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Assemblies of dendrimers and proteins on carbon and gold electrodes

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Abstract

Dendritic macromolecules of two adjacent (G3.5 and G4) generations have been used to modify gold or carbon electrodes. The structure and stability of deposited films have been explored by quartz crystal microbalance (QCM), Surface Plasma Resonance (SPR) and electrochemistry. Dendrimers have been shown to adsorb spontaneously on electrode materials as compressed macromolecular films. They are able to inhibit (G3.5) or promote (G4) electroactive anionic species such as $Fe(CN)_6^{3-/4-}$ used as a probe system. Mixed protein/dendrimer assemblies have been constructed with proteins differing in charge, nature of the prosthetic groups and sizes such as lysozyme, cytochrome c, polyhemic cytochrome c_3 or glucose oxidase. Generally, the stability of adsorbed films seems to be limited to one dendrimer/protein bilayer. Owing to the satisfactory stability of composite cytochrome c_3/G3.5 or glucose oxidase/G4 films, biosensing applications are described for metal bioremediation and glucose detection, respectively.

Keywords: Dendrimer modified electrodes; QCM; Cyclic voltammetry; SPR; Cytochromes; LBL assemblies; Metal bioremediation

1. Introduction

A large variety of applications in analytical chemistry and molecular device technologies requires highly ordered, ultrathin coatings with surface properties controllable at the molecular level. Research in these fields is focused on supramolecular polymeric materials and abilities to form organized superstructure at the interfaces [1]. Synthetic or natural polyelectrolytes [2,3], alone or in combination with nanocomponents such as metal colloids [4], clays [5,6], silica [7], dendrimers [8] or other inorganic particles [9] provide a wide selection of materials that can be fit together into functional multilayers. Layer-by-layer (LBL) assembly is one of the most promising methods for thin film deposition [10,11]. It has been successfully applied to construct thin films where oppositely charged polyelectrolytes or charged particles including polymers and proteins or other biomolecules are involved in multilayer assemblies [12]. Such self-assemblies are governed by a delicate balance between adsorption/desorption equilibria [11].

Dendrimers [13,14] are promising candidates for such functional coatings. They constitute a unique class of materials with a cascade, tree-like architecture, having well-defined geometrical sizes and surface functionality. They possess three basic architectural components: a core, an interior of shells (generations) consisting of repeating branch-cell units, and terminal functional groups (Scheme 1).

Dendrimers are capable of self-organizing into superstructures at the interfaces [1,15]. These polymers distinguish from other synthetic macromolecules by their globular shape resulting from their perfectly highly branched architecture and their monodisperse nature. Their size, molecular weight, and chemical functionality can be easily controlled through the methods used for their preparation [13]. In this context, they possess a unique surface of multiple chain ends and the number of surface groups can be precisely controlled as a function of synthetic generation. Interestingly, dendrimers of adjacent generations bear oppositely charged surfaces at controlled pH, depending on the nature of carboxylic or amine surface groups, thus opening the way for techniques based on electrostatic self-assemblies [16]. The globular shape and multifunctional periphery make them well suited for the construction of

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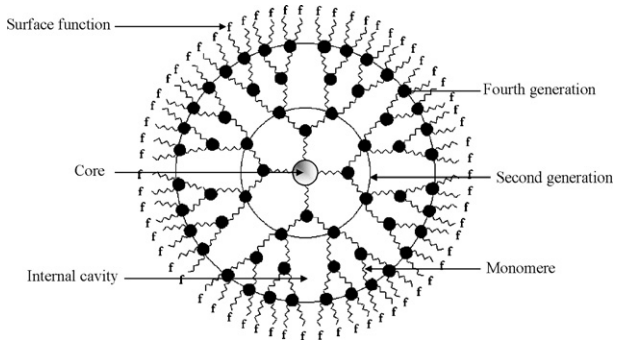

Scheme 1. Architectural components of a dendrimer.

multilayer nanocomposite materials. Because dendrimers provide multiple conjugation sites, a densely functionalized and structurally stable architecture can be easily obtained. Few studies are devoted to the behavior of dendrimers at the interfaces [8,17,18]. Only few examples of formation of multilayer self-assembled films from dendrimers have been reported [16]. A possibility is the exploitation of dendritic macromolecules as a building block for fabrication of composite supramolecular films with internal superstructure [19]. Nevertheless, several basic problems have to be solved in particular the possibility for dendrimer molecules of different generations to build alternating films via electrostatic interactions and the surface morphology and microstructure of multilayer dendrimer films.

The multilayered configuration based on dendrimers offers advantages for several applications including catalysis, encapsulation and controlled release, chemical and biological sensors, and biomimetic materials. Because of the interior void structure of dendrimers [20], the resulting multilayer film puts up only minimal diffusion restriction for analytes and electron-transferring substances ensuring desired enzymatic and electrocatalytic reaction over the whole range of multilayers. Dendrimer-modified electrodes have been used for the detection of halogenated organic acids [21] and for the construction of protein-modified electrodes [22–25].

Basing on their properties and physicochemical behavior [26], dendrimers have been referred to as "artificial proteins" [27,28]. For this reason, they exhibit a great interest to be used as protein mimicry for better understanding interactions in electron-carrier chains. Moreover, electroactive proteins in interaction with dendrimers offers a marked advantage as electrochemical probes for controlling the construction and extent of multilayer assemblies. We report here results on fabrication of self-assembled composite multilayer films built from dendrimers of adjacent generations (in this article G3.5 and G4) and from G3.5 or G4 dendrimers and polyions or proteins. Electrochemistry, Surface Plasma Resonance (SPR) and quartz crystal microgravimetry (QCM) have been used in this article for investigating the microstructure and morphology of dendrimer/polymer or protein hetero-assemblies. We report also on the possibility of constructing dendrimer/enzyme multilayers on electrode surfaces for use as a biosensing interface. The multilayer films were prepared by using G4 PAMAM or G3.5 PAMAM dendrimers, and redox proteins such as cytochrome c_3 or glucose oxidase.

2. Experimental section

2.1. Materials

Polyamidoamine (PAMAM) dendrimers (generations G3.5 and G4) were obtained from Aldrich as 10 wt.% solution in methanol and used without further purification. Freshly prepared 0.1% dendrimer solutions in Milli-Q water (corresponding to a concentration of 80 μg ml^{-1}) were used for all the deposition experiments. They were stored at 4 °C between experiments.

Molecular weights of dendritic macromolecules are 10,550 and 14,215 [16] for G3.5 and G4 dendrimers, respectively. G3.5 and G4 dendrimers possess surface carboxylic or amine groups, respectively. These surface groups are able to dissociate or to be protonated in aqueous solutions and thus dendrimer macromolecules gain either negative or positive net charges. For this reason, G3.5 dendrimer solutions were alkalinized before to be used and G4 dendrimer solutions were acidified [16]. The number of charged surface groups is 64 for the two dendrimers.

When necessary, gold electrodes were modified with sodium 3-mercapto 1-propanesulfonate (MPS, Aldrich) or cystamine dihydrochloride (Aldrich). 4-aminobenzoic acid (4-ABA) used for modifying glassy carbon surface was purchased from Aldrich.

Lysozyme from chicken egg white was obtained from Sigma. Horse heart cytochrome c (type VI) from Sigma was purified by chromatography on carboxymethylcellulose (CM-52, Whatman). Cytochrome c_3 from *Desulfovibrio vulgaris* Hildenborough (*Dv*H) was prepared and purified in our laboratory as previously described [29]. Glucose oxidase from *Aspergillus niger* was purchased from Sigma. All the other chemicals were of reagent grade.

2.2. Apparatus

Cyclic voltammetry (CV) and square-wave voltammetry (SWV) were obtained using an EG and G 6310 Electrochemical Impedance Analyzer modulated by EG and G PAR M 270/250 software. SWV voltammograms were performed using 2 Hz as the square-wave frequency, 2 mV as the scan increment, and 25 mV as the pulse height amplitude. A three-electrode system consisting of a Metrohm Ag/AgCl/NaCl (sat.) reference electrode, a gold wire auxiliary electrode, and the working electrode was used throughout. Working electrodes were constructed from 1-mm diameter gold wire, or 4-mm diameter rod of pyrolytic graphite (PG) (Le Carbone Lorraine, Paris), or 3-mm diameter rod of glassy carbon (GC) (Tokay) inserted in resin casings. Working electrodes were first polished with ultrafine emery paper, and then with 0.05 μm alumina slurry. Unless otherwise specified, all reported potentials refer to the Ag/AgCl/NaCl (sat.) reference electrode. Potentials versus the standard hydrogen electrode (SHE) can be obtained by adding 210 mV. Prior each experiment, the solutions were deoxygenated by bubbling high-purity nitrogen. All experiments were carried out at room temperature (about 23 °C) under a high-purity nitrogen atmosphere.

QCM studies were performed using a MAXTEK PM-710 plating monitor coupled with a MPS-50 sensor probe. The MAXTEK quartz resonators were made from AT-cut quartz crystals (resonance frequency, 5 MHz) covered by evaporated gold on both faces (apparent electrode area of 0.316 cm^2). Quartz resonators were used without any washing/polishing/electrochemical pretreatment. For QCM measurements, one side of the resonator was covered with the dendrimer or protein solution of interest for a selected period, washed with water or buffer solution, dried with a hair-drier (without warming) and the frequency was measured. The mass increase resulting from adsorption onto the resonator surface was estimated from the Sauerbrey equation [30] using the following relationship between adsorbed mass ΔM (ng) and frequency shift ΔF (Hz), by taking into account the characteristics of the resonator:

$$\Delta M = -17.6 A \Delta F$$

where A corresponds to the apparent area of the quartz microbalance electrode. In these conditions, a decrease in frequency of 1 Hz results from a mass increase of 17.6 ng cm^{-2} provided that shifts in frequency can be ascribed exclusively to mass effects and not to changes in solution density or viscosity [31]. The thickness d (nm) of the deposited film (adsorbed on one side of the resonator) can be estimated from the relationship $d = 0.176\ \Delta F/\rho$ where ρ (g cm^{-3}) is the density of the deposited film. The values of ρ currently used are 1.2 g cm^{-3} for polymer films and 1.3 g cm^{-3} for protein films [32,33].

A BIAcore 1000 was used for SPR measurements. A clean BIAcore Au sensorchip was mounted into the SPR flow system. The solutions of dendrimers and proteins were successively pumped through the cell for 15 min each, with 15 min of buffer between, using a flow rate of 1 μl/min. The sensorgram resulting from changes in the resonance signal was recorded.

2.3. Preparation of the modified electrodes and film assembly

Gold electrode surfaces used for voltammetry experiments were polished successively with emery paper and 0.05 μm alumina slurry to obtain a mirror finish, and then sonicated in a water bath. Modification of the gold surface was accomplished by dipping the polished electrode in 1 mM MPS ethanolic solution for 15 h [3] followed by rinsing in pure ethanol and water (MPS-modification generating a negatively charged surface) or in 10 mM cystamine dihydrochloride for 1 h followed by rinsing in water (cystamine-modification generating a positively charged surface) [23]. The modification of GC surfaces was based on the electrochemical oxidation of amine-containing compounds (here 4-ABA) which produces a covalent linkage between the nitrogen atom of the amine and carbon sites of the carbon surface [32]. The polished electrode was introduced to a 3 mM 4-ABA + 0.1 M $LiClO_4$ solution in ethanol, and scanned over the range [+0.40 − +0.93 V] at 10 mV s^{-1} for 5 min (about 3 scans). Deprotonation of the 4-ABA provided a negatively charged surface [34].

After the formation of a positively (or negatively) charged precursor film on the electrode surface, LBL deposition based on electrostatic attractions was utilized to build up multimolecular films from dendritic macromolecules and protein molecules. Each immersion was performed for 20 min, then the electrode surface (electrochemical experiments) or the resonator surface (QCM experiments) was rinsed with water and dried.

3. Results and discussion

3.1. Formation of dendrimer layers on polished electrode surfaces

The adsorption of dendrimers (which are non electroactive compounds) on electrode surfaces has been monitored indirectly by detecting the extent of the electrochemical response of $Fe(CN)_6^{3-/4-}$ couple chosen as an electroactive test probe. It has been shown in previous works [35–38] that ferricyanide electrochemistry is noticeably affected by specific surface interactions which can be further influenced by surface modification. In particular, the electrochemical behavior of such an anionic system can be either promoted or inhibited through positively or negatively charged species, respectively. An additional advantage is that dendrimer surfaces are very porous toward the $Fe(CN)_6^{3-/4-}$ couple as has been established from previous electrochemical ac-impedance measurements [39].

Polished gold electrode was treated for 10 min with a 0.1% G3.5 (or G4) dendrimer aqueous solution and then transferred to the electrochemical cell containing 1 mM ferricyanide in 10 mM Tris chloride buffer, pH 7.6. Typical CV curves obtained for a polished untreated, and G3.5- or G4-treated gold electrode, respectively, are shown in Fig. 1.

$Fe(CN)_6^{3-/4-}$ behaves as a moderately reversible electrochemical system at the polished gold electrode as currently observed in such experimental conditions, exhibiting a peak separation of 168 mV. The reversibility is poorer at the G3.5-modified electrode (peak separation, 190 mV). In contrast, when using the G4-modified electrode, the peak separation is decreased to 65 mV, which is consistent with a virtually reversible, one-electron transfer as it can be expected for $Fe(CN)_6^{3-/4-}$ couple. Such results explain on the basis of spontaneous adsorption of dendrimers onto the gold surface generating unfavorable/

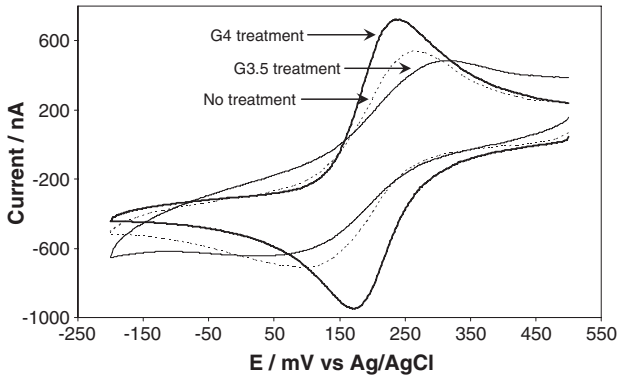

Fig. 1. Cyclic voltammograms of 1 mM $Fe(CN)_6^{3-/4-}$ in 10 mM Tris chloride buffer, pH 7.6 at a gold electrode: without pre-treatment (dotted line), after a G4 dendrimer treatment (bold line), after a G3.5 dendrimer treatment (fine line). Scan rate, 20 mV s^{-1}.

favorable electrostatic interactions between the negatively charged $Fe(CN)_6^{3-/4-}$ couple and the thus-modified electrode. When using the G3.5 dendrimer-treated electrode, the electrochemical system becomes slower because of the presence of negatively charged material at the gold surface. In contrast, positively charged G4 dendrimer "promotes" the response of $Fe(CN)_6^{3-/4-}$. The kinetic of the G4 dendrimer adsorption onto gold electrode surface has been studied by monitoring the dependence of the CV peak currents on the time of treatment by dendrimer. A rapid increase is noted during the first minute of treatment and then a leveling off is attained after about 10 min. Such a profile corresponding to the progressive adsorption of dendrimers on the gold surface was confirmed from SPR measurements. In consequence, all the depositions of dendrimer in this work were done using 10 min of treatment time.

Our experimental results agree with previous data on the spontaneous formation of dendrimer self-assembled monolayer on gold [39,40]. It has been assumed that a strong van der Waals interaction with the gold surface takes place as a result from the high surface area of dendrimer molecules like in the case of polymers. Most importantly, a large number of amine groups are able to adsorb to the gold surface, and, by analogy to polydentate metal–ion ligand interaction, stabilize the dendrimer layer [39]. As shown above, when a gold surface has been modified with G3.5 dendrimer, a marked inhibition of the $Fe(CN)_6^{3-/4-}$ couple is observed. Inhibition is abolished and CV peaks are fully restored through a subsequent treatment with G4 dendrimer thus reflecting an inversion for the overall surface charge. Actually, two possible fates of surface G3.5 dendrimer can occur on exposure to a solution of G4 dendrimer: the negatively charged dendrimer is either stripped off the surface which becomes free of any adsorbed material or adds to oppositely charged G4 to form an adsorbed mixed G3.5/G4 bilayer. QCM measurements have been able to settle in favor of a LBL buildup based on G3.5/G4 combination because of the linear decrease in frequency observed upon each surface treatment. Accordingly, SPR experiments proved the formation of multilayers consisting of negatively charged G3.5 and positively charged G4 dendrimer. Indeed, when the gold chip was flushed sequentially with G3.5 and G4 dendrimer solutions, intermitted by running buffer, the resonance signal increased at each alternating deposition. LBL deposition of oppositely charged polyions on dendrimer layer platform can be also performed: thus, the G4-promoted electrochemistry of ferricyanide is inhibited through the treatment of the G4-modified gold surface with a polyanion (e.g. poly(styrenesulfonate)) but is restored after a further treatment with a polycation (e.g. poly(ethyleneimine)).

Similar results have been gained when working at dendrimer-modified PG or GC electrodes. Dendrimers are hydrophilic and carbon electrode surfaces possess (especially after polishing with alumina) hydrophilic areas with relatively high densities of C–O functional groups. Even in the absence of favorable electrostatic interactions, adsorption can occur provided the dendrimer molecules are able to adopt a sufficiently distorted conformation. Actually, it has been shown that it was possible to distort the dendrimers to different extents, especially those of the lowest generations which exhibit a higher surface mobility [39] and a flexible structure [20].

3.2. Formation of dendrimer layers on pre-treated electrode surfaces

An alternative for preparing dendrimer self-assembled monolayers is to use functionalized electrode surfaces. Such "pre-treated" surfaces are expected to combine robustness and organization of covalently bound species with the specific properties of dendrimers [21].

Gold substrate was modified using MPS (as described in Section 2.3) to generate a negatively charged metal surface. Positively charged G4 dendrimer was expected to be retained on the MPS-modified surface through favorable electrostatic interactions. Typical CV curves obtained after such modifications are given in Fig. 2 for a 1 mM ferricyanide solution.

When using the MPS-modified gold electrode, a strong inhibition of the electrochemical couple is observed. In contrast, the system becomes quasi-reversible (peak separation, 84 mV) at the G4/MPS-modified electrode, thus denoting the presence of G4 dendrimer at the electrode surface. Subsequently, layer-by-layer depositions of oppositely charged G3.5 and G4 dendrimers were carried out to build multilayer assemblies. QCM technique was used concomitantly with electrochemical approach to control the film construction. Results are shown in Figs. 2 and 3. When the G4/MPS-modified electrode was treated with a G3.5 dendrimer solution, $Fe(CN)_6^{3-/4-}$ couple became slower (peak separation, 176 mV), thus suggesting that negatively charged G3.5 dendrimer has been adsorbed on the subjacent G4 dendrimer layer. This result can be validated from QCM measurement which shows a linear decrease in frequency after the G3.5 dendrimer deposition (Fig. 3).

Gold substrate was also modified by using cystamine (see Section 2.3) to generate a positively charged surface. In this case, G3.5 adsorbs on the pre-deposited cystamine layer as established from electrochemical and QCM measurements. Nevertheless, multilayers deposited on the cystamine/G3.5/G4 assembly become instable when the number of layers is tentatively increased (>3).

4-ABA has been used to modify glassy carbon substrate (as described in Section 2.3) thus generating a negatively charged electrode surface onto which positively charged G4 dendrimer could be deposited. A fairly reversible CV response is observed

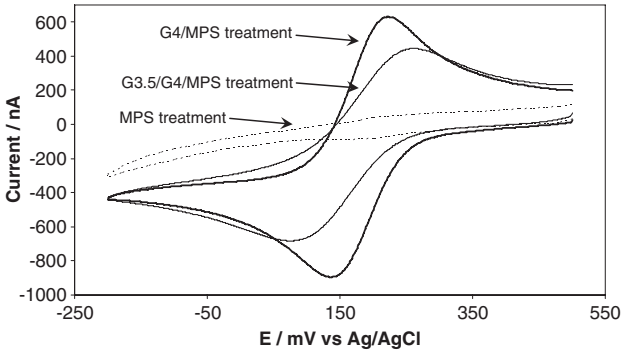

Fig. 2. Cyclic voltammograms of 1 mM $Fe(CN)_6^{3-/4-}$ in 10 mM Tris chloride buffer, pH 7.6 at a gold electrode: after a MPS treatment (dotted line), after a G4 dendrimer/MPS treatment (bold line), after successive G3.5/G4 dendrimer/MPS treatments (fine line). Scan rate, 20 mV s^{-1}.

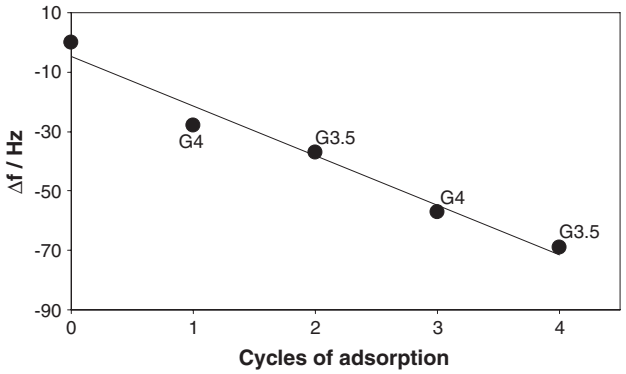

Fig. 3. Dependence of the QCM frequency shift on the number of alternate adsorption cycles from G4 and G3.5 dendrimer solutions. The zero is arbitrary.

at the polished untreated GC electrode for $Fe(CN)_6^{3-/4-}$ couple (peak separation, 162 mV) (Fig. 4).

After the CG surface has been submitted to the 4-ABA treatment, the electrochemical response of $Fe(CN)_6^{3-/4-}$ couple becomes totally inhibited (because of unfavorable electrostatic interactions between the modified electrode surface and ferrocyanide anions). When the 4-ABA-modified electrode is treated with G4 dendrimer, reversibility is partially restored, and a subsequent treatment with G3.5 dendrimer leads to virtually inhibited system.

3.3. Structure of dendrimer layers

The strategy based on the direct adsorption of dendrimers on electrode surfaces appears as simple and versatile, and, in fact, it does not need pre-treatments. Stable layers can be deposited via polydentate interactions with gold and other materials on electrode surfaces. Subsequent LBL deposition can be performed either with oppositely charged dendrimers or with mixed dendrimer/polyions (e.g. PSS or PEI). QCM measurements have been used to quantify the dendrimer coverage and estimate the thickness of dendrimer layers. Decreases in frequency are of -15 ± 2 Hz for G4 and G3.5 dendrimer, corresponding to a thickness of 2.2 ± 0.3 nm, in good agreement with previous data (2.8 ± 0.2 nm) obtained from ellipsometric measurements [39]. It has been concluded that dendrimer monolayers are easily prepared by immersing a gold substrate in a dendrimer solution. Considering the bulk dimension of G3.5 and G4 dendrimer (about 4.5 nm for the bulk-phase diameter) [20], it is suggested that adsorbed dendrimers are not spherical (as they are in aqueous solution), but rather compressed against the gold surface in an oblate configuration [41], in agreement with results obtained from other approaches [42–44]. It is a consequence of the relative flexibility of G3.5 and G4 dendrimer molecules. Screening and barrier properties of dendrimer layers in aqueous electrolyte solutions have been examined in previous works from impedance analyses [39]. In the case of the $Fe(CN)_6^{3-/4-}$ couple, the dominance of the Warburg impedance has indicated that the redox reaction remained diffusion-controlled for G4 dendrimer coatings. $Fe(CN)_6^{3-/4-}$ anions can easily penetrate the surface-confined dendrimers as well as the interstices between them. Nevertheless, the reversibility of the $Fe(CN)_6^{3-/4-}$ electrochemical system is strongly dependent (i.e. promoted or inhibited) upon the presence of positively or negatively deposited dendrimers. On the contrary, we have observed from CV experiments with $Ru(NH_3)_6^{3+}$ and methylviologen (MV^{2+}) that none of these redox systems was affected in peak heights and potential values by the presence of adsorbed dendrimers, even when up to five alternate successive G4/G3.5 dendrimer layers were assembled on the electrode surface. It can be concluded that the resistance to diffusion resulting from the presence of G3.5/G4 dendrimer coatings is negligible.

Evidence that dendrimers remain adsorbed even in very negative potential range has been obtained indirectly by examining the behavior of a strongly adsorbed electroactive compound, MV (i.e. the fully reduced form of MV^{2+}) at dendrimer-modified PG electrodes. The oxidation CV peak of MV

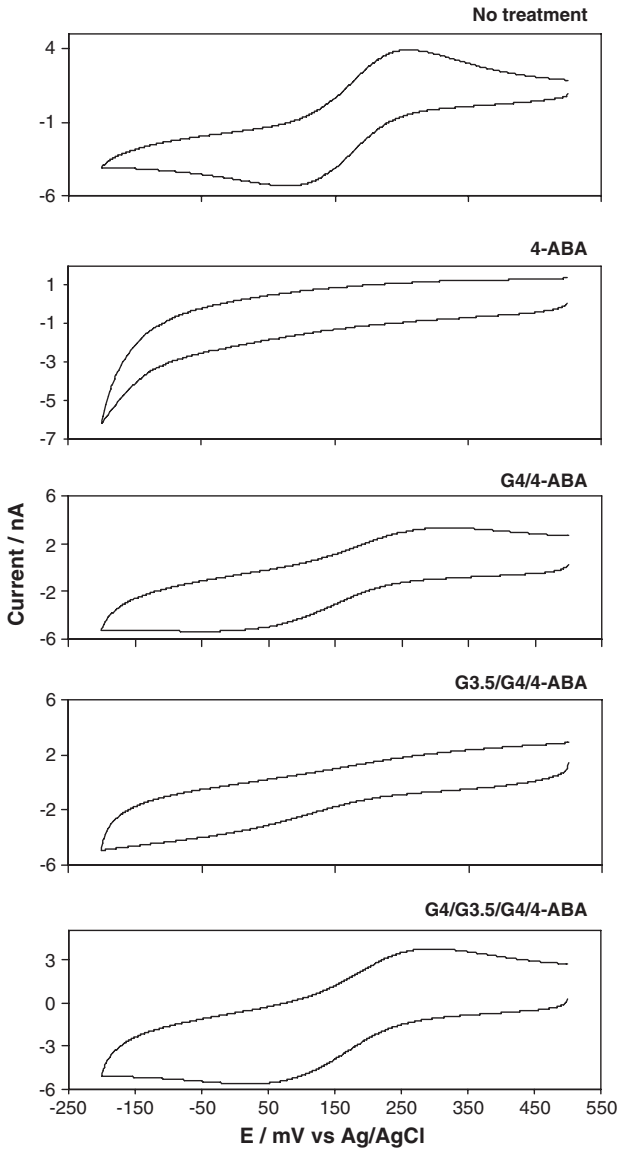

Fig. 4. Cyclic voltammograms of 1 mM $Fe(CN)_6^{3-/4-}$ in 10 mM Tris chloride buffer, pH 7.6 at a glassy carbon electrode successively from up to down schemes: without pre-treatment, after a 4-ABA treatment; then, the 4-ABA modified electrode is successively treated with G4 dendrimer, then with G3.5 dendrimer, and then with G4 dendrimer. Scan rate, 20 mV s^{-1}.

exhibits a spike-shaped profile characteristic of adsorption phenomena at polished PG electrodes. The spike profile is abolished to rounded peak at G3.5 or G4 dendrimer modified electrodes thus suggesting that stable pre-coating has been formed on the electrode surface.

Results confirm the significant role of electrostatic interactions in the formation of compact layer-by-layer molecular structures. In both cases, association based on electrostatic compatibility is obtained. It can be assumed as in the case of multilayer dendrimer films [16] that molecules do not preserve their shape. In fact, screening of electrostatic interactions between adjacent dendrimer layers can be observed only by placing low molar weight intermediates between them.

3.4. Formation of dendrimer/protein assemblies

Because of their size-scaling properties and physicochemical behavior, dendrimers have been used as globular protein replacements [45]. Highly branched dendrimer macromolecules are of great interest in the assembly of thin films. For these reasons, it is important to study interactions between dendrimers and "true" protein molecules and the stability of composite dendrimer/protein assemblies. As a consequence, using dendrimer/enzyme multilayered films can be useful for biosensing applications. We have investigated here the construction of several types of proteins as "participants" in film assemblies, differing in global charge, nature of the prosthetic group and size, i.e. non-electroactive lysozyme, electroactive mono- and multihemic cytochromes (cytochrome c and cytochrome c_3), and glucose oxidase.

3.4.1. Lysozyme and G3.5 dendrimer assembly

Lysozyme is a rather compact protein (MW ~ 14,000) with a high isoelectric point (pI=10.5–11). The molecule carries a net charge of +9 at pH 5.6. It is a good candidate to be associated with negatively charged G3.5 dendrimer. Because of the non-electroactivity of lysozyme, film assemblies have been monitored from QCM measurements, using 0.05 mg ml^{-1} lysozyme solution and 80 μg ml^{-1} G3.5 dendrimer solution, respectively. Instability was observed at the MPS-modified electrode for successive MPS/lysozyme/G3.5 treatments. In contrast, reproducible and "stable" results were obtained when using a cystamine pre-treated gold electrode subsequently modified with G3.5 dendrimer and lysozyme through alternate depositions. In this case, QCM monitoring shows a relatively good linearity for changes in frequency with the number of adsorption steps (Fig. 5), suggesting a linear increase in film mass with increasing number of layers.

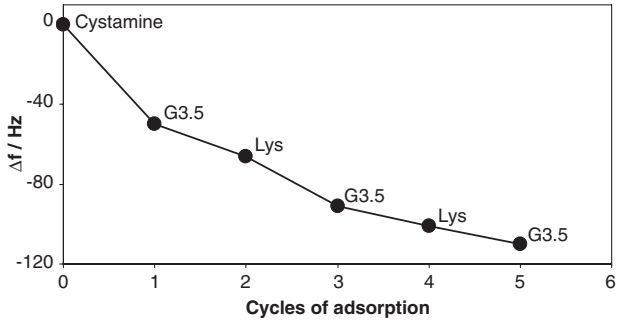

Fig. 5. Dependence of the QCM frequency shift on the number of alternate adsorption cycles on a cystamine pre-treated gold resonator from G3.5 dendrimer and lysozyme solutions. The zero is arbitrary.

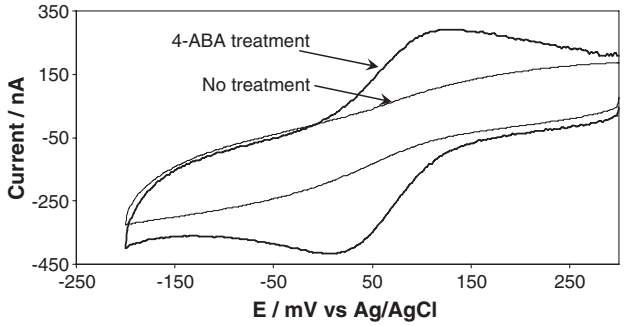

Fig. 6. Cyclic voltammograms of 160 μM cytochrome c in 10 mM Tris chloride at a glassy carbon electrode: in the absence of pre-treatment (fine line), after a 4-ABA treatment (bold line). Scan rate, 20 mV s^{-1}.

For every layer of adsorbed protein, the decrease in QCM frequency is of − 15±2 Hz. This change corresponds to a film mass increase of 264 ng cm^{-2} and a 2.2±0.3 nm thickness for each deposited lysozyme layer. Taking into account crystallographic data on the lysozyme molecule (3×3×4.5 nm) [46], it could be concluded that either sub-monolayers are fixed at each step of the assembly process or protein molecules adopt a more flattened conformation as it is the case for dendrimer molecules (see Section 3.3). In previous study on the adsorption of lysozyme [47], it was suggested that no large structural changes occurred when the protein was deposited onto negatively charged mica surfaces. The same conclusion was attained when studying AQ-29D polymer/lysozyme layers deposited on a gold surface [48], probably because of the flexibility of AQ-29D polymer chains, in contrast with dendrimers which have a more rigid conformation.

3.4.2. Cytochrome c and G3.5 dendrimer assembly

The surface of the GC electrode was first modified with 4-ABA (as described in Section 2.3 [34]) to create a negatively charged interface. Cytochrome c, a highly positively charged protein at neutral pH (isoelectric point pI=10.5), is expected to favorably adsorb on the electrode surface and thus to provide a new positively charged surface for further adsorption of negatively charged G3.5 dendrimer. As shown in Fig. 6, no CV signals are observed for a 160 μM cytochrome c solution when using a polished untreated GC electrode.

The presence of cytochrome c is revealed from well-shaped CV peaks at E_{pc}=+17 mV, E_{pa}=+109 mV (which correspond to a midpoint potential of +63 mV, i.e. +273 mV when referred to the SHE) when using a 4-ABA-modified GC electrode owing to favorable electrostatic interactions between the electrode surface and the protein. Film-transfer experiments performed using more sensitive SWV technique show (from the presence of a peak at E_p=+54 mV, i.e. +264 mV versus the SHE, curves not given) that cytochrome c remains attached to the electrode surface even when the working electrode is transferred into a pure buffer solution. In a second step, the cytochrome c/4-ABA-modified GC electrode

was treated with G3.5 dendrimer solutions. Unfortunately, very poor and unstable signals were observed after this treatment.

Spontaneously adsorbed G3.5 dendrimer (see Section 3.1) was also used to modify polished gold or PG electrodes and thus to provide negatively charged surfaces capable of fixing cytochrome c. Though it has been established that cytochrome c adsorbs on a G3.5 dendrimer layer (from (i) the inhibition of the electrochemical response of $Fe(CN)_6^{3-/4-}$ couple when a G3.5-modified PG or gold electrode has been treated with a cytochrome c solution, (ii) decreases in QCM frequency for a G3.5 dendrimer-modified resonator treated with cytochrome c, and (iii) SPR measurements), no stable assemblies were obtained upon repetition of the treatment. Moreover, no "promotion" of the electrochemical response of cytochrome c was detected at an electrode modified with G3.5 dendrimer contrary to what it could be expected when modifying the electrode surface with a negatively charged species. Similarly, additional experiments have established that no promoted response was observed for spinach ferredoxin (a highly acid protein, pI ~ 3.5) at G4 dendrimer-modified electrodes. It can be assumed that the dendrimer framework is cumbersome enough to exert a shielding effect toward the electron transfer between the electrode and the redox center of a protein. Reinforcing this hypothesis, it has been established in previous work [49] on redox-active dendrimers that an incomplete sampling of pendant redox sites occurs especially in the case of the largest molecules (G4 dendrimer).

In consequence, it was not possible to construct stable assemblies containing sandwiched electrochemically active cytochrome c layers.

3.4.3. Cytochrome c_3 and G3.5 dendrimer assembly

Cytochrome c_3 from *Desulfovibrio vulgaris* Hildenborough (*Dv*H) is a highly basic (isoelectric point pI=10.5) tetrahemec-type cytochrome (MW ~ 15,000) characterized by non-identical low redox potentials ranging from −400 to −165 mV (versus a standard hydrogen electrode) [50]. Because of its basic character, cytochrome c_3 is positively charged at neutral pH; the net charges of the molecule are +8 and +4 for the oxidized and reduced forms, respectively. As in the case of cytochrome c, generating negatively charged surfaces could serve as platforms for building up mixed cytochrome c_3/G3.5 dendrimer assemblies. For this reason, GC surface was first modified with 4-ABA (as previously described) and then submitted for 15 min to alternate treatments with (20 μM) cytochrome c_3 or G3.5 dendrimer solutions. After each treatment, the modified electrode was transferred into 10 mM Tris chloride buffer solution at pH 7.6 before CV scanning. From the presence of a quasi-reversible couple at E_{pc}=−535 mV, E_{pa}=−516 mV corresponding to the immobilized protein [51], CV curves in Fig. 7 show that cytochrome c_3 is able to attach to the 4-ABA-modified GC surface, but a major amount of protein seems to be pulled off from the electrode surface after a new treatment with G3.5 dendrimer.

In another approach, a gold surface was first modified with G3.5 dendrimer (see Section 3.1) to display a negatively charged surface. A stable and persistent adsorbed bilayer was obtained when the G3.5 dendrimer-modified electrode was

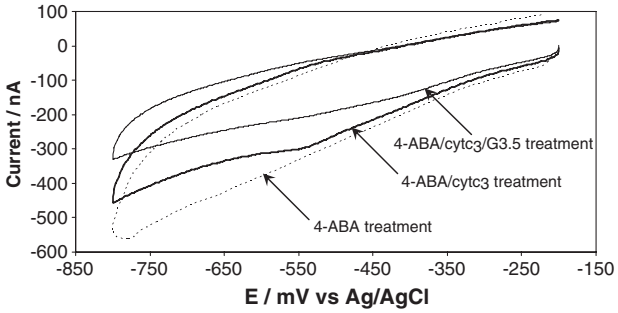

Fig. 7. Cyclic voltammograms at a 4-ABA modified glassy carbon electrode transferred into 10 mM Tris chloride buffer at pH 7.6: after a treatment with a 20 μM cytochrome c_3 solution (bold line), after successive treatments with cytochrome c_3 and then G3.5 dendrimer (fine line). The dotted fine line was obtained for the simple 4-ABA treated GC electrode. Scan rate, 10 mV s^{-1}.

treated with cytochrome c_3. Nevertheless, when repeating the G3.5 dendrimer/cytochrome c_3 treatment, destabilization of the adsorbed layers was observed. Thus, stable G3.5 dendrimer/cytochrome c_3 assembly seems to be limited to only one bilayer.

As has been established from CV and SWV experiments (one characteristic CV curve is given in Fig. 8A), the electrochemical response of cytochrome c_3 is maintained at either G3.5- or G4 dendrimer-modified gold electrode in contrast with the total inhibition observed for cytochrome c in the same conditions. Simply, a decrease of about 20% in CV peak heights is detected at modified electrodes. Since no interruption in electron transfer to/from the dendrimer-modified electrode is observed for cytochrome c_3, it is suggested that the differences in the behavior of both hemic proteins stem from the distance between the electrode surface and the redox sites. Cytochrome c has only one electroactive active site which is supposed to be far enough from the electrode surface to be able to exchange electrons. In contrast, cytochrome c_3 possesses four hemes relatively exposed to the external solution, and possibly sufficiently close to the electrode to participate to the electron transfers.

Owing to the good stability of the assembled composite bilayer on gold surface, the mixed dendrimer/cytochrome c_3 configuration has been examined from the viewpoint of biosensing application. It was established in previous works [52–54] that polyheme c-type cytochromes exhibit metal-reductase properties. The performance and efficiency of modified electrodes have been evaluated and compared in the presence of a soluble Fe(III) complex, ammonium Fe(III) citrate, acting as the soluble substrate [55]. The same test-system has been selected here for probing the biosensing properties of dendrimer/cytochrome c_3 assembly. A polished gold electrode was modified successively with G3.5 dendrimer and then cytochrome c_3. After the bilayer assembly was prepared, the modified electrode was dipped into 10 mM Tris chloride solution at pH 7.6. Typical CV curve is shown in Fig. 8A (dotted line).

Increasing concentrations of a stock solution of Fe(III) citrate complex were added into the electrochemical cell and the corresponding CV curves were recorded. Typical cyclic voltammogram in the presence of Fe(III) is given in Fig. 8A (solid line). The shape of the voltammogram clearly shows that the reduction of Fe(III) catalyzed by cytochrome c_3 occurs. Increasing catalytic

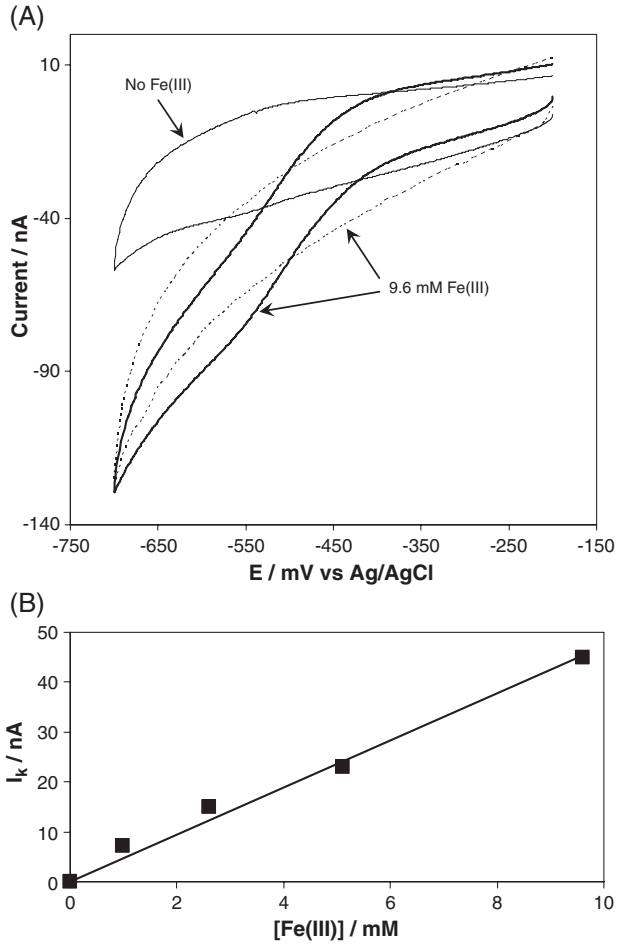

Fig. 8. (A) Cyclic voltammograms in 10 mM Tris chloride buffer, pH 7.6 at a G3.5 dendrimer/cytochrome c_3 modified gold electrode: in the absence of Fe(III) citrate complex (fine line), in the presence of 9.6 mM Fe(III) citrate complex (bold line). The dotted line was obtained at the untreated gold electrode in the presence of 9.6 mM Fe(III) citrate complex. (B) catalytic current (I_k) dependence on Fe(III) citrate complex concentration. Scan rate, 5 mV s^{-1}.

currents have been shown to develop in the 0–10 mM concentration range investigated in this work (Fig. 8B). Catalytic current remains unchanged after 2 days (the electrode was stored in 10 mM Tris chloride buffer pH 7.6 in the dark). The stability observed for the G3.5 dendrimer/cytochrome c_3-modified gold electrode compares advantageously with previous data gained using a LBL-modified gold electrode based on the assembly of cytochrome c_3 and poly(styrenesulfonate) which was destabilized after about 1 h [55].

3.4.4. Glucose oxidase and G4 dendrimer assembly

Multilayered films prepared from G4 dendrimer and glucose oxidase (GOx) were assembled on a polished gold surface basing on the layer-by-layer technique. The first step of the construction of the dendrimer/enzyme assembly consisted in the spontaneous adsorption of G4 dendrimer on a polished gold electrode (see Section 3.1). Then, favorable electrostatic conditions were offered to negatively charged GOx (p*I* 4.05 [56]) to adsorb onto the thus-formed G4 dendrimer layer. After the formation of the bilayer assembly, the catalytic activity of the immobilized enzyme was assayed in a solution of 0.37 mM ferrocene methanol (FcMeOH), used as a redox mediator, in 0.05 M phosphate buffer, pH 8.0, in the presence of glucose. Typical cyclic voltammograms obtained after the treatment of a gold electrode successively with G4 dendrimer or (16 mg ml^{-1}) GOx solutions in the absence and in the presence of 0.45 M glucose are shown in Fig. 9A.

In the absence of glucose, the quasi-reversible reduction/reoxidation of FcMeOH is observed at E_{pc}=+197 mV, E_{pa}=+276 mV. We have verified that the immobilized bilayer had no effect on the electrochemical reversibility of the mediator. A catalytic anodic current is detected after the addition of glucose (0.45 M glucose for the CV curve given in Fig. 9A). The catalytic current (i_{cat}) increases upon increasing glucose concentration in the range 0–0.20 M (Fig. 9B). The stability of the bilayer G4 dendrimer/enzyme modified electrode was evaluated from day-by-day calibrations using reproducible experimental conditions (as those given above). When not in use, the modified electrode was stored in 10 mM Tris chloride buffer, pH 7.6, in the dark at 4 °C. Decrease in catalytic current was noted over 1 day (probably because of the release of poorly retained enzyme), but then the response of the modified electrode was found to be maintained over at least 1 week. Using the rate constants of the catalytic activity of glucose oxidase either in homogeneous

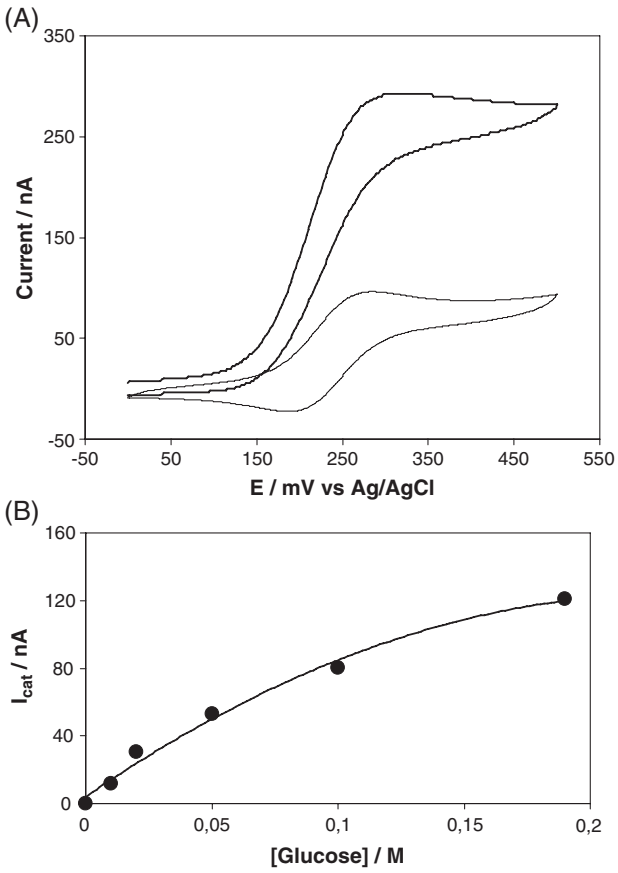

Fig. 9. (A) Cyclic voltammograms at a G4 dendrimer/GOx modified gold electrode of 0.37 mM ferrocene methanol in 0.05 M phosphate buffer, pH 8.0, in the absence (fine line) and in the presence (bold line) of 0.45 M glucose. Scan rate, 2 mV s^{-1}. (B) Dependence of the catalytic current on the glucose concentration.

solution or immobilized as evaluated in previous works [57,58], the surface concentration of active enzyme Γ_E was estimated to be 0.4×10^{-12} mol cm^{-2}. This is a value noticeably lower than that (1.8×10^{-12} mol cm^{-2}) measured in previous work [23] using multilayered assemblies of dendrimer and IO_4^- modified glucose oxidase on a cystamine pretreated gold surface. An estimation of the concentration of glucose oxidase immobilized on a gold surface pretreated with G4 dendrimer has been obtained from QCM measurements. The values of 2.6×10^{-12} mol cm^{-2} for the surface coverage by glucose oxidase and 3.5 nm for the layer thickness have been calculated from the decrease ($\Delta F=-24$ Hz) in frequency. This can be compared with the expected value for a close packed monolayer of glucose oxidase (4.7×10^{-12} mol cm^{-2}) and the average Stokes radius (4.3 nm) based on the known dimensions of the molecule [59,60]. The estimate for Γ_E obtained from electrochemical measurements (0.4×10^{-12} mol cm^{-2}) is significantly lower than the value of coverage obtained from QCM data and particularly than the value corresponding to a compact film. It is concluded that the active glucose oxidase layer behaves as a sub-enzyme monolayer, either because the electrode surface is not totally occupied or/and because a fraction of the enzyme molecules on the surface is inactive or not properly oriented in the presence of dendrimer due to conformational factors. Similar conclusions have been suggested in previous works on other LBL assemblies containing GOx [23,61].

The stable G4 dendrimer/GOx bilayer attached to the gold surface constitutes a platform for further step-by-step building up of a multi-bilayer assembly. The formation and stability of the deposited layers can be monitored concomitantly from QCM measurements and from the electrochemical detection of the catalytic current triggered by the immobilized glucose oxidase. The dependence of the catalytic current on the number of deposited bilayers is given in Fig. 10A.

When a second bilayer is formed from the successive depositions of G4 dendrimer and glucose oxidase on the first G4/GOx bilayer, the catalytic current increases linearly in accordance with the doubling of the amount of intercalated enzyme. However, when trying to carry on deposition of subsequent layers, linearity is not any more obeyed. The same observation can be made from QCM data in Fig. 10B. Departure from linearity is noted after the fourth adsorption cycle.

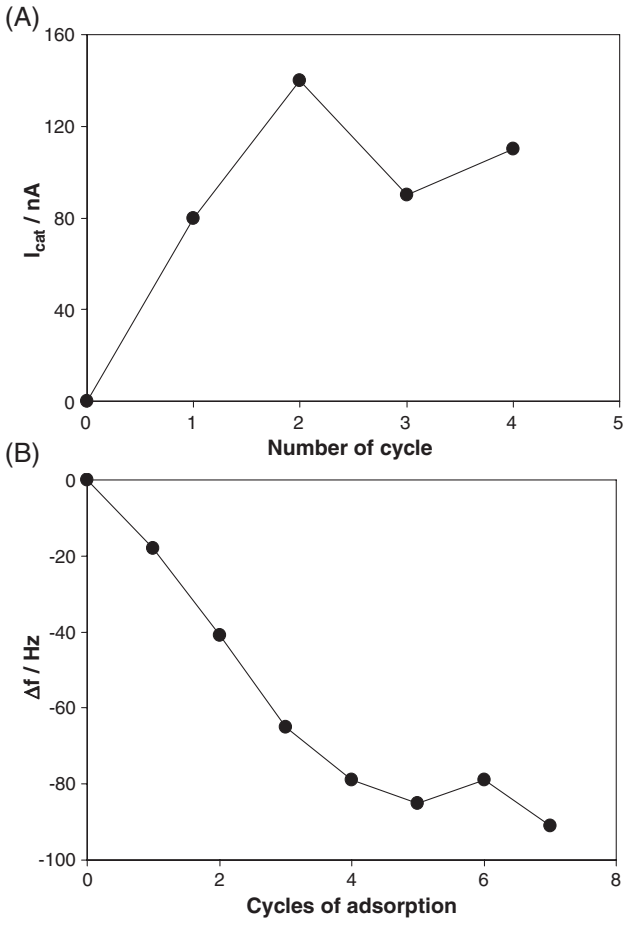

Fig. 10. (A) Dependence of the catalytic current on the number of adsorption cycle. The current was measured for each G4 dendrimer/GOx deposited successively on the gold surface. The cyclic voltammograms were performed at 2 mV s^{-1}. (B) Dependence of the QCM frequency shift on the number of alternate adsorption cycles from G4 dendrimer and glucose oxidase solutions. The zero is arbitrary.

4. Concluding remarks

Results from electrochemical, SPR and QCM experiments have confirmed that G3.5 and G4 dendrimers have the capability to adsorb spontaneously either on bare or on pre-treated gold surfaces covered with positive (for G3.5) or negative (for G4) charges. Fixation can also occur on carbon (PG or GC) material and on 4-ABA-pre-treated glassy carbon. The results of studies of dendrimer adsorption on a gold surface allow a conclusion to be made about compressed state of dendrimers and the formation of multilayers in accordance with previous reported data [15,39]. Composite films of the G3.5G4 (or G4G3.5) types can be built up essentially by electrostatic LBL self-assembly of these macromolecules of two generations differing by the charge of their surface groups. A tendency to form compressed layers is observed also for composite films. The assemblies tend to become unstable when the number of layers is increased. It has been suggested [15] that non-homogeneous surface distribution of functional groups may promote non-homogeneous surface adsorption. In this case, layer-by-layer growth would be replaced with “island” growth, resulting in the decrease of interaction strength between the terminal groups of dendrimers.

A poor stability has been noted in this work for the assemblies involving cytochrome c and G3.5 dendrimer, though the presence of opposite global charges on the two molecules, respectively. Similar conclusions have been reported in previous works on other proteins, e.g. for GOx/lysozyme composite layer [62]. It has been assumed that direct assembly of oppositely charged protein molecules was difficult because electrostatic attraction cannot be maximized with globular proteins. In the case of cytochrome c, it is known that the molecule develops a dipolar moment (325 Debye) [63] that must force the positive patch of cytochrome c to face the G3.5 dendrimer layer and to point the negative one on the outside. As a consequence,

cytochrome c could be more weakly retained and less favorable conditions offered to a new layer of G3.5 dendrimer to be adsorbed. In contrast, DvH cytochrome c_3 molecule exhibits a more homogeneous distribution of positive charges without privileged orientation governed by a dipolar moment. It is thus suggested that a better stabilization of the G3.5 dendrimer/cytochrome c_3 assembly is attained because of an enlarged contact of face-to-face positively/negatively charged sites. Gold electrodes modified with composite cytochrome c_3/G3.5 dendrimer films have been shown to exhibit attractive properties in biosensing applications, especially to explore problems related to bioremediation. Satisfactory results have been also gained using composite GOx/G4 assemblies immobilized on gold surfaces for studying the catalytic activity of the enzyme. Further studies have to be carried out to quantitatively control access of small molecules (of substrate) to the underlying electrode surface. Also important for future applications, experiments can be performed to exert active control over intra-dendrimer mass transport, e.g. by modulating the pH. We are investigating all these points.

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