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Biosensors and Bioelectronics 18 (2003) 1491–1499
BIOSENSORS & BIOELECTRONICS
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Encapsulation of glucose oxidase microparticles within a nanoscale layer-by-layer film: immobilization and biosensor applications

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Received 27 May 2002; received in revised form 15 January 2003; accepted 8 March 2003

Abstract

We report on an immobilization strategy utilizing layer-by-layer encapsulated microparticles of enzymes within a nanoscale polyelectrolyte film. Encapsulation of glucose oxidase (GOD) microparticles was achieved by the sequential adsorption of oppositely charged polyelectrolytes onto the GOD biocrystal surface. The polyelectrolyte system polyallylamine/polystyrene sulfonate was used under high salt conditions to preserve the solid state of the highly water soluble GOD biocrystals during the encapsulation process. The resulting polymer multilayer capsule of about 15 nm wall thickness is permeable for small molecules (glucose), but non-permeable for macromolecules thus preventing the enzyme from leakage and at the same time shielding it from the outer environment e.g., from protease or microbial activity. Decrease of the buffer salt concentration leads to the dissolution of the enzyme under formation of μ-bioreactors. The spherical μ-bioreactors are bearing an extremely high loading of biocompound per volume. Encapsulated GOD was subsequently used to construct a biosensor by nanoengineered immobilisation of μ-bioreactor capsules onto an electrode surface. The presented approach demonstrates a general method to encapsulate highly soluble solid biomaterials and an immobilization strategy with the potential to create highly active thin and stable films of biomaterial.

Keywords: Encapsulation; Layer-by-layer; Enzyme; Crystal; Immobilization

1. Introduction

Encapsulation was 'invented' by nature, approximately 2–3 billion years ago and is one of the biggest inventions ever. Encapsulation creates single compartments, the basis for the cellular evolution and the starting point of all life forms on Earth. Such a single compartment represents a microenvironment separated from outer environmental factors. The details of the appearance of encapsulated biomaterials (the first cells) are still unknown, but we can learn from nature that encapsulation can concentrate, shield and protect biomolecules in a defined volume and creates a microenvironment.

Encapsulation as well as immobilization of biomaterials is of high interest for various industrial applications and in life sciences. In previous developed methods, biomaterials were almost encapsulated as a solution. This work describes a general approach for the encapsulation of biomaterials as a solid and an immobilization strategy based on this encapsulated biomaterials. The enzyme glucose oxidase (GOD) was chosen as a model substance representing a highly water soluble micro particular biomaterial.

State-of-the-art encapsulation techniques can be classified in accordance to the morphology of the product or by the employed physical or chemical method. The most important methods are: interfacial polymerization, solvent evaporation and matrix entrapment.

The interfacial polymerization method based on the polymerization of two different monomers at a water–organic solvent interface. One monomer is dissolved in the water phase and the other is dissolved in the organic

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0956-5663/03/$ - see front matter
doi:10.1016/S0956-5663(03)00119-2

solvent. The method leads to a core-capsule product that encapsulates a liquid core. The disadvantage is the need of aggressive chemicals and organic liquids causing denaturation of sensitive biomolecules. The solvent evaporation method is a physical method, no chemical reaction takes place. But it imparts the same disadvantages for biomolecules as the previous process. The capsule-core product of both methods contains only 30–75% encapsulated substance. Matrix entrapment is based on the fixation of substances in a meshwork of a gel with pore sizes smaller than the biomolecule to be entrapped. The method is suitable for macromolecules, particles, microbes, organelles and cells (Vorlop et al., 1992). The advantage of gel matrix encapsulation is the low toxicity of the gel compounds for the entrapment of living organisms. Low molecular weight substances are not efficiently prevented to diffuse out of the gel. The gel matrix takes usually up a substantial part of the entire volume of the encapsulate. Beside the above-discussed methods, methods based on: coacervation, extruding of plasticized substances (Van Lengerich, 1998), transport of substances into pre-produced capsules (Thomas and Kim, 2000) or combination thereof are also used. Method overviews with a focus on biomedical and pharmaceutical applications are given by Lim (1985) and Chien (1992).

The here-presented approach uses the layer-by-layer technology applied under high salt conditions to encapsulate microparticles of a biomaterial. Originally, the layer-by-layer technology was not developed as an encapsulation technique. It was developed as a surface coating, modification and engineering tool (Decher and Hong, 1991) on macroscopically flat surfaces and was later applied on colloidal particles (Keller et al., 1995). The layer-by-layer technique is based on attractive electrostatic forces between a charged surface (template) and an oppositely charged polyelectrolyte. The theory of the absorption process is complex (Arys et al., 2000; Joanny, 1999); in addition to electrostatic forces entropy effects and van der Waals forces are involved. Beside synthetic polyelectrolytes many other charged molecules e.g., cytochrome *c*, myoglobin, lysozyme, GOD (Lvov et al., 1995) or nanoscopic objects e.g., gold and SiO_2 nanoparticles (Liz-Marzán et al., 1996; Lvov et al., 1997) are suitable for deposition and incorporated into the polymeric films. Combinations of different templates with synthetic and natural polyelectrolytes and nano/microparticles can be applied leading to a pool of potential products (Bertrand et al., 2000; Caruso, 2001).

The technique was further developed (Caruso et al., 2000a) to coat or encapsulate microcrystalline biomaterials (catalase). A scheme of the layer-by-layer process for the encapsulation of microparticular biomaterials (e.g. microcrystals of catalase or GOD) is given in Fig. 1. Encapsulation is achieved by the sequential adsorption of oppositely charged polyelectrolytes onto the

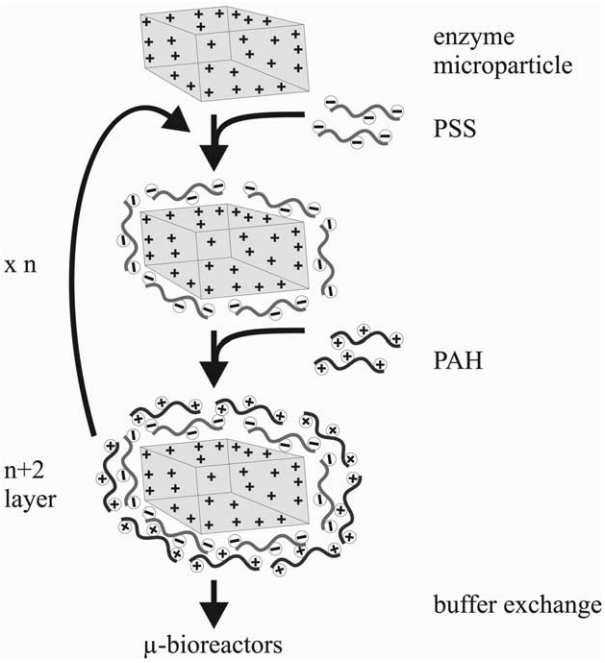

Fig. 1. Principle of the layer-by-layer coating enzyme microparticles.

biocrystal/microparticle surface. The challenge of the method is to keep highly water soluble biomaterials (e.g. the glycoprotein GOD) in their solid state while coating multiple layers of polyelectrolyte in an aquatic buffer system. Successful encapsulation was carried out at high salt conditions to preserve the solid state of biomaterials. An intrinsic mechanism of the layer-by-layer coating process is its self-limitation. Deposition of polyelectrolyte stops after the system reaches the state of the lowest surface energy and biggest entropy gain. Therefore, the amount of deposed material is limited and is independent on the incubation time (assumed the time was sufficiently long, 2–20 min) and polyelectrolyte concentration (assuming excess polyelectrolyte is present). The capsule wall thickness increases approximately 1.7 nm (Estrela-Lopis et al., 2002) with every coating step. Layer thickness can be fine controlled by the polyelectrolyte material and the coating conditions, e.g., the ionic strength of the buffer (Lvov et al., 1998). Consequently, capsule permeability can be controlled by the number of layers and the conditions under which the layers are formed. Capsules with tailored properties and a thickness of only 8–20 nm can be prepared. In comparison, other encapsulation methods lead to capsules with a thickness typically in the micrometer range.

Followed encapsulation, microparticles of biomaterials can be immobilized on a solid support, e.g., for biosensor applications. A scheme of state-of-the-art immobilization methods and a new approach, based on layer-by-layer immobilization of encapsulated solid biomaterials is given in Fig. 2. The new approach has the potential to combine the advantages of the state-of-

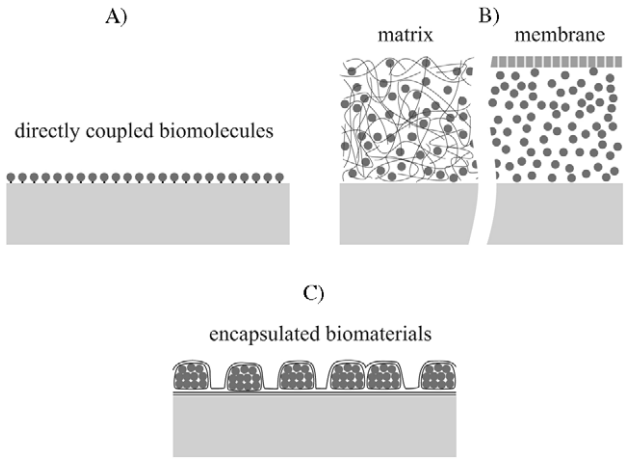

Fig. 2. State-of-the-art biosensor immobilization methods (A and B) and our approach based on layer-by-layer immobilization of solid encapsulated biomaterials (C). The thickness of the biomaterial layer for (A) is in the range of 5 to 100 nm and for (B) about 100 to 500 μm. The maximum thickness of the biomaterial layer for (C) depends on the size of the biomaterial particles and is in the range of 1 to 20 μm.

the-art methods (Fig. 2A) and (Fig. 2B), direct contact and high biomaterial loading respectively, with a low diffusion barrier for a fast sensor response (Fig. 2C). The maximum thickness of the biomaterial layer for the new approach (Fig. 2C) depends on the size of the biomaterial particles and is in the range of 1–20 μm.

The greatest limitation of biosensors is still their lack in stability. This problem was addressed in the past by the immobilization of an extremely high amount of biomaterial. This method was practicable in some cases, but it is not an elegant answer to the problem, in particular for expensive biomaterials. The new approach presented in Fig. 2C has the potential to enable relative stable biosensors. The amount of biomaterial is much bigger than in methods based on direct immobilization (Fig. 2A) thus enhancing stability. Furthermore, encapsulation can shield biomaterials from outer destructive factors (e.g. microorganisms or proteases) as demonstrated on encapsulated catalase (Caruso et al., 2000a).

The combined approach of encapsulation and subsequent immobilization of solid GOD microparticles was applied to construct an amperometric biosensor. Non-encapsulated enzymes were already used by others in the ordinary layer-by-layer process to coat electrodes (Forzani et al., 2000; Cosnier et al., 1999; Anzai et al., 1998). The result is a multilayer consisting of polyelectrolyte and enzyme monolayers with properties very close to the method described in Fig. 2B. An interesting variation is the incorporation of redox active polymers in combination with oxidoreductases to create reagentless biosensors (Narvaez et al., 2000). The advantage of the here-presented method is the use of an encapsulated solid enzyme to immobilize a relatively high amount of biomaterial in a thin film very close to the sensor surface.

2. Materials and methods

2.1. Materials

Poly-(allylamine-hydrochloride) (PAH), $M_w = 8000$–11 000 Da, poly-(sodium-4-styrenesulfonate) (PSS), $M_w = 70\,000$ Da and poly-acrylic-acid (PAA) $M_w =$ 8000 Da, were obtained from Aldrich, USA. GOD was obtained from Sigma, USA. PSS was dialyzed against Milli-Q water (M_w cut-off = 2000) and lyophilized before use. PAH–FITC was prepared as described by us previously (Caruso et al., 2000b). All polyelectrolyte solutions and washing buffers were filtered through a 0.22 μm membrane filter prior use.

2.2. GOD encapsulation

GOD powder (specific activity 180 U mg^{-1}) was intensively milled for 5 min by hand in a small agate mortar. Thirty milligram of GOD particles were washed once with 1 ml of a chilled (4 °C) solution of 90% saturated ammonium sulfate of pH 5 (coating buffer). The suspension was centrifuged (Hettich EBA 12R, 500 g, 4 min, 4 °C) and the washing solution was discarded. The polymer layers were then assembled onto the enzyme particles by the sequential deposition of PSS and PAH (or PAH–FITC). The first layer was deposited by adding a 0.5-ml aliquot of PSS solution ($\sim$0.7 mg ml^{-1} in coating buffer) to the enzyme particle suspension, occasionally shaking the suspension, and allowing 15 min for adsorption. The excess polyelectrolyte was removed by two repeated centrifugation and redispersion cycles in chilled coating buffer. The next layer, PAH (5 mg ml^{-1} in coating buffer), was deposited by using the same procedure as described for PSS. Further PSS and PAH layers were deposited in identical fashion until the desired number of polymer multilayer was achieved. The GOD particles are very fragile and sensitive to dissolve in buffers with a lower salt concentration than 90% saturated ammonium sulfate. Zeta-potential measurements to confirm polyelectrolyte coating are not possible with highly soluble biomaterials. A particle suspension in pure water is needed to measure an accurate value of the zeta-potential. The particles will dissolve immediately under these conditions. The same problem occurs with particles having only a small number coated layer. The shell stability of crystals coated with only one or two layers is not high enough to withstand dispersion in pure water. As an alternative method, FITC conjugated PAH was deposited and polyelectrolyte coating was conformed due to an increase in fluorescent light intensity.

2.3. Immobilization of encapsulated GOD

Screen-printed electrodes (SensLab, Leipzig, Germany) were chosen as a substrate for immobilization. Electrodes were pre-coated with four layers of alternatively charged polyelectrolyte (PAH: 25 mg ml^{-1} in 0.1 M NaCl, pH 6; PAH–FITC: 10 mg ml^{-1} in 0.1 M NaCl, pH 6; PSS: 25 mg ml^{-1} in 0.1 M NaCl, pH 6). PSS was chosen as the first layer, due to its hydrophobic character and better interaction with the hydrophobic electrode surface. The last layer was positively charged PAH. The electrode head was dipped into the polyelectrolyte solution over night at room temperature allowing the molecules to diffuse into the pored surface structure of the electrode. The electrode was washed intensively with water and the coating procedure was repeated with an alternative charged polyelectrolyte. The coating success was verified by a change in the wetting characteristic (from hydrophobic to hydrophilic for the first PSS layer) and by fluorescent microscopy (increase in fluorescent light intensity after each PAH–FITC coating). A concentrated suspension of encapsulated GOD microcrystals with an outer layer of PSS was incubated on the electrode for 6 h at 4 °C. Little water was added to decrease the shielding effect of salt to the Coulomb force between the surface and the enzyme particles. Unabsorbed GOD crystals were removed by dipping the electrode in coating buffer. The adsorbed crystals were over-coated with four alternating layers of polyelectrolyte (in coating buffer), starting with a layer of PAH.

2.4. Immobilization of dissolved GOD

Ten milligram of polycarbamoylsulfonate (PCS) stock solution (35.6%, SensLab) was weighed in a 500 μl reaction tube and diluted with 10 μl water. Twenty milligram GOD was diluted in 36 μl water (100 U μl^{-1}). An aqueous solution of 3% polyethylenimine (PEI) was prepared. Ten microliter of the PCS solution was mixed with the same volume of a GOD solution (GOD solutions with 10, 1, 0.1, 0.05 and 0.01 U μl^{-1} were used to measure the electrode characteristic in an enzyme loading experiment) and an aliquot of 3 μl PEI was added (the PEI raised the pH to 5). The solutions were immediately mixed carefully by avoiding the production of air bubbles in the mixture. An aliquot of 0.1 μl was transferred to the working electrode of the screen-printed electrode and was allowed to air dry over night.

2.5. Fluorescent microscopy

Microphotography was carried out with a fluorescent microscope (OLYMPUS CX-40 with U-LH 50 HG lamp house, Japan) equipped with a trinocular tube and camera (OLYMPUS OM-1 with PE 2.5 × photo ocular, Japan). Images were taken on negative film (FUJICOLOR 200, Fuji, Japan).

2.6. FIA system set-up

A screen-printed three-electrode amperometric GOD biosensor was mounted in a flow-throw cell (SensLab). The electrode was connected to a potentiostat (Biometra EP300, Göttingen, Germany) and polarized to +600 mV vs. the internal Ag/AgCl reference electrode in 50 mM potassium phosphate buffer containing 0.1 M KCl. The electric current of the electrode was measured for glucose samples with a concentration of 1–10 mM in the same buffer. The current measured in the sample buffer without glucose was set to zero. The samples were transported to the electrode with a peristaltic pump (Ismatec IPC, Germany) at a constant carrier flow of 0.2 ml min^{-1}. An inject/load valve (Knauer, Germany) was used to switch between buffer flow and sample flow. The maximum peak value calculated as the peak current minus the baseline current was used as the result. For signal response measurements, the buffer was alternating switched to the glucose sample buffer for a period of 20 s and the sensor response was recorded. The system allowed the measurement of two parallel electrodes at the same time. All components were connected to a personal computer for their programmed control with FIABOLO software (Dipl. Ing. Feldbrügge, Münster, Germany).

3. Results and discussion

3.1. Layer-by-layer encapsulation of GOD microparticles

Despite the fact that GOD is classified as a glycoprotein and is therefore highly water soluble it was chosen as a model system in this work (Hecht et al., 1993). The high water solubility of GOD is in contrast to the needs of suitable templates for the layer-by-layer technique. The solubility of proteins is strongly affected by the pH and the salt concentration. Increased salt concentration can lead to fallout of protein from solution and can prevent the solubilization of crystalline proteins. The solubility of salt depends on the temperature, making it a problem to work at low temperature and high salt concentration to keep crystalline enzymes in suspension. Ammonium sulfate is an exception; its solubility is nearly independent from the temperature. The solubility of ammonium sulfate is 4.1 M at 25 °C (767 g $liter^{-1}$) and 3.9 M at 0 °C (Cooper, 1981). Ammonium sulfate is frequently used to isolate proteins by 'salt-fractionating'. In this work, ammonium sulfate was used to prevent solid GOD from solubilization during its encapsulation by polyelectrolyte coating. The drawback

of high salt concentration is that the solubility of polyelectrolytes is limited. Polyelectrolyte concentrations of about 3 mg ml^{-1} were found to be ideal in most coating experiments mainly on latex particles as a template. Coating conditions must be established which prevent the template from solubilization and at the same time enables the application of sufficient polyelectrolyte. Coating conditions can be fine tuned by the parameters: temperature, salt concentration, organic solvent concentration and pH. It was found that a 90% saturated ammonium sulfate solution at 4 °C is suitable to protect GOD microparticles from solubilization. Stable capsules could be produced by using the PSS/PAH polyelectrolyte system. A solution of 5 mg ml^{-1} PAH and a saturated solution of PSS ($\sim$0.7 mg ml^{-1}) in 90% saturated ammonium sulfate at 4 °C were used for these experiments. Fig. 3A shows the encapsulated GOD particles coated with four layers of polyelectrolyte [$(PSS/PAH)_2$]. No change in morphology was registered for coated microparticles. After water was added to reduce the salt concentration spherical μ-bioreactors were created (Fig. 3B). Some capsule fragments are visible in the picture (lower right corner). It seems that the encapsulated big crystals collapsed after water addition and only small crystals formed stable μ-bioreactors. A detailed picture of a representative group of GOD μ-bioreactors is presented in Fig. 4. It is clearly seen that more fluorescent light is originated from the fluorescent-labeled capsule material and that the capsule established a discrete border to the surrounding buffering media. The outstanding properties of such μ-bioreactors can be summarized as follows: 5–50 μm diameter in size, spherical shape, extremely thin wall thickness of 8–20 nm, extremely high protein density in the interior, high permeability for small molecules, no permeability for macromolecules, quantitative recovery of enzyme activity in the capsule, extremely high ratio of enzyme activity to volume, extremely high ratio of biomass to capsule wall mass. Calculations of the approximate enzyme activity per volume and the number of encapsulated enzymes per capsule are as follows: an encapsulated rhombic crystal/microparticle with 20 μm side lengths has a volume of 8000 μm^3 and a

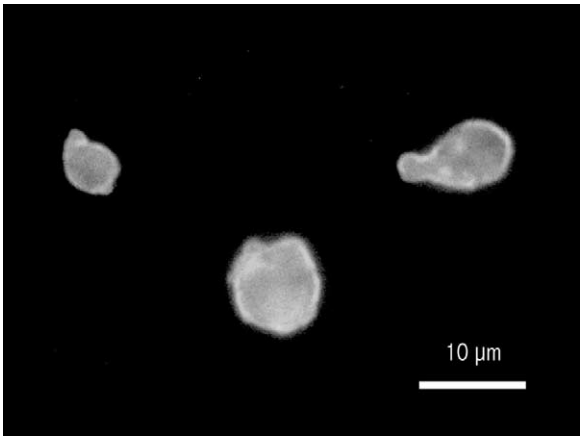

Fig. 4. Fluorescent micrograph of GOD μ-bioreactors. Crude GOD particles were coated with 2 layer pairs of PSS/PAH polyelectrolyte. A change in shape is seen after the addition of water. 'Spherical' μ-bioreactors were formed due to the dissolution of the GOD in the interior of the capsule.

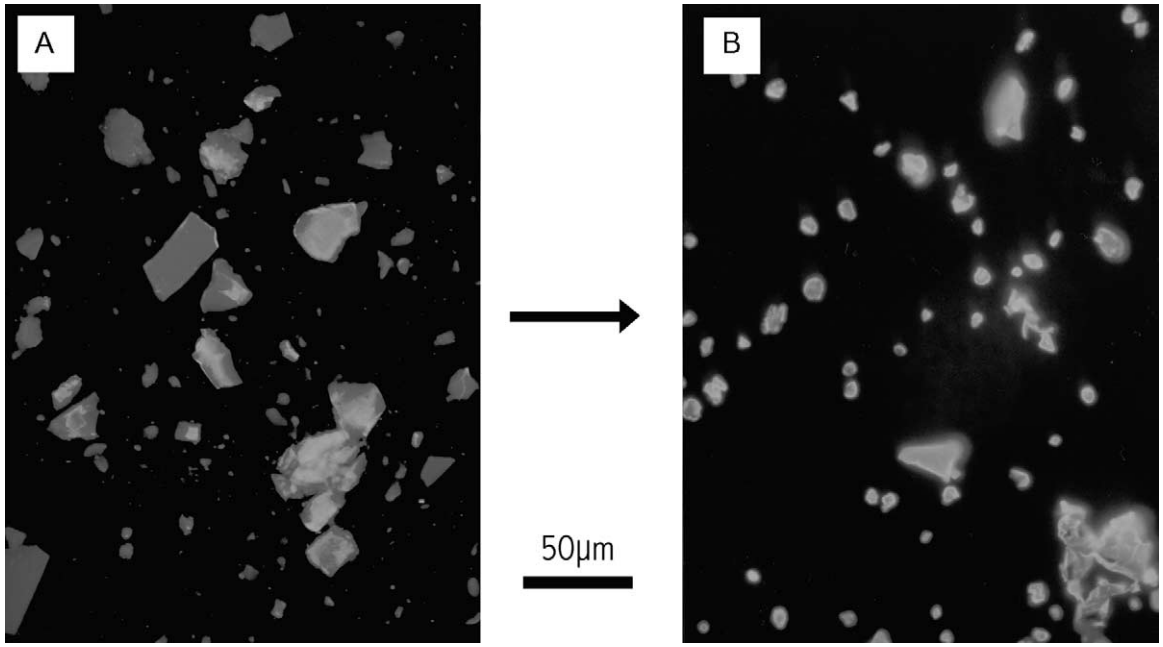

Fig. 3. A) Fluorescent micrograph of PSS/PAH coated GOD particles in 90% saturated ammonium sulfate solution (4 layers, including one PAH-FITC layer). B) Formation of 'spherical' μ-bioreactors was observed after the addition of water. The water lowers the salt concentration resulting in the dissolution of solid GOD.

surface area of 2400 μm^2. The approximate density of the solid enzyme is around 0.8 g ml^{-1} ($=0.8\times10^{-6}$ µg μm^{-3}). Each encapsulated crystal/microparticle bears a biomass of around 6.4×10^{-3} µg ($=6.4$ ng). The number of encapsulated GOD (240 000 Da) molecules is about 1.6×10^{10} molecules per capsule. With an activity of 180 U mg^{-1}, an enzyme mass of 6.4 ng corresponds to an activity of around 1.2×10^{-3} U $capsule^{-1}$. Around 830 capsules represent the activity of 1 U.

Assuming that the surface area remains nearly constant during the dissolution of the solid core and a spherical shaped µ-bioreactor is formed, the volume of the capsule is about 11 000 μm^3. The volume is increased by about the factor 1.3, resulting in an approximate concentration of 70% biomaterial in the capsule. The volume increase is due to the osmotic pressure caused by the inflowing water. The system tries to achieve the state of its lowest energy (small difference between inner pressure and outer pressure of the capsule). This is possible by increasing the capsule volume, the highest volume (surface area is constant) is represented by a spherical shape. The volume of the capsule wall material is about 24 μm^3 (assuming a wall thickness of 10 nm). The ratio of capsule volume to capsule wall volume is about 333 for capsules with solid core and 450 for capsules with a dissolved core. The density of the organic polyelectrolyte and the biomaterial is in the same range. Therefore we can conclude that the ratio of biomass (GOD) to capsule wall mass is also about 333.

In detail, the final protein concentration depends on more factors. The elasticity of the capsule is unknown; therefore the concentration is lower in case the capsule is stretched under the osmotic pressure. Only some capsules are of perfect spherical shape (immobilized capsules are more deformed). The GOD contains around 20% buffer salts and other materials; the protein concentration is therefore lower. However, compared to other encapsulation methods the capsule wall mass is extremely small and contains less than 1% of the entire mass.

3.2. Novel immobilization approach

Encapsulated GOD microparticles were immobilized onto a surface by using a layer-by-layer approach as described in Fig. 2C. To verify the biofunctionality after immobilization a screen-printed carbon electrode was chosen as surface material. In this way an amperometric GOD biosensor was constructed. The electrode was pre-coated with four layers of polyelectrolyte with an outer positively charged layer of PAH. Encapsulated GOD microparticles with an outer layer of negatively charged PSS were incubated with the pre-coated electrode and the GOD was immobilized due to attracting coulomb forces. The electrode was washed and over-coated with four layers of polyelectrolyte to ensure a strong fixation of the GOD capsules. A fluorescence micrograph of the working electrode is shown in Fig. 5. The surface of the electrode is covered with a layer of encapsulated GOD microparticles. The fluorescent light originates from the GOD itself and from the FITC conjugated polyelectrolyte (PAH–FITC) used to encapsulate the enzyme. The solubilization of the immobilized solid GOD was studied after the addition of water. Fig. 6A shows the solid enzyme immobilized onto the electrode surface in a 90% saturated ammonium sulfate buffer. Fig. 6B shows the immobilized enzyme after water was added to reduce the salt concentration and to dissolve the enzyme. Solubilization was observed in less than a minute. It must be mentioned that fluorescent light intensity originated from a substance depends on the environment. Therefore, the intensity is different for a substance present in its solid state or dissolved in a liquid. A direct comparison of fluorescent light intensity between the Fig. 6A and B is therefore not possible. The FITC conjugated capsules encapsulating the enzyme are clearly seen in Fig. 6B and a slight change in morphology to a more spherical shape was observed. The diameter of the capsules is about 10 µm, which is also the maximum thickness of the entire enzyme capsule film on the electrode surface. The surface coverage with capsules is about 50–60%.

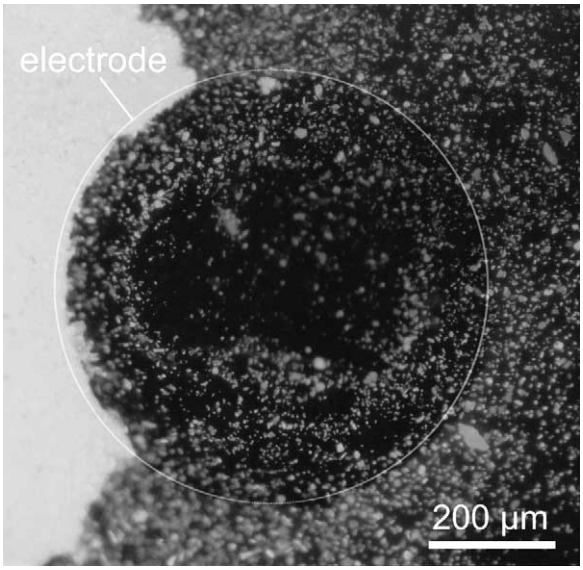

Fig. 5. Encapsulated GOD microparticles are immobilized by a layer-by-layer process onto the surface of a platinum/carbon electrode for the construction of an amperometric biosensor. The electrode is indicated in the fluorescent micrograph as a circle with solid GOD (lighter particles) on the surface.

3.3. Amperometric measurements

Following the immobilization of the GOD capsules, amperometric measurements were carried out to de-

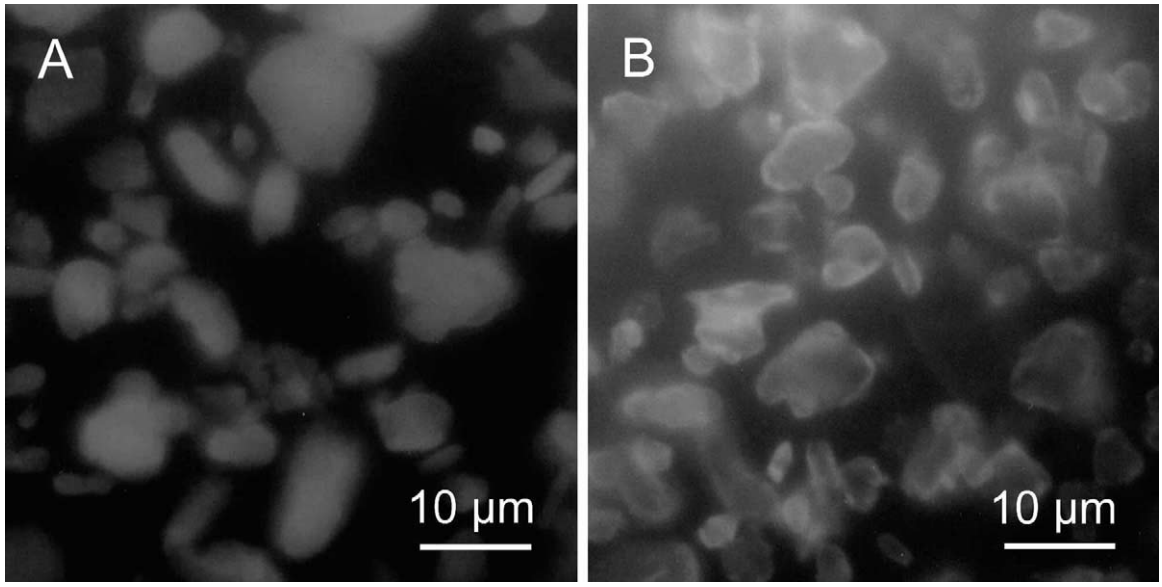

Fig. 6. Fluorescent micrograph of immobilized GOD. A) Before the addition of water. B) After water is added to dissolve the solid enzyme. FITC-labeled capsules containing the dissolved GOD are clearly seen. Capsules are fixed to the surface by coulomb forces and coated over by 4 layers of polyelectrolyte.

monstrate the biofunctionality of GOD and the visibility of the method for biosensor construction. Measurements were carried out in a FIA set-up with 5 mM glucose solution as analyte. Fig. 7A shows the signal response curve of the novel biosensor. The buffer stream was switched alternating between pure buffer and a glucose solution for a 20-s period and the corresponding signal was registered. Due to the very thin coverage of the electrode with polyelectrolyte layers of approximately 2×10 nm (pre-coating and over-coating) and GOD µ-bioreactor capsules of about 10 µm thickness, the diffusion barrier of the electrode is expected to be very low. Evidence is the fast response of the sensor signal to changes in the glucose concentration. In comparison, a glucose sensor prepared by the PCS method shows a slower response (Fig. 7B). The PCS/GOD sensor shows a higher signal, due to the larger amount of biomaterial immobilized and the accumulation of peroxide in the PCS matrix during measurement. In a 20-s period of time the PCS/GOD sensor could not

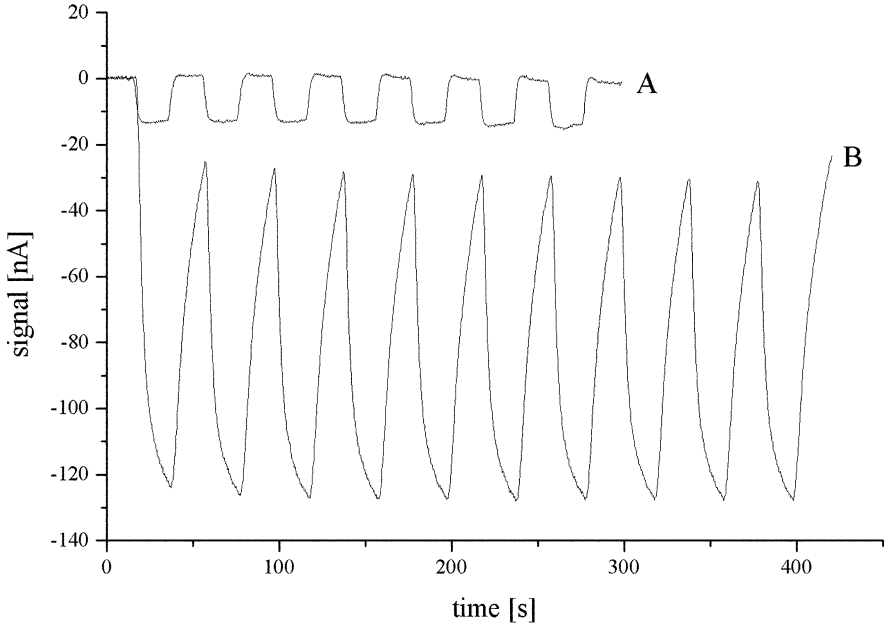

Fig. 7. Signal response curve of the µ-bioreactor based glucose biosensor (A), and reference measurements (B) with a conventional biosensor based on matrix entrapment of GOD in PCS polymer.

reach a steady-state signal and the signal could not reach the base line after the sample was switched back to pure buffer. In comparison, the response time of the novel constructed biosensor was much faster. The maximum signal as well as the base line could be reached in about 5 s. The immobilized PCS/GOD layer can be clearly seen on the electrode with the naked eye, its thickness is approximately 100 μm. The GOD particles immobilized by the layer-by-layer approach are invisible to the naked eye due to the extremely thin layer and less material on the surface. The sensor gave a linear response to glucose concentrations from 0 to 2 mM followed by a non-linear current increase until 5 mM glucose is reached.

4. Conclusions

The challenge in biosensor development is to isolate or engineer a biocompound and to create a microenvironment for the immobilized biocompound on the transducer surface that preserved the full bio-functionality of the biocompound over a long time. Encapsulation of biomaterials in a cell-like microcapsule with consecutive immobilization was demonstrated as an alternative approach to meet those challenges. Due to the solid biomaterial template used in the layer-by-layer encapsulation process, the prepared capsules carrying the highest possible loading of biocompounds. The bioactivity to volume ratio is higher than with all other techniques and unsurpassable, because the crystalline/solid state represents the highest possible density for any biomaterial. The capsule wall comprises less than 1% of the entire capsule mass. The microcapsule size is determined by the size of the template and is fixed before the encapsulation process is started. This means the capsule size can be actually predicted and measured before the encapsulation takes place. The nonaggressivity and biocompatibility of the wall materials and the fact that only the capsule comes in direct contact with the substrate while immobilized make the technique perfectly suitable for biomolecule immobilization where sensitivity of the biocompound is often a serious problem. Solid GOD was successfully encapsulated and immobilized onto a solid support. As the GOD is highly soluble in water, we demonstrated the use of high salt concentration in the layer-by-layer technique for its encapsulation. Our approach enables the immobilization of a relatively high amount of biomass very closed to a surface thus reducing the diffusion barrier. The biofunctionality of immobilized microcapsules was demonstrated by the construction of a biosensor. A very fast sensor response was demonstrated compared to sensors prepared by thick-film matrix entrapment. The out-diffusion of substrates and enzymatic generated products from the 'biomembrane' is often the limiting factor if a high frequency of measurements is desired. The out-diffusion characteristic was found as good as the in-diffusion characteristic of the sensor. The permeability of the capsule wall is controllable by its thickness (or layer number) and the capsule wall material. This behavior can lead to a control of the in- and out-flow of substrates and products respectively and also the in-flow of inactivating molecules/ions (e.g. heavy metals) and the outflow of cofactors (e.g. FAD). We believe that the application of microencapsulated enzymes in biosensors is pointing a promising way to implement novel properties and to increase biosensor lifetime.

Acknowledgements

This work was supported by the German/Hong Kong Joint Research Scheme sponsored by the Research Grants Council of Hong Kong and the German Academic Exchange Service (DAAD). We thank Frank Caruso for valuable discussions and Henryk Kalisz for his help in the preparation of GOD microparticles.

References

Anzai, J., Takeshita, H., Kobayashi, Y., Osa, T., Hoshi, T., 1998. Layer-by-layer construction of enzyme multilayers on an electrode for the preparation of glucose and lactate sensors: elimination of ascorbate interference by means of an ascorbate oxidase multilayer, Anal. Chem. 15, 70 (4), 811–817.

Arys, X., Jonas, A.M., Laschewsky, A., Legras, R., 2000. Supramolecular polyelectrolyte assemblies. In: Supramolecular Polymers. Marcel Decker, pp. 505–564.

Bertrand, P., Jonas, A., Laschewsky, A., Legras, R., 2000. Ultrathin polymer coatings by complexation of polyelectrolytes at interfaces: suitable materials, structures and properties. Macromol. Rapid Commun. 21 (7), 319–348.

Caruso, F., 2001. Nanoengineering of particle surfaces. Adv. Mater. 13 (1), 11–22.

Caruso, F., Trau, D., Möhwald, H., Renneberg, R., 2000a. Enzyme encapsulation in layer-by-layer engineered multilayer capsules. Langmuir 16 (4), 1485–1488.

Caruso, F., Yang, W., Trau, D., Renneberg, R., 2000b. Microencapsulation of uncharged low molecular weight organic materials by polyelectrolyte multilayer self-assembly. Langmuir 16 (23), 8932–8936.

Chien, W.Y., 1992. Novel Drug Delivery Systems, Marcel Dekker, ISBN 0-8247-8520-7.

Cooper, T.G., 1981. Biochemische Arbeitsmethoden, Walter de Gruyter, Berlin, New York ISBN 3-11-007806-6, pp. 348–352.

Cosnier, S., Stoytcheva, M., Senillou, A., Perrot, H., Furriel, R.P., Leone, F.A., 1999. A biotinylated conducting polypyrrole for the spatially controlled construction of an amperometric biosensor. Anal. Chem. 71 (17), 3692–3697.

Decher, G., Hong, J.D., 1991. Buildup of Ultrathin Multilayer Films by a Self-Assembly Process: II. Consecutive Adsorbtion of Anionic and Cationic Bipolar Amphiphiles and Polyelectrolytes on Charged Surfaces, Berichte der Bunsen-Gesellschaft für Physikalische. Chemie 95, 1430.

Estrela-Lopis, I., Leporatti, S., Moya, S., Brandt, A., Donath, E., Möhwald, H., 2002. SANS Studies of polyelectrolyte multilayers on colloidal templates. Langmuir 18 (21), 7861–7866.

Forzani, E.S., Solis, V.M., Calvo, E.J., 2000. Electrochemical behavior of polyphenol oxidase immobilized in self-assembled structures layer-by-layer with cationic polyallylamine. Anal. Chem. 72 (21), 5300–5307.

Hecht, H.-J., Schomburg, D., Kalisz, H., Schmid, R., 1993. The 3D structure of glucose oxidase from *Aspergillus niger*. Implications for the use of GOD as a biosensor enzyme. Biosensors Bioelectron. 8, 197–203.

Joanny, J.F., 1999. Polyelectrolyte adsorption and charge inversion, European. Phys. J. B 9 (1), 117–122.

Keller, S.W., Johnson, S.A., Brigham, E.S., Yonemoto, E.H., Mallouk, T.E., 1995. Photoinduced charge separation in multilayer thin films grown by sequential adsorbtion of polyelectrolytes. J. Am. Chem. Soc. 117, 12879–12880.

Lim, F., 1985. Biomedical Applications of Microencapsulation, CRC Press, ISBN 0-8493-5440-4.

Liz-Marzán, L.M., Giersig, M., Mulvaney, P., 1996. Synthesis of nanosized gold-silica core-shell particles. Langmuir 12 (18), 4329–4335.

Lvov, Y., Ariga, K., Ichinose, I., Kunitake, T., 1995. J. Am. Chem. Soc. 117, 6117.

Lvov, Y., Ariga, K., Onda, M., Ichinose, I., Kunitake, T., 1997. Alternate assembly of ordered multilayers of SiO_2 and other nanoparticles and polyions. Langmuir 13 (23), 6195–6203.

Lvov, Y., Onda, M., Ariga, K., Kunitake, T., 1998. Ultrathin films of charged polysaccharides assembled alternately with linear polyions. J. Biomater. Sci. Polym. 9 (4), 345–355.

Narvaez, A., Suarez, G., Popescu, I.C., Katakis, I., Dominguez, E., 2000. Reagentless biosensors based on self-deposited redox polyelectrolyte-oxidoreductases architectures. Biosensors Bioelectron. 15 (1–2), 43–52.

Thomas, M., Kim, Y.D., 2000. Patent US 6045835, Method of encapsulating flavors and fragrances by controlled water transport into microcapsules.

Van Lengerich, 1998. Patent WO 9818610, Embedding and Encapsulation of Controlled Release Particles.

Vorlop, K.D., Muscat, A., Beyersdorf, J., 1992. Entrapment of microbial cells within polyurethane hydrogel beads with the advantage of low toxicity. Biotechnol. Tech. 6, 483–488.