a similar immobilization procedure to form reactive PEG layers which permit the preparation of protein-based sensor chips. An allergen, β-lactoglobulin, was used as a test protein to examine the feasibility of the method. To avoid nonspecific interactions between the negatively charged protein and unreacted amino groups of poly-β-CD-NH_2, a monomeric derivative of β-CD carrying one amino group per cavity (β-CD-NH_2) was used in this work to functionalize gold chips with β-CD cavities. After reaction of MUA-modified surfaces with NHS, the coupling of β-CD-NH_2 to NHS-ester functions (Fig. 1) was carried out on the SPR instrument. As expected, the increase in reflectivity resulting from the binding of β-CD-NH_2 to the surface (ΔR between levels 1 and 3 in Fig. 8), $\Delta R \cong 0.01$, was lower, than values reported for poly-β-CD-NH_2 ($\Delta R \cong 0.06$[3]), since β-CD-NH_2 is a monomer (MW = 1150 g/mol) whereas poly-β-CD-NH_2 is a polymer (30,000 g/mol). However, the ratio of reflectivities of 6 is to be compared to a mass ratio of 26, indicating that the effective molar binding efficiency is 4.3 times higher in the case of the β-CD-NH_2 than in

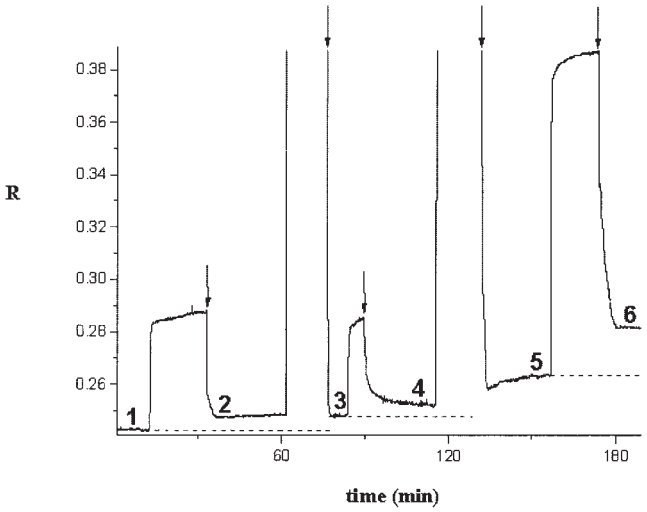

Figure 8 Sensorgram (reflectivity *R* versus time) illustrating the functionalization of the gold surface with β-CD cavities, the immobilization of Ad-PEG-COOH by formation of inclusion complexes, and the coupling of β-lactoglobulin. Step 1: coupling of β-CD-NH_2 (10 g/L in water) to MUA-functionalized gold surface. Step 2: deactivation of residual NHS-ester functions with 0.5 mol/L ethanolamine, pH = 7.8. Step 3: immobilization of Ad-PEG-COOH (1 g/L in water). Step 4: activation with NHS (0.05 mol/L in water) and EDC (0.2 mol/L in water). Step 5: coupling of β-lactoglobulin (0.2 g/L in PBS). Arrows indicate rinsing steps (water or PBS).

the case of the poly-β-CD-NH_2. This latter polymer is a copolymer of β-CD and epichlorohydrine,[19] which contains 12 β-CD cavities per chain.[3] So, it can be demonstrated that the β-CD concentration near the interface in the present work is 2.8 times lower than that in our previous procedure and is lower than other values reported in the literature.[20,21]

The next step concerned the adsorption of Ad-PEG-COOH onto β-CD-functionalized chips. As illustrated in Figure 8, an increase in reflectivity was observed after passing the aqueous polymer solution (1 g/L) for 7 min in the flow cell and rinsing with water ($\Delta R \cong 0.005$ between levels 3 and 4; same value for three different chips). This result confirms that the formation of inclusion complexes between PEG derivatives and β-CD cavities takes place with a rapid kinetics. Nevertheless, the increase in reflectivity was five to six times, instead of only 2.8, lower than values determined previously for the adsorption of a Ad-PEG-OCH_3 with the same molecular weight (MW = 5000 g/mol) onto poly-β-CD-NH_2-modified surfaces.[3]

It was demonstrated by HPLC that the formation of inclusion complexes with β-CD cavities was not significantly hindered by the presence of COOH groups at the other end of the PEG chain. Thereby, the lower increase in reflectivity observed with Ad-PEG-COOH when compared with results obtained previously with Ad-PEG-OCH_3 is probably due to the type of β-CD interface rather than to the PEG derivative itself. The higher binding capacity of polymeric β-CD layers is probably due to the topography of the interface, since cavities carried by a flexible polymer chain are more easily accessible to adamantyl-modified PEGs than cavities linked to stiff gold surfaces.

Coupling of β-lactoglobulin to Ad-PEG-COOH-modified gold chips

PEG-modified gold chips were evaluated for the binding of β-lactoglobulin (pI = 5.5) after activation of COOH functions with NHS and EDC. The coupling reaction was performed at pH = 7.4. In these conditions, residual carboxylic groups are negatively charged. So, nonspecific ionic interactions between the negative protein and the Ad-PEG-COOH-coated surface were avoided. As a proof, the reflectivity level was the same before and after passing, at pH = 7.4, a β-lactoglobulin solution on a non activated surface (data not shown). As illustrated in Figure 8, the increase in reflectivity measured after washing with water was between 0.018 and 0.02. Although coupling conditions were not optimal,[4] these results obtained for three different chips confirm the feasibility of the method.

CONCLUSIONS

It was demonstrated by affinity HPLC that the complexation constants between β-CD cavities and adamantyl-modified PEGs were similar for Ad-PEG-COOH and Ad-PEG-OCH_3 polymers. This result was taken as an advantage to immobilize biocompatible COOH-modified PEG layers onto gold chip according to a rapid procedure. The resulting sensing layers allowed the coupling of β-lactoglobulin, a negatively charged protein. It should be noticed that these PEG-based interfaces could be easily applied to the binding of other proteins. Moreover, the efficiency of the procedure in terms of binding capacity could be improved by using a longer spacer arm between β-CD cavities and the surface.

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