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COLLOIDAL AND MOLECULAR ASSEMBLIES FOR BIOENGINEERING APPLICATIONS
Paula Pescador Álvarez
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Colloidal and molecular assemblies for bioengineering applications

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Paula Pescador Álvarez

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Que el present treball que porta per títol

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i que presenta na Paula Pescador Álvarez per optar al grau de Doctora per la Universitat Rovira i Virgili, ha estat realitzat sota la nostra direcció i que tots els resultats presentats i la seva anàlisi són fruit de la investigació realitzada per l'esmentada doctoranda.

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List of Abbreviations

ϵ	Extinction coefficient
Abs	Absorbance
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
CLSM	Confocal laser scanning microscopy
DL	Degree of labelling
E_{GOx}	Efficiency of GOx-coupled enzyme activity assay
E_{HRP}	Efficiency of HRP-coupled enzyme activity assay
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GOx	Glucose oxidase
HRP	Horseradish peroxidase
K_M^{app}	Apparent Michaelis constant
K_M	Michaelis constant
LbL	Layer-by-Layer

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M_w	Molecular weight
oxHRP	Oxidised horseradish peroxidase
PAH	Poly(allylamine hydrochloride)
PBS	Phosphate buffered solution
PDADMAC	Poly(diallyldimethylammonium chloride)
PEGDGE	Poly(ethylene glycol) diglycidyl ether
PEI	Poly(ethyleneimine)
pI	Isoelectric point
POs	Osmium-based redox polymer
PSS	Poly(styrenesulfonate)
Rho	5−(and 6)−carboxytetramethylrhodamine
SSC	Side scatter
TEM	Transmission electron microscopy

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Chapter 1

Introduction

1.1 The Layer-by-Layer technique

The layer-by-layer (LbL) coating of charged surfaces with oppositely charged materials is a powerful and versatile approach for the fabrication of functional molecular assemblies and interfaces. The key advantages of this technique over other existing methodologies for the preparation of thin films, such as self-assembled monolayers (SAM) or Langmuir-Blodgett (LB), are its unparalelled flexibility in combination with its simplicity and inexpensiveness. With simple instrumentation and easy preparation steps, it is possible to assemble highly complex and stable architectures with nanoscale control over their composition and structure.

The origins of this technique can be found in the work of Iler, who in 1966 reported on the sequential deposition of colloidal particles on solid substrates [1]. In 1991, Decher and Hong [2, 3] demonstrated that polyelectrolytes could also be electrostatically assembled onto solid supports in a sequential fashion, establishing the basis for a rapid development of

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the technique. In the original approach, a charged surface is exposed in alternating order to solutions of polycations or polyanions. After each deposition step the surface charge is reversed, allowing for the adsorption of a new layer of oppositely charged species, and in this way a polyelectrolyte multilayer film is assembled [4]. By simply varying the number of adsorbed layers, different film thicknesses can be easily specified. The thickness of a polyelectrolyte bilayer is typically close to 1 nm, and therefore nanometer-scale control can be achieved. Moreover, the thickness and physicochemical properties of the films are influenced by parameters like pH [5, 6], ionic strength [7], temperature [8] or solvent polarity [9], and can be varied in a controlled way by adjusting the experimental conditions. Thus, finely tuned structures can be assembled with nanometric resolution.

Initially, the most commonly employed polyelectrolytes were synthetic polyanions like poly(styrenesulfonate) (PSS), poly(vinylsulfonate) (PVS), poly(acrylic acid) (PAA) and polycations like poly(allylamine hydrochloride) (PAH), poly(diallyldimethylammonium chloride) (PDADMAC) and poly(ethyleneimine) (PEI). However, the same approach was found to be applicable to many other inorganic and organic materials. A large number of publications have described the assembly of multilayer films incorporating nanoparticles, crystals, dyes, proteins, nucleic acids, polysaccharides and a wide variety of other functional materials as layer constituents (see [10] for a recent review). Furthermore, in the last years it has become fully apparent that the LbL method is not restricted to charged species. Other driving forces apart from electrostatics can be employed for the construction of this kind of controlled multilayers, such as hydrogen bonding, covalent bonding or affinity interactions [10, 11]. This has opened the way to an even broader range of materials that can be used for the sequential assembly of ultrathin films with tailor-made functional properties.

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The LbL approach is particularly suitable for the integration of biomolecules into functional multilayers, since layer buildup is carried out under mild conditions, and many relevant biomaterials (proteins, DNA, lipids) are charged molecules, which further facilitates the process. Several studies have demonstrated that biomolecules and other biologically important species can be incorporated into multilayer films while retaining or even improving their biological functions. Some examples include the layer-by-layer immobilisation of nucleic acids, lipid vesicles, polypeptides and different bioactive proteins into functional LbL-based devices for sensing and biomedical applications [10, 12].

1.2 Assembly of LbL films on colloidal substrates

The versatility of the LbL methodology is not limited to the choice of layer components. A further advantage of the technique is that solid substrates of virtually any size and shape can be coated with functional films in a simple and controlled fashion. Of particular interest has been the extension of the LbL method to the surface modification of colloidal particles [13, 14]. Colloid science is a long established field of research which involves several disciplines, from chemistry and physics to biology and engineering. Apart from their interest from a fundamental point of view, the technological applications of colloids and colloidal suspensions have been exploited for centuries, and nowadays they are widely used in many industrial processes, including catalysis, separations and diagnostics. However in the last years, colloids have emerged as powerful tools particularly suited to meet the challenge of creating tailored building blocks for the rapidly evolving fields of bio- and nanotechnology [15]. As in the case of planar supports, biomolecules have been extensively used as layer components in the prepa-

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ration of LbL coated colloids, owing to the increasing demand for designed functional materials. At a fundamental level, LbL coating of particles provides a path to the creation of biomimetic cellular models and other systems to study the organisation of matter from the nano- to the microscale [16]. At the same time, technologically-driven applications such as miniaturised reactors, sensors or drug delivery systems have also found a solid ground for development in the LbL surface modification of colloidal substrates [17–19].

LbL coated particles can be prepared following similar protocols to those used for planar substrates, although some requirements need to be met in order to prevent flocculation [20]. In the most general approach, a colloidal suspension is added to a polyelectrolyte solution, and polyelectrolyte molecules are adsorbed through electrostatic interaction on the surface of the particles. Adsorption is carried out in an excess of polyelectrolyte, in order to allow complete coverage of the particles. After the first deposition step, excess polyelectrolyte can be removed by centrifugation/wash cycles or by filtration [13, 21, 22]. A schematic representation of the general coating procedure is shown in Figure 1.1. Silica particles are a commonly used substrate for colloid surface modification because of their mechanical and chemical stability, good monodispersity and spherical shape. This kind of particles are also easy to process due to their high density, which reduces the time and centrifugation speed required for sedimentation and the derived problems of aggregation. Other types of colloidal materials used for modification include polystyrene, melamine formaldehyde, calcium carbonate and alginate spherical particles, but also metal nanoparticles and nanorods [15].

Despite the enormous advances in the last decades in the development of new methods and materials for nanofabrication, some issues remain to be solved in order to complete the transition from the nano- to the micro- or macroscale, allowing to exploit the unique properties of nanomateri-

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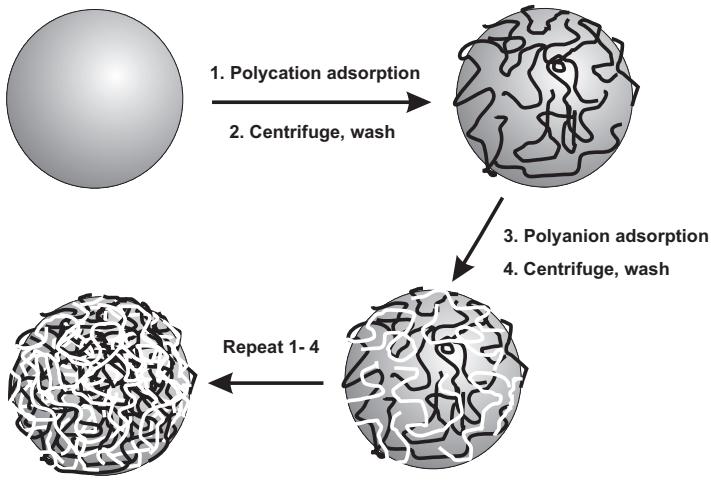

Figure 1.1: Scheme of the general procedure for LbL assembly of polyelectrolytes on the surface of a charged colloidal substrate.

als for the development of "real world" functional devices. In this sense, the LbL technique represents one of the most exciting alternatives due to its superior flexibility, but also because it does not rely in the use of sophisticated equipment and is therefore comparatively inexpensive. In particular, the LbL functionalisation of colloidal particles provides a new route for the creation of composite architectures which allow the integration of nanoscale-defined materials into two- and three-dimensional structures. Multiple functionalities can be assembled on the surface of core-shell particles [23] or within hollow capsules, obtained after dissolution of a LbL-coated sacrificial core [14]. These in turn can be arranged on a substrate in a controlled way by a number of lithographic and non-lithographic methods [24–26], and here again the LbL technique provides simple and versatile routes for selective deposition taking advantage of the same functionalities introduced in the films [27–29]. Thus, the LbL surface modification of

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colloids has already demonstrated its potential for the creation of tailored nano- and microstructures, and ultimately opens the way not only to functional microsystems but also to the fabrication of macroscopic devices.

1.3 LbL electrochemical interfaces

One of the fields in which the LbL technique has represented a major advance is the development of electrochemical systems. The precise control of the film architecture allowed by this approach, combined with the almost unlimited flexibility in the choice of components, offer unique opportunities for the assembly of functional materials ranging from solid-state electrolytes [30, 31], proton exchange membranes and electrodes for fuel cell applications [32,33] to electrochromic and photovoltaic devices [34,35]. Electrochemically active materials can be readily incorporated into multilayer films, together with organic or inorganic species which provide additional functionalities. Furthermore, critical parameters such as film thickness, mass transport and electrical conductivity can be precisely tuned, allowing an increased control over the performance of the system [36]. In particular, LbL assemblies have found many successful applications in the area of electrochemical biosensors. These devices are of great interest because they provide an interface between biological functions and electronic signal-transduction processes. Moreover, these systems are well suited to meet the challenges in the development of new miniaturised, low-cost, integrated biodetection platforms [37]. LbL assembly offers the ability to incorporate biomolecules and biologically relevant materials into ultrathin films without compromising their stability and activity. Furthermore, spatial control over the film architecture is a powerful tool for the study and optimisation of charge and mass transport processes.

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Several publications have demonstrated the applications of LbL assemblies in electrochemical biosensing, and some recent reviews are available [38,39]. Most of these studies correspond to the preparation of enzyme electrodes, but other successful approaches include the development of electrochemical immunosensors and DNA sensors. Enzymatic biosensors have been fabricated with a large number of different enzymes for the detection of biologically relevant substrates. Of these, sensors based in the enzyme glucose oxidase are by far the most widely studied, due to the need for reliable glucose monitoring systems for diabetic patients. In some of these electrochemical biosensors, diffusional electron mediators are required for signal detection, while more sophisticated approaches incorporate electron mediators as layer components or even achieve direct electron transfer between enzymes and electrodes, allowing for the fabrication of completely integrated devices. Spatial control by varying the number of active or spacing layers provides a way to optimise the catalytic and detection efficiency of these systems.

1.4 Scope of this work

In the present work, the layer-by-layer technique is employed to assemble multilayer films of enzymes and polyelectrolytes on the surface of micrometer-size silica particles. Two different enzymes, glucose oxidase (GOx) and horseradish peroxidase (HRP), are co-immobilised together with polyelectrolyte precursor and intermediate layers. In the resulting multilayer films, a sequential enzymatic reaction takes place, with the conversion of glucose to gluconic acid and hydrogen peroxide catalysed by GOx and the subsequent reduction of hydrogen peroxide to water catalysed by HRP. The sequential LbL approach allows to explore the influence of different poly-

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electrolyte combinations on the immobilisation and functionality of the enzyme layers. Flow cytometry, confocal and transmision microscopy and spectrophotometric measurements provide information about the interaction between the different layer components, as well as the stability of the colloidal substrates and the interaction of the multilayer film with a solution containing the different enzyme substrates and products.

Electrochemical functionality is further added to these multilayer films with the incorporation of an osmium-based redox polymer to the structure. The osmium centers of the polymer are able to exchange electrons with the active centers of the immobilised HRP molecules, but also with the surface of an electrode. In this way, the specific chemical events taking place at the redox centers of the enzymes are transduced into an electrical signal. The nanostructured silica microparticles, coated with redox enzymes and polymers, assume a multiple role in the final system. On one hand, they act as immobilisation substrates and high surface area carriers for the creation of enzymatic microreactors. Furthermore, the LbL-coated colloids are embedded in a redox polymer film and immobilised on the surface of gold electrodes, acting as building blocks for the fabrication of a functional electrochemical device for the detection of glucose.

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Chapter 2

Assembly and characterisation of enzyme layers on silica colloids

2.1 Introduction

The fabrication of organised films incorporating functional biomaterials in a controlled fashion is an active area of research due to the multiple applications of these systems in biotechnology, analytical biochemistry and biomedical engineering [1]. In the last years, the layer-by-layer (LbL) technology has acquired increasing relevance in this field due to its extreme versatility and the possibility to control the assembled films and their properties at the molecular level. The LbL technique can be applied not only to planar supports but also to colloidal particles or capsules, with additional advantages such as a high surface area or loading capacity and the possibility to finely tune the permeability of the system and the diffusion

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of different substances through the assembled structures [2, 3]. In particular, layered architectures containing different enzymes and polyelectrolytes have been studied with the aim to develop sensing devices and enzymatic reactors [4–13]. Within this range of complex films with tailored enzymatic activity, multienzymatic systems are particularly interesting for biocatalysis applications, and some examples can be found in the literature. Onda and coworkers demonstrated the sequential reactions of glucoamylase/glucose oxidase and glucose oxidase/horseradish peroxidase in multilayer films alternated with poly(styrenesulfonate) (PSS) and poly(ethyleneimine) (PEI) assembled on flat substrates [14, 15]. Working also with planar supports, Disawal et al. developed multicomponent films in which the sequential action of urease and arginase catalysed the decomposition of L-arginine to ammonia [16]. Fewer attempts have been made to fabricate multienzymatic films on colloids. Caruso and Schüler coated polystyrene particles with multiple layers of glucose oxidase (GOx) and horseradish peroxidase alternated with PSS and PEI and observed the resulting coupled enzymatic reaction on the surface of the colloids, although the catalytic efficiency of this system was very low when compared with the activity of the equivalent single-enzyme films [17]. More recently, the same enzyme couple was incorporated into hollow microspheres with dextran sulfate/protamine or poly(styrenesulfonate)/poly(allylamine hydrochloride) as constituents of the capsule wall [18,19]. In both cases the activity of the enzymes was only a fraction of that observed for equivalent amounts of the proteins in free solution. In these studies it was established that the amount and distribution of the immobilised enzymes and their observed catalytic activity were affected by the choice of polyelectrolytes and the immobilisation conditions (concentrations of coating solutions, pH, ionic strength, etc). However the precise behaviour of enzyme multilayers on polyelectrolyte microparticles

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has not been investigated thoroughly. Especially in the case of multienzymatic films, there are only very few reports on the parameters controlling the efficiency of the sequential reaction of the different components.

In the present work, the well known and biotechnologically relevant glucose oxidase/horseradish peroxidase enzyme couple was chosen as a model system for a detailed study of the formation of multienzyme films on the surface of micrometer-size silica colloids. For each of the individual enzymes, the amount of immobilised protein and its catalytic activity were determined and compared for films incorporating different combinations of polyelectrolytes. Different immobilisation strategies, involving not only alternating deposition of protein and polyelectrolyte layers but also precomplexation of enzymes and polymers and covalent binding were examined. Furthermore, an additional functionality was introduced to the multilayer films with the incorporation of an osmium-based redox polymer, opening the way to the development of an electrochemical interface. For the final bienzymatic GOx/HRP films, the influence of the construction parameters on the efficiency of the sequential reaction between both enzymes was investigated. Taking advantage of the properties of the silica microparticles chosen as solid support for the multilayer films, the powerful technique of flow cytometry was employed for the characterisation of the coated colloids in terms of enzyme loading and stability of the colloidal suspensions. Additional techniques such as UV-vis spectroscopy, confocal microscopy and transmission electron microscopy were used to characterise the multienzymatic colloids.

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2.2 Materials and methods

2.2.1 Materials

Poly(sodium 4-styrenesulfonate) (PSS), M_w 70000; poly(allylamine hydrochloride) (PAH), M_w 70000; poly(diallyldimethylammonium chloride) (PDADMAC), M_w 200000-350000, 20% solution in water; glucose; o-dianisidine hydrochloride; 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS); hydrogen peroxide (H_2O_2); potassium dihydrogen phosphate; dipotassium hydrogen orthophosphate trihydrate; sodium chloride; sodium acetate; sodium carbonate; sodium bicarbonate, and sodium periodate were obtained from Sigma-Aldrich. Glucose oxidase (GOx, 135 U mg^{-1}) and horseradish peroxidase (HRP, 250 U mg^{-1}) were purchased from Biozyme. Fluorescein isothiocyanate (FITC) and rhodamine (Rho) EZ-label protein labelling kits were obtained from Pierce. Silica (SiO_2) particles (3 μm diameter) were supplied by Microparticles GmbH.

A cationic-based redox polymer, poly[(vinylpyridine)$Os(bpy)_2Cl$] partially quaternised with bromoethylamine (POs) was synthesised as described elsewhere [20–22]. A positively charged polyelectrolyte with the same structure as POs but no osmium redox centers (Binder) was also synthesised [7, 23]. All aqueous solutions were prepared using purified water from a Millipore Milli-Q water purification system.

Figure 2.1 shows the structures of the different polyelectrolytes employed as precursor and intermediate layers (PAH, PSS, PDADMAC), as well as those used to prepare enzyme-polyelectrolyte complexes (POs and Binder).

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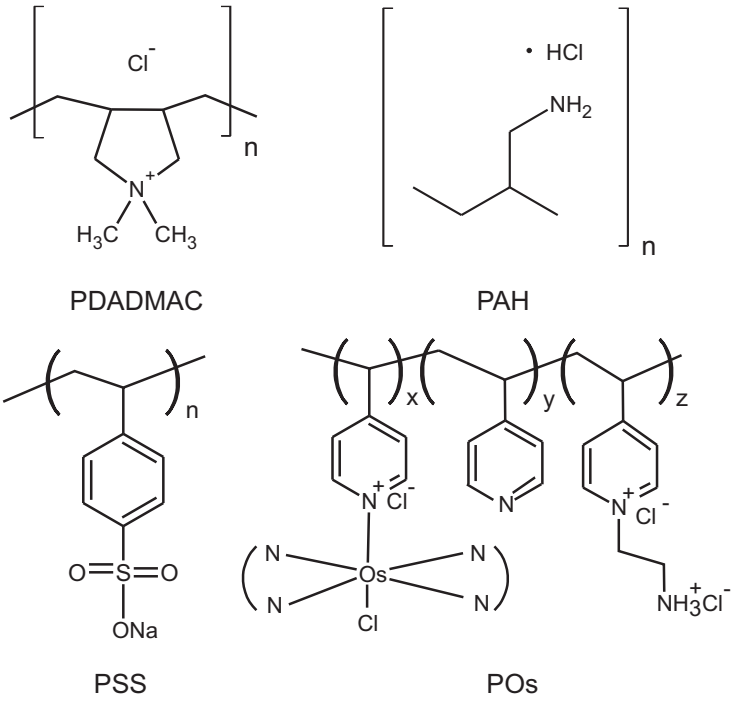

Figure 2.1: Chemical structures of the different polyelectrolytes used in this work. The polymer "Binder" has the same structure as POs except that no osmium redox centers are present.

2.2.2 Assembly of enzyme layers on colloids

Two different approaches were used for the immobilisation of enzymes on the particle surface. The first one consists simply in adding the polyelectrolyte-coated colloids to a solution of enzyme and incubating the dispersion for a defined amount of time to allow the assembly to proceed. In the second strategy, the enzyme is premixed with a polyelectrolyte prior to assembly on the colloids. In this way, enzyme-polyelectrolyte complexes are formed

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which are then used to coat the particles. In order to ensure a reproducible surface for enzyme immobilisation, the particles were coated with four to five precursor polyelectrolyte layers (using either the PAH/PSS or PDADMAC/PSS polyelectrolyte pairs) before the assembly of the enzyme layer. The procedure used to assemble the polyelectrolyte multilayers was similar to that described previously in the literature. First, SiO_2 particles were dispersed in water and centrifuged for 1 minute at 1000 g. This wash/centrifugation cycle was repeated for three times. Next, the colloidal dispersion was added to a 1 mg mL^{-1} solution of polyelectrolyte containing 0.5 M NaCl. The polyelectrolyte was allowed to adsorb onto the particles for 10 minutes under constant stirring, and afterwards excess polyelectrolyte was removed by three wash/centrifugation cycles in 0.1 M NaCl.

Enzyme layers

For the assembly of GOx and HRP layers, polyelectrolyte-coated particles were added to a 1 mg mL^{-1} enzyme solution in 0.1 M carbonate buffer (pH 8.2). Under these conditions GOx has a net negative charge (isoelectric point, pI, GOx=4.2), and it was assembled on colloids coated with either PAH or PDADMAC as the outermost layer. Oxidised HRP (oxHRP) was prepared as follows: 2 mg HRP were dissolved in 100 μL of a 0.1 M bicarbonate solution (pH=8.5). Next, 50 μL of a 12 mg mL^{-1} aqueous solution of sodium periodate were added, and the mixture was incubated in the dark for 1 hour. The enzyme solution was purified by gel filtration using Sephadex 25 columns (mini Quick Spin, Roche) and extensively dialysed against milliQ water and buffer. oxHRP was adsorbed on colloids coated with a top layer of POs or Binder. The concentrations of GOx and HRP/oxHRP were determined spectrophotometrically using an extinction

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coefficient ϵ=267200 M^{-1} cm^{-1} at 280 nm for GOx and the Soret extinction coefficient of 102000 M^{-1} cm^{-1} at 403 nm for HRP/oxHRP. After 1 hour incubation, excess enzyme was removed by 5 wash/centrifugation cycles and the particles were redispersed in 0.1 M NaCl. Subsequent polyelectrolyte layers were assembled in the same way as described above.

Premixed Enzyme-Polyelectrolyte layers

The adsorption of enzyme-polyelectrolyte complexes was conducted as follows: 1 mg mL^{-1} solutions of POs or Binder and enzyme were mixed in the appropriate volumes to obtain the desired final weight ratios of each component. POs and Binder solutions were prepared in 0.1 M NaCl and enzymes were dissolved in 50 mM carbonate buffer (pH=8.2). For the preparation of GOx:Binder coated colloids, the final ratios in the mixture were 65 wt % Binder, 35 wt % GOx. For oxHRP, a mixture of 85 wt % POs or Binder and 15 wt % enzyme was used. The mixtures were allowed to react for 10 minutes. After this time the solutions were centrifuged to remove any possible precipitates and transferred to new tubes. Polyelectrolyte-coated colloids were added to the solution and incubated for 10 minutes. The particles were then washed 5 times and redispersed in 0.1 M NaCl. All enzyme-polyelectrolyte complexes were adsorbed on PSS, since the overall charge of the complexes was positive as confirmed by microelectrophoresis measurements performed on a Malvern Zetasizer 3000 HS.

2.2.3 Flow cytometry measurements

Labelling of GOx and oxHRP with FITC or Rho was performed according to the instructions provided by the manufacturer. Briefly, the enzymes were dissolved in 50 mM borate buffer (pH 8.5) and the appropriate amount

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of FITC or Rho dissolved in dimethylformamide was added. The mixture was incubated for 1 hour at room temperature. After completing the labelling reaction, excess fluorescent dye was removed by gel filtration using Sephadex 25 columns. The degree of labelling of the enzymes (DL, moles dye:moles enzyme) was determined spectrophotometrically using ϵ=267200 M^{-1} cm^{-1} for GOx and ϵ=20720 M^{-1} cm^{-1} for oxHRP at 280 nm. For the quantification of immobilised FITC-labelled enzymes, the measured fluorescence intensity was converted to molecules of equivalent fluorescein according to the relationship obtained by measuring five-peak Spherotech Sphero^{TM}FITC calibration particles (3 μm diameter). Flow cytometry was performed on a Becton Dickinson FACScalibur flow cytometer using an excitation wavelength of 488 nm. For each sample 10000 single colloids were measured. The data were analysed using the WinMDI v2.8 software written by Joseph Trotter.

2.2.4 Enzyme activity assays

Activity tests were performed on a Hewlett Packard 8453 UV/Vis spectrophotometer. To measure the activity of adsorbed GOx layers, 500 μL of a 0.21 mM *o*-dianisidine solution in phosphate buffer (pH 7.0), 125 μL of a 10%(w/v) β-D-glucose solution (previously allowed to mutarotate for at least 12 hours) and 25 μL of a HRP solution in phosphate buffer containing approximately 60 units mL^{-1} were mixed and air equilibrated until the absorbance at 436 nm was constant. GOx-coated colloids were then added to the mixture and the increase in absorbance at 436 nm for 3 minutes was recorded immediately after mixing. For the determination of HRP activity, HRP-coated colloids were added to a mixture of 580 μL of a 9.1 mM ABTS solution in potassium phosphate buffer (pH 7.0) and 20 μL of a 0.3% (w/w)

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H_2O_2 solution. Immediately after mixing, the increase in absorbance at 405 nm was recorded for 1.5 minutes. Within experimental error, the same particle concentration was used for each assay: approximately 2 x 10^6 colloids in a volume of 2 μL were added to the reaction mix.

2.2.5 Transmission Electron Microscopy (TEM)

TEM measurements were carried out on a JEOL 1011 microscope operated at 120 kV. Samples were prepared by depositing a drop of a particle suspension onto a carbon-coated copper grid and were imaged after allowing them to air-dry.

2.2.6 Confocal laser scanning microscopy (CLSM)

Imaging was performed on a Leica TCS-SP2 confocal laser scanning microscope using an Ar/Kr laser (λ=488 nm) and a He/Ne laser (λ= 543nm) as excitation source and a 63x planapochromatic oil immersion objective. Images were recorded in sequential mode.

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2.3 Results and Discussion

2.3.1 Characterisation of GOx and GOx-Binder layers

Flow cytometry

The technique of flow cytometry was used to characterise both qualitatively and quantitatively the assembly of enzyme or enzyme-polyelectrolyte complexes on the surface of silica particles. In flow cytometry, multiple physical and/or chemical characteristics of cells or other particles are measured as they flow in a fluid stream. While many different parameters can be studied by this technique, flow cytometry is mainly based in measurements of fluorescence and light scattering using a laser beam as a source of light. The forward scatter (FSC) signal corresponds to the amount of light scattered at small angles to the incident beam, and gives information about the size of the particles. The amount of light scattered at large angles, called orthogonal or side scatter (SSC) is related to the surface roughness and in general to the structural complexity of the particles. The amount of fluorescence emitted is in turn proportional to the amount of fluorescent material present in the particles [24]. An advantageous feature of this technique is that the characteristics of each particle are individually measured, but at the same time thousands of particles can be analysed in only a few seconds, providing robust statistics.

Particle aggregation Figure 2.2 shows the fluorescence intensity and forward scatter of particles coated with four precursor layers, in this case $(PDADMAC/PSS)_2$, and one layer of GOx:Binder complex. Different particle populations can be distinguished, corresponding to individual particles and aggregates of two or more particles. The fluorescence intensity and the

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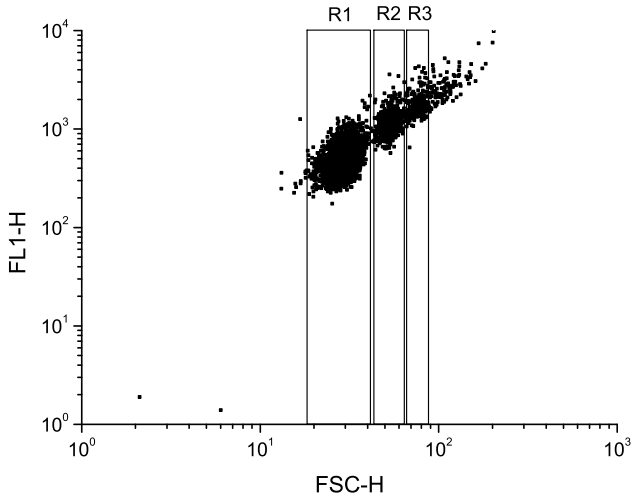

Figure 2.2: Fluorescence intensity (FL1, green channel) of SiO_2 particles coated with GOx:Binder complex, shown as a function of the forward scatter (FSC). The region R1 corresponds to 10000 single particles, while R2 are doublets and R3 contains the triplets. Some aggregates of higher order can be observed. 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4.

forward scatter of a doublet are twice those of the single particles, and so on. Aggregation is a common problem when coating colloidal particles with polyelectrolytes, and in order to avoid it some general principles should be observed. For example, it has to be ensured that the concentration of polyelectrolyte in the solution is large enough to allow complete coverage of the surface of the particles. If this is not the case, aggregation will occur due to the interactions between colloids with different charge density. It is also common to observe certain degree of aggregation after the centrifugation steps following deposition of each polyelectrolyte layer [2].

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The degree of aggregation of the particles varied depending on the nature of the top layer. This has been previously reported in the literature for the particular case of the PAH/PSS polyelectrolyte pair [25]. In the present work, for colloids coated only with precursor PAH/PSS or PDADMAC/PSS, the highest percentage of aggregated particles was observed with PAH as the outer layer (25-30%). When the top layer was PDADMAC, a stronger cationic polyelectrolyte than PAH, the aggregation was noticeably lower (10-15% of the particles), while less than 5% of the particles formed aggregates with PSS as the outer layer. Similarly, when negatively charged GOx was assembled on PDADMAC, the degree of aggregation was 5% or less. The causes for the differences in aggregation are not completely understood. Prediction of the behaviour of a particular system is difficult, since the outcome is determined by a complex interplay of electrostatic and steric effects together with the hydrophilic-hydrophobic balance of the different polyelectrolytes involved. For example, the amino groups of PAH are easily deprotonated, and in that state it tends to behave as a rather hydrophobic species. In addition, the PAH molecule is highly flexible, which further facilitates intra- and intermolecular interactions. Both PDADMAC and PSS are strong polyelectrolytes with a linear configuration, but in the case of PDADMAC the charges are effectively screened by its methyl groups, while in PSS they are much more exposed. Thus, for each polyelectrolyte pair the interactions taking place and the resulting layer configurations can be quite different. The conditions used for layer assembly (pH and ionic strength of the solutions, adsorption time, stirring) have to be optimised in order to obtain stable colloidal suspensions with reasonable degrees of aggregation.

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In the case of particles coated with enzyme-polyelectrolyte complexes, the weight ratios of the two components in the coating solution had to be adjusted. For the preparation of GOx:Binder complexes, the enzyme solution was added to a tube already containing the appropriate amount of Binder polymer. For solutions with a ratio of Binder to GOx lower than 1.5, the particles were completely aggregated after the first centrifugation step and could not be resuspended, although no precipitation was apparent in the coating solution. Thus, a mixture of 60% Binder and 40% GOx by weight was used to coat the colloids, and under these conditions the degree of aggregation was between 5 and 10%. These observations can be interpreted as follows: depending on the ionic strength of the solution and on the enzyme loading, the spatial configuration of the polymer and its interaction with the oppositely charged enzyme molecules will vary. In addition, the behaviour of complexes in solution may differ from that of complexes adsorbed on the surface of the particles. It may be assumed that in order to obtain stable suspensions of coated colloids, each enzyme molecule must be completely surrounded or "screened" by polymer molecules. If this is not the case, attractive electrostatic interactions can take place between neighbouring particles, leading to aggregation. Assuming a molecular weight of ≈ 63000 for Binder and 160000 for GOx, it can be estimated that the maximum molar ratio of enzyme to polymer in the coating solution is approximately 1:4, which would correspond to complexes formed by four molecules of polymer per enzyme molecule.

Quantification of immobilised glucose oxidase Figure 2.3a shows the fluorescence intensity of individual particles coated with FITC-labelled GOx in different configurations. When GOx was assembled as the outermost layer on PDADMAC, a strong fluorescence signal was obtained,

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corresponding to a dense GOx coverage on the surface of the particles. In contrast, for GOx adsorbed on PAH only a very low fluorescence was detected. The reason for this difference is not straightforward. The interactions of proteins and polyelectrolytes are complex and include electrostatics, van der Waals forces, dipolar or hydrogen bonding and hydrophobic effects [26,27]. Parameters such as pH and ionic strength strongly influence protein adsorption. In the case of PAH/GOx and PDADMAC/GOx, it is clear that apart from the electrostatic attraction between the negatively charged protein and the two different polycations, other factors must be operating which depend on the characteristics of each polyelectrolyte and which lead to very different global interactions. In addition, as mentioned before the different composition of the precursor layers results in different physical configurations of the assembled films. It has been reported that PAH/PSS layers have a more compact structure than those formed by PDADMAC/PSS [28, 29]. Especially in relatively high ionic strength solutions such as the ones used in this work, the porosity and roughness of the two kinds of films may also differ substantially, which could have an effect on the interaction with the adsorbing GOx molecules [28, 30].

Premixed enzyme-polyelectrolyte complexes were also used to coat the particles. For Binder:GOx complexes, the intensity of the fluorescence signal when the complex was deposited on PAH/PSS was higher than for the pair PDADMAC/PSS (in all cases with PSS as the outermost layer). After adsorbing GOx or GOx:Binder, an additional polyelectrolyte layer of opposite charge was deposited on top of the enzyme layer (Figure 2.3b). The fluorescence signal decreased drastically in the case of PDADMAC/GOx layers, losing over 70% of the intensity. No change was observed for PAH/GOx layers, where the low initial level of fluorescence of these particles suggests negligible enzyme coverage and therefore re-exposure to PAH should not

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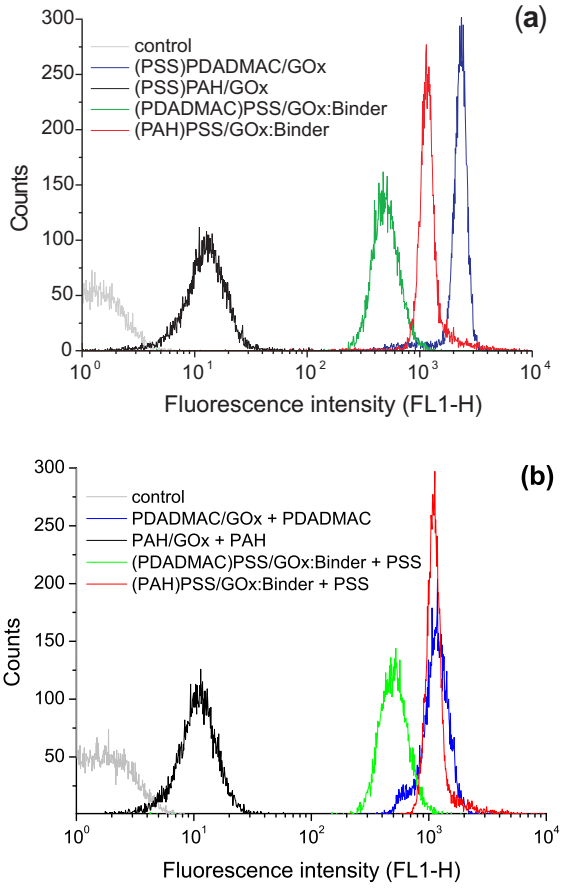

Figure 2.3: (a) Fluorescence intensity of particles coated with GOx or GOx:Binder complexes in different configurations. (b) The same particles after coating with one additional polyelectrolyte layer. A strong decrease in fluorescence is observed for $(PSS/PDADMAC)_2/GOx$ particles upon adsorption of a further layer of PDADMAC (blue line), while for the particles coated with GOx:Binder complexes (red and green lines) the fluorescence intensity values do not show significant changes after deposition of a PSS top layer.

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result in adsorption of a new layer. For Binder:GOx complex layers both on PAH/PSS and PDADMAC/PSS, the fluorescence remained practically unchanged after adsorption of an additional PSS layer.

The removal of a substantial amount of the adsorbed species upon deposition of a subsequent polyelectrolyte layer is a well known phenomenon described in the literature [31–35]. This removal is generally understood to proceed via solubilisation of adsorbed chains through the formation of water-soluble polyelectrolyte complexes. However the specific behaviour of each system depends strongly on the type of polyelectrolytes involved and other factors like the ionic strength and pH of the adsorption solutions. For the particular case of GOx and HRP adsorbed on colloids, Caruso and Schüler [17] reported that up to 80% of the initially deposited enzyme was removed by the next layer of polyelectrolyte. This indicates that a large fraction of the enzyme molecules are only weakly adsorbed on the underlying surface, and in the next coating step, their interaction with the incoming polyelectrolyte in solution is stronger, resulting in desorption. The same authors observed that complexes of GOx:PAH or HRP:PSS could be successfully immobilised on colloids and were not desorbed upon coating with further polyelectrolyte layers. In the present work, the redox polymer POs and its non-osmiated equivalent, Binder, were used to form enzyme-polyelectrolyte complexes. These polymers were originally developed by Heller and coworkers [20–22, 36, 37] for application in enzyme amperometric biosensors, and in particular glucose sensors based on glucose oxidase. They were specifically designed to complex the enzyme molecules, forming stable electrostatic adducts which can be then immobilised on the surface of an electrode. As shown by flow cytometry measurements, it is also possible to coat particles with GOx:Binder complexes, which are not removed by subsequent polyelectrolyte layers.

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In order to have an estimation of the amount of GOx immobilised in the different configurations, the amount of fluorescein equivalents on each particle was calculated from a relationship established by measuring the fluorescence of commercially available FITC calibration particles. As the degree of labelling of the enzyme is known, fluorescein equivalents can be converted to enzyme molecules. Converting this again to moles of enzyme and dividing by the surface area of the 3 μm particles (2.8 x 10^{-7} cm^2), an approximation for the enzyme coverage was made (Table 2.1). The molecule of glucose oxidase is an ellipsoid of approximate dimensions 77 x 60 x 52 Å with a Stokes radius of 4.3 nm [38,39]. Assuming a maximum random packing density of 60% [40], the concentration of glucose oxidase corresponding to a monolayer is 1.7 pmol cm^{-2}. According to this estimation, the initial surface coverage obtained immobilising GOx on PSS/PDADMAC is approximately 2.5 times that of a saturated monolayer, suggesting enzyme aggregation, but after adsorption of an additional PDADMAC layer the measured value is close to the theoretical concentration for a monolayer. When the colloids were coated with PSS/PAH precursor layers, only 2-3 % of the maximum surface coverage was achieved after incubation with GOx. For GOx:Binder complexes, approximately full monolayer coverage was obtained on PAH/PSS and around 65% of that value was adsorbed on PSS/PDADMAC.

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Enzyme activity

The activity of GOx was measured spectrophotometrically by following the oxidation of o-dianisidine upon reaction with hydrogen peroxide:

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{GOx}} \text{D-glucono-1,5-lactone} + H_2O_2 \quad (2.1)$$

$$H_2O_2 + \text{reduced o-dianisidine} \xrightarrow{\text{HRP}} 2H_2O + \text{oxidised o-dianisidine} \quad (2.2)$$

Activity assays were carried out with known concentrations of GOx in solution in order to establish a linear relationship between the measured activity (expressed as ΔAbs_{436} min^{-1}) and the amount of enzyme present. To take into account the effect of enzyme complexation on its activity, the same calibration was done with GOx that was premixed with Binder in solution. The amounts of each component in the solution were the same as used for particle coating, 40% weight GOx and 60% weight Binder. GOx:Binder complexes showed no loss of activity compared to the free enzyme in solution. The effect of complexation on the activity of enzymes can be rather variable. On one hand, different studies have shown that the electrostatic interaction of enzymes with a countercharged polyelectrolyte can restrict the segmental motion of the protein chains enzymes and lead to increased thermostability and extended shelf life [41–44]. Other authors found that enzymes may lose part of their activity under these conditions [45–47]. For the particular case of GOx and HRP, Caruso et al. reported a loss of 60-70% of the activity when these enzymes were complexed in solution with PAH and PSS, respectively [17]. For the same system, but with slightly different preparation and assay methods, Stein and coworkers found no decrease in the activity of the complexed enzymes [19]. In the case of GOx:Binder complexes no deactivation was expected, since this polymer

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was designed for the express purpose of complexing enzymes while retaining their activity [37].

Following the same experimental procedure, the activity of the assembled GOx and GOx:Binder layers was tested, with all samples containing approximately 2 x 10^6 particles. The measured values of ΔAbs_{436} min^{-1} were then converted to picomoles of enzyme, and dividing by the surface area of the particles, the concentration of immobilised enzyme was estimated. The results of these calculations are summarised in Table 2.1. From the comparison with the surface coverage values measured by flow cytometry, it appears that GOx retains most of its activity after immobilisation on PSS/PDADMAC, either alone (on PDADMAC) or complexed with Binder (on PSS). For the PAH/PSS pair activity measurements give values of coverage 25% lower than the fluorescence method, suggesting that some of the activity is lost in this configuration. Moreover, when an additional polyelectrolyte layer is adsorbed on top of GOx or GOx:Binder, in all cases the measured enzyme activity is much lower than that expected from fluorescence measurements, even taking into account the partial desorption of GOx molecules observed for non-complexed enzyme. PDADMAC/GOx/PDADMAC layers retained 15% of the activity before the last PDADMAC layer, while for GOx:Binder/PSS the residual activity was 22% for complexes adsorbed on PDADMAC/PSS and only 5% with PAH/PSS precursor layers.

This loss of activity has been observed in several studies with enzyme-polyelectrolyte assemblies both on colloids and on planar supports [8, 17, 19, 48–51]. One of the possible explanations for this phenomenon is the limited diffusion of glucose through polyelectrolyte films. A decrease of four orders of magnitude has been reported for the diffusivity of glucose through polyelectrolyte films comprised of 7 (PAH/PSS) bilayers as com-

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pared to its diffusivity in water (6.9 x 10^{-6} cm^2 s^{-1}) [52]. However in the present system severe glucose diffusion limitations seem unlikely, since only one extra polyelectrolyte layer (estimated thickness 0.5-1.5 nm) is adsorbed on top of the GOx:Binder complex. Alternatively, adsorption of polyelectrolytes on top of enzyme layers may cause blocking of the enzyme catalytic centers, either directly or by inducing changes in the conformation and/or spatial distribution of the enzyme molecules [17, 53]. This could explain why the decrease in activity seems to be different depending on the particular combination of polyelectrolytes used, since the configuration of the multilayer structure is determined by the nature of the polymers involved. The underlying polyelectrolyte layers may also affect the enzyme activity, as suggested by the results for GOx:Binder on PAH/PSS, since it is known that the layers can interpenetrate. In this sense, the highly flexible PAH molecule could be more likely to interact with enzyme molecules and reduce the accessibility of their active centers than linear polymers like PSS or PDADMAC. Finally, the rearrangement of the film upon further polyelectrolyte adsorption may lead to changes in the enzyme microenvironment, resulting in decreased activity. In this case the effect would also vary for each combination of polyelectrolytes.

2.3.2 Characterisation of HRP- and enzyme-polymer layers

The assembly of HRP layers on silica colloids was studied in the same way as described above for glucose oxidase. However there were some differences in the methodology followed to immobilise this enzyme. In the case of HRP, the aim of this study was not only to immobilise the enzyme on the colloids, but also to electrically connect the adsorbed enzyme molecules to the surface of an electrode, taking advantage of the specific properties of

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Multilayer configuration	**Flow cytometry**	**Activity assays**
(PSS)PAH/GOx	0.04±0.001	n.d.
(PSS)PDAD/GOx	4.69±1.49	4.33±0.14
(PAH)PSS/GOx:Binder	1.80±0.26	1.35±0.12
(PDAD)PSS/GOx:Binder	1.1±0.2	1.14±0.11
(PSS)PAH/GOx/PAH	0.04±0.002	n.d.
(PSS)PDAD/GOx/PDAD	1.34±0.69	0.20±0.04
(PAH)PSS/GOx:Binder/PSS	1.95±0.37	0.1±0.012
(PDAD)PSS/GOx:Binder/PSS	0.92±0.2	0.21±0.02

Table 2.1: Estimation of GOx coverage (in pmol cm^{-2}) on the surface of SiO_2 particles (n.d.: not detected).

a conducting redox polymer incorporated into the film. For this purpose, the chosen strategy was to co-immobilise HRP with the osmium-based redox polymer POs. In a study with HRP and the same redox polymer, in which layers of enzyme and polyelectrolytes were alternatingly adsorbed on the surface of gold electrodes, the amount of enzyme adsorbed on either POs or PSS was not enough to allow detection of electrocatalytic currents [7]. It is also possible that the adsorbed enzyme molecules were not effectively connected ("wired") to the redox polymer. Consequently, a different method of co-immobilisation was employed, involving chemical cross-linking of the enzyme and the redox polymer. Namely, alcohol functions in the oligosaccharide groups of the enzyme are oxidised by periodate to aldehydes, which are then linked to amino groups of the redox polymer through the formation of Schiff bases. The cross-linking facilitates the contact between the active centers of the enzyme and the osmium centers of the redox polymer. In this way, an effective electrical connection can

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be established, with the redox polymer shuttling electrons from the surface of an electrode to the immobilised enzyme [23]. The covalent binding of HRP to the redox polymer results also in the formation of very stable films, able to withstand considerable shear stress without being removed from the surface of an electrode [54]. The catalytic activity of the enzyme is only slightly decreased by the periodate oxidation procedure. Activity tests performed with periodate-oxidised HRP (oxHRP) showed that it retained approximately 90% of the activity of the native enzyme.

The formation of oxHRP:polymer complexes in solution was carried out as described above for glucose oxidase, but using either Binder or the redox polymer POs. In the case of Binder a maximum enzyme to polymer weight ratio of 1:3 was found to produce stable suspensions of coated colloids, whereas for POs this ratio was decreased to 1:9, due to the different molecular weight of the two polymers. Assuming a molecular weight of ≈180000 for POs, both weight ratios correspond to an enzyme to polymer molar ratio of 1:2. The observed particle aggregation at higher enzyme loadings could be due to interactions between the oxidised enzyme and the polymer molecules adsorbed on the surface of neighbouring particles. Because the enzyme:polymer complexes are positively charged, the layers were adsorbed in all cases on particles coated with PSS as the top layer, but the cationic polyelectrolyte was either PAH or PDADMAC.

Quantification of immobilised HRP Transmission electron microscopy was used to confirm the presence of adsorbed enzyme:polyelectrolyte complexes on the surface of the particles (Figure 2.4). Subsequent characterisation was carried out by flow cytometry and colorimetric enzyme activity assays. Figure 2.5 shows the fluorescence intensity measured for particles coated with FITC-labelled oxHRP and Binder adsorbed in the different

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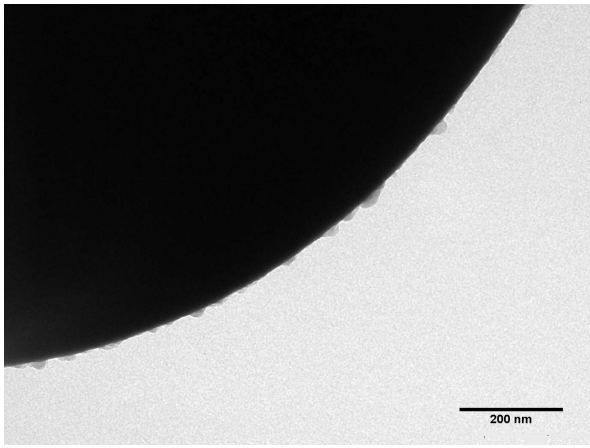

Figure 2.4: TEM micrograph of silica particles coated with four precursor polyelectrolyte layers and one layer of HRP:POs complexes. The presence of adsorbed material is apparent from the increased surface roughness in comparison with non-coated silica particles (image not shown).

configurations tested. All samples were prepared with Binder instead of POs, since the osmium atoms of the redox polymer cause quenching of the fluorescence through the external heavy-atom effect [55]. The fluorescence intensity was very similar for the two methods of immobilisation, but in both cases the measured values when PDADMAC/PSS were used as precursor layers were higher than for PAH/PSS. Fluorescence intensity measurements were converted to picomoles of immobilised enzyme in the same way as described previously for glucose oxidase. Estimations based on the geometrical dimensions of HRP allow to calculate a theoretical surface concentration of 3.3 pmol cm^{-2} for a saturated monolayer of enzyme on a flat surface [56, 57]. According to this, approximately 45% of a full monolayer of oxHRP was adsorbed on PAH/PSS and 65% of that value was immobilised on PDADMAC/PSS-coated colloids (Table 2.2).

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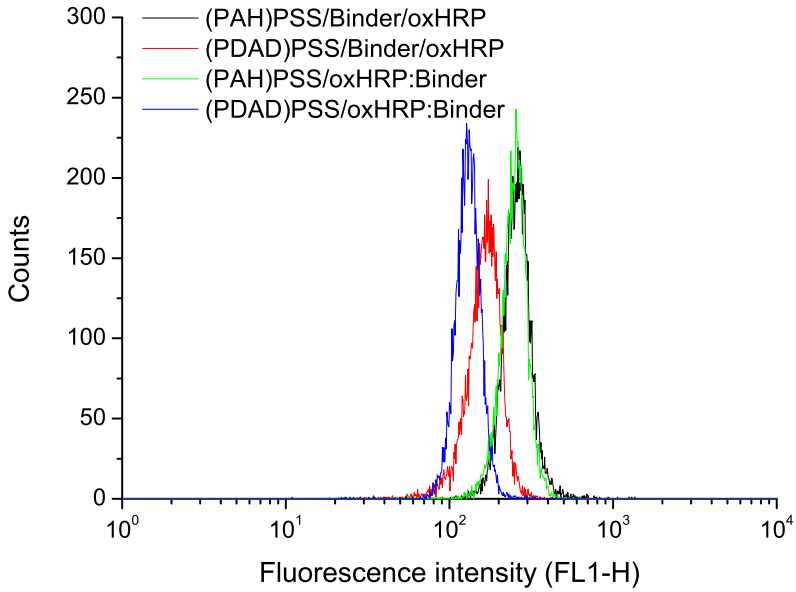

Figure 2.5: Fluorescence intensity of particles coated with Binder and oxHRP in different configurations. Slightly higher amounts of enzyme were adsorbed on PAH/PSS (black and green lines) than on PDADMAC/PSS precursor layers (blue and red).

Activity assays In order to evaluate the functionality of the different layer configurations, the activity of horseradish peroxidase was measured spectrophotometrically using ABTS as substrate at pH 7.0:

$$H_2O_2 + \text{ABTS} \xrightarrow{\text{HRP}} \text{oxidised ABTS} + 2H_2O \tag{2.3}$$

As in the case of glucose oxidase, the activity of known concentrations of oxHRP in solution was measured and related to the amount of enzyme.

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Multilayer configuration	**Flow cytometry**	**Activity assays**
(PAH)PSS/Binder/oxHRP	1.52±0.52	1.59±0.12
(PDADMAC)PSS/Binder/oxHRP	2.24±0.36	2.64±0.13
(PAH)PSS/Binder:oxHRP	1.52±0.44	1.66±0.1
(PDADMAC)PSS/Binder:oxHRP	1.94±0.31	2.16±0.14
(PAH)PSS/oxHRP:POs	n.m.	1.78±0.11
(PDAD)PSS/oxHRP:POs	n.m.	2.38±0.12

Table 2.2: Estimation of oxHRP coverage (in pmol cm^{-2}) on the surface of SiO_2 particles (n.m.: not measured).

The same was done for mixtures of Binder/oxHRP and POs/HRP in a 1:3 and 1:9 enzyme to polymer mass ratio, respectively. Again it was found that complexation with these polymers did not drastically affect the activity of the enzyme, with only 5-10% loss with respect to the activity of free oxHRP in both cases. Activity assays were then performed with particles coated with oxHRP and Binder or POs, in order to check whether the different composition of the two polymers had any influence on the amount of enzyme that is complexed and immobilised on the colloids. The assays were carried out with 2 x 10^6 particles per sample, and from the measured slope (ΔAbs_{405}/min) the concentration of immobilised enzyme was calculated. It was assumed that in the case of oxHRP:POs coated colloids the small amount of immobilised redox mediator should not interfere with the colourimetric activity assay, since the concentration of the diffusional co-substrate (ABTS) is much higher. The results obtained from the activity of particles coated with oxHRP:Binder and oxHRP:POs were practically the same, which suggests that similar amounts of enzyme are immobilised in both cases. Table 2.2 shows the estimated values of enzyme coverage. From

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the good agreement with flow cytometry data obtained for oxHRP:Binder coated particles, it was concluded that the immobilised oxHRP remained fully active on the surface of the colloids.

2.3.3 Bienzymatic colloids

Once the conditions for efficient adsorption of each enzyme were known, the next step in the construction of the final system was the co-immobilisation of both glucose oxidase and horseradish peroxidase on the colloids. In this way a sequential reaction can take place on the surface of each particle, with a GOx layer generating hydrogen peroxide which can diffuse towards the HRP layer to be reduced to water. In view of the results obtained with the individual enzymes, PDADMAC/PSS was chosen as the most convenient polyelectrolyte pair for the adsorption of the precursor layers. In order to assemble the GOx and HRP layers on the colloids, two different alternatives were explored:

- GOx and HRP separated by an intermediate layer
- GOx and HRP co-immobilised on the same layer

GOx and HRP in separate layers

In the first configuration, a layer of GOx:Binder complex was deposited, and a PSS layer was adsorbed on top to form the required negatively charged surface. Next, the particles were incubated in a solution containing oxHRP:POs complexes to produce the final GOx:Binder/PSS/HRP:POs bienzymatic multilayer. In both cases the ratios of enzyme and polymer used were the same as for single-enzyme coated colloids. To investigate the functionality of in these constructs, activity tests for both enzymes were

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performed and the results were expressed as ΔAbs min^{-1} cm^{-2}, taking into account the total surface area of the colloids. In the case of glucose oxidase, a standard assay as described in the experimental section was carried out first, followed by a second assay in which no horseradish peroxidase solution was added to the reaction mix (*GOx coupled assay*). In this situation, only the H_2O_2 molecules reduced by the immobilised HRP will produce a detectable colourimetric signal through the corresponding oxidation of o-dianisidine (eq. 1.2). An efficiency parameter E_{GOx} was established, which relates the results of both assays. This parameter represents the fraction of H_2O_2 that is collected by the immobilised HRP out of the total amount of H_2O_2 that is produced by the immobilised glucose oxidase. Similarly, a standard activity test for HRP was performed, followed by a second assay in which glucose was added to the reaction mix (in the same final concentration used for the glucose oxidase assay), but no hydrogen peroxide was supplied (*HRP coupled assay*). The efficiency parameter E_{HRP} represents the fraction of the maximal HRP activity (i.e. the activity measured in absence of substrate or cosubstrate limitations) that is measured when the substrate H_2O_2 is produced by the immobilised glucose oxidase. Finally, glucose oxidase assays were carried out with the same concentrations of GOx and HRP as measured on the colloids, but with both enzymes free in solution.

$$E_{GOx} = \frac{\text{activity coupled GOx assay}}{\text{activity standard GOx assay}} \quad \text{x} \quad 100 \tag{2.4}$$

$$E_{HRP} = \frac{\text{activity coupled HRP assay}}{\text{activity standard HRP assay}} \quad \text{x} \quad 100 \tag{2.5}$$

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For each enzyme the surface coverage on the colloids was estimated as described above from calibration curves obtained with the enzymes in solution. The activity of the immobilised GOx measured by the standard assay corresponded to a coverage of only 0.12 pmol cm^{-2}, reflecting again the likely blocking of the enzyme active centers and possible glucose diffusion limitations caused by the layers deposited on top. The estimated HRP coverage was 2.7 pmol cm^{-2}, in good agreement with the values obtained for the single-enzyme coated colloids. These values correspond to a GOx:HRP ratio of 1:11.5 in units cm^{-2} and a molar ratio of 1:22.7. In the GOx coupled assays, the rate of formation of product was not linear within the measurement time of 1 minute. As will be explained later in detail, this reflects the accumulation of peroxide in the bulk and the corresponding increase of the rate of consumption with time. However from the comparison of the final values of absorbance for the standard and coupled assays, an efficiency E_{GOx} of 40.6±3.7% was calculated, indicating that a significant amount of the H_2O_2 produced by GOx is not converted by the immobilised HRP (Figure 2.6). The efficiency obtained from the HRP coupled assay was only 2.33±0.16%, showing again that under these conditions the rate of formation of product is severely diminished. Recently, the feasibility of the coupled reaction between both immobilised enzymes was demonstrated in a similar multi-enzyme GOx/HRP film adsorbed on polystyrene particles [17]. However the measured GOx activities were orders of magnitude lower than the corresponding values for monoenzyme multilayers, which was attributed to the low amount of HRP immobilised within the film. It should be also noted that the multienzyme films in that work were composed by two inner layers of HRP and two outer layers of GOx, i.e. in the opposite order as the films assembled in the present work. Different studies with multienzymatic assemblies have shown that the structure of the film

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and the position of each enzyme layer within the film significantly influence the catalytic activity of the system [14, 16, 58]. In similar systems with GOx and HRP co-immobilised within hollow capsules with polyelectrolyte walls, a remarkable decrease in catalytic activity (70-85%) was observed when compared to the activity of the free enzymes [18, 19]. The proposed reasons for this decrease included the formation of complexes between the enzymes and the capsule constituents and also limitations to the diffusion of glucose through the polyelectrolyte film.

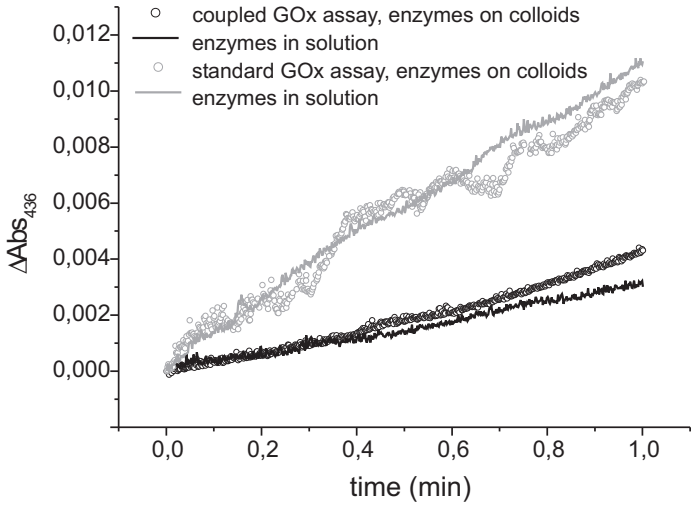

Figure 2.6: Time course of the conversion (absorbance at 436 nm) of the sequential reaction catalysed by GOx:Binder and oxHRP:POs complexes assembled in separate layers on PDADMAC/PSS coated particles. The values of absorbance obtained from the coupled GOx assay (black circles) were significantly lower than for the standard assay (grey circles), showing that only a fraction of the H_2O_2 generated at the GOx layer is converted at the HRP layer. Solid lines correspond to the same assays performed with equivalent amounts of both enzymes free in solution (see text).

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A scheme of the reaction of HRP with hydrogen peroxide and ABTS is depicted in Figure 2.7, where a conventional ordered ping-pong mechanism with two substrates and two products is assumed [59].

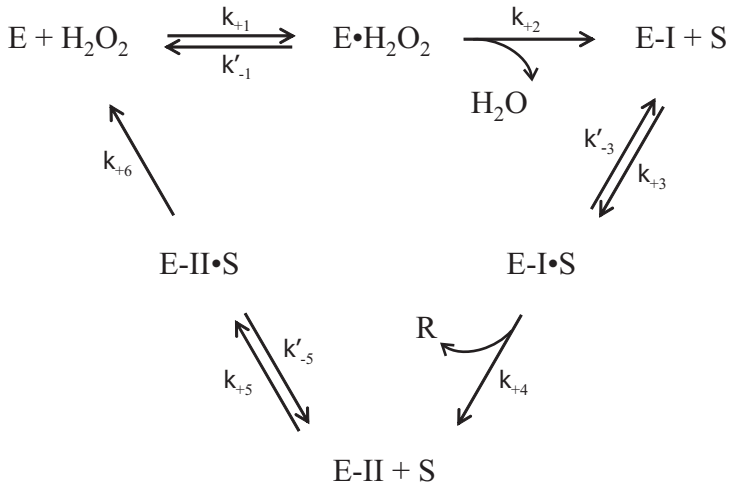

Figure 2.7: Mechanism of reaction of HRP with H_2O_2 and ABTS. The catalytic cycle starts with the oxidation of the native enzyme E (iron formal oxidation state: +III) by H_2O_2 to give the oxyferryl π-cation radical heme ($[Fe^{IV} = O]^{\bullet +}$ intermediate, E-I. The enzyme returns to the resting Fe^{III} state by two successive single electron-transfer reactions with the reducing cosubstrate S; in the first of these two steps the porphyrin radical cation of E-I is reduced to give the intermediate oxyferryl E-II.

In the presence of H_2O_2, ABTS is oxidised to generate the metastable radical $ABTS^{\bullet +}$. When the initial concentrations of H_2O_2 and ABTS are much higher than that of the enzyme, i.e. $[H_2O_2]_0$, $[ABTS]_0 \gg [HRP]_0$, the initial rate of production of the radical can be described by the expression:

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$$\upsilon_0 = \frac{V_{max}[H_2O_2]_0[ABTS]_0}{K_M^{ABTS}[H_2O_2]_0 + K_M^{H_2O_2}[ABTS]_0 + [H_2O_2]_0[ABTS]_0} \tag{2.6}$$

where V_{max} is the maximum steady-state rate of production and $K_M^{H_2O_2}$, K_M^{ABTS} are the Michaelis constants towards peroxide and ABTS, respectively. In terms of the reaction rate constants, these correspond to:

$$\begin{aligned} V_{max} = 2k_{+2}k_{+6}[HRP]_0/(k_{+2} + k_{+6}) = 2k_{cat}[HRP]_0 \\ K_M^{ABTS} = k_{cat}/k_{+5} \\ K_M^{H_2O_2} = k_{cat}/k_{+1} \end{aligned} \tag{2.7}$$

Rearranging eqn. 1.6 leads to:

$$\upsilon_0 = \frac{V_{max}^{app}[H_2O_2]_0}{K_M^{app} + [H_2O_2]_0} \tag{2.8}$$

with

$$\begin{aligned} V_{max}^{app} = \frac{2k_{cat}[ABTS]_0[HRP]_0}{K_M^{ABTS} + [ABTS]_0} \\ K_M^{app} = \frac{K_M^{H_2O_2}[ABTS]_0}{K_M^{ABTS} + [ABTS]_0} \end{aligned} \tag{2.9}$$

When $[ABTS] \gg K_M^{ABTS}$, as is the case for the measurements described in this work, the approximation can be made that $V_{max}^{app} \approx V_{max}$ and $K_M^{app} \approx K_M^{H_2O_2}$. Under these conditions, and for sufficiently high concentrations of H_2O_2, the rate-determining step in the catalytic cycle is the reduction of E-II, since

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for ABTS, as for many other peroxidase substrates, this reaction is much slower than the reduction of E-I, i.e. $k_{+2}k_{+6}/(k_{+2}+k_{+6}) = k_{cat} \approx k_{+6}$. As the concentration of hydrogen peroxide becomes smaller, however, the rate-determining step tends to be the formation of E-I, and when $[H_2O_2] \ll K_M^{H_2O_2}$ the kinetics are first order with $[H_2O_2]$:

$$v_0 = \frac{V_{max}[H_2O_2]_0}{K_M^{H_2O_2}} \tag{2.10}$$

In the case of the bienzymatic colloids, the observed rate of conversion of peroxide in the coupled HRP assay is much slower than that observed in the standard assay, when the concentrations of peroxide and ABTS are not limiting. As described above, this low rate of conversion corresponds to very low concentrations of hydrogen peroxide, even though it is being produced in the vicinity of the enzyme molecules. On the other hand, the results of the GOx coupled assays show that only a fraction of the produced peroxide is actually consumed, although the total peroxidase activity in units cm^{-2} is higher than the total GOx activity. The reasons for this can be based on topological considerations, taking into account the spatial configuration of the system. The HRP molecules do not cover the whole surface of the particles, and they are not necessarily homogeneously distributed. At the same time, the produced peroxide will rapidly diffuse from the GOx layer in all directions, since it has been reported that polyelectrolyte layers do not significantly affect H_2O_2 transport [60]. However only those H_2O_2 molecules which come into contact with the active centers of the HRP molecules will be able to react. Further time restrictions apply, in the sense that enzyme molecules which are already "busy" cannot accept new substrate molecules until the first catalytic cycle is over. The fraction of peroxide that is not

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collected by HRP in first instance will diffuse into the bulk solution. The peroxide that has diffused into the bulk may still be consumed at the surface of the colloids. Thus, the measured H_2O_2 conversion would correspond to two different processes taking place simultaneously: molecules that are consumed as they diffuse through the HRP layer, and molecules that have diffused into the bulk and again come into contact with the HRP on the colloids.

Measurements were performed with GOx and HRP in solution in equivalent amounts to the immobilised system (see Figure 2.6). This would approximately correspond to a situation where all the peroxide produced escapes into the bulk, altough in the case of the colloids the distribution of HRP is much less homogeneous. However the results show that for these concentrations of free GOx and HRP, the conversion of peroxide is even more inefficient within the time scale of the experiment, with a value of $E_{GOx} = 29.15 \pm 0.13$. At the low peroxide concentration present in the bulk, the reaction with HRP is controlled by H_2O_2 diffusion, resulting in very slow reaction rates. This can be compensated if the concentration of peroxidase is large enough, as in the case of the standard GOx assay. However in the coupled assay, the combination of substrate and enzyme concentration results in a rate of H_2O_2 consumption that is lower than the rate of production. The increased efficiency observed for the immobilised configuration suggests that a substantial amount of the produced peroxide is collected as it diffuses through the immobilised HRP layer, and that most of the observed HRP activity corresponds to the H_2O_2 molecules consumed in this process rather than to those diffusing back from the bulk solution.

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GOx and HRP in the same layer

Previous results showed that covering the GOx layer with further polyelectrolyte or enzyme layers greatly reduces the observed GOx activity. Therefore, it was speculated that if GOx and HRP could be co-immobilised as the top layer on the colloids, the amount of hydrogen peroxide produced would be increased, and this could also increase the overall activity of the system (i.e. the total amount of H_2O_2 converted by the immobilised HRP). In order to test this hypothesis, a mixture of POs, GOx and HRP was prepared and used to coat the particles. The three components were added sequentially as follows: the appropriate volume of GOx was pipetted first into tubes already containing POs, and immediately after mixing, the corresponding amount of HRP was added to the solution.

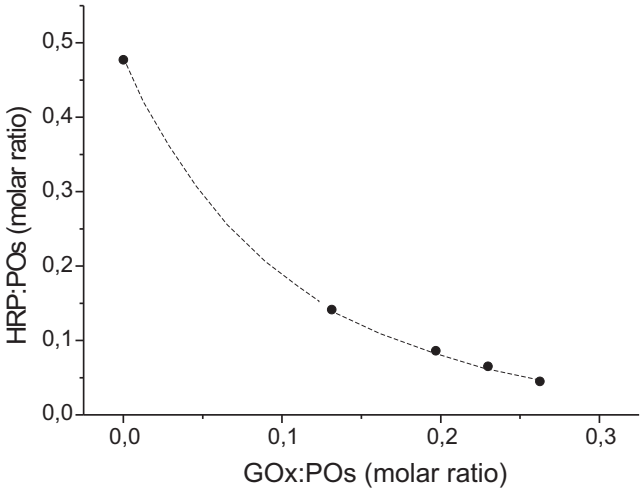

Figure 2.8: Relationship between the amounts of GOx and HRP in the coating solutions used to adsorb POs:GOx:HRP complexes on colloids. The amount of POs was constant for all the mixtures. The curve is drawn to guide the eye.

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For a constant volume of POs, the amounts of GOx and HRP in the mixture were varied in order to obtain different proportions of the two immobilised enzymes on the colloids. The maximum amount of HRP which allowed to obtain stable colloidal suspensions decreased along with increasing amounts of GOx in the coating solution (Figure 2.8). This reflects the fact that HRP is immobilised through covalent binding to amino groups of the redox polymer: on one hand, when HRP is added to POs molecules already complexed with GOx, it seems likely that a lower fraction of amino groups will be available for the second enzyme. On the other hand a high proportion of HRP in the POs/GOx/HRP complexes seems to increase the probability of destabilising interactions when the complexes are immobilised on the surface of the colloids. Bienzymatic colloids were also prepared using fluorescently labelled enzymes complexed with Binder instead of POs. Flow cytometry measurements and confocal microscopy imaging confirmed that both enzymes were successfully immobilised on the surface of the particles (Figure 2.9).

The activity of the bienzymatic colloids coated with POs:GOx:HRP complexes was investigated in the same way as described in the previous section. Table 2.3 summarises the results of the different activity tests performed. Standard enzyme assays showed that the HRP coverage decreased along with increasing GOx coverage, as expected from the composition of the coating mixtures. The values of E_{GOx} obtained from the coupled GOx assays decreased with increasing GOx coverage, corresponding to increasing GOx:HRP ratios, and they were in all cases lower than the value observed for the bilayer configuration (Figure 2.10). In other words, although more peroxide is being produced, a lower fraction of it is converted. This is reasonable because for lower HRP coverages, the probability of the diffusing

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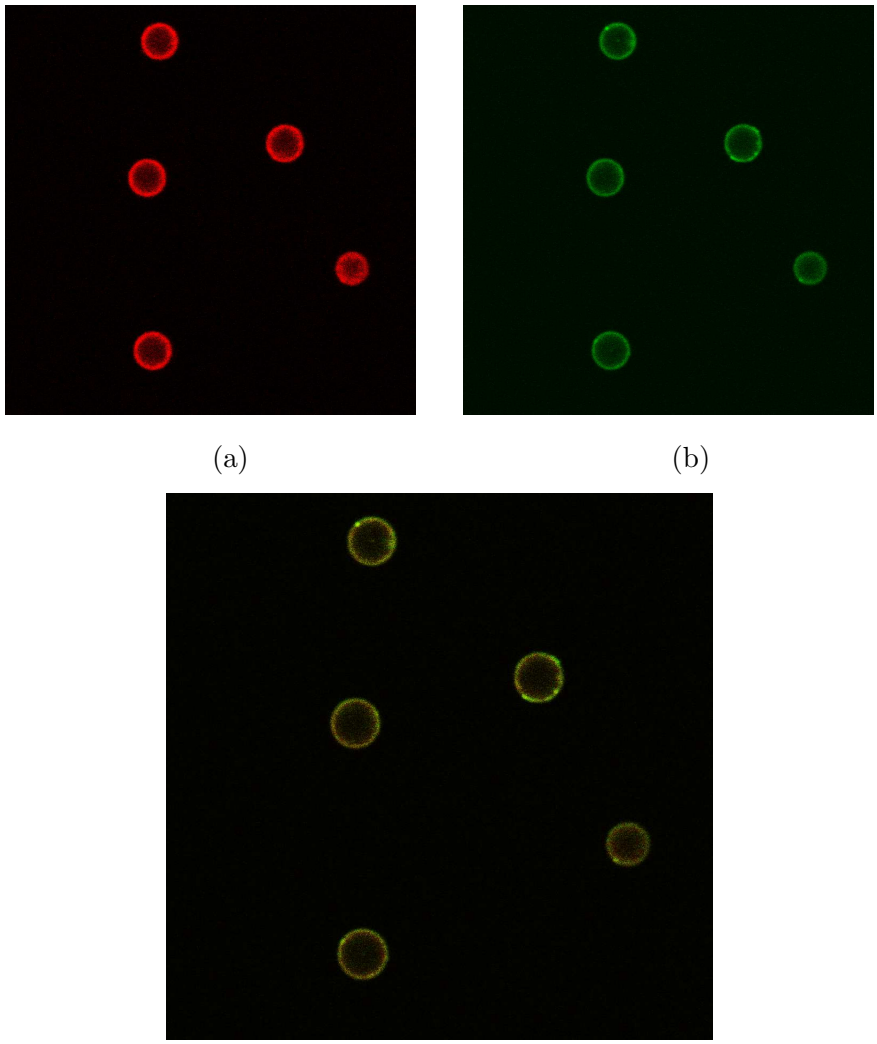

(a) (b)

(c)

Figure 2.9: CLSM images of SiO_2 particles coated with a mixture of GOx (rhodamine labelled) and oxHRP (FITC labelled) complexed with Binder. (a) GOx-Rho, (b) oxHRP-FITC, (c) Overlay of both images showing the presence of both enzymes in the layer.

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	Standard assay			Coupled
Configuration	**GOx**[a]	**HRP**[b]	**ratio**[c]	**Activity**[d]
Separate layers	2.59x10^{-3} (0.12)	2.97x10^{-2} (2.7)	0.087 (0.044)	0.049
Same layer	6.96x10^{-3} (0.32)	1.03x10^{-2} (0.94)	0.67 (0.34)	0.11
	1.23x10^{-2} (0.57)	7.65x10^{-3} (0.69)	1.61 (0.82)	0.16
	1.67x10^{-2} (0.77)	1.03x10^{-3} (0.09)	16.19 (8.24)	0.02
	1.68x10^{-2} (0.78)	6.27x10^{-3} (0.57)	2.69 (1.37)	0.13
	2.27x10^{-2} (1.05)	2.42x10^{-3} (0.22)	9.37 (4.77)	0.06

Table 2.3: Results of activity tests performed with bienzymatic colloids. [a] GOx coverage in units cm^{-2}. [b] HRP coverage in units cm^{-2}. The values in parentheses represent the respective enzyme coverages in pmol cm^{-2}. [c] Ratio of GOx:HRP units, molar ratio in parentheses. [d] Measured activity in coupled HRP assays using glucose as substrate, expressed as ΔAbs $min^{-1}cm^{-2}$.

peroxide molecules to come into contact with available enzyme active centers will be lower. Conversely, the same combination of factors, i.e. higher amounts of H_2O_2 and lower amounts of HRP, results in increased values of E_{HRP} for increasing GOx:HRP ratios.

The increase in efficiency E_{HRP} is sharp for the lower GOx:HRP ratios, where the peroxide collection efficiency is relatively high (as seen from E_{GOx} values), and as described above, the rate of peroxide conversion will increase linearly with the concentration of H_2O_2. Due to the limitations in the total amount of protein that can be immobilised on the colloids, the higher GOx:HRP ratios corresponded to layer configurations in which the amount of HRP was decreased but there was little or no increase in the GOx coverage, which explains the small increase in the values of E_{HRP}. In terms of absolute activity, the opposite trends in efficiency give rise to the following behaviour: the overall amount of hydrogen peroxide converted increases initially with increasing GOx:HRP ratios, as the local concen-

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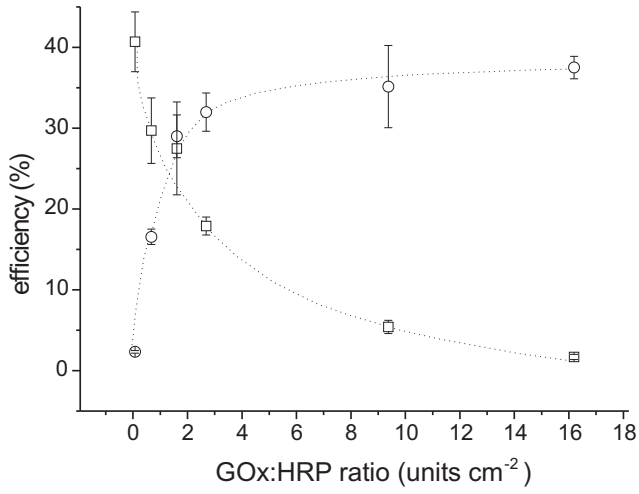
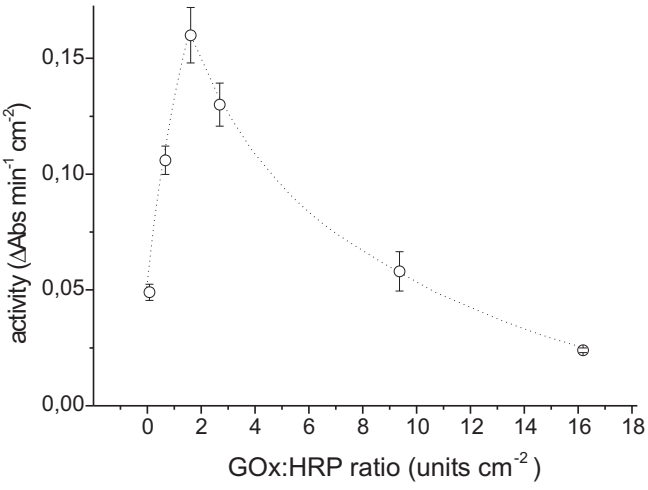


(a)



(b)

Figure 2.10: (a) Values of E_{GOx} (squares) and E_{HRP} (circles) obtained from the standard and coupled enzyme activity assays for different GOx:HRP ratios. (b) Absolute activity measured in HRP coupled assays. Curves are drawn to guide the eye.

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tration of H_2O_2 within the layer increases, and reaches a maximum for a GOx:HRP activity ratio of ≈1.6. This corresponds to a molar ratio of ≈0.82, so that there is at least one molecule of HRP per molecule of GOx. However for higher ratios, as the HRP coverage decreases, the maximum theoretical activity of the colloids will also decrease. Moreover, since now the number of peroxide-producing GOx molecules greatly exceeds that of HRP molecules, more and more peroxide will escape into the bulk solution. These factors explain the progressive decay in the observed activity for increasing GOx:HRP ratios.

In spite of these limitations, for all but one of the tested single-layer bienzymatic films the particles showed more activity than the colloids coated with GOx and HRP in separate layers, demonstrating the advantages of the former methodology. For the best configuration, the overall measured activity was 3 times higher than that of the bilayer films, although the amount of immobilised HRP was approximately 4 times lower. Moreover, the efficiency calculated from GOx coupled assays with equivalent amounts of both enzymes free in solution was only 6.78±0.27%, whereas the colloidal system showed a value of $E_{GOx} = 27.5 \pm 5.76\%$ (Figure 2.11). Thus, when GOx and HRP are immobilised in the same layer on the colloids, the increase in efficiency compared to the enzymes in solution is even higher than for the bilayer configuration.

2.4 Conclusions

Multilayer films of polyelectrolytes and enzymes were assembled on the surface of silica colloids. The influence of different supporting and alternating polyelectrolytes on the amount of immobilised enzymes and their catalytic activity was examined. The polyelectrolyte pair PDADMAC/PSS

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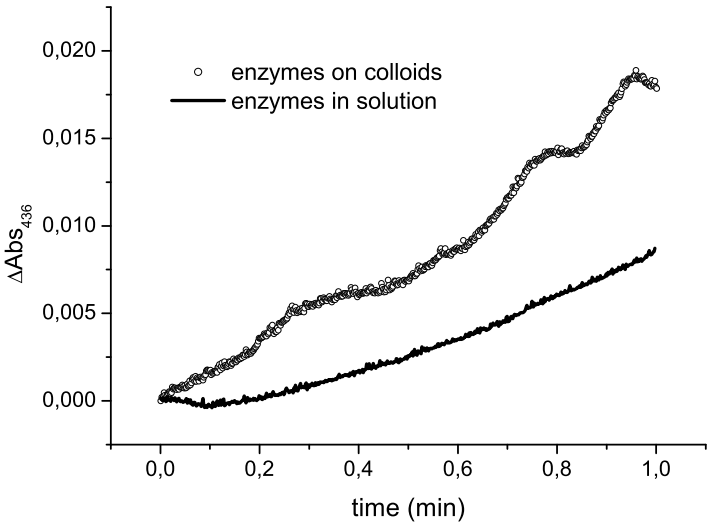

Figure 2.11: Time course of the conversion (absorbance at 436 nm) of the sequential reaction catalysed by GOx:oxHRP:Binder complexes assembled on PDADMAC/PSS coated particles (GOx coupled assay). The amount of converted H_2O_2 is larger for the colloidal system (GOx:HRP activity ratio ≈1.6) than for approximately equal amounts of both enzymes free in solution.

was found to be a suitable support for the enzyme layers. Precomplexation of glucose oxidase and horseradish peroxidase with the osmium redox polymer POs or the homologous non-osmiated polymer allowed for the deposition of high-density protein layers with good retention of the catalytic activity. This is a promising result in the way to develop an electrochemical interface through the immobilisation of the colloids on the surface of an electrode, as will be described in the next chapter. However the adsorption of further polyelectrolyte layers on top of the assembled GOx greatly reduced

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the observed activity, presumably due to blocking of the enzyme active centers. This limited the efficiency of the bienzymatic colloids in which a sequential reaction took place between an inner GOx:Binder layer and an outer HRP:POs layer. In order to circumvent this problem, POs:GOx:HRP complexes were formed and successfully assembled as a single layer on the surface of PDADMAC/PSS coated particles. In the optimised configuration, these constructs showed three times more activity than the system with GOx and HRP immobilised on separate layers. Because the total amount of both enzymes that can be immobilised on the surface of the colloids is limited, it was not possible to assemble films having simultaneously high GOx and HRP activities. As a result of the ratio between the specific activities of both enzymes and of the spatial configuration of the films, in all cases a significant fraction of the produced peroxide escaped into the bulk solution and was not consumed. However it was found that the co-immobilisation of GOx and HRP on the surface of the silica colloids substantially improves the peroxide collection efficiency of HRP with respect to the behaviour of equivalent amounts of both enzymes in solution.

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Chapter 3

Electrocatalytic activity of enzyme-coated colloids

3.1 Introduction

Electrical connection of redox enzymes to the surface of an electrode is a convenient transduction strategy which allows the detection and study of specific chemical events taking place at the active centers of the catalyst by converting them into electrical signals [1, 2]. Thus, enzyme electrodes are widely used because they combine highly specific and sensitive biorecognition elements with relatively simple and inexpensive detection methods [3]. In the last decades, this kind of systems have been especially important in the field of analytical sensors. However the generation of electrical signals allows also for integration into a variety of bioelectronic devices ranging from electrically driven bioreactors to optical memories or biofuel cells [4].

A central topic in the development of bioelectronic systems is the design of an appropriate electrical connection between biomaterials and conduc-

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tive supports. In particular for redox enzymes, electrical communication between the protein and the electrode interface can be achieved in some cases by direct electron transfer, although usually the shielding of the enzyme redox center by the surrounding protein shell prevents the process or renders it prohibitively slow. More commonly, electrical connection is achieved through the use of redox mediators which shuttle electrons in the required direction. Redox mediators can be present as freely diffusing molecules in the electrolyte solution or they can be co-immobilised together with the enzymes on the surface of the electrode. The first type of mediation is often more efficient and higher responses can be achieved, however immobilised redox mediators have the major advantage of allowing to develop integrated, self-contained reagentless devices. Perhaps the most successful examples of this approach are the redox hydrogels introduced by Degani and Heller [5], in which a polymer backbone with covalently-bound redox centers is used simultaneously as immobilisation matrix and electrical "wire" for the enzymes. According to electron transfer theory [6], the donor-acceptor distance plays a key role in the control of the electron transfer rate, and therefore the spatial configuration of redox-enzyme based electrodes has a substantial influence on the performance of the system. In redox hydrogels, although extremely high current densities can be achieved, it is known that the overall electron transfer rate is often limited by the distance between the enzyme active sites and the redox centers of the polymer [7]. Once this first electron exchange step is accomplished, the rate of homogeneous charge transport through the film is further controlled by the segmental motion of the polymer backbone (allowing electron self-exchange between redox centers) and by the transport of counterions required to mantain electroneutrality. In the last years, the layer-by-layer technique has been employed for the assembly of well-organised enzyme-polyion films on the

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surface of electrodes, since it allows control of the composition and thickness of the films down to the nanometer level [8]. Hodak et al. and Hou et al. [9, 10] first fabricated electrostatically assembled multilayers of glucose oxidase alternated with a ferrocene-modified cationic polymer which acted as redox mediator. A similar approach was taken by other groups in studies with osmium-based redox polymers and different enzymes, like glucose oxidase, horseradish peroxidase, polyphenol oxidase, lactate oxidase, pyruvate oxidase, fructose oxidase and alcohol oxidase [11–13]. Although some of these studies showed that not all the enzyme molecules were effectively "wired" by the co-immobilised redox mediator, it was also found that organised multilayers can be comparatively more efficient than hydrogel configurations in terms of current density [14].

In a study with myoglobin and cytochrome $P450_{cam}$, Lvov and coworkers [15] demonstrated that thin films assembled by layer-by-layer deposition of proteins and polyions can also facilitate direct electron transfer between redox enzymes and gold electrodes. Again it was found that the electroactivity of the enzyme layers strongly depended on the distance from the electrode. Interestingly, by using rougher electrode surfaces the number of electroactive protein layers was extended from 2 to 7, which was attributed to a decrease in the enzyme-electrode distances due to enhanced mixing of neighbouring layers [16]. Following the extension of the layer-by-layer technique from planar to colloidal surfaces, two groups reported the fabrication of electroactive films incorporating enzyme-coated particles prepared through layer-by-layer immobilisation [17–21]. In their studies with hemoglobin and myoglobin, Hu and coworkers found that the protein-particle films exhibited better direct electron transfer and electrocatalytic properties than the corresponding protein-polyelectrolyte films prepared without particles. Although in the first configuration the distance between

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the enzymes and the electrode surface is increased, the more porous structure of these constructs allows more efficient mass transport of counterions and reaction substrates though the film. Combined with the high enzyme loading capacity of the particles, this results in a larger fraction of electroactive proteins and an improved overall performance of the system.
In the present work, enzyme-coated colloids were used to fabricate electroactive films on the surface of electrodes. In an effort to combine the advantages of this approach with those of mediated electrochemistry, an osmium-based redox polymer was incorporated to the structure in order to facilitate electron transfer between the immobilised enzyme and the surface of the electrodes. The functionality of different colloid- and film configurations was examined. As a first step, the electrocatalytic activity of the constructs was demonstrated by measuring the electroreduction of hydrogen peroxide. A more sophisticated system was also explored, based on the sequential action of co-immobilised glucose oxidase and horseradish peroxidase. This multienzymatic platform allowed for the electrochemical determination of glucose at low applied potentials and under aerobic conditions.

3.2 Materials and methods

Enzymes and other reagents were obtained as described in Chapter 2. The crosslinker poly(ethylene glycol) diglycidyl ether (PEGDGE) was purchased from Polysciences. Potassium ferro- and ferricyanide (reduced and oxidised forms, respectively) were obtained from Aldrich. Hydrogen peroxide (30% w/w in water) was supplied by Sigma. Electrochemical measurements were carried out in a conventional three-electrode electrochemical cell using a computer controlled potentiostat Autolab PGSTAT10 with the GPES

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software from EcoChemie. Potentials were measured against a potassium chloride-saturated silver/silver chloride electrode, and a platinum mesh was used as counter electrode. The supporting electrolyte for electrochemical measurements was 0.1 M sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl. Measurements were performed at 25 °C. The working electrodes were gold disks of 2 mm diameter. Prior to modification, the gold electrodes were polished successively with 0.3 and 0.1 μm alumina slurry (Buehler) followed by sonication and extensive rinsing in deionised water. The real surface area of the electrodes (geometric area 0.03 cm^2) was determined by performing potential scans between -0.2 and 1.8 V in 2 M sulphuric acid at a scan rate of 0.2 V s^{-1}. Integration of the reduction peak at 0.8 V yielded the corresponding values of charge passed through the electrode. Using a ratio of charge/area of 400 μC cm^{-2}, the real area of the gold disks was estimated to be 0.05 cm^2 (roughness factor 1.6).

3.2.1 Immobilisation of colloids on electrodes

In order to cover the surface of the gold electrodes with the enzyme-coated colloids, a 2.5 μL drop of the particle suspension was deposited on the electrode and allowed to dry. The particle concentration of the suspensions was adjusted so that the total number of colloids contained in the drop would be sufficient to achieve maximum coverage of the surface of the electrodes. Assuming a theoretical maximum coverage of 60% for randomly packed particles and a projection area of 7 μm^2 for each particle, approximately 270000 colloids can be immobilised on the surface of the electrode in a monolayer configuration. This would correspond to a surface area of 0.076 cm^2, roughly 2.5 times the geometric surface area of the gold disk. The final concentration of the particles was therefore adjusted to approximately

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120000 particles per microliter. The suspensions were prepared either in 0.1 M NaCl or in a 1 mg mL^{-1} aqueous solution of POs containing 10% wt. PEGDGE. In the last case, the total weight of redox polymer and PEGDGE applied to the electrode surface was calculated to yield a film of 50 μg cm^{-2}. Electrodes prepared with suspensions in POs-PEGDGE were left to cure overnight in order to allow the crosslinking reaction to proceed. All electrodes were extensively rinsed in milliQ water before use to remove excess salt and weakly adsorbed material.

3.2.2 Scanning electron microscopy

SEM images were acquired with a FEI Quanta 600 microscope at an acceleration voltage of 20 kV. Electrodes were prepared in the same way as described for the electrochemical measurements. The electrodes were then attached to a support inside the microscope chamber and the surface of the gold disk with the immobilised colloids was imaged.

3.3 Results and Discussion

In the previous chapter the co-immobilisation of glucose oxidase and horseradish peroxidase on the surface of polyelectrolyte coated colloids was described. The functionality of both enzymes after immobilisation was confirmed by colorimetric activity tests performed with the colloids in solution. The next objective was to create an electrochemical interface by immobilising the modified colloids on the surface of an electrode. The proposed electrochemical detection scheme is based on the electrocatalytical reduction of hydrogen peroxide by the enzyme horseradish peroxidase, with a co-immobilised osmium redox polymer acting as cosubstrate for the en-

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zyme and relaying electrons from the surface of the electrode to the active centers of the HRP molecules.

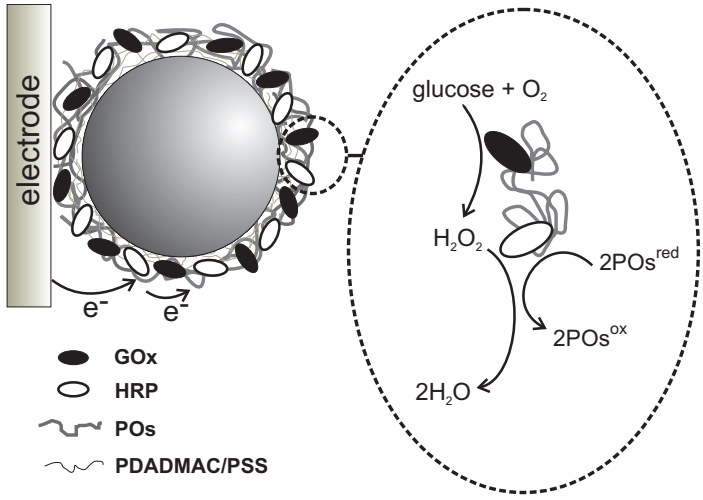

Figure 3.1: Schematic representation of an electrode modified with POs/GOx/HRP-coated colloids. A sequential reaction takes place within the enzyme layer, involving also electron transfer between HRP and the co-immobilised redox polymer POs. The polymer in turn is able to exchange electrons with the surface of the electrode, generating an electrochemical signal.

Electrocatalytic cycle for H_2O_2 reduction For enzyme electrodes modified with HRP and osmium redox polymer, the sequence of reactions taking place in the film can be described as follows [1, 22]:

$$\mathrm{HRP} + \mathrm{H_2O_2} \underset{\mathrm{k_{-1}}}{\overset{\mathrm{k_1}}{\rightleftharpoons}} \mathrm{HRP \cdot H_2O_2} \tag{3.1}$$

$$\mathrm{HRP \cdot H_2O_2} \xrightarrow{\mathrm{k_2}} \mathrm{HRP\text{-}I} + \mathrm{H_2O} \tag{3.2}$$

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$$\text{HRP-I} + \text{R}_{\text{red}} \xrightarrow{k_3} \text{HRP-II} + \text{R}_{\text{ox}} \tag{3.3}$$

$$\text{HRP-II} + \text{R}_{\text{red}} \xrightarrow{k_4} \text{HRP} + \text{R}_{\text{ox}} \tag{3.4}$$

where HRP is the native ferriperoxidase (Fe^{III}), HRP·H_2O_2 is the enzyme-substrate precursor complex, HRP-I is the oxyferryl π-cation radical compound I ($[Fe^{IV} = O]^{\bullet+}$, HRP-II is the oxyferryl compound II ($Fe^{IV} = O$), and R_{red} and R_{ox} represent the reduced and oxidised forms of the osmium centers in the redox polymer, respectively. At high substrate concentrations inhibition of the enzyme can take place either by irreversible conversion into an inactive verdohaemoprotein (P670) or through the formation of oxyperoxidase, also designated by compound III. This last pathway is not irreversible, since compound III may reenter the catalytic cycle by spontaneous decomposition, yielding the native enzyme, or by reduction to compound I by the osmium cosubstrate. Under the experimental conditions used in this work both inhibition pathways should be negligible, since the hydrogen peroxide concentration remains well below the saturation level. However for electrodes modified with bienzymatic colloids, the situation is further complicated by the fact that the substrate for the immobilised HRP, hydrogen peroxide, must be produced by another immobilised enzyme, glucose oxidase. In this case the reaction scheme is the following:

$$\text{GOx-FAD} + \text{glucose} \longrightarrow \text{GOx-FADH}_2 + \text{gluconolactone} \tag{3.5}$$

$$\text{GOx-FADH}_2 + \text{O}_2 \longrightarrow \text{GOx-FAD} + \text{H}_2\text{O}_2 \tag{3.6}$$

where GOx-FAD represents the oxidised form of the flavin adenine dinucleotide cofactor at the active site of the enzyme, and $GOx\text{-}FADH_2$ is the reduced form. The produced hydrogen peroxide may then diffuse and reach

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the HRP molecules in the vicinity, starting the catalytic cycle described in equations 3.1-3.4. If these HRP molecules are effectively connected or "wired" by the osmium redox polymer, upon applying an adequate potential a current can be measured corresponding to the reduction of the oxidised osmium centers at the surface of the electrode.

3.3.1 Characterisation of colloid-modified electrodes

In order to test the feasibility of establishing electrical connection between the enzyme-coated colloids and the surface of an electrode, the first question to be addressed was the procedure for immobilisation of the colloids. When a drop of the modified particles suspended in NaCl was allowed to dry on bare gold electrodes, a considerable amount of colloids remained attached to the surface even after washing the electrodes under vigorous stirring, as observed by scanning electron microscopy. However the distribution of the colloids was quite irregular and frequent aggregates could be observed (Figure 3.2a). When cyclic voltammetry was performed with these electrodes, two peaks corresponding to oxidation and reduction of the osmium redox polymer could be identified, but the peak currents were of only a few nanoamperes even at fast scan rates (50 mV s^{-1}), indicating low efficiency of electroechemical connection with the electrode or a low coverage of osmium redox centers, and the peak splitting ΔE_p was around 70 mV, corresponding to very slow charge-transfer kinetics on the electrodes (Figure 3.2b). In this type of redox polymers, the main mechanism of charge transfer is through collisions between osmium redox centers tethered to the polymer backbone. Thus, electron transfer through the film depends on the mobility of the lateral chains carrying the redox sites. In the case of the polymer-coated colloids, the few osmium centers which are in direct

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contact with the surface of the electrode should be able to exchange electrons rapidly, but "diffusion" of electrons through the rest of the layer will depend on self-exchange reactions between the remaining redox sites of the polymer, which in turn depends on the segmental mobility. If the configuration of the redox polymer layer and its interaction with the underlying polyelectrolytes and the co-immobilised enzyme molecules restricts the mobility of the osmium centers, charge transfer through the redox polymer will become slow, as observed for the electrodes described above. In addition, it is well known that the glycoprotein shell surrounding the active centers of the enzymes acts as an electrical insulator. Thus, when a film is created by mixing a conducting redox polymer with enzymes, high amounts of protein make the films increasingly resistive. Assuming that the ratio of polymer to enzyme is the same on the colloids as on the coating solutions, approximately one third of the film assembled on the surface of the colloids is made up of protein. Combined with the factors outlined above, this could also contribute to decrease the conductivity of the POs-enzyme film.

Colloid-polymer films The second strategy employed for immobilisation of the colloids consisted in resuspending the particles in a solution of POs and the crosslinking diepoxide PEGDGE, which can react with amino groups present both in the enzymes and in the redox polymer. The purpose of this procedure was to increase the amount of redox polymer in contact with the electrode surface and to decrease the relative proportion of insulating enzyme in the film, making it more conducting. At the same time, more redox centers should also be available to mediate the electron transfer between the surface of the electrodes and the active centers of the enzymes. It has been shown before for electrostatic assemblies that increasing the concentration ratio of polymer to enzyme significantly increases the

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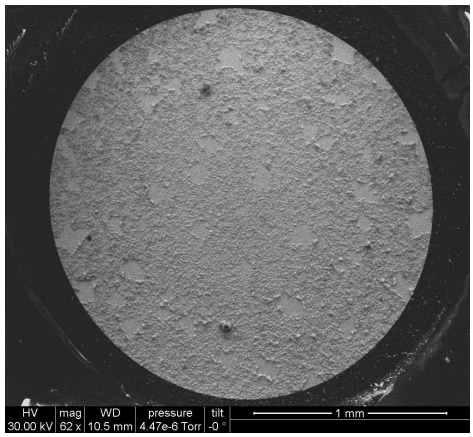
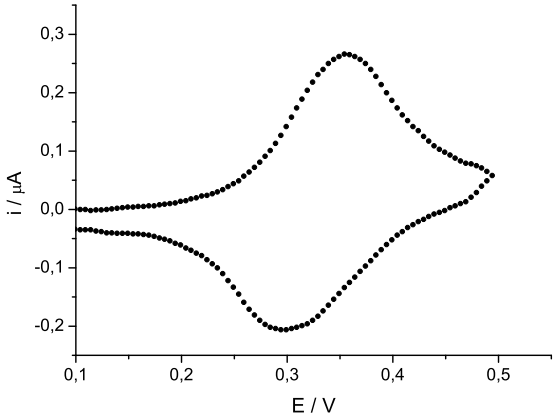


(a)



(b)

Figure 3.2: (a) SEM image of bienzymatic POs/GOx/HRP-coated silica colloids deposited on the surface of a gold electrode, showing an irregular distribution. (b) Cyclic voltammetry in 0.1 M phosphate buffer, 0.15 M NaCl, pH 7.0, at 0.05 V s^{-1}. The large separation between the oxidation and reduction peaks is indicative of slow charge transfer kinetics.

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fraction of electrically connected or "wired" enzyme [14, 23, 24].

As seen in Figure 3.3, electrodes prepared in this way showed a relatively homogeneous coverage with the particles regularly arranged on the gold surface. This suggests that a more uniform distribution is reached when the particles are embedded in the polymer as the mixture is left to dry on the surface of the electrodes. In order to estimate the amount of colloids immobilised on the surface, images of 200x200 μm sections of the gold disks were taken and the particles contained in each of these sections were counted with the help of the image analysis software ImageJ. It should be noted that the imaging was performed after several electrochemical measurements had been conducted with these electrodes in vigorously stirred solutions. The results showed that an average of 292000 particles (RSD=21%, n=31) remained attached to the surface of the electrodes.

Figure 3.4 shows a typical cyclic voltammogram for a gold electrode modified with enzyme-coated colloids and POs. A pair of well defined peaks are observed at about +0.325 V, characteristic of the reversible oxidation of osmium redox sites. At low scan rates (1 to 3 mV s^{-1}) the voltammograms showed an almost symmetric wave typical of a surface-confined electroactive species, with peak splitting ΔE_p = 12 mV. At higher scan rates (5 to 50 mV s^{-1}) a tailing of the wave indicates diffusional control of the charge transfer, with the peak splitting correspondingly increasing to a value around 23 mV which remains constant within this range of scan rates. A surface concentration of redox centers of 5.75 x 10^{-9} mol cm^{-2} was calculated by integration of cyclic voltammograms at low sweep rate (2 mV s^{-1}), with all of the osmium centers in the film being oxidised and reduced within the time scale of the measurements. According to the estimations of the amount of immobilised enzyme, this corresponds to a Os to HRP ratio of ≈1300 for the monolayer configuration and ≈2700 for the bilayer config-

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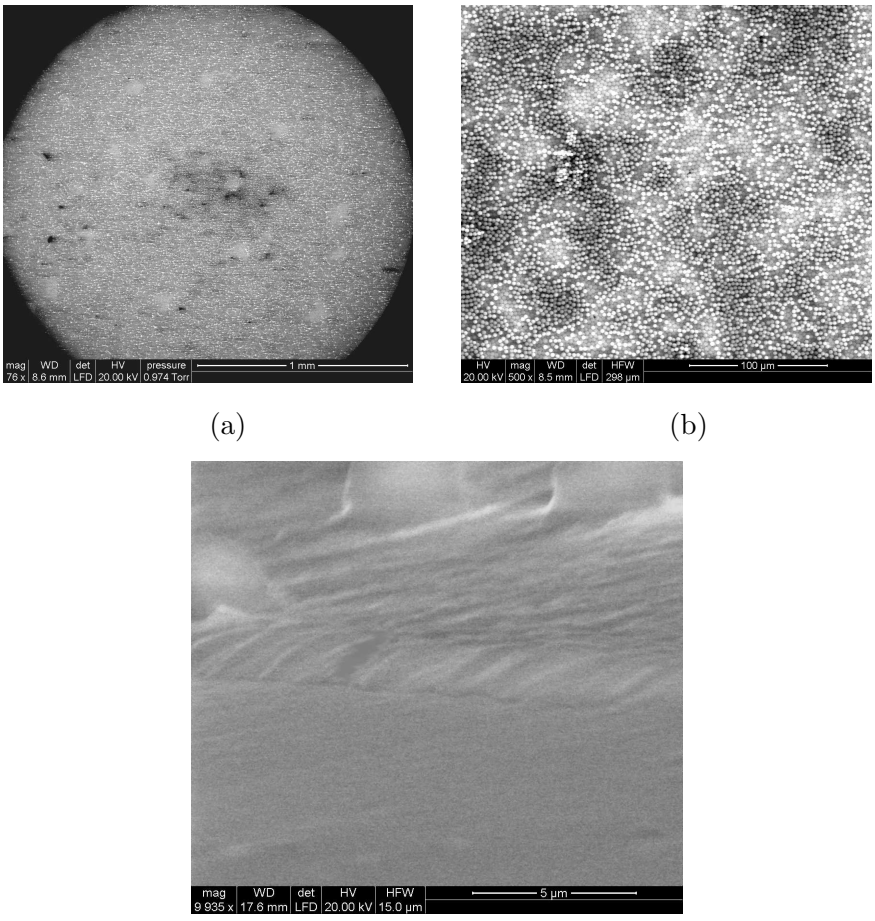

(a) (b)

(c)

Figure 3.3: SEM images of a gold electrode modified with bienzymatic POs/GOx/HRP-coated silica colloids entrapped in a POs matrix. (a) Surface of the 2 mm diameter gold disk, (b) Section of the electrode surface at higher magnification, (c) Section at the edge of the gold disk, showing the colloids embedded in a polymer layer.

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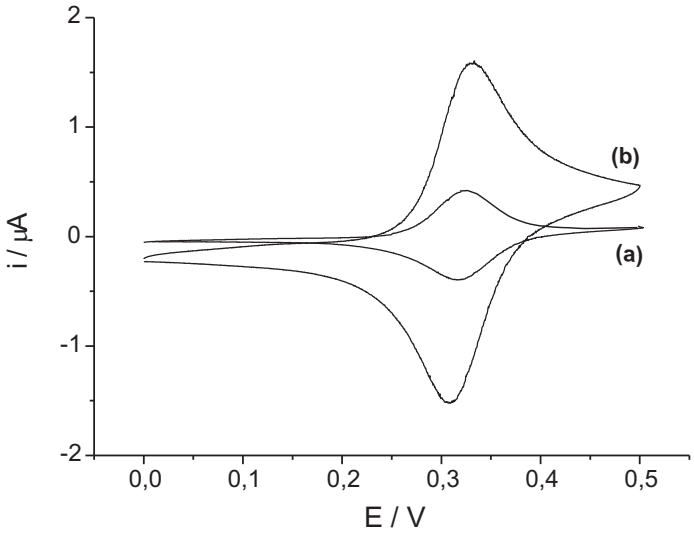

Figure 3.4: Cyclic voltammograms at (a) 0.002 and (b) 0.01 V s^{-1} of a typical POs/SiO_2 film immobilised on the surface of a gold electrode. The observed features are characteristic of the reversible oxidation of the osmium redox polymer (0.1 M phosphate buffer, 0.15 M NaCl, pH 7.0).

uration. When the high molecular weight redox polymer is deposited on an electrode, a three-dimensional hydrophylic network is formed. If particles are incorporated in these films, part of the hydrogel will be confined to the channels formed between the particles. The diameter of these channels depends on the dimensions of the particles. If the channels are small, the increased viscosity of the gel in these regions may reduce the diffusivity of ions, reactants and products, having a negative effect on the electron transport characteristics of the film [25]. However the behaviour of the electrodes coated with the POs/colloids film shows the fast charge transfer kinetics typical of the osmium redox polymer, implying rapid movement of counterions through the redox hydrogel [26]. This suggests that the pres-

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ence of the non-conducting silica particles does not substantially hinder charge transport through the colloid-polymer film.

3.3.2 Electrocatalytic activity of colloid-POs electrodes

Response to hydrogen peroxide

Electrodes were prepared using the two different colloid configurations described in the previous chapter: bilayer (GOx and HRP separated by a PSS layer) and monolayer (GOx and HRP in the same layer) [see Figure 3.5]. The performance of both types of electrodes is compared in the next sections. The electrocatalytic behaviour of the enzyme-coated colloids immobilised on gold electrodes was evaluated by performing amperometry at a controlled potential. In the presence of the corresponding substrates and cosubstrates, the catalytic activity of the enzymes generates a current that is measured as a function of time. Owing to its simplicity, this electrochemical technique is the most employed one for the detection of hydrogen peroxide at enzyme-based electrodes. The first step to investigate the electrochemical behaviour of the colloid-coated electrodes was to establish whether effective electrical connection had been achieved between horseradish peroxidase and the osmium redox polymer, and consequently between the enzyme and the surface of the electrode. Thus, the steady state response of the electrodes was measured at an applied potential of 0 V in the presence of increasing concentrations of hydrogen peroxide. Measurements were carried out under different hydrodynamic conditions, either in quiescent solutions or under vigorous stirring. For the measurements in quiescent solutions, the appropriate volumes of hydrogen peroxide were added to the cell before connecting the working electrode, and afterwards the response of the electrode was measured. In the case of the stirred so-

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lutions, the electrodes were allowed to reach a baseline after application of potential. From that moment on, successive injections of substrate were made to bring the concentration in the cell to the desired values, each time waiting for a steady state current before making the next injection.

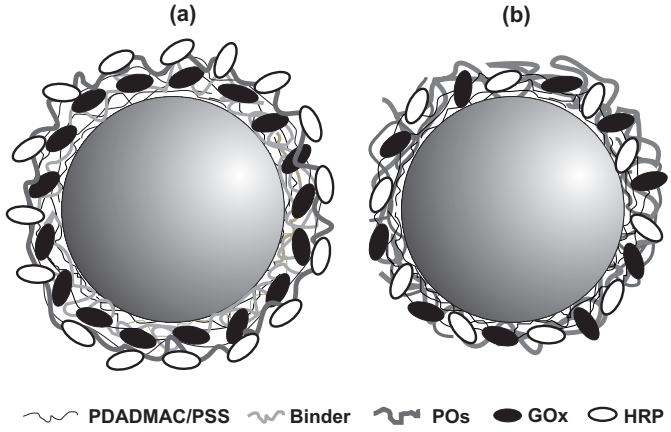

Figure 3.5: Schematic representation of the two different colloid configurations. (a) Bilayer colloids, with a GOx/Binder layer and a HRP/POs layer separated by an intermediate PSS layer. (b) Monolayer configuration, with POx/GOx/HRP adducts immobilised as a single layer.

Bilayer configuration As shown in Figures 3.6 and 3.7, upon addition of hydrogen peroxide to the cell a reduction current was developed, demonstrating the existence of electrical connection between the immobilised HRP and the osmium redox polymer. The results also show the influence of substrate mass transport on the observed steady state current. For redox hydrogels incorporating HRP and the same osmium polymer used in this work, with an estimated film thickness of 0.8 μm, significant mass transport effects were found even for high peroxide concentrations ($\leq 600\mu$M)

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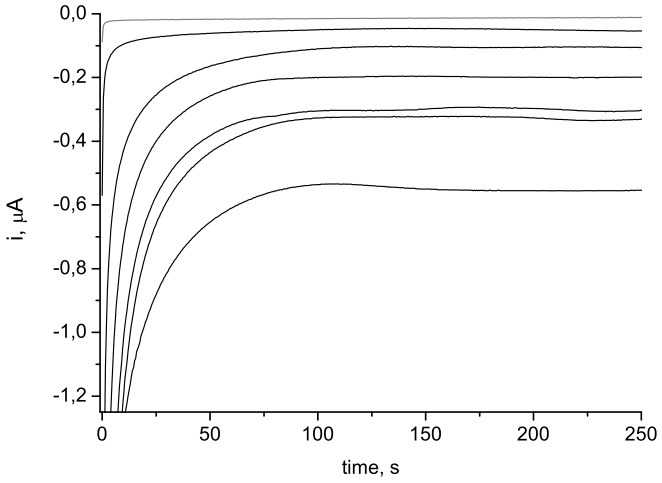

Figure 3.6: Chronoamperometric response of a bilayer colloid-POs film to increasing H_2O_2 concentrations in a quiescent solution, at an applied potential of 0 V. From top to bottom: blank (0.1 M phosphate buffer, 0.15 M NaCl, pH 7.0), 10μM, 20μM, 40μM, 80μM, 100μM, 200μM hydrogen peroxide.

and high rotation speeds (1500 rpm) when the film was immobilised on the surface of a rotating disk electrode [1]. Even for extremely thin films comprised of two electrostatically assembled bilayers of glucose oxidase and an osmium redox polymer, with a thickness of only a few nanometers, Pishko and coworkers reported the existence of mass transfer limitations [27]. Under convective diffusion conditions, the response of the electrodes to peroxide injections was fast and the maximum current density was 30.25 μA cm^{-2}. This value compares well with those reported in other studies for a single HRP layer in LbL assemblies of this enzyme with osmium redox polymers (10.2 and 12.7 μA cm^{-2}) [13,28,29]. A Hanes-Woolf linearisation

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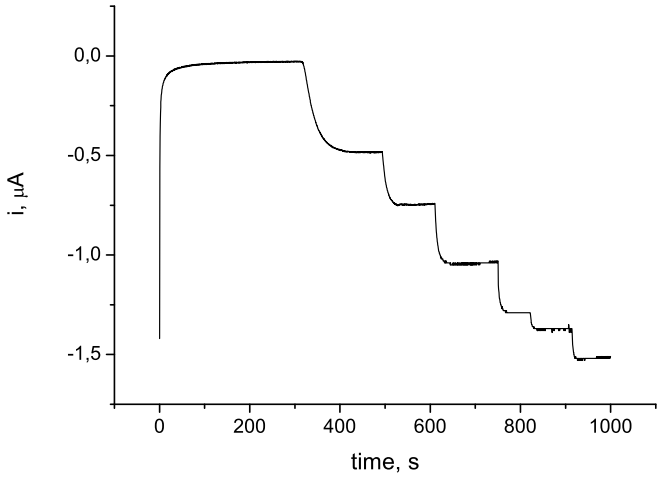

Figure 3.7: Chronoamperometric response of a bilayer colloid-POs film to successive H_2O_2 injections in a stirred solution, bringing the concentration in the cell from 10 to 200μM (0.1 M phosphate buffer, 0.15 M NaCl, pH 7.0, working potential 0 V).

of the data from the calibration curve was used to estimate the value of the apparent Michaelis-Menten constant K_M^{app} (Figure 3.8). Starting from the electrochemical form of the Michaelis-Menten equation, if the ratio of substrate concentration [S] to the observed catalytic current i is plotted against [S], the x-intercept will take the value of -K_M^{app}:

$$i = \frac{i_{max}[S]}{K_M^{app} + [S]} \tag{3.7}$$

$$\frac{[S]}{i_{max}} = [S]\frac{1}{i_{max}} + \frac{K_M^{app}}{i_{max}} \tag{3.8}$$

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In this way a value of K_M^{app}=27.2 μM was obtained for this electrode configuration. It must be pointed out that in the case of immobilised enzymes, K_M^{app} is not an intrinsic property of the enzyme itself, but rather of the complete system in a particular environment [26], and thus it can differ considerably from the value observed for the enzyme in solution (27-128 μM with different electron donors in solution [30]). In this kind of enzyme electrodes, K_M^{app} is a phenomenological parameter which defines the concentration of substrate that gives half the maximum current response, and it is influenced by construction parameters like enzyme immobilisation method and total amount of enzyme and redox polymer, as well as by substrate diffusion. Furthermore for the case of HRP, if the one-substrate form of the Michaelis-Menten equation is used to approximate the two-substrate case (H_2O_2 as substrate and the redox polymer as cosubstrate), K_M^{app} contains also cosubstrate terms and does not necessarily approach the value of $K_M^{H_2O_2}$ (see equation 2.9 in the previous chapter, p. 43). However this parameter provides useful information about the range of concentrations over which the response of the electrode is approximately linear. For the electrodes modified with bilayer GOx/HRP colloids, the slope of the calibration curve with H_2O_2 in the 0-25 μM range allowed to calculate a sensitivity of 0.72 A M^{-1} cm^{-2}. This value is one order of magnitude higher than those reported in other studies for a single HRP layer in LbL assemblies of this enzyme with similar osmium redox polymers [13, 28, 29]. In fact it is closer to the values found for hydrogels of HRP and redox polymer, which reach sensitivities over 1 A M^{-1} cm^{-2} [1, 31]. However the high sensitivity of these colloid-modified electrodes is accompanied by a relatively narrow linear range.

The sensitivity of an enzyme electrode is related to the amount of active immobilised enzyme and to its catalytic activity. In the above mentioned

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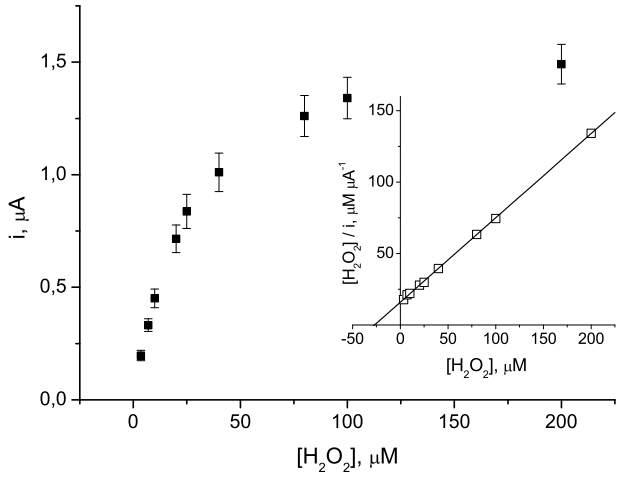

Figure 3.8: Steady-state response curve of bilayer-colloid electrodes (n=6) to H_2O_2 in a stirred solution. Inset: Hanes-Woolf plot of these values showing the linear dependence of the current on the hydrogen peroxide concentration. From the x-intercept of the plot, an apparent Michaelis constant K_M^{app}=27.2 μM was estimated. 0.1 M phosphate buffer, 0.15 M NaCl, pH 7.0, applied potential 0 V.

studies the specific activity of the peroxidase was similar or superior to that of the HRP used in this work, and therefore should not be a determining factor in the observed currents. On the other hand, the amount of enzyme that can be immobilised in redox hydrogels is much superior to that present even in multilayer LbL assemblies, which accounts at least in part for the higher sensitivities. However Calvente and coworkers reported that, of the total HRP entrapped in a redox polymer matrix (290 pmol cm^{-2}), only about 1% was effectively "wired" and thus contributed to the observed currents [1]. Calvo and Wolosiuk reached similar conclusions for LbL films of glucose oxidase and an osmium redox polymer built on flat supports [23].

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Interestingly, these authors also found that when the concentration ratio of osmium centers per enzyme molecule was increased from 3 to 100, the wiring efficiency correspondingly increased from 1 to 30%.

For the colloid-modified electrodes, when a freely diffusing mediator, in this case ferrocyanide, was added to the hydrogen peroxide solution, the observed catalytic current was substantially increased (Figure 3.9). This suggests that again for this kind of electrocatalytic films, part of the enzyme molecules are catalytically active but are not effectively wired by the osmium redox polymer. Although the reaction rates between the enzyme and the two mediators are different, it has been recently reported that in the case of POs/HRP redox hydrogels the rate of enzyme oxidation, rather than electron exchange with the redox polymer, is the limiting step in the response of the electrodes [1]. A more detailed characterisation would be necessary in order to obtain quantitative estimations of the wiring efficiency, especially when different redox mediators are involved. Recently, a number of methods have been described for the kinetic analysis of enzyme-polymer films which allow to evaluate the proportion of wired enzyme, although they eventually rely on parameter values obtained for the enzyme in solution [1, 9]. However a series of assumptions are made in terms of substrate mass transport and homogeneity of the electrocatalytic film which are not applicable under the experimental conditions used in this work.

The reason why not all of the immobilised enzyme is effectively wired is most likely the limited access of the osmium redox centers to the active centers of the enzyme molecules. In all these types of architectures, polymer and enzyme have a relatively random orientation with respect to each other and also to the surface of the electrode. However the osmium centers, bound to the polymer backbone, must approach the enzyme molecules in a specific orientation in order to increase the probability of electron ex-

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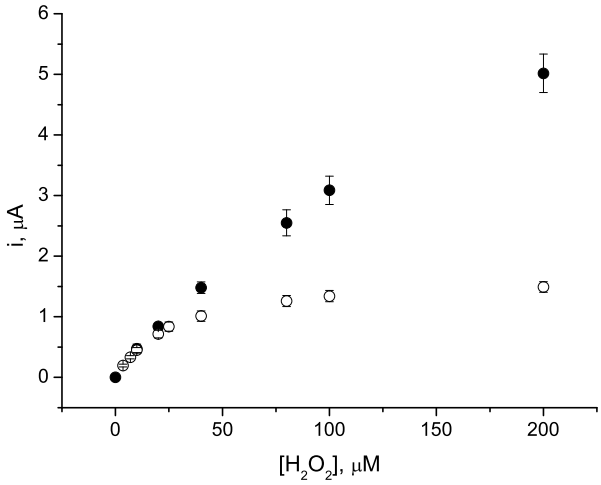

Figure 3.9: Response of bilayer-colloid electrodes (n=6) to increasing concentrations of hydrogen peroxide: ○ In the absence of soluble redox mediator, • In the presence of 10 mM potassium ferrocyanide. 0.1 M phosphate buffer containing 0.15 M NaCl, pH 7.0, working potential 0 V, stirred solution.

change, since according to Marcus theory [6] electron transfer is a distance-dependent phenomenon. Many studies have underlined the importance of an adequate orientation of the molecules in the process of electron transfer between redox enzymes and mediators [9, 11, 14, 24, 32–34]. As pointed out before, if the mobility of the redox centers is restricted, for example, by strong electrostatic interactions of the polymer with surrounding molecules, interactions with the enzyme will be less likely to occur with the adequate positioning. This has been suggested for the case of assemblies of negatively charged glucose oxidase and cationic redox polymer [23]. In the mentioned study of a POs/HRP hydrogel by Calvente and coworkers, the positively

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charged native enzyme was chemically modified in order to give it an overall negative charge. This has been shown to increase the total amount of immobilised enzyme through the strong electrostatic interaction with POs [13], but it could also limit the mobility of the redox centers close to the enzyme molecules. As in the case of the present work, PEGDGE was also added to the films in order to promote crosslinking; however this procedure has little effect on the segmental motion of the polymer and on the electrochemical behaviour of the enzyme electrodes [35, 36]. On the other hand, increasing the ratio of polymer to enzyme may increase the amount of osmium centers able to interact with the enzyme molecules, and therefore the probability of interactions with the proper distance and orientation. In the case of the colloid-modified electrodes, this ratio was about tenfold higher than in the POs/HRP hydrogels for the monolayer colloid configuration. In sum, the high sensitivity observed for the colloid-modified electrodes could be due to a higher fraction of "wired" enzyme in comparison with similar architectures described in the literature. This in turn could be related to the high osmium to enzyme concentration ratio in these structures. Because HRP is chemically linked to the cationic redox polymer but retains its overall positive charge, the absence of attractive electrostatic interactions might result in an increased mobility of the osmium redox centers, which could also lead to an increased wiring efficiency.

Monolayer configuration The response of the electrodes prepared with monolayer POs/GOx/HRP colloids is shown in Figure 3.10. As in the case of the bilayer configuration, the effect of hydrogen peroxide mass transport is evident from the difference between the measurements performed in stirred or quiescent solutions. The maximum current density reached with these electrodes was 16.8 μA cm^{-2}, and a K_M^{app}=2.97 μM was calculated by

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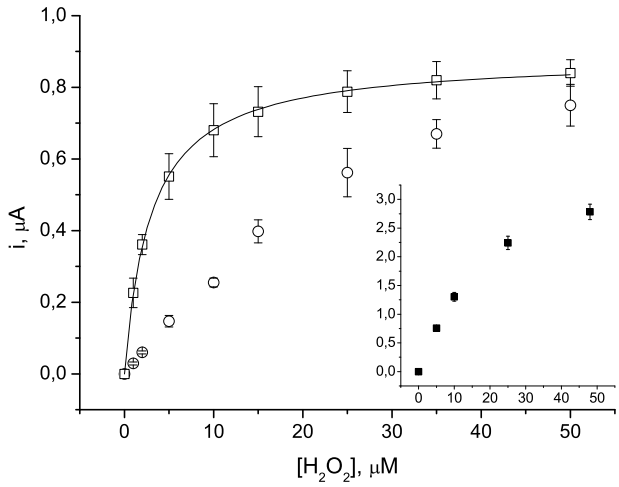

Figure 3.10: Dependence of the current on hydrogen peroxide concentration for monolayer-colloid electrodes poised at 0 V: □ Stirred solution, ○ Quiescent solution, ■ In the presence of 10 mM potassium ferrocyanide, stirred solution. The higher currents observed in the presence of the diffusional mediator indicate that not all the HRP molecules are effectively "wired" by the osmium redox polymer. 0.1 M phosphate buffer, 0.15 M NaCl, pH 7.0.

non-linear fit to the Michaelis-Menten equation. The currents obtained in the lower range of peroxide concentration are similar for both systems, but with the monolayer configuration the response is rapidly saturated. These results seem reasonable considering that much less HRP is immobilised in the monolayer configuration than in the bilayer system. In a study with immobilised HRP and a diffusional mediator, Andrieux and coworkers found that mono- and multilayers of enzyme produced similar responses at low peroxide concentration due to kinetic control by substrate diffusion, but for higher concentrations the current increased with the number of immobilised

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layers, as did the values of K_M^{app} and therefore the dynamic range of the biosensors [37]. In the case of the colloid-modified electrodes, it is apparent that increasing the total amount of immobilised enzyme also increases the amount of wired HRP. In terms of wiring efficiency, a comparison between the monolayer and the bilayer configuration can only be speculative with the available information; however the fact that with approximately four times less immobilised HRP the maximum current produced by the monolayer electrodes reaches half that of the bilayer electrodes suggests that the former configuration is more efficient.

3.3.3 Bienzymatic system: Response to glucose

The response of the electrodes to glucose injections was investigated in a similar way as described for hydrogen peroxide. Increasing concentrations of glucose were added to the cell and the amperometric response was measured at an applied potential of 0 V. Measurements were done alternating between a stirred and quiescent solution. The resulting calibration curves are shown in Figure 3.11. The maximum observed current for the electrodes prepared with monolayer colloids was higher than that of the bilayer colloids by a factor of approximately three. This is in agreement with the results obtained with the two different colloid configurations in solution, where particles with both enzymes immobilised in the same layer showed three times more activity than particles with the two enzymes separated by a PSS layer. Control measurements performed under an argon atmosphere showed no response to glucose, confirming that the observed current under air corresponds to the hydrogen peroxide produced by glucose oxidase in the presence of oxygen (Figure 3.12). The response of the bilayer electrode was linear up to a glucose concentration of 20 mM, whereas the monolayer

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electrode showed a slightly narrower range, up to 15 mM glucose.

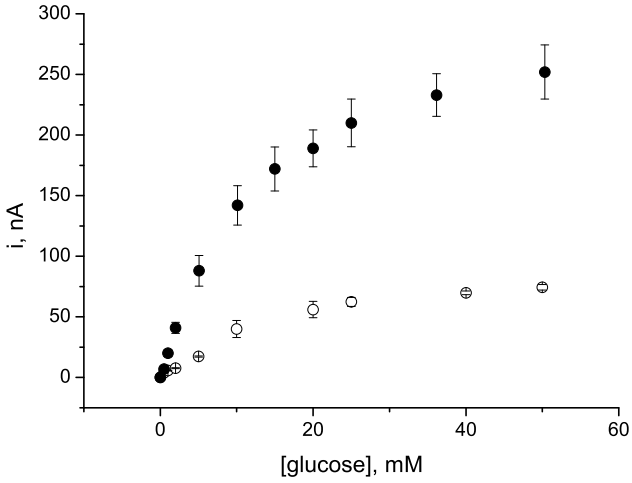

Figure 3.11: Steady-state response curves of POs-colloid films in the presence of glucose at an applied potential of 0 V: • Monolayer colloids, ○ Bilayer colloids. 0.1 M phosphate buffer, 0.15 M NaCl, pH 7.0.

For this last system a sensitivity of 0.42 μA mM^{-1}cm^{-2} was estimated, with a limit of detection of 0.5 mM at a signal to noise ratio of 3. This covers the normal range of glucose concentrations in blood, between 4 and 7 mM, and almost the entire range required for medical sensing systems, from 2 to 19 mM [38]. Moreover, the low applied potential (0 V) required for detection of glucose in the GOx/HRP system minimises the interference effects caused by easily oxidisable compounds (ascorbate, urate, acetaminophen) present in physiological fluids. These are relevant aspects when considering the possibility of future practical applications.

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A typical chronoamperometry measurement for a monolayer-configuration electrode is shown in Figure 3.12. In the lower range of glucose concentrations the response of the electrode is very similar with and without stirring; however as the glucose concentration is increased, the observed current is approximately 10% higher in the quiescent solution. This suggests that when the solution is stirred, the increased mass transport rate causes a larger fraction of the produced peroxide to escape into the bulk solution [39, 40], therefore decreasing the observed signal.

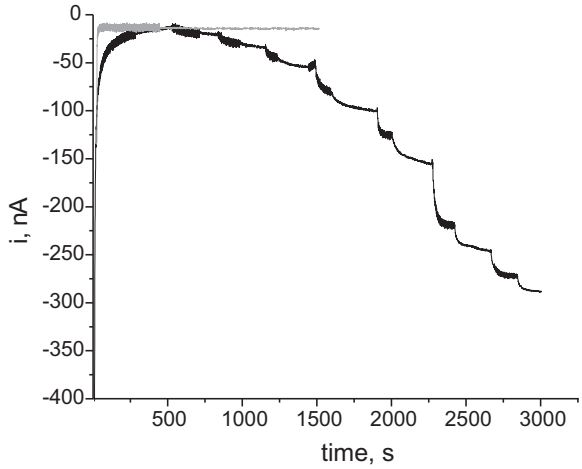

Figure 3.12: Chronoamperometric response of POs-colloid modified electrode (monolayer configuration) in the presence of glucose at an applied potential of 0 V. Black line: Successive injections of glucose, bringing the concentration in the cell from 0.5 to 50 mM, under air. The hydrogen peroxide produced by GOx is reduced by the immobilised HRP, which is then regenerated by the osmium redox polymer. Grey line: 25 mM glucose, under argon. When no oxygen is present, no hydrogen peroxide is produced, and therefore no reduction current is observed.

The response of the colloid-modified electrodes to glucose was also examined in the presence of 10mM ferrocyanide (Figure 3.13). In this case

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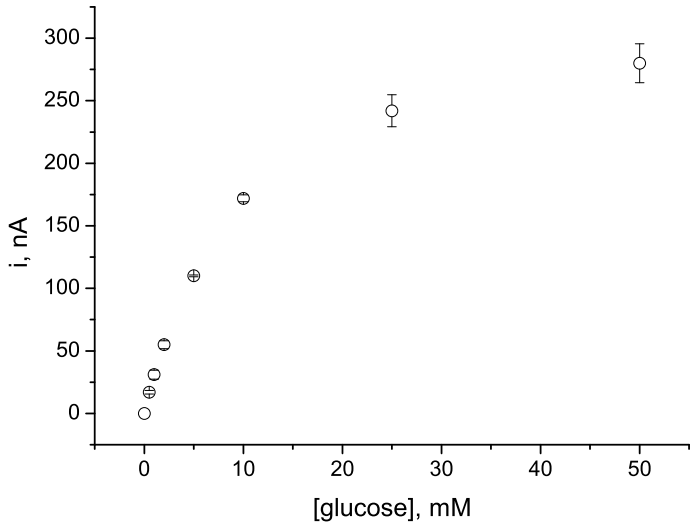

Figure 3.13: Steady-state response curve of POs-colloid modified electrodes (monolayer configuration) at 0 V in the presence of glucose and 10 mM potassium ferrocyanide. The observed currents are similar to those obtained when only the redox polymer is present (see Figure 3.11), showing that the system is limited by the low concentration of hydrogen peroxide, and not by the wiring efficiency. 0.1 M phosphate buffer, 0.15 M NaCl, pH 7.0.

the observed currents were comparable to those obtained with POs as the redox mediator. The amount of hydrogen peroxide that is collected by HRP within the film is not enough to bring about the kinetic control by electron transport in the case of the immobilised mediator, and therefore the wiring efficiency of the system does not come into play. The situation is similar to that observed with bilayer and monolayer electrodes in hydrogen peroxide solutions, where at low H_2O_2 concentrations a higher amount of "wired" enzyme does not produce higher currents. These results show that

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in the colloid-modified electrodes the response to glucose is limited by the first catalytic step, i.e. the production of peroxide, which in turn is limited by the glucose oxidase activity of the colloids and the peroxide collection efficiency of the immobilised HRP. Theoretically, if these two parameters can be improved, higher current densities could be obtained, as seen from the response of the electrodes to hydrogen peroxide injections.

Test for GOx short-circuiting In the bienzymatic mode of operation, the response of the electrodes corresponds to the detection of H_2O_2 produced through the catalytic action of GOx over glucose. But in the presence of the osmium mediator, a competing reaction may take place which would interfere with this scheme:

$$\text{GOx-FAD} + \text{glucose} \longrightarrow \text{GOx-FADH}_2 + \text{gluconolactone} \tag{3.9}$$

$$\text{GOx-FADH}_2 + 2\ \text{Os(III)} \longrightarrow \text{GOx-FAD} + 2\ \text{Os(II)} + 2\text{H}^+ \tag{3.10}$$

The oxidised centers of the redox polymer compete with O_2 for the reduced form of the enzyme. GOx-$FADH_2$ is oxidised by two Os(III) sites, which are then reduced to Os(II) with the corresponding release of two protons. Under these conditions, with the osmium redox centers acting as electron acceptors, no hydrogen peroxide is generated. In the case of GOx/HRP bienzyme electrodes this represents a short-circuiting of the system, since it is based on the current generated by electroreduction of hydrogen peroxide.

Figure 3.14 illustrates the short-circuiting reaction that can take place at the surface of the electrode. A diagnostic test for the existence of short-circuit can be made by poising the electrode at a potential where the os-

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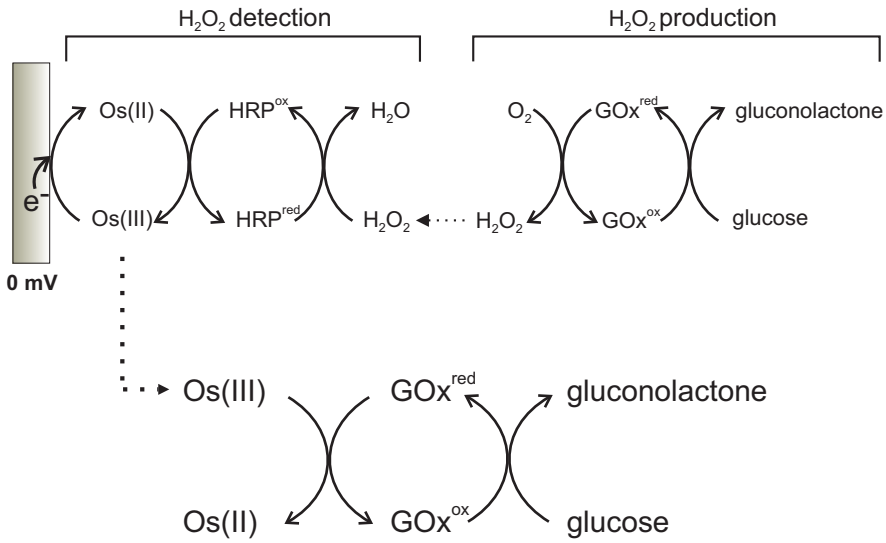

Figure 3.14: Short-circuiting reaction of glucose oxidase with the oxidised form of the osmium redox polymer. If the enzyme is oxidised by the Os(III) centers instead of by oxygen, no hydrogen peroxide is produced.

mium centers of the polymer are in the oxidised state, e.g. +0.5 V: at this potential hydrogen peroxide is not electroreduced by the wired HRP, but if GOx is electrically connected to the redox polymer, an oxidation current is observed. Another typical manifestation of this short-circuiting is the suppression of the electroreduction current at 0 V and high glucose concentrations, with the current first reaching a maximum and then decreasing with increasing concentrations of glucose. Ohara et al. [41] found that when GOx and HRP were co-immobilised within a crosslinked redox polymer network, part of the $FADH_2$ centers of GOx were oxidised by the osmium centers in the polymer, causing a decrease of the current due to the competition between oxygen and the redox polymer for the $FADH_2$

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electrons. The same was observed for electrodes in which a layer formed by biotin-labelled GOx and avidin was immobilised on top of a H_2O_2-sensing hydrogel of HRP and osmium redox polymer [42].

Different strategies have been developed in order to electrically insulate the hydrogen peroxide producing and sensing components in this type of bienzyme electrodes. Kenausis and coworkers [43] introduced a cellulose acetate membrane between the wired peroxidase and the glucose oxidase layer. This membrane effectively insulated the immobilised GOx from the redox polymer, but it also caused the sensitivity of the electrode to drop by 50%. Calvo and coworkers [14] exploited the advantages of the LbL technique, which allows nanometric control of the spatial organisation of the films. These authors incorporated a non-redox polyacrylic acid (PAA) layer on top of a multilayer assembly of peroxidase and an osmium redox polymer. The PAA layer acted as a physical barrier between this assembly and a top glucose oxidase layer and prevented the short-circuiting of the system. In contrast to hydrogel-based bienzyme electrodes, the current densities achieved in this configuration were higher than those observed for a directly wired glucose oxidase layer.

In the present work, no oxidation currents were detected at +0.5 V in the presence of glucose and under an argon atmosphere with either the bilayer or the monolayer electrodes. This shows that in both cases the immobilised GOx is electrically insulated from the osmium redox polymer. In the bilayer electrodes, the PSS layer assembled between the GOx and HRP/POs layers is expected to prevent contact between the redox polymer and the $FADH_2$ centers of glucose oxidase. However in the case of the monolayer electrodes, where both GOx and HRP are complexed with the redox polymer, the absence of short-circuiting reaction with GOx is somewhat surprising. Moreover, when GOx-POs complexes without HRP were

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immobilised on the surface of the colloids, the oxidation of glucose could be detected with the electrodes poised at +0.5 V, with the redox polymer relaying electrons from the $FADH_2$ centers of GOx to the surface of the electrode (Figure 3.15).

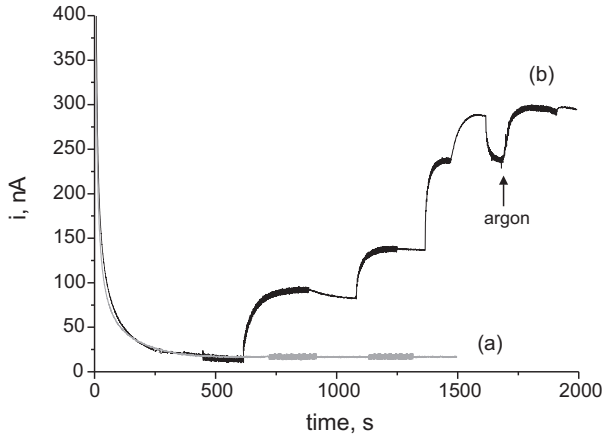

Figure 3.15: Chronoamperometric response of POs-colloid modified electrode to increasing concentrations of glucose (1, 2 and 5 mM) at an applied potential of +0.5 V, alternating between stirred and quiescent solution. (a) Bienzymatic GOx/HRP colloids (monolayer configuration). The absence of an oxidation current shows the electrical insulation between GOx and the redox polymer. (b) Monoenzymatic colloids coated with GOx-POs complexes. The measured current corresponds to the electrocatalytic oxidation of glucose mediated by the osmium redox polymer. When oxygen is removed from the solution by bubbling argon, the catalytic current increases, showing the competition between oxygen and POs for the reduced $FADH_2$ centers of glucose oxidase.

This suggests a different layer configuration for GOx-POs and GOx-HRP-POs complexes. In the case of the GOx-HRP-POs layers, the "screening" interactions that allow for the formation of stable complexes could result in a configuration where the osmium centers are not able to penetrate the enzyme, and therefore electron transfer cannot take place. The fact

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that one of the enzymes in the complexes is wired by the polymer while the other remains electrically insulated could be related to the different structure and accessibility of the active centers and the specific mechanism of electron transfer proper to each enzyme. The redox-active center of horseradish peroxidase has a relatively peripheral location [44], while the structure of the FAD binding site for GOx is a deep, funnel-shaped pocket [45]. In this sense, it should be noted that direct electron transfer between GOx and the surface of an electrode is very difficult to achieve, with only a few examples found in the literature, whereas efficient electron transfer between electrodes and HRP has been frequently reported since the seventies [46]. Thus, in the case of glucose oxidase an adequate positioning of the osmium centers could be even more critical for the establishment of electrical connection with the polymer, and any restrictions to the mobility of the redox polymer chains (due to interactions between the three molecules in the GOx/HRP/POs complexes) could prevent the effective "wiring" of the enzyme.

3.4 Conclusions

Electroactive films were fabricated on the surface of gold electrodes through the immobilisation of enzyme-coated silica colloids and an osmium redox polymer. The performance of bilayer (GOx and HRP adsorbed in separate layers) and monolayer colloids (GOx and HRP in the same layer) was compared. The electrocatalytic reduction of hydrogen peroxide in solution confirmed the electrical "wiring" of the immobilised horseradish peroxidase, with the redox polymer shuttling electrons from the active centers of the enzyme to the surface of the electrodes. A fast and sensitive response to peroxide was obtained, although the narrow linear range limited the ap-

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plicability of this system to low hydrogen peroxide concentrations (up to 25 μM). Electrodes prepared with bilayer colloids developed higher current densities due to the higher amount of HRP immobilised in this configuration with respect to monolayer colloids. Moreover, for both kinds of electrodes the measured current densities were an order of magnitude higher than those achieved for layer-by-layer films of HRP and osmium redox polymer, which suggests that a larger fraction of the immobilised enzyme is electroactive in the case of the colloid-polymer films. This may be due to the higher osmium to enzyme ratio in these constructs, which could increase the probability of interaction between the redox sites of the polymer and the active centers of HRP.

The sequential reaction of glucose oxidase and horseradish peroxidase in multienzymatic colloids was also detected electrochemically, with the electrocatalytic reduction of the hydrogen peroxide produced within the film in the presence of glucose and oxygen. As expected from the behaviour of the colloids in solution, higher currents were obtained for the films incorporating colloids with GOx and HRP co-immobilised in the same layer. The response of the electrodes was linear up to a concentration of glucose of 15 mM at an applied potential of 0 V, with a sensitivity of 0.42 μA mM^{-1} cm^{-2}. The current densities developed in the bienzymatic mode of operation were considerably lower than with direct injection of hydrogen peroxide, showing that the response of the electrodes is limited by the first catalytic step of H_2O_2 production and by the collection efficiency of the HRP immobilised within the film. The absence of short-circuiting reactions with the electrodes poised at an oxidising potential indicates that even in the monolayer configuration, where both GOx and HRP are precomplexed with POs prior to immobilisation on the surface of the colloids, glucose oxidase was not electrically "wired" by the osmium redox polymer.

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Chapter 4

Conclusions and future work

The most relevant conclusions and perspectives opened by this work can be summarised as follows:

- The immobilisation of GOx/HRP/POs adducts on the surface of PDADMAC/PSS coated silica microparticles was a successful strategy for the construction of enzyme microreactors. In these systems, the sequential reaction of both immobilised enzymes was demonstrated, with a superior overall efficiency than that observed for an equivalent system in solution. However the amount of hydrogen peroxide converted, and therefore the observed activity, was limited by the total amount of both enzymes as well as by the relative GOx:HRP ratio. Although it was found that the immobilisation of further layers on top of a GOx layer decreased the activity of this enzyme, the assembly of multiple layers of GOx and HRP on the surface of the particles should be examined. In this way the maximum theoretical GOx activity as well as the HRP collection efficiency could be increased, although at the same time further limitations due to sub-

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strate and product diffusion are expected. Nevertheless, it should be evaluated whether a trade-off between these two opposed trends may be reached in order to achieve a higher overall efficiency.

- The creation of an electrochemical interface between the colloidal microreactors and the surface of an electrode was achieved through the incorporation of the redox polymer POs. However, when POs was only present as a layer constituent on the surface of the colloids, the efficiency of this electrochemical connection was very low, presumably because only those osmium centers in direct contact with the electrode surface were able to exchange electrons effectively. Further studies should be undertaken in order to establish the causes for this limitation, in terms of the geometry of the system and the properties of the multilayer film. Experiments should be carried out with silica particles of smaller diameter in order to explore the effects of the particle size on the efficiency of electrical communication. Another interesting concept to be evaluated would be the incorporation of conductive metal nanoparticles as film constituents, in order to further facilitate charge transport processes at the colloid-electrode interface.

- A more successful strategy was found with the immobilisation of composite colloid-polymer films on the surface of the electrodes. The larger amount of redox polymer in direct contact both with the enzyme layer and with the electrode surface substantially improved the electrochemical communication between the different elements of the system. In this way, a functional electrochemical biosensor for the detection of glucose and hydrogen peroxide was demonstrated. However, limitations in the fraction of effectively "wired" enzyme as well as significant mass transport limitations were apparent for these composite

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films. Additional experimentation should be carried out, making use of rotating disk electrodes and voltammetric analysis, in order to develop a useful model for the determination and optimisation of the kinetic behaviour of the system.

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