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TOPICAL REVIEW

Encapsulation of biomolecules in silica gels

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Abstract

A wide variety of biomolecules, ranging over proteins, enzymes, antibodies and even whole cells, have been embedded within sol–gel glasses. They retain their bioactivity and remain accessible to external reagents by diffusion through the porous silica. Sol–gel glasses can be cast into desired shapes and are optically transparent, so it is possible to couple optics and bioactivity to make photonic devices and biosensors. The high specificity and sensitivity of enzymes and antibodies allows the detection of traces of chemicals. Entrapped living cells can be used for the production of metabolites, the realization of immunoassays and even for cell transplantation.

1. Introduction

Combining advanced materials with biology is becoming one of the most innovative research fields. During the past few decades, the development of biomaterials was mainly driven by medical applications in the realization of implants. Such materials had to exhibit good mechanical properties and to be biocompatible in order to be implanted in the human body to replace damaged tissues or bones [1, 2]. The industrial development of biotechnologies was another important factor in the search for new materials. They require the immobilization of active biospecies such as enzymes or whole cells on solid substrates. Combining fragile biomolecules with tough materials has thus become a highly innovative research field. Up to now, bioencapsulation has remained restricted to organic polymers; the high-temperature processing of glasses is not compatible with fragile biomolecules. However, silica could offer some advantages such as improved mechanical strength and chemical stability. It does not swell in aqueous or organic solvents, preventing leaching of entrapped biomolecules. Silica is not a food source for micro-organisms, it is not toxic and it is biologically inert. Thus techniques for the immobilization of enzymes or cells at the surface of porous glasses have already been being developed for more than twenty years, using coupling of chemical agents; but such covalent attachment requires chemical modifications that may affect the bioactivity of enzymes and cells.

Therefore physical encapsulation within sol–gel glasses could offer new opportunities for biotechnologies

The so-called sol–gel process, developed over the past two decades, is a new method allowing the room temperature synthesis of silica glasses. The recent extension of this process to the entrapment of functionally active biomolecules demonstrated that it is possible to introduce and retain labile biological activity within silica gels. First reports on the sol–gel encapsulation of enzymes were published in the early 1970s [3], but the real development of this process began with the work of the Jerusalem group in 1990 [4]. Since then, hundreds of scientific papers have been published [5–8]. Metalloproteins, enzymes, antibodies and even whole cells have now been encapsulated within sol–gel glasses. Bioactivity together with the flexibility to shape materials make these new hybrid materials attractive for demanding applications in biotechnology, medicine, environmental technology and even sensors and photonics! Many patents have been lodged, initiatives are being created and the first products are now commercially available [9].

2. Biomineralization and sol–gel encapsulation

2.1. Sol–gel synthesis of silica

The 'sol–gel process' is actually close to the biomineralization processes. Biogenic materials are formed on a huge scale by living organisms ranging from Protista to human beings. Silica is present in all living organisms and, after carbonates, it is the second most abundant mineral formed by organisms. Silica structures with precisely controlled morphologies are produced on a huge scale by single-cell organisms such as diatoms [10, 11].

Biomineralization occurs in water under mild conditions, at neutral pH and ambient temperature. Biogenic silica is formed from solute molecules of silicic acid $Si(OH)_4$ [12]. Condensation occurs between the silanol groups (Si–OH) of two molecules, leading to the formation of an oxo bridge (Si–O–Si) while one water molecule is removed:

$$Si{-}OH + HO{-}Si \Rightarrow Si{-}O{-}Si + H_2O. \quad (1)$$

A silica network made of corner-sharing $[SiO_4]$ tetrahedra is thus obtained when all Si–OH groups have reacted and the overall reaction can be written as

$$Si(OH)_4 \Rightarrow SiO_2 + 2H_2O. \quad (2)$$

The concentration of silica dissolved in seawater (a few mg l^{-1}) is actually too small for condensation to occur spontaneously. In the case of diatoms, silica is formed inside a silicon deposition vesicle (SDV) where it is genetically controlled by proteins and polysaccharides [13].

Chemists currently use alkoxide precursors $Si(OR)_4$, where R is an alkyl group, rather than silicic acid $Si(OH)_4$ [4, 14]. The most frequently used alkoxide for bioencapsulation is the commercially available tetramethyl orthosilicate, $Si(OMe)_4$, also called TMOS ($Me = CH_3$).

Silica is then formed according to a two-step reaction:

- Silicic acid is first formed upon hydrolysis, by adding water:

$$Si(OMe)_4 + 4H_2O \Rightarrow Si(OH)_4 + 4MeOH. \quad (3)$$

- Condensation follows spontaneously to give silica (equation (2)) and the overall reaction is

$$Si(OMe)_4 + 2H_2O \Rightarrow SiO_2 + 4MeOH. \quad (4)$$

Silica particles grow progressively as condensation proceeds, leading to the formation of colloidal solutions (sols) and gels. These gels can be partially dried at room temperature, giving a porous network of hydrated amorphous silica $SiO_2{\cdot}nH_2O$ (xerogel) with pores ranging typically between 1 and 10 nm in diameter. Bulk glasses can be obtained upon densification around 1000 °C [15].

Sol–gel chemistry provides a versatile method for the low-temperature synthesis of silica. Viscosity increases progressively with the size of silica particles and shaped materials can be processed directly from the solution. Thin films can be deposited by dip-coating or spin-coating onto any kind of substrate (glasses, ceramics, metals, polymers). Fibres can be drawn from viscous gels and nanospheres synthesized from colloidal dispersions [16].

2.2. Organic–inorganic hybrids

The mild chemical conditions involved in the sol–gel synthesis of metal oxides provide versatile access to hybrid organic–inorganic materials. The intimate mixing of organic molecules and alkoxides in the precursor solution allows organic and inorganic components to be associated at the molecular level. Organic molecules can be simply embedded within the silica matrix or chemically linked via Si–C bonds [17]. The mean size of organic and inorganic phases can be of the order of a few nanometres. Therefore hybrid materials are transparent and can be used for optical applications. Moreover, due to their improved mechanical properties, they can be shaped (films, fibres, nanospheres, . . .) and polished down to one nanometre in surface roughness.

The recent development of sol–gel optics takes advantage of the optical properties of organic dyes together with the hardness and optical transparency of silica. A large number of organic dyes have been trapped within sol–gel silica matrices [18]. Sol–gel matrices offer several advantages for optical applications. Interaction between dyes and the oxide matrix prevents aggregation that could quench the luminescence. The solubility of organic dyes can be increased significantly and larger amounts of dyes can be used. Moreover, these interactions can be tailored in order to improve the optical response of trapped dyes [19].

2.3. Bioencapsulation

The ability to form hybrid silica glasses under aqueous, room temperature conditions (at which proteins and cells are active) opens up the possibility to extend sol–gel processing to the encapsulation of biologicals. However, sol–gel conditions are mild enough for organic molecules but still remain too harsh for biomolecules. Alcohol and acid pH for instance lead to the denaturation of most proteins. Therefore the sol–gel process has to be adapted to biomolecules and bioencapsulation is currently performed in two steps. The first step is the hydrolysis of TMOS in the presence of an acid in order to hydrolyse all alkoxy groups (equation (3)). Biomolecules are then added to the $Si(OH)_4$ hydrolysed aqueous solution in the presence of a buffer with a pH of $\approx$7. Condensation is then quite fast and the silica network grows around the biomolecules that remain entrapped within the porous gel. The whole process occurs at room temperature within a few minutes and without denaturation of most proteins. A novel class of bioactive materials is thus obtained, made up of biomolecules physically trapped within silica matrices.

3. Bioactivity in confined nanopores

Enzymes are biological catalysts, which are responsible for the chemical reactions of living organisms. They are far more efficient than our catalysts and enzyme immobilization is a key point for the development of biotechnologies. Their high specificity and huge catalytic power is due to the fact that the geometry of their active site can fit exactly that of the substrate according to the so-called 'lock-and-key' model. Therefore even small changes in the enzyme conformation upon immobilization could reduce their catalytic activity drastically. In fact most enzymes have been shown to exhibit similar reactivity in wet gels and in aqueous solutions, suggesting that their structure does not change upon encapsulation. Silica gels provide an aqueous environment close to that observed in biological media and the porous sol–gel matrix allows the free flow of small substrates to the protein [5, 6]. Therefore the catalytic activity of entrapped enzymes may be close to that observed in aqueous suspensions and follows the usual Michaelis–Menten law [4].

3.1. Stabilization by limited diffusion

Enzymes retain their bioactivity in sol–gel glasses but, because of the diffusion through the pores, the kinetics of the reactions are usually slower than with free enzymes. This is not always a drawback and the porous silica network may protect encapsulated proteins against denaturation. Environmental effects that otherwise destroy the enzyme catalytic activity may be greatly reduced by the limited diffusion of external reagents through the pores of the matrix [20–22]. Enzymes often exhibit increased stability when encapsulated in sol–gel glasses. Their catalytic activity in aqueous solution may be lost fairly rapidly whereas, once immobilized, enzymes may be stable for weeks and even months. The half-life of entrapped glucose oxidase or acid phosphatase for instance was shown to be two orders of magnitude longer in gels than in solution [23]. It has been observed that the activity of lactase, an unstable enzyme, decays rapidly when removed from its natural environment, whereas it remains stable for at least two months in silica gels [24]. Cytochrome c appears to be stabilized against external denaturation agents by sol–gel encapsulation. Protein aggregation is avoided and even the drying process does not affect the protein which can be regenerated by rehydration [25].

3.2. Restricted mobility in nanopores

Upon encapsulation, proteins are encased by the hydrated silica in a cage tailored to their size. The silica matrix constrains the motions of encapsulated proteins and may prevent irreversible structural deformations. Trapped proteins can still undergo the necessary conformational changes for binding and release of substrates, but it appears that the sol–gel matrix slows the kinetics of unfolding and refolding of protein chains and greatly limits conformational changes. The stabilizing effect observed upon encapsulation may result from the ability of the silica matrix to restrict the conformational flexibility of the protein and to promote structural rigidity in the internal water [26–27]. Spectroscopic studies of trapped carbon-monoxide-bound myoglobin (COMb) show that encapsulation slows the kinetics of acid-induced unfolding of myoglobin and dramatically slows the kinetics of refolding [28].

Translational motions of encapsulated proteins are expected to be eliminated but rotational mobility may be preserved. Dielectric relaxation measurements show that encapsulated cytochrome c retains its rotational freedom. A slight increase in the rotational activation energy ($\Delta E \approx 1.1$ kcal mol^{-1}) is observed, but the biomolecule remains free to rotate

in order to align its dipole with the applied electric field [29]. Time-resolved fluorescence anisotropy experiments performed with acrylodan-labelled bovine and human serum albumin suggest very little effect of encapsulation [30]. However, this is not a general case; the decay of the fluorescence anisotropy measured for magnesium-substituted myoglobin for instance shows a significant decrease, suggesting that encapsulation impedes rotational diffusion of proteins [31]. The dynamics of antibodies sequestered within sol–gel silica matrices has been studied using anti-dansyl antibodies from rabbit (anti-DAN). In this system, dansyl serves simultaneously as a hapten and a fluorescent probe. This solvatochromic molecule is sensitive to subtle changes of its environment. Both the binding affinity and the rotational reorientation dynamics have been studied. The rotational mobility decreases significantly but the binding affinity (10^8 M^{-1}) is only fivefold less than in aqueous solutions and remains constant for at least eight months [32].

In fact, the sol–gel procedure can be tailored to optimize the stability of proteins in the precursor solution, before encapsulation. This was achieved by adjusting the level of Ca^{2+} present during the entrapment of the calcium-binding protein oncomodulin. Fluorescence experiments show that the protein, entrapped in a fully folded state in the presence of excess Ca^{2+}, is partially unfolded when the level of Ca^{2+} decreases. Calcium can be used to maintain the structure and stability of the protein during entrapment. The protein is then protected against denaturation by chemical reagents such as alcohol produced during the hydrolysis of alkoxides [33].

Steric hindrance is not the only cause for the limited mobility of entrapped enzymes. Electrostatic interactions may also occur between silicate sites and specific residues on the protein surface. Silica surfaces are negatively charged above the point of zero charge (pH $\approx$ 3) and electrostatic interactions mainly depend on the isoelectric point (IEP) of the protein. Experiments performed with three different oxidases, glucose oxidase (IEP = 3.8), glycolate oxidase (IEP = 4.6) and lactate oxidase (IEP = 9.6), show that only glucose oxidase retains its activity upon encapsulation. Electrostatic interactions decrease the catalytic activity of the two other positively charged oxidases. However, the detrimental effects of these electrostatic interactions can be reduced by complexing the enzyme with a polyelectrolyte that shields the critical charged sites and lactate oxidase can be stabilized by complexing with the weak base PVI (poly-(*N*-vinylimidazole)) [34, 35].

3.3. Chemically controlled nanopores

Confinement within silica gels does more than just protect enzymes against denaturation. It can also provide a chemical surrounding that favours the enzymatic activity. The ability to tailor the matrix properties, by modifying the sol–gel chemistry, enables optimization of the bioactivity of encapsulated enzymes. Hybrid materials can be used to control the polarity of the internal environment within the nanopores. Hydrophobic hybrid organic–inorganic materials can then be produced that are more suitable for the encapsulation of lipophilic enzymes that would not remain functional in polar matrices [36, 37].

Lipases provide a nice example showing how chemical control of the sol–gel matrix can be used to improve enzymatic activity. Lipases catalyse hydrolysis and esterification reactions. In aqueous media they hydrolyse fats and oils into fatty acids and glycerol whereas esterification reactions occur in organic media. In fact, most lipases are interface-activated enzymes. In an aqueous solution, an amphiphilic peptidic loop covers the active site just like a lid. At a lipid/water interface, this lid undergoes a conformational rearrangement, which renders the active site accessible to the substrate [38]. Their activity in hydrophilic silica matrices is rather

poor but they can be almost 100 times more active when trapped within a hybrid silica matrix. Using hybrid precursors such as $RSi(OMe)_3$ or, to a lesser extent, adding polymer additives such as polyethylene glycol (PEG) or polyvinyl alcohol (PVA) provides organic groups (R) that offer a lipophilic environment and could interact with the active site of lipases to increase their catalytic activity [39–41]. Such entrapped lipases are now commercially available and offer new possibilities for organic chemistry, the food industry and oil processing.

4. Protein-based photonic devices

Many biological molecules exhibit unique optical properties that could be very useful for photonics applications. However, these properties have been mainly studied in solutions that are not very convenient for the realization of optical devices. Solid materials or films would be more suitable and there is considerable interest in the possibility of designing optical biodevices based on solid-state materials. Sol–gel processing could be an answer to this problem. It provides silica matrices with high optical quality together with good thermal and mechanical stability. In fact, a large variety of organic dyes have already been trapped within silica xerogels. Sol–gel optics has led to the development of new materials for luminescent devices, lasers, photochromic coatings and non-linear materials [19]. Photochemical hole burning (PHB) and reverse saturable absorption (RSA) have been observed in sol–gel films containing porphyrin and phthalocyanines [42, 43].

4.1. Photochromic bacteriorhodopsin

Photoactive proteins have been extensively studied in view of their light-transducing properties, but the possibility of using these proteins as active components of photonic devices is quite new. Assembling these molecules into a geometric structure that provides rigidity and stability is critical for practical applications. Bacteriorhodopsin (bR) is a photochromic protein produced by halobacteria for its photosynthetic capability [44]. Its photochromic properties (sensitivity and reversibility) are far beyond those of synthetic materials. Moreover, they can be genetically modified to a large extent leading to a whole class of photochromic materials [45].

Bacteriorhodopsin consists of 248 amino acids arranged in seven transmembrane α-helix bundles. It converts light into chemical energy: under light irradiation, bR undergoes a conformational change that pumps protons across the membrane, from the cytoplasm inside the cell to the external environment. The resulting pH gradient is used as an energy source for the synthesis of ATP from ADP. Several steps are involved in the photocycle. The step whereby the proton is released results in a shift in the optical absorption of more than 150 nm. It absorbs at 410 nm and turns back to a ground state absorbing at 570 nm, with a 10 ms relaxation time. Therefore, bR could be used as a photochromic component in optical recording materials. Many attempts have been made to immobilize this protein on solid substrates in order to use its properties in the design of optoelectronic devices [46, 47]. Almost 100 patents have been lodged during the past 20 years using bR for photochromic devices, optical recording or data storage [48].

In fact, it has been shown that bR retains its light-sensitive properties when encapsulated within sol–gel glasses [49, 50]. The proton pumping function is not affected by the sol–gel matrix, and circular dichroism experiments suggest that bR is encapsulated along with its membrane lipids. When excited by a laser, entrapped samples show a decrease in absorbance at 570 nm and an increase at 410 nm as long as the laser irradiation is maintained. These results are quite reproducible and the optical activity of the protein persists for many cycles.

The average half-time to maximum change is even shorter in the sol–gel glass than in an aqueous suspension. Transparent films can be deposited and the use of bR as a molecular switch in optoelectronic sol–gel devices now seems to be technically feasible [49, 51].

4.2. Photoactive phycoerythrin

Phycoerythrin (PE) is another light-transducing protein that could be a promising candidate for signal transduction and biosensor applications. This bulky, water-soluble protein is found in the outer membrane of marine algae [52]. It is able to absorb and transfer light energy at low intensity levels to chlorophyll for photosynthesis with efficiency greater than 90% [53]. PE displays very intense fluorescence at 575 nm (20 times more than fluorescein) with high quantum yields and a large Stokes shift (2.7 times that of fluorescein) and is already widely used as a fluorescent marker in biomedical research [54].

As for bR, phycoerythrin retains its optical activity when encapsulated within sol–gel glasses. It even exhibits enhanced stability against photodegradation. In solution, PE degrades approximately 60% faster than when encapsulated in sol–gel. Even strong two-photon-induced fluorescence is observed, suggesting that this material could be used for 3D optical memories [55]. However, such a result cannot be extended to any other light-transducing proteins. It appears that other phycobiliproteins (phycocyanin, allophycocyanin) undergo significant changes in conformation in sol–gel silica. They tend to aggregate and cannot be used in optical devices. The reason for such behaviour is not yet clearly understood, and further studies are required to get a better understanding of protein–silica interactions [56].

5. Biosensors

5.1. Optical biosensors

Silica gels provide a transparent mesoporous matrix in which sensing molecules are entrapped and into which smaller analytes may diffuse. Many reactions that occur in solution can also be accomplished in the pores of the silica gel that can be used to quantitate reactions that generate a colour change. The first sol–gel sensors doped with organic dyes to be described were for pH or metal-ion sensing. Several configurations have been explored, such as doped monoliths, coated optical fibres, planar waveguides and miniaturized probe-tip sensors [57, 58].

Sol–gel biosensors were developed later and most of the initial activity was centred on metalloproteins. They can easily and reversibly form complexes and be reduced or oxidized by addition of appropriate reactants to the solution bathing the sol–gel. The first optical biosensors were based on the affinity of haemoglobin (Hb), myoglobin (Mb) or cytochrome c for O_2, CO or NO [59–62]. Ca^{2+}-ion-sensitive optical biosensors have also been made with aequorin, a bioluminescent protein found in jellyfish, immobilized in a sol–gel glass. The luminescence of this protein is specifically triggered by calcium ions and has been used for the determination of the presence of calcium in human sera and milk [63].

Enzymes are also used for optical biosensing. Interesting results have been obtained in the biodetection of nitrates using a sol–gel-immobilized nitrate reductase. Characteristic changes in the UV/visible absorption spectrum of the nitrate reductase allow the detection of nitrates in the μmol l^{-1} range [64]. This is important, as nitrate fertilizers are increasingly used in agriculture and their detection is essential for environmental and human health.

In principle, optical detection requires some change in the optical spectra of reacting biospecies. However, this is not always the case. Ammonia, for instance, is produced

by the enzymatic hydrolysis of urea in the presence of urease without giving any coloured reaction. Nessler's reagent (K_2HgI_4) is therefore added; this that forms a coloured product with ammonia. Following the optical absorbance at 405 nm provides a combination of rapid response, high storage stability, good run-to-run stability and simplicity in fabrication [65].

5.2. Electrochemical biosensors

Optical methods can be used to quantitate reactions that generate a colour change, whereas electrochemical detection is suitable for redox reactions. Biomaterials that can be used in electrochemical biosensors include enzymes, antibodies, antigens, oligonucleotides and DNA fragments. Systems that employ enzymes as bioactive interfaces are by far the most extensively studied assemblies in bioelectronics. These biocatalysts offer high specificity and turnover. They open the way to tailoring sensitive and specific enzyme-based biosensor devices.

However, electrochemical detection requires the attachment of enzymes to the electrode [66]. The chemical means to support the biomaterials include the immobilization of redox enzymes on electrodes by means of polymers, membranes, carbon paste and sol–gel matrices. Sol–gel processing holds great promise for the immobilization of biomolecules onto physical transducers, and electrochemical sensors based on enzyme electrodes have been extensively developed during the past decade. In such devices an electrode transducer converts the redox reaction that occurs at the active site of the enzyme into an electrical signal. The immobilization of the biological entity onto a transducer surface is thus a key feature [67, 68]. Bioreceptors can either be immobilized on the surface of electrodes or trapped within conductive materials. They may suffer from fouling and contamination of the surface during long-term operation. Therefore polishable or disposable biosensors are currently made. They can be either renewed or discarded after a few measurements.

Composite ceramic carbon electrodes (CCE) in which an enzyme-loaded carbon powder is mixed into the sol–gel solution have been extensively developed [69, 70]. These electrodes can be prepared in virtually any desired shape: as thick supported films useful for disposable electrodes, as discs or rods which can be used as renewable surface electrodes [71] and even in the form of microtips ($\approx$10 μm) [72]. Hydrophobic electrodes have been made using hybrid silica precursors such as methyltrimethoxysilane, $MeSi(OMe)_3$. Water penetration is then limited to a very thin layer at the outermost section of the hydrophobic electrode and they can be renewed by a simple polishing step [73, 74]. CCE can be deposited as thick films by screen-printing using carbon inks made of enzymes and carbon powders dispersed into the sol–gel silica, with a binder such as hydroxylpropyl cellulose [75]. Printing inks for glucose biosensors based on glucose oxidase (GOD) were made using ferrocene as a mediator and polyvinyl pyrrolidone (PVP) as a binder [76]. This process offers a one-step fabrication of disposable enzyme electrodes.

Sandwich configurations in which enzymes are deposited between two sol–gel silica layers of controlled porosity have been developed. They offer several advantages compared to homogeneously encapsulated enzymes due to rapid diffusion of the substrate through the upper porous layer and a shorter enzymatic reaction along with high enzyme loading. They combine fast response with high enzyme loading [77].

Most of the research activities on sol–gel-derived electrochemical biosensors have focused on the design of amperometric enzyme electrodes. However, conductimetric biosensors have also been realized. They are based on the catalytic activity of urease in the hydrolysis of neutral urea into charged ammonium and hydrogen carbonate ions, which increase the overall

conductivity of the solution:

$$NH_2CONH_2 + 3H_2O \Rightarrow 2NH_4^+ + HCO_3^- + OH^-. \quad (5)$$

The sol–gel-immobilized urease is deposited on a microfabricated interdigitated-array gold electrode. This biosensor shows good reproducibility and long-term storage stability when used for the determination of urea in urine samples. The coupling of sol–gel immobilization and microfabrication technology can offer mass production of durable biosensors [78].

Thermometric biosensors have also been developed for monitoring enzyme reactions based on changes in enthalpy. Injection of glucose results in change of the heat content of the circulating buffer, recorded as a thermometric peak by a sensitive thermistor. This system was used to detect glucose in fruit juice, sodas and human blood serum [79].

5.3. Glucose biosensors

The most efficient sol–gel biosensors are now based on the catalytic activity of enzymes. The high specificity and rate of reaction, often two to three orders of magnitude faster than those for corresponding uncatalysed reactions, make enzymes excellent candidates for use in biosensing. A large number of enzymes have been encapsulated within sol–gel glasses. Several biosensors have been developed for the detection of pollutants such as organophosphorus pesticides [80–82]. However, glucose oxidase (GOD) is by far the most widely used enzyme for biosensing. Glucose is very important in clinical analysis for the diagnosis and treatment of diabetes and in biotechnology and the food industry [83]. GOD catalyses the oxidation of glucose by molecular oxygen into hydrogen peroxide and gluconic acid:

$$C_6H_{12}O_6 + O_2 \Rightarrow C_6H_{10}O_6 + H_2O_2. \quad (6)$$

Glucose biosensors based on GOD represent a good example of the numerous possibilities offered by the sol–gel process. Glucose detection can be achieved via the consumption of O_2, the formation of H_2O_2 or the redox reaction at the active site of GOD (figure 1):

(i) Oxygen depletion can be electrochemically followed via an oxygen-sensitive electrode. The sol–gel film containing GOD is deposited onto the Pt cathode of a Clark electrode, between two polymeric films. The oxygen concentration is measured by amperometric titration at an imposed potential and the enzyme activity is determined via the decrease in oxygen content upon the injection of glucose into the cell. Such a sensor is highly sensitive, as oxygen depletion at the electrode is used to measure the amount of converted glucose. It can be easily regenerated, by washing the electrodes with a glucose-free solution [84, 85]. The oxygen consumption can also be optically evidenced via the fluorescence of an oxygen-sensitive ruthenium complex. A planar thin-film sandwich device was constructed in which the O_2 concentration in the Ru-doped sol–gel film is monitored by a superficial sol–gel layer containing the GOD enzyme [86].

(ii) Hydrogen peroxide, H_2O_2, is a product of many reactions catalysed by oxidases. It is therefore essential for chemical, biological and even clinical analyses [87–90]. Amperometric methods can be used to follow its formation [91–94] but optical detection is usually preferred. A coloured reaction is obtained by adding a horseradish peroxidase (HRP) that catalyses the oxidation of an organic dye. Such reactions have been performed in bulk sol–gel glasses doped with both enzymes (GOD, HRP) and the dye. The whole glass becomes coloured when dipped into a glucose solution, showing that the enzymatic activity is distributed throughout the gel and that the colour change is not due to surface reactions alone. The optical spectra are almost the same for the gel and the solution and

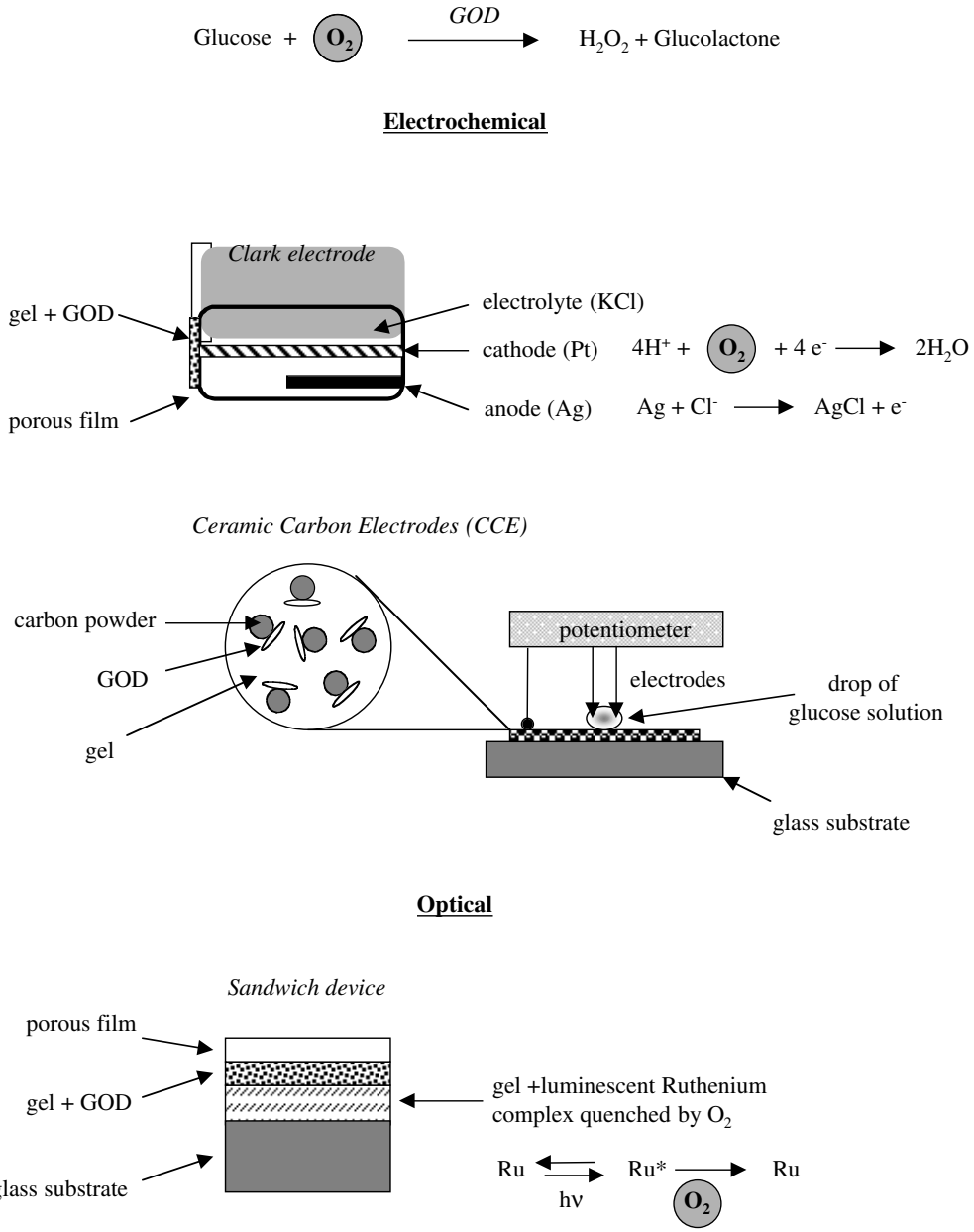

Figure 1. Examples of experimental devices used for glucose oxidase activity monitoring. Measurements of O_2 consumption can be followed by electrochemical [71, 85] and optical [86] methods.

a fair correlation is observed between optical density and the amount of glucose in the solution [95–97].

(iii) The direct electron transfer between the redox-active site of enzymes and the electrode is usually prohibited since the redox centre is sterically insulated by the protein matrices. Molecular mediators such as ferrocene or ferricyanide are then added in order to transport

electrons from the active site of the enzyme to the electrode. The reduced mediator is regenerated at the anode and the measured current is proportional to the amount of converted glucose [98, 99]. In some devices the ferrocene mediator is even covalently bonded to the silica network in order to prevent leaching [100].

5.4. Antibody-based affinity biosensors

The recognition of antigens by antibodies is highly specific and sensitive. It is currently used for the realization of immunoassays for medical applications, but antibodies could also be used for the detection of chemicals. However, small molecules do not exhibit antigenic properties. They have to be bound to a macromolecular carrier such as bovine serum albumin (BSA) to induce an immune response and stimulate the production of antibodies. These antibodies are then able to recognize specifically the target analyte and can be used as immunosensors for the detection of traces of chemical species. However, as for enzymes, sensors require the immobilization of antibodies which in many cases is a lengthy, multistep process. Sol–gel immobilization offers a simple and versatile method for entrapment.

The specific antigen–antibody fixation can be detected via optical or electrochemical measurements. The first attempt to encapsulate antibodies in sol–gel matrices was made with antifluorescein antibodies. These antibodies have a very high affinity for fluorescein in aqueous solution and it was shown that, when trapped in sol–gel matrices, they retain most of their activity and remain able to fix fluorescein specifically. This reaction can be optically detected via the emission spectrum of fluorescein. Fluorescence is red-shifted and its intensity decreases [101, 102]. Recently, optical detectors for trinitrotoluene (TNT), a commonly used explosive, were described. Anti-TNT antibodies, trapped within optically transparent sol–gel glasses, retain their ability to bind TNT. Immunoassays, performed with a fluorescent probe, allowed the detection of traces of the order of ppm and even differentiating between TNT and TNB (trinitrobenzene) [103]. Antibodies have also been used for electrochemical immunosensors in conjunction with screen-printing technologies. Antigen-containing thick-film electrodes were employed with an alkaline phosphatase-labelled antibody. Screen-printing allows one-step mass production of low-cost antigen-containing strips. Encapsulated antigens are readily accessible to the labelled antibodies and the dispersed graphite powder offers facile detection of the phenolic product via cyclic voltammetry [104].

The high binding constants for the association of antibodies to antigens make them ideal for chromatographic separation. Immunoaffinity purification is a form of chromatography in which antibodies are used to bind a specific molecule from a mixture of compounds. The bound molecule is then released with an eluting solution. Sol–gel-encapsulated antibodies have been used for the detection of various chemicals such as dinitrobenzene (DNB) [105] and dinitrophenyl (DNP) [106]. The immunoaffinity purification of such chemicals is based on the entrapment of antibodies within a sol–gel matrix. They are able to recognize nanograms of nitroaromatic compounds. Sol–gel immunoaffinity columns may even exhibit binding capacities higher than those of agarose gels.

More interesting was the application of sol–gel immunosensors for the analysis of pesticides and environmental contaminants [107]. The extensive use of pesticides and their persistency in the geosphere causes environmental problems, mainly for drinking water resources. The maximum level allowed for a single pesticide by European rules is 0.1 μg l^{-1} (0.1 ppb). Highly sensitive detection methods are thus required and immunochemical reactions are good candidates for use in pesticide analysis. The detection of traces of atrazine, a widely used herbicide, was carried out using monoclonal anti-atrazine antibodies produced by a mouse.

These antibodies were trapped within a SiO_2 sol–gel chromatographic column. Nanograms of an atrazine solution were then poured through the column; up to 90% of the whole atrazine was fixed by trapped antibodies. The reaction is reversible and atrazine molecules can be eluted from the gel and titrated by classical enzyme-linked immunosorbent assays (ELISA) [108, 109]. Anti-isoproturon-doped sol–gel powders have also been used to determine isoproturon, another widely used herbicide, in seawater and potatoes. This process was shown to be more sensitive than the traditional HPLC methods, which usually need a preconcentration step to reach low detection limits [110].

6. Whole cells in sol–gel glasses

6.1. Whole cells for chemical applications

Many intracellular microbial enzymes are produced in quantities large enough to be used in industrial processes. However, the cost for their isolation and purification can be quite high. It would therefore be of interest to be able to encapsulate directly whole cells such as yeast or bacteria in order to avoid tedious separation and purification procedures. Bacteria could behave as a 'bag of enzymes' and retain enzymes within their natural surrounding in order to preserve their stability and avoid leaching during repetitive operations. Cell entrapment in polymeric gels is a very popular and effective immobilization method and the metabolic activity of cells is used in a large variety of processes in the food industry, waste treatment, production of chemicals or drugs and even cell transplantation [111]. Both natural and synthetic polymers (alginate, agarose, ...) are currently used for cell encapsulation. The number of papers describing the sol–gel encapsulation of whole cells is very limited, but they show that the process is feasible.

The first paper was actually published in the late 1980s, by Carturan *et al* reporting the immobilization of yeast spores (*Saccharomyces cerivisiae*) into thin SiO_2 layers [112]. Yeasts are used for the conversion of sugar and carbohydrates into ethyl alcohol and CO_2. They are currently employed in the fermentation of beers and the raising of bread. Sol–gel-encapsulated yeast cells follow the well-known Michaelis–Menten law and exhibit almost the same activity as in a solution. The sample can be reprocessed by solvent exchange in water to remove fermentation by-products and then impregnated with fresh food. Bioactivity can be sustained for repeated cycles over several months [113, 114]. Encapsulated yeast cells have also been used for environmental protection and metal recovery [115]. They are able to accumulate heavy metals (Hg^{2+}, Cd^{2+}, ...) from aqueous solutions. The fine porosity of the gel allows nutrients to reach the cell and by-products to escape [116, 117].

Following the same idea, plant cells (*Coronilla vaginalis*) have been deposited onto a glass fibre fabric and immobilized by sol–gel under a gas flow of silicon alkoxide. They remain able to produce secondary metabolites such as umbelliferone and marmesin for several months [118]. Such results could be very important as plant cells are known to produce a large variety of chemical compounds that could be used for medical purposes, in the food industry or in perfumes [119]. Chlorophyll was immobilized within sol–gel mesoporous silica films. These entrapped photosynthetic pigments are more stable against light than in aqueous solutions and could be used for *in vitro* biomimetic devices for solar energy conversion [120].

The sol–gel encapsulation of the bacterium *Escherichia coli* was reported recently [121]. The cellular organization and the enzymatic activity of trapped bacteria can be preserved for many days as long as the gel remains wet (figure 2). They even seem to be stabilized by encapsulation. This might be due to the fact that the sol–gel matrix protects the cell and prevents

lysis in the absence of nutrients. Another bacterium, *Pseudomonas* species-nominated strain ADP, was isolated from a herbicide spill site and immobilized in sol–gel glasses. It has the ability to rapidly metabolize atrazine, a moderately biodegradable herbicide, and could be used for the biodecontamination of water and soils. Entrapped cells lose much of their activity upon immobilization, but partial activity could be restored by amendment of nutrients, suggesting that bacteria may remain alive, at least for some time (less than 75 days at 4 °C) [122].

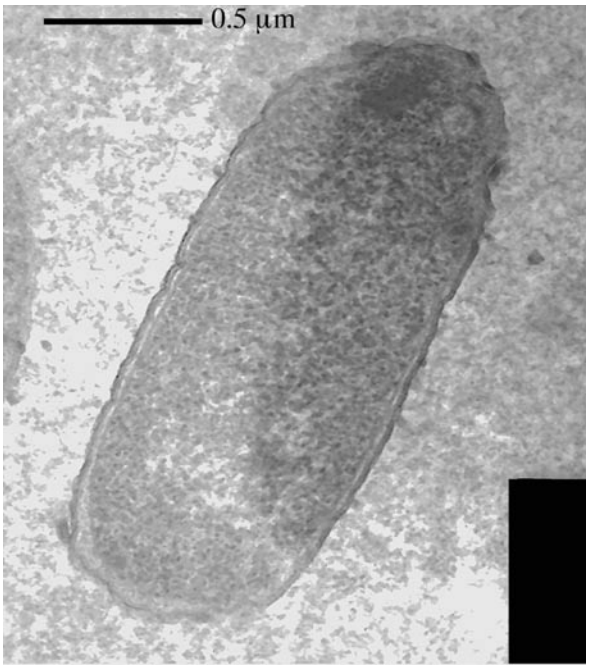

Figure 2. Ultrastructural observations by transmission electron microscopy of thin slices of wet gels show that the cellular organization of *Escherichia coli* cells is preserved in the gel [121]. The capsule, cell wall and plasma membrane are not destroyed. The porous texture of the silica matrix around entrapped cells can also be seen in this micrograph.

6.2. Whole cells for medical applications

6.2.1. Sol–gel encapsulation and drug delivery. The controlled release of active agents from an inert matrix has become increasingly important for oral, transdermal and implantable therapeutic systems. The possibility of embedding bioactive compounds into silica matrices offers new and interesting perspectives for drug carriers. In fact, only a small number of studies have been made. Experiments were performed with the calcium antagonist 'nifedipine' to evaluate the suitability of sol–gel materials. This well-known drug is commonly used to treat hypertension and angina pectoris [123]. The liberation of nifedipine from sol–gel composites can be controlled to a high degree via a chemical modification of the sol–gel matrix. Water-insoluble additives reduce the release rate, whereas soluble or swelling penetration enhancers (sugars, cellulose) accelerate the rate as a result of enlargement of the pore size [124].

Other experiments performed with silica gels doped with benzoic acid show that this antibacterial compound is released only in the presence of moisture (release on demand) [125, 126]. Impregnation with such a product causes a silicification of a wood structure that

enhances its resistance to biodeterioration, mechanical loading and fire. The first commercial product for wood preservation is already available (tradename Sebosil, produced by Feinchemie GmbH Sebnitz/Germany).

Nanoencapsulation within inorganic materials could have some potentiality for drug carriers. Such a process could also be used for vaccine formulation. Silica nanoparticles have been made using the aqueous core of reverse micellar droplets as host reactors. Peroxidase entrapped into silica nanoparticles shows higher stability against temperature and pH changes compared to free enzymes. Such silica nanoparticles could encapsulate active compounds and be used as drug carriers. Their administration in the body would be possible without any risk of allergic or proteolytic reactions because of their nearly zero leachability [127].

6.2.2. Immunoassays. The immune system is based on the recognition of antigens by antibodies. Antibodies are produced by the plasma cells as a response to the aggression of foreign micro-organisms (virus, fungi, bacteria, ...). These immunoglobulins exhibit a typical Y shape with two fixation sites that can bind specifically the corresponding antigens. Antigen–antibody recognition is currently used in medicine for the realization of immunoassays. The first attempt to realize sol–gel immunoassays was performed with the pathogenic protozoon (*Leishmania donovani infantum*) that is responsible for a disease called Leishmania. Whole cell parasites harvested from a culture medium were trapped within sol–gel matrices and used as antigens for blood tests. As for bacteria, transmission electron microscopy shows that the cellular organization of the parasites is well preserved and that the plasma membrane is unaltered. This is very important as the antigenic determinants recognized by antibodies (epitopes) are on the outside surface of the membrane. Immunoassays were performed by the so-called ELISA test, widely used in parasitology [128]. Leishmania cells are trapped within sol–gel silica directly inside the 96 microwells of a microtitre plate. Human or dog sera from infected patients are then poured into the wells of the microplate. Specific antibodies bound to Leishmania cells in gel are detected via an enzyme conjugate, horseradish peroxidase (HRP) that gives a coloured reaction with an organic dye (ortho-phenylenediamine, OPD) involving H_2O_2. The reaction can be followed by optical density measurements performed through the microwells of the plate. The optical density of positive tests decreases regularly with dilution showing that mainly specific antigen–antibody interactions are involved (figure 3). Non-specifically bound immunoglobulins are washed out giving a very low residual coloration in negative sera [129, 130].

6.2.3. Cell transplantation. An emerging approach to treating diseases makes use of living cells encapsulated within porous membranes that shield the cells from immune attack. Organic hydrogels and biopolymers are currently being developed for such applications but recent works show that sol–gel silica could also be used for cell transplantation.

The first experiments were made in the USA with pancreatic islets of Langerhans. These cells are known to produce insulin in response of glucose. *In vitro* experiments show that their insulin secretory capacity is maintained upon sol–gel encapsulation. Trapped islets still respond to glucose addition [131, 132]. *In vivo* experiments have even been performed via the transplantation of encapsulated islets into a diabetic mouse. The cells continue to function and to mimic a natural organ. Urinary excretion of glucose fell to almost zero within a few days of transplantation and remained close to zero for almost three months. In addition, both blood sugar and blood insulin concentrations were normal. The fine porosity of the gel protects transplanted islets against antibody aggression but allows nutrients to reach the cell and by-products to escape. One month after transplantation, the surgically removed transplant

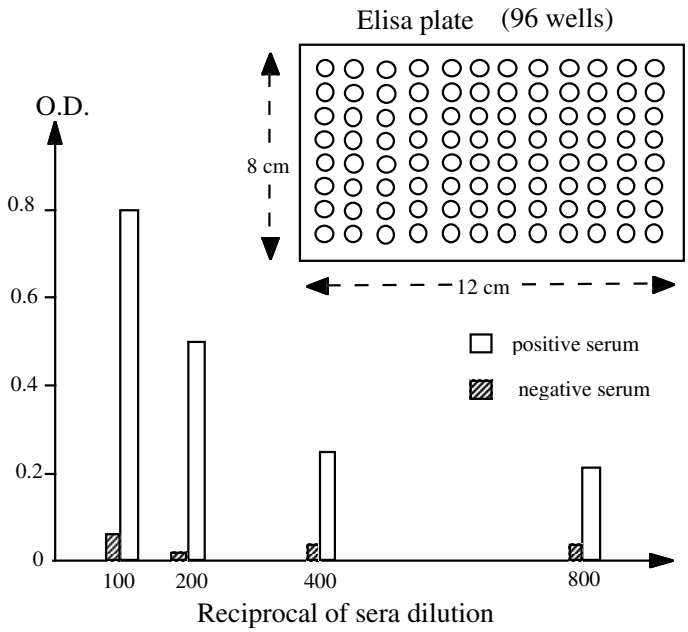

Figure 3. Immunoassays performed by the ELISA test. Sera are poured into the 96 microwells of a microtitre plate filled with silica containing Leishmania cells. Specific antigen–antibody interactions are optically detected via an enzymatic reaction directly through the microwells [129–130].

showed no evidence of fibrosis. Such encapsulated cells are under development at 'Solgene Therapeutics LLC' in California. Biogel transplants, if viable for extended lengths of time, could emerge as a viable treatment for diseases such as diabetes [133].

Another process, called 'biosil', was developed in Italy for the encapsulation of swine hepatocytes and rat liver. In this process, living cells are deposited onto a substrate and then partially covered by a silica film via a gas phase. They have already been used as a bridge to liver transplantation for a patient with fulminant hepatic failure [134].

7. Conclusions

Adsorption, covalent binding to solid surfaces, entrapment in polymer hydrogels and micro-encapsulation have long been used to immobilize biomolecules and whole cells. However, these methods are not generic and there is a lack of a universal technology. Despite its infancy, sol–gel encapsulation is perhaps the single most facile and generic immobilization technology available today. Sol–gel glasses offer several advantages compared to organic polymeric matrices, which are nowadays widely used in biotechnology. Biomolecules are trapped inside hard porous glasses that do not swell in water and protect biospecies against external aggression (pH, temperature, solvents, antibodies, ...). However, repeatability and long-term stability should still be addressed fully.

Sol–gel encapsulation does not destroy the cellular organization of micro-organisms. This might be one of the major advances of sol–gel chemistry and some promising examples show that living cells can be immobilized within sol–gel matrices. Silica gels have been shown

to be biocompatible. They can be used as supports for the culture of bacteria [135] and fungi [136]. The high porosity of silica gels favours water retention and nutrient diffusion. However, the usual alkoxide route in which alcohol is produced as a by-product is not really cytocompatible. Therefore several studies are being performed in order to find safer sol–gel procedures, either based on removing alcohol before encapsulation [137] or based on using aqueous precursors [138, 139].

Cell encapsulation aims to entrap viable cells and micro-organisms which retain their viability within polymeric gels. Immobilized cells grow within the support material by cell division and as a result microcolonies are formed. They provide their own space by expanding and exerting pressure on the surrounding matrix. This often results in protrusions of colonies through the surface. This may occur within soft organic polymers but would be much more difficult to achieve with hard silica gels. In principle, cell proliferation should be possible only on the surface of the gel [140] and the budding of yeast cells was actually observed at the surface of silica gels [112].

Achieving viability of cells within sol–gel matrices appears to be a real challenge. However, some recent experiments show that *E. coli* bacteria retain their metabolic activity and are even able to grow when trapped inside a gel matrix [141]. The formation of acetate was observed when lactate was added to a gel containing anaerobic sulphate-reducing bacteria, providing evidence of the metabolic conversion of lactate to acetate. Encapsulated bacteria survive the gelation procedure and are able to continue normal metabolic activity within the gel matrix. This activity decreases with time but can be regenerated by immersion in nutrient solution, even after several weeks [142]. Progress has still to be made in order to improve the viability and growth of cells within silica gels, but these first results are really promising. In spite of the dearth of industrial applications at present, there is great promise for the commercialization of sol–gel biological catalysts, sensors and photonic devices.

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