15 Sol–Gel Immunoassays and Immunoaffinity Chromatography

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15.1 INTRODUCTION

The sol–gel process and its use in the encapsulation of biomolecules of various kinds have recently seen important developments. Since its first introduction over two decades ago, sol–gel encapsulation has opened intriguing new ways to immobilize biological materials that offer an immense potential for the design of a large variety of applications. A great number of biomolecules, including enzymes, antibodies (Abs) (monoclonal, polyclonal, recombinant, and catalytic), DNA, RNA, and live animal, plant, bacterial, and fungal cells as well as whole protozoa have been encapsulated and then tested and implemented as optical and electrochemical sensors as well as core components of diagnostic, chromatographic, and catalytic devices.

In the past decade, several comprehensive reviews have been written on the sol–gel process, especially on the encapsulation of the above biomolecules and living cells [1–7]. The main topics covered include detailed explanations of the sol–gel process itself, information on the entrapment of proteins via the sol–gel process and on various types of sol–gel-derived biocomposites, a description of the behavior of sol–gel-entrapped biomolecules (distribution, conformation,

dynamics, accessibility, activity, kinetics, etc.), details of the applications of sol–gel based biocomposites (analytical, biomedical, biophysical, biosynthetic), and conjectures regarding the future development of sol–gel bio-immobilization.

Despite the large number and diverse nature of the biomolecules that have been, so far, entrapped in the sol–gel matrix, most of the recent reviews focus on the entrapment of enzymes, which represent the largest and most studied group of entrapped biomolecules [1,2,4,5]. Other biomolecules, including Abs, have drawn less attention, although the number of studies that have been carried out and the progress the field has achieved in the past decade as well as their potential for practical applications is immense. The present review focuses on entrapment of Abs and various antigens (Ags) in sol–gel matrices and their applications for irnrnunoassay (IA) and immunoaffinity chromatography (IAC). For the convenience of the reader, a brief introduction to the sol–gel process, biomolecule entrapment, and the recent innovations in the field of biomolecule encapsulation in this matrix is presented. Detailed reviews covering the above issues as well as topics related to the properties of sol–gel encapsulated biomolecules, their conformation, dynamics, accessibility, reaction kinetics, stability, and the recent developments in their application are available [1,2,4,5], and the reader is referred to them for further information.

15.1.1 The Sol–Gel Process

The term sol–gel refers to a chemical process where metallic or semi-metallic alkoxide precursors or their derivatives form composites at moderate temperatures through a chemical reaction that involves hydrolysis followed by condensation–polymerization (Figure 15.1). Most sol–gels are silicon-based oxides, although other oxides such as aluminum silicates, titanium dioxide, zirconium dioxide, and many other oxide compositions are also employed [8–11]. Silica oxides (SiO_2) sol–gel matrices can be composed in a wide range of physical properties (e.g., porous texture, network structures, surface functionalities) and under a wide variety of processing conditions (e.g., ambient temperatures, moderate pH values, and short gelation time), making silica alkoxides the most favorable group of sol–gel precursors. These matrices may take the form of porous wet gels, ambigels, aerogels, xerogels, or organically modified sol–gels (termed ormosils). The resulting matrix has high surface area and porosity, inertness and stability to chemical and physical agents, and optical clarity in the visible and UV ranges.

15.1.2 Entrapment of Biomolecules in Sol–Gel Matrices

Inorganic gels have been studied for over a century. In the past two decades, however, the sol–gel process underwent a significant development when it turned into a generic methodology for the incorporation of bioactive molecules. Although the first report on the entrapment of enzymes appeared in the mid-1950s [12], it was only three decades later that the importance of this finding was realized by Avnir et al. who entrapped a series of enzymes in a silica-based matrix [13]. Since then, a large body of work has emerged that encompasses the entrapment of a wide variety of biological molecules and even whole cells [1,2,4,5,14–16].

To achieve successful entrapment in sol–gel matrices, it is necessary to maintain the active conformation of the biomolecule within the matrix, to optimize the configuration of the doped sol–gel to provide the utmost performance, and to ensure that the entrapped biomolecule gains access to substrates, ligands, or analytes. The preparation of biologically doped materials via the sol–gel process fulfills the above requirements because the encapsulation is based on the growth of the polymer chains around the biomolecule, thus minimizing its denaturation. The sol–gel process can be carried out in aqueous solutions where hydrolysis and condensation–polymerization conditions occur at room temperature and at ranges of pH and ionic strength that are favorable for the biomolecule. Moreover, unlike the process in other polymers, the formation of the polymer backbone (e.g., Si–O–Si in the polysiloxane-based polymers) does not involve intermediates that could

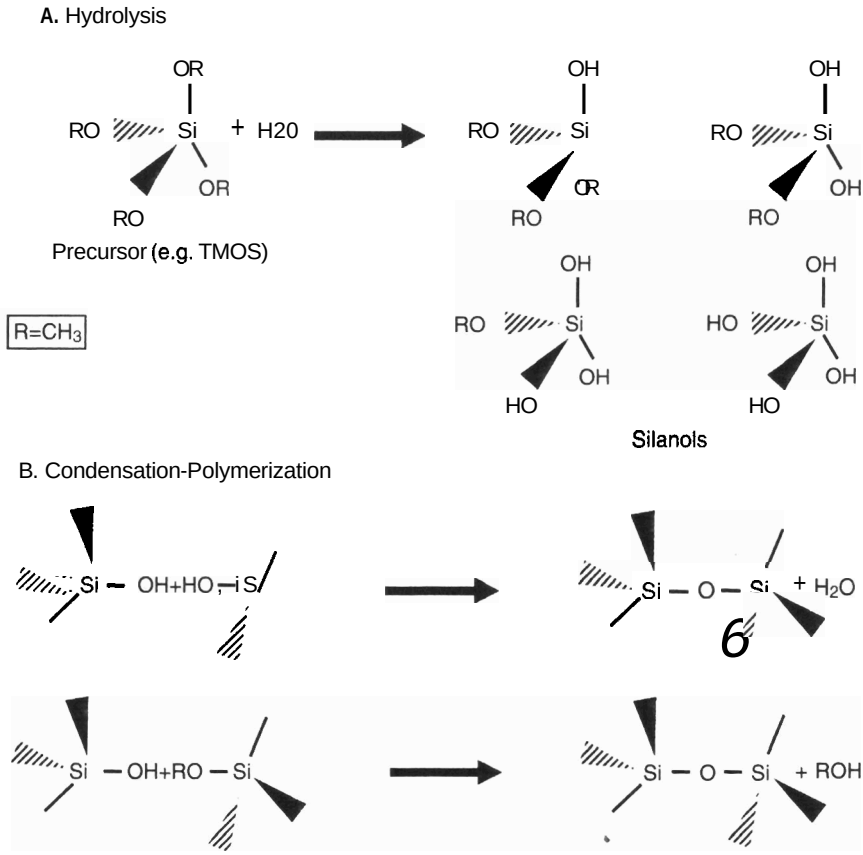

FIGURE 15.1 General scheme of the sol–gel process. TMOS: Tetramethoxysilane.

interact via a stable covalent bond with the protein being entrapped and potentially lead to its denaturation. Also, the pore size of the matrix can be controlled so that it is possible to obtain pores large enough to allow analytes, ligands, and substrates to reach the biomolecules, and the matrix can be modified to include residues and additives that modify the internal environment so as to improve bioactivity.

Entrapment of biomolecules is usually carried out according to the scheme shown in Figure 15.2. The precursor is first hydrolyzed (usually under acidic conditions that minimize the rate of siloxane condensation in the case of silicon oxides) to form an aqueous sol, then the hydrolyzed precursor is mixed with an aqueous solution of the biomolecules (in an appropriate buffer and at a pH that is suitable for bioactivity). The process results in condensation–polymerization of the hydrolyzed precursor followed by gelation of the aqueous sol to form a wet gel within which the biomolecules are entrapped. The initial gels have high water content and large pores, but over a period of time (days to weeks), further condensation occurs, strengthening the network. Further dehydration of the wet gel results in shrinkage of the polymer, collapse of its porous structure, and formation of a dry gel designated xerogel (Figure 15.2). As indicated above, most sol–gels are silica alkoxides, mainly because the hydrolysis and condensation rates of silica alkoxides are slow, enabling each step to be controlled independently and the kinetics to be optimized for specific needs.

Sol–gel biocomposites can be prepared from inorganic (silica or other metal or semi-metal) alkoxide precursors of the general formula $X(OR)_4$ where X is a metal or a semi-metal residue or

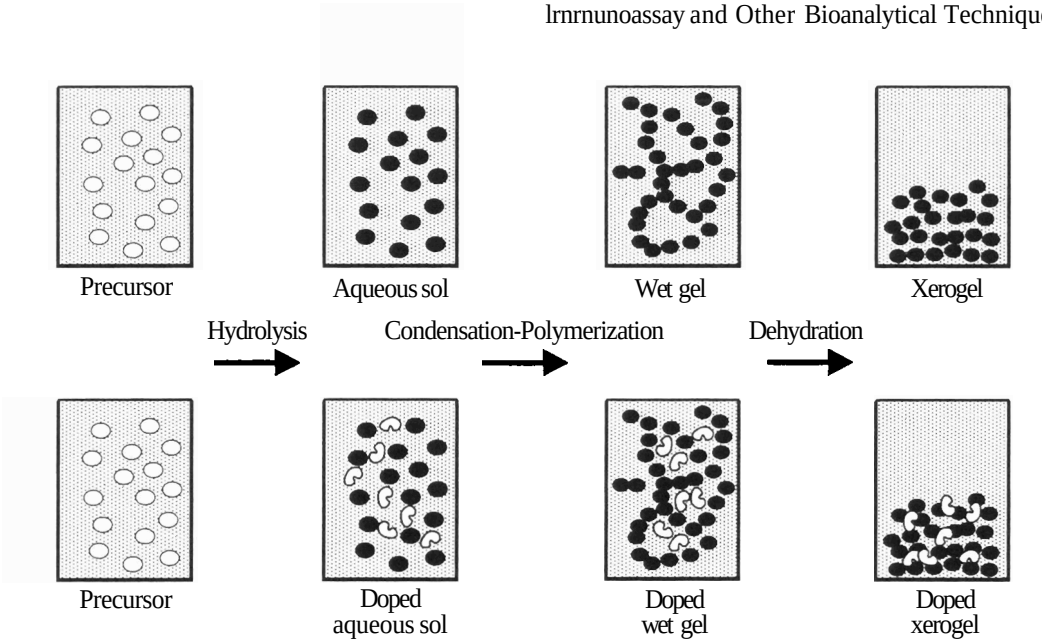

FIGURE 15.2 Scheme of the gelation process (upper panel) and biomolecule entrapment (lower panel).

from combined organic and inorganic materials of the general formula $YX(OR)_3$ where Y is an organic group such as ethyl or methyl, giving tetraethylorthosilicate (TEOS) or tetramethylsilane (TMOS). In addition, it is possible to encapsulate, together with the entrapped biomolecules, additives that can be beneficial for the stability and activity of the molecule. Such additives may be hydrophobic moieties, polymers (e.g., polyethylene glycol, glycerol, polyvinylimidazole, etc.), surfactants, liposomes, polysaccharides (dextran, cellulose, and chitosan), cofactors (e.g., redox modifiers), or even biological or synthetic additives. These compounds can be directly mixed with the sol–gel precursors before gelation to form hybrid organic–inorganic gels. The additives may affect the physical properties of the gel by changing its rigidity, mechanical stability, pore size, and optical or electrochemical clarity, and they may also affect the interactions of the gel with the entrapped biomolecules, thus providing higher overall activity. The nature of such gels and their effects on the entrapped biomolecules are discussed in detail in several reviews [1,4,5].

15.1.3 Characteristics of Entrapped Biomolecules

Progress in the encapsulation of biomolecules resulted in entrapment of a large variety of molecules such as enzymes, Abs, regulatory proteins, transport proteins, membrane-bound proteins, and nucleic acids [1,4,5]. Overall, these studies revealed that the entrapped biomolecules are surrounded by the porous gel network in a capsule- or cage-like manner. Depending on the nature and concentration of the entrapped molecule, the specific precursor and the additives used to form it may be homogenously dispersed in the pore solvent or adsorbed onto the silica in a variety of orientations, groups, or aggregates. A detailed review is presented by Jin and Brennan [1]. Entrapped biomolecules are not physically adsorbed or attached to the polymer texture although such interactions may occur naturally and could affect the activity. The interactions between the biomolecule and the inorganic, organic, or hybrid composite material and the additives in the gel determine the degree to which the biomolecule retains its native properties.

A variety of methods were employed to determine and characterize the bioactivity of entrapped biomolecules. They included measurements of activity by electrochemical and spectrophotometric methods, measurement of the absorbance changes that accompany ligand/substrate/Ag/Ab binding, use of fluorescence methods to probe ligand binding by regulatory proteins, and detailed studies of

the interactions of Abs with their respective Ags. These studies revealed that the biomolecules are strongly encapsulated within the matrix and cannot diffuse out; the molecules retain their activity; their stability is enhanced; and they can react with compounds that diffuse into the highly porous matrix. Although the moderate temperatures and the mild hydrolysis and condensation–polymerization conditions that characterize the entrapment process allow the biomolecules to be entrapped without being denatured, conformational studies of entrapped molecules have revealed that conformational changes do occur in the course of the sol–gel entrapment. The enhanced stability of the entrapped biomolecules and the physical and chemical properties of the matrix are among the reasons for the attractiveness of the sol–gel approach to immobilization in general and to that of proteins in particular. Detailed information on the conformational stability of sol–gel-entrapped molecules and their dynamics, protein–gel interactions, response times for entrapped proteins, and their influence on the gelation process are all described by Jin and Brennan and by Pierre [1,5].

15.1.4 Applications of Entrapped Biomolecules

Doped silicate materials have been applied in a variety of fields. The activity in this field has been quite intense in recent years and has resulted in many biomaterials with diverse applications. Some of the applications of sol–gel-derived biomaterials include the successful immobilization of numerous biotechnologically important enzymes, the construction of optical and electrochemical biosensors for clinical, industrial, environmental and domestic use, enzymatic electrodes, stationary phases for affinity chromatography, generation of immunosorbents, solid-phase extraction (SPE) materials, controlled-release agents and solid-phase biosynthesis, construction of bioactive optical components, biocatalytic paints and films, preparation of biomatrices that can be applied for environmental studies and functional proteins, DNA and RNA biochips, etc. The ability to shape gels in a variety of formats (blocks, thin films, microarrays, columns, fibers, and powders) that are compatible with a variety of applications has turned out to be very important for industrial use, and a number of original designs based on these formats have extended the applicability of these encapsulated materials. Further details on some of the above applications are presented in [1,2,5] and in the references therein.

This review focuses on one group of biologically important molecules that have drawn the attention of many research groups: sol–gel-entrapped Abs and Ags for use in the development of IAs and IAC devices. The studies that have been performed in this connection and the applications of this approach are described below.

15.2 SOL-GEL IMMUNOASSAYS AND IMMUNOCHROMATOGRAPHY

15.2.1 Solid Phase Immunoassays—General Aspects

Solid phase IAs are well-established methods in many fields. This powerful assay technology crosses discipline boundaries and is applied extensively as a research and diagnostic tool in both applied research and basic science as well as in medical, agricultural, and environmental studies. Solid-phase IAs are based on the fact that Abs or Ags can be immobilized on solid-phase matrices, thus enabling a simple and quick separation of free (unbound) analytes from the complexes immobilized on the solid surface. This simple feature made it possible to develop the method for quantitative detection of analytes and was, apparently, the most important reason for the rapid increase in its popularity and its wide application. Since the development of the first IA in the mid-1960s, hundreds of assays have been developed for native and synthetic molecules, and currently, they form one of the most generic diagnostic methods. The rapidity, sensitivity, simplicity, and cost-effectiveness of the method have made it an attractive tool, and it has been optimized

for a large number of analytes. It also has been automated, and it now serves as a routine procedure in monitoring a wide variety of analytes in the fields of medical, environmental, and food sciences.

As indicated above, solid phase IAs are based, first and foremost, on the successful immobilization of Abs or Ags onto or within a suitable solid surface. The requirements for useful immobilization include high density of the immobilized molecule (high surface-to-volume ratio), high activity, good orientation of the adsorbed molecule, long-term stability under potentially adverse reaction conditions, good accessibility for analytes, rapid response time, and resistance to leaching or desorption. Several methods of immobilizing molecules on inorganic, organic, and polymeric surfaces have been reported; they include physical adsorption, covalent binding to the surface, entrapment in semi-permeable membranes, and micro-encapsulation in polymer microspheres or hydrogels (for a detailed review see [17]). Although some of the techniques have been successful, none of them are generic; namely, they could not be applied to a wide variety of molecules and had to be carefully optimized based on the chemical and physical properties of the entrapped molecule and the entrapping matrix. As a result, those techniques could be used, in most cases, for only a limited number of molecules and applications. Problems related to low surface loading, long preparation procedures, leaching and desorption of molecules, difficulties in controlling molecule orientation, and instability of the immobilized molecule resulted in the need to invest substantial efforts in the optimization of the immobilization protocol for each molecule, and this limited the applicability of IAs The emergence of the sol–gel technology, with all its advantages, and the reports on successful entrapment of biomolecules in such matrices opened the road to implementation of the method for the development of IAs in general and for those employed for food and environmental monitoring, in particular.

Immunochemical methods such as enzyme IA have become increasingly important during recent years for the determination of pesticides and other xenobiotics [18–23]. Many commercial kits for assaying pesticides are available, and several hundred assays have been described in the literature [24]. A major problem associated with the use of Abs in immunochemical assays for environmental or agricultural monitoring is their limited stability that can be a source of variation or deterioration in the test performance quality. Fruit and vegetable extracts as well as soil samples contain organic and inorganic substances that may directly interfere with Ab–Ag binding or impair it indirectly by denaturing the Ab or complexing with it, thus decreasing the efficiency of the assay and its monitoring capability. On top of the problems associated with matrix interference, some of the assays suffer from low reproducibility, slow reaction times, and high costs, and for many cases, they need to be used in on-site monitoring (in the field, packing houses, contaminated sites, etc.). Introduction of the sol–gel method for biomolecule entrapment, the unique nature of alkoxides polymer chemistry, and the advantages that are introduced by this method have opened up new possibilities for such applications and have enabled the development of simple, highly sensitive, highly reproducible, and cost-effective assays for off-line, on-line, and on-site use (that, in some cases, have proved to be significantly better than the standard existing ones).

A summary of the sol–gel based IAs developed in the course of the past two decades for environmental, agricultural, and medical applications is presented below.

15.2.2 Sol–Gel Based Solid Phase Immunoassays

Encapsulation of Abs and Ags in sol–gels has been well documented in the literature (see lists of references in Table 15.1 and Table 15.2), although less than a dozen IAs (assays that can be applied for monitoring real samples) have been developed in over a decade. Key issues in the development of a functional sol–gel based IA focused on optimization of the configuration of the doped sol–gel to provide the utmost performance, immobilization of biomolecules at a high density in a manner that will render them high activity, long term stability, good accessibility to analytes, rapid response time, and resistance to leaching. Indeed, those were the main issues that were addressed in most of the studies as indicated below. Early studies focused mainly on demonstrating the ability of

TABLE 15.1
Sol–gel Entrapped Antibodies Applied for Immunoassays

Entrapped Antibody	Immobilization Matrix	Sol–Gel Form	Method of Detection	Application	Tested Parameters	References
Anti-progesterone	TEOS/3-aminopropyl trimethoxysilane	Slurry suspension	Liquid scintillation counter	IA-POC	Binding affinity, leaching, effect of pH, entrapment capacity, comparison with ELISA	[29]
Anti-fluorescein	TMOS	Monolith	Spectrofluorimeter	IA-POC	Dose response, effect of gel aging, drying and storage on analyte binding and affinity	[28]
Anti-fluorescein	TEOS	Aerosol derived thin-films	Spectrofluorimeter	IA-POC	Binding activity as a function of storage time, leaching, non-specific binding, regenerability	[35]
Anti-dansyl	TEOS	Monolith	Spectrofluorimeter and time resolved fluorometry	IA-POC	Binding affinity, stability upon storage, mobility of entrapped Ab within the gel	[26]
Anti-TNT	TMOS	Monoliths & xerogels	Spectrofluorimeter	IA-POC	Dose and time response, comparison of binding in wet gels vs. xerogels, cross reactivity, stability	[25]
Anti-laminin	Aluminum isopropoxide	Thin-film capacitive electrochemical electrode	Voltametric analyzer	IA-POC	Optimization of binding conditions, time and dose response, binding capacity, non-specific binding, selectivity, reproducibility, comparison with SiO_2 sol–gel derived immunosensors	[34]
Anti-cortisol	TMOS	Monoliths & thin-films	Spectrofluorimeter	IA-POC	Binding (specific, non-specific). conformational changes of entrapped Ab, dose and time response binding, leaching	[27]
Anti-isoproturon (herbicide)	TMOS	Powder placed in a flow through spectrofluorimeter	Flow — injection Spectrofluorimeter	IA	Binding capacity, flow rate, leaching, recovery from spiked sea water and spiked food samples.	[36]
Fluorescein labeled anti-D dimer (a fibrin fragment)	TMOS	Coated tip of an optical fiber immunosensor & monoliths	Spectrofluorimeter	IA	Binding, LOD, dose response, aging, leaching, regenerability, determination of analyte in spiked human plasma and spiked whole blood samples	[31]

(continued)

TABLE 15.1 *(Continued)*

Entrapped Antibody	Immobilization Matrix	Sol–Gel Form	Method of Detection	Application	Tested Parameters	References
Anti-complement 3 (C3)	MTMOS/graphite	Squeezed paste in a form of an amperometric immunosensor	Electrochemical analyzer	IA	Optimization of operating conditions, sol–gel-graphite ratio, working potentials, sensitivity, reproducibility, non-specific adsorption, stability, detection range and LOD of C3, determination of C3 in real human serum samples	[32]
Anti-gentamicin (antibiotic)	TMOS/PEG	Slurry column in the form of a continuous flow displacement immunosensor	Spectrofluorimeter	IA	Effect of PEG on activity, non-specific binding, leaching, dose response, LOD column regeneration, stability, flow rate, recovery from spiked serum samples and real patient samples, validation with analytical methods	[33]
Anti-gentamicin (antibiotic)	Magnetite containing spherical silica (TMOS) nanoparticles	Magnetite-silica nanoparticles in solution	Spectrofluorimeter	IA	Binding capacity, recovery from spiked blood samples	[30][a]

The table divides the experiments into two groups: (A) experiments that proved the concept (POC) that sol–gel entrapped Abs can be used as immunoassay (IA) devices (such assays are termed IA-POC and involved binding of synthetic standard analytes); (B) experiments where the entrapped Ab was used to monitor analytes from spiked or real samples (such assays are termed IA). Abbreviations: IgG: immunoglobulin; LOD: Limit of detection; MTMOS: methyltrimethoxysilane; PEG: Polyethylene glycol; TEOS: Tetraethylorthosilicate; TMOS: Tetramethoxysilane.

[a] Unbound and bound analytes were separated with a magnet. In all other experiments, separation was by washing.

TABLE 15.2
Sol–Gel Entrapped Antigens Applied for Immunoassays

Entrapped Antigen	Immobilization Matrix	Sol–Gel Form	Method of Detection	Application	Tested Parameters	References
Rabbit-IgG	TEOSI hydroxypropyl cellulose/graphite	Thick-film amperometric electrode & electrochemical immunosensor	Voltmeter and ampermeter	IA-POC	Optimization of binding conditions, Ag loading. LOD. measurement range, different gel microstructures, reproducibility	[40]
Echinococcus granulosus Ag	TMOS	Gel cast in 96-well microplate	ELISA reader	IA	Detection of ***Echinococcus granulosus*** Abs in sera of infected patients	[16,38]
Leishmania whole cells	TMOS	Gel cast in a 96-well microplate	ELISA reader	IA	Characterization of the physical properties of the sol–gel matrix, ultrastructure of entrapped cells, storage detection of ***Echinococcus granulosus*** Abs in sera of infected patients, correlation with ELISA	[16]
Toxocara canis Ag	TEOS/polyvinyl alcohol	Sol–gel cast in 96-well microplates	Spectrophotometer (colorimetric signal)	IA	Determination of detection range, comparison of sol–gel based IA with conventional ELISA, detection of Abs in infected serum samples	[37]
CA19-9 (carbohydrate tumor marker)	Titanium isopropoxide (Ti based sol–gel)	Thick-film electrochemical electrode	Electrochemical analyzer	IA	Optimization of immunosensor preparation and working conditions, dose response analysis, selectivity, reproducibility, stability, measurements of CA19-9 in real serum samples from patients	[41]
Schistosorna japonicum Ag	TMOS/ BSA/graphite	Fiber-like electrochemical electrode	Electrochemical and polarographic analyzer	IA	Binding conditions, non-specific adsorption, binding capacity, effect of incubation temperature, dose response, reproducibility. sensor stability, detection of Abs in infected serum samples	[42]

(continued)

TABLE 15.2 *(Continued)*

Entrapped Antigen	Immobilization Matrix	Sol–Gel Form	Method of Detection	Application	Tested Parameters	References
HCVand EBV derived peptide	Semicarbazide 3-aminopropyl trimethoxysilane	Slide microarrays	Fluorescence array scanner	IA	Non-specific binding, sensitivity, specificity, cross reactivity, reproducibility, comparison with ELISA, stability in storage, detection of HCV and EBV Abs in human serum	[39]

The table divides the experiments into two groups: (A) experiments that proved the concept (POC) that sol–gel entrapped Abs can be used as immunoassay (IA) devices (such assays are termed IA-POC and involve binding of Abs by the entrapped Ags); (B) experiments where the entrapped Ag was used to monitor Abs from spiked or real samples (such assays are termed IA). Abbreviations: BSA: Bovine serum albumin; CA: Carbohydrate antigen; EBV: Epstein-Barr virus; HCV: hepatitis C virus; IgG: immunoglobulin; LOD: Limit of detection; TEOS: Tetraethylorthosilicate; TMOS: Tetramethoxysilane.

entrapped molecules to function within gels; later studies extended the scope of their analysis to more detailed characterization of the properties of entrapped biomolecules and even whole cells (such studies are referred to in the tables as proof of concept, POC IAs). More recently, studies have begun to focus on the implementation of sol–gel entrapment of molecules and whole cells for generation of sol–gel based IA devices for monitoring real samples (real IA).

A quick overview of the main issues that were addressed by the POC-IA studies reveals that they focused on the optimization of assay conditions (pH, sample volume, flow rate, effect of additives), kinetic parameters and thermodynamic analysis of Ab–Ag interactions (binding affinity, time kinetics), non-specific binding, cross reactivity and selectivity, reproducibility, regenerability, leaching, effects of gel drying and storage time on bioactivity (aging and stability), interaction of dopant and/or analyte with the matrix, effects of the dopants on pore size and of the pore size on bioactivity, conformation of the entrapped molecule and its mobility, and comparison of the sol–gel activity with that of other IA formats (e.g., enzyme linked immunosorbent assay, ELISA). The more applied studies extended their focus beyond the above to the examination of issues such as assay capacity, detection range, and detection limits relative to those of other IAs, ability to use sol–gel based IA for monitoring analytes in real samples, recovery, and validation against other analytical methods.

Regardless of their final purpose, whether POC or real IA, all of the above studies revealed that the entrapped biomolecules were active within the biogels and obeyed basic binding and kinetic rules (all Abs listed in Table 15.1), retained their specificity (e.g., [25]), were very stable after exposure to denaturing factors such as extreme pH values, high temperatures, organic solvents, etc. and retained their binding activity for several months (e.g., [25,26]). The biomolecules did not exhibit any detectable rotational reorientation (e.g., [26,27]), and their binding affinity was lower than that in solution (e.g., [26,28]) although, in some cases, the affinity did resemble that in solution (e.g., [29,30]). The non-specific binding of the assay could be reduced to a minimum by appropriate choice of matrix (e.g., [27]), the detection limit was within the necessary range for analytical or diagnostic purposes (e.g., [27,30–34]), and in most of the studies, aging did not affect the binding properties of the Abs (e.g., [26,31,35]).

Two main formats of sol–gel based IA were developed: those where Abs were immobilized1 entrapped (Ab format, Table 15.1) and those where Ags were immobilized/entrapped (Ag format, Table 15.2). In each format, competitive and non-competitive assays were designed. In the first format, Abs were entrapped, and Ags that either exhibited self-fluorescence or were labeled with an enzyme, a fluorescent tag, or a radioactive isotope were tested for their binding to the entrapped Ab in the absence (non-competitive assay [26,28,31,34,35]) or the presence (competitive assay, [25,27,29,30,32,33,36]) of a free Ag. In the non-competitive assay, the decay of fluorescence was monitored; in the competitive assay, the decrease in enzymatic activity, fluorescence, or radioactivity (that resulted from an increase in the amount of free Ag that competed on binding to the entrapped Ab) was measured. In the second format, Ags were entrapped in the sol–gel matrix, and Abs of unknown samples (usually patient sera) were detected by a tagged secondary Ab conjugated either to an enzyme or to a fluorescent tag (non-competitive assay [16,37–39]), or by competition with a tagged primary Ab (competitive assay [40–42]).

Both IA formats employed a wide variety of entrapping matrices. Most assays were based just on silica alkoxides (TMOS or TEOS). Others used modified or derivatized alkoxides (3-aminopropyl trimethoxysilane, semicarbazide 3-aminopropyl trimethoxysilane, methyltrimethoxysilane) or silica alkoxides in combination with other additives (polyethylene glycol, PEG, hydroxypropyl cellulose, polyvinyl alcohol). Composites based on Al (aluminum isopropoxide) or Ti (titanium isopropoxide) were also employed. Graphite was added for generation of electrochemical electrodes. Both IA formats used a wide variety of sol–gel forms: slurry suspension, monoliths, aerosol-derived thin films, xerogels, gels cast in microplates, and a variety of electrochemical electrodes (thin-film capacitive electrodes, thick-film and fiber-like electrochemical electrodes, and amperometric electrodes). A whole array of immunosensors (optical, amperometric, and continuous flow) was generated as well as magnetite-containing spherical

silica nano-particles and slide microarrays. The Ab format IA has been used to monitor environmental as well as medically related analytes, whereas the Ag format IA has been used only for medical applications. Examples of the different formats and their applications are presented below, and summarized in Table 15.1 and Table 15.2.

15.2.2.1 Sol–Gel Entrapped Ab Immunoassays (Ab Format)

The first study on entrapped Abs was reported in 1984 by Venton et al. [29]. It demonstrated the successful entrapment of anti-progesterone Abs in a polysiloxane copolymer prepared from a 3:1 mixture of TEOS and 3-aminopropyl trimethoxysilane. The study revealed that entrapped Abs retained their ability to bind Ag molecules with an apparent Ka equal to that of free antiserum (although only 50% of the entrapped Abs retained their activity). The study also revealed that the Abs did not leach out of the matrix, and they were stable at a wide range of pH values.

This study was followed almost a decade later by the work of Wang et al. [28] who entrapped anti-fluorescein Abs in a monolithic format and also confirmed the concept that sol–gel entrapped Abs retain their binding activity and affinity to their analytes (in this case, fluorescein). The group carried out a detailed quantitative examination of the influence of the encapsulation method and also of the aging, drying, and storage of the gel on the affinity constant of the Ab-analyte interaction. The study revealed that storage time and conditions affected the affinity of the sol–gel encapsulated Abs if the gel was kept dry, but the affinity could be retained if the samples were stored wet in the cold. Contrary to the findings of Venton et al. [29], encapsulation of antifluorescein resulted in a decrease in binding affinity by about two orders of magnitude compared with that obtained in solution.

Two other Abs were entrapped in a monolith sol–gel format: anti-TNT [25] and anti-dansyl [26]. The anti-TNT Abs were able to detect analytes at a ppm level, and they retained their ability to differentiate between TNT and other trinitroaromatic analogues. The sol–gel entrapped Abs exhibited better relative stability than Abs immobilized by means of surface attachment, and they proved to be highly stable upon exposure to denaturing conditions. Satisfactory activity and enhanced stability of the entrapped Abs was also demonstrated with the anti-dansyl Abs [26].

Despite the successful development of monolith-based IAs, such assays suffer from a limitation that could affect measurements of real samples because of the inherently long response time associated with the slow diffusion of the analyte. In order to overcome the above limitation and to improve the response time and accessibility of the analytes to the entrapped sensing molecule, thin film IAs were developed [27]. Zhou et al. entrapped anti-cortisol Abs in thin films and compared their activity with that of monolith-entrapped Abs, finding that both formats could be used as optical-sensing IAs. The data also indicated that the Abs retained their activity in both formats in a dose responsive manner, that the sensitivity of the assay was satisfactory (within the physiologically relevant range), and that the entrapped Abs showed only small changes in their secondary structure. A comparison between the sensing properties of the monolith and thin-film forms revealed that the thin films were more effective and gave improved accessibility of Ags to the encapsulated Abs, leading to a significant reduction in the required assay time. However, these advantages were gained at the expense of a decrease in signal intensity (although not a major one) and wider variability between assays. Another study was carried out by Jordan et al. [35] who applied the thin-film approach to the entrapment of anti-fluorescein Abs, and they quantified their response as a function of storage time. Although the study did not compare the time kinetics of thin-film reactions with those of monolith assays, it revealed that the Ab retained affinity for its hapten, that the binding was retained for over three months, and that the response time increased as a function of storage time (because of the particular structure of the device that was used, selecting Ab subpopulations whose behavior changed as a function of time). Partial regeneration of the device was possible for several cycles using a mild chaotropic reagent.

The thin-film sol–gel method was also applied to the development of capacitive label-free IAs with γ-alumina sol–gel entrapped Abs [34]. Capacitive immunosensors have been extensively studied as novel label-free IAs that offer high sensitivity and rapid testing that are used with ordinary instrumentation. Successful implementation of this method required formation of an electrically insulated film in order to allow the capacitive measurements and the formation of thin (nanometer size) films of the embedded Ab layer. In their study [34], Jiang et al. described the use of y-alumina-based sol–gel (that is especially suitable for the formation of thin films because of the inherently high surface area of the matrix that enables significant reduction of the thickness of the formed films) for the development of a capacitive immunosensor. A multi-channel capacitive sol–gel-derived anti-human immunoglobulin (IgG) and anti-laminin IA was constructed on the basis of this system to illustrate the application. The device was able to measure Ags (human IgG and laminin) with the accuracy required for diagnostic analysis, it showed a low detection limit (lower than those of SiO_2 sol–gel-derived capacitive immunosensors or conventional ELISAs), demonstrated reproducible linear responses to the respective Ags, and was found to be selective and specific. This is the only example of a sol–gel matrix based on alumina alkoxide in this format.

Biosensors offer considerable advantages, especially when on-site monitoring is necessary. There are several formats of biosensors; the most common based on sol–gel technology are electrochemical immunosensors. Electrochemical immunosensors combine simple, portable, low-cost electrochemical systems with specific and sensitive IA procedures, representing a promising approach to clinical, biochemical, and environmental analyses. An example of a sol–gel amperometric immunosensor is presented in the study of Liu et al. [32]. The authors describe the generation of an amperometric immunosensor based on anti-complement C3 Ab entrapped in a mixture of sol–gel-BSA-graphite composite. The doped sol–gel paste (that was squeezed into a polyvinyl chloride tube) formed an arnperometric immunosensor that was used in a competitive binding assay to detect C3 in human serum (with the aid of a C3-horse-radish peroxidase (HRP) labeled conjugate). The immunosensor was found to be sensitive in the range required for clinical analysis, stable, highly reproducible, and renewable.

Another application of sol–gel entrapped Abs is in the formation of immunosensors that can monitor analytes in real time. Flow-injection IAs or electrochemical immunoelectrodes offer many advantages for real time monitoring. Analysis can be performed under continuous flow and automated on-line performance in the laboratory or on-site without the need to use costly and complex instrumentation. This is especially important for environmental and forensic monitoring. Two studies address the implementation of sol–gel entrapped Abs for the formation of continuous-flow displacement immunosensors. In the first study [36], anti-isoproturon Abs were entrapped in TMOS that was crushed into a powder and applied in a flow-through spectrofluorimeter to monitor the herbicide isoproturon in spiked sea water and potato extracts. The method was found to be effective, rapid, and more sensitive than HPLC; matrix effects were minimized, the overall analysis was simple, and the immunosensor could be used for up to two months without changes in sensitivity. It is interesting to note that this assay is the only sol–gel based IA that was developed for environmental monitoring of real samples, highlighting the fact that although IAs are currently quite widely employed in environmental, forensic, and food-exposure studies [18–24], introduction and implementation of sol-gel based IAs to these fields is somewhat slow and lags behind that in the pharmaceutical and biomedical fields. This is very well reflected in view of the fact that, out of all the sol–gel IAs that have been developed, only two (the above study and the POC-IA on TNT [25]) are for environmental diagnostics. One possible explanation may lie in the need to develop Abs to diverse and sometimes lipophylic analytes that is not always a straightforward matter and to monitor diverse and complex matrices such as sediments, fatty foods, and crude acetonic extracts of fruits and vegetables, etc. that are much more complicated than body fluids.

A flow-injection IA was also introduced by Yang et al. [33] who used anti-gentamicin in a silicon sol–gel based matrix that contained PEG in the form of a slurry column. The IA was based on the competition between gentamicin and fluorescence-labeled gentamicin that competed for the

entrapped Ab. The method was found to be efficient, the columns could be reused many times, non-specific binding was low, and it was possible to determine analytes in patients' serum in amounts close to those obtained with the commonly used IAs. A further verification of sol–gel entrapped anti-gentamicin Abs was presented by the same group [30] where the Abs were entrapped in magnetite-containing spherical nanoparticles and were used in a magnetic-separation IA system. The mechanically stable particles that are nanometer in size and magnetically separable were tested for their ability to bind fluorescent gentamicin in solution. The data revealed that the entrapped Abs retained their ability to bind gentamicin and that the magnetite-based IA was quantitative and behaved in a manner similar to that of IAs that used Abs in solution. The assay was also employed to analyze the recovery of spiked serum samples, and the method showed satisfactory recovery and reproducibility, thus indicating the high potential for the use of magnetite-containing spherical silica nanoparticles as improved biosensor devices in a micro-scale fluid system or in vivo for biomedical monitoring (e.g., drug release, drug metabolism, intake, etc.).

A very interesting application of the sol–gel method was reported by Grant and Glass [31] who entrapped fluorescent anti-D dimer Abs (a fibrin product resulting from the degradation of fibrin clots) at the tip of an optical fiber and used the device for monitoring the D-dimer analyte in spiked human plasma and whole blood. The study demonstrated the feasibility of application of the method in spiked PBS, human plasma, and whole blood. The sensor showed clinically relevant sensitivity, low leachability, some regenerability, and stability for about a month. Although several features (such as lifetime and regenerability) still need to be improved, the method offers an intriguing diagnostic application in monitoring thrombolysis in stroke patients undergoing thrombolytic therapy and the possibility of additional new intracatheter applications.

The sol–gel method was also used for the development of IAs and immunosensors where the Abs were not encapsulated in the matrix itself but rather adsorbed or covalently bound to the solid-phase surface. Although this topic is beyond the scope of this review, a few examples are listed. In two studies [43,44], the sol–gel method was employed to form a novel potentiometric immunosensor that comprised a two-dimensional sol–gel layer onto which nanoparticles and Abs (anti-diphtheria and anti-cortical hormone Abs) were adsorbed. In another study [45], a continuous-flow displacement immunosensor was formed from anti-RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) Abs covalently immobilized on TEOS. In other studies, a hybrid inorganic-organic composite based on polyvinyl alcohol and polysiloxane was used to covalently immobilize Ags obtained from the bacterium Yersinia *pestis* [46] and anti-2,4-D monoclonal Abs (MAbs) adsorbed via anti-mouse IgG onto a sol–gel treated glass capillary surface was used in a competitive chemiluminescent reaction to develop a highly sensitive IA for the detection of the herbicide [47].

15.2.2.2 Sol–Gel Entrapped Ag Immunoassays (Ag Format)

The first study on the development of an IA of the Ag format was reported by Livage et al. in 1996 [16,38] (Table 15.2). An Echinococcus granulosus cyst fluid Ag was entrapped in a sol–gel matrix, cast in a 96-well microplate, and used for the detection of anti-Echinococcus granulosus Abs in sera of infected patients. The study proved the ability of the assay to detect relatively large molecules (Abs) with a sensitivity that did not significantly differ from that of the classical ELISA.

In another study [16], whole protozoa cells (Leishmania) were entrapped in a sol–gel matrix in a microplate format and were used to detect *Leishamnia* Abs in infected patients. The study revealed that the entrapment of whole-cell organisms within sol–gel matrices did not destroy their cellular organization and that the gelation process could be controled to ensure that the pores were large enough to accommodate a whole cell and to allow diffusion of large molecules such as IgGs in a manner that enabled detection of Abs in infected human patients.

A similar study was performed by Coelho et al. [37] with a Toxocara *canis* Ag entrapped in a hybrid sol–gel composite consisting of TEOS and polyvinyl alcohol (that enabled the Ag to be retained more strongly in the matrix by its covalent binding via glutaraldehyde). Detection of

Toxocara canis Abs in unknown samples was determined with a secondary anti-human IgG conjugated to HRP by means of a chromogenic substrate. The study revealed that the assay could detect Abs in infected patients and that the sensitivity of the assay was significantly better than that of conventional ELISA.

The first report on the formation of a disposable thick-film electrochemical immunosensor based on sol–gel technology was published by Wang et al. in 1998 [40]. The group entrapped an Ag (rabbit IgG) that competed with an analyte in solution for binding of Abs (anti-rabbit IgG) conjugated to a reporting enzyme. The study demonstrated that the Abs could readily access the embedded Ag despite the thick gel film, the immobilized Ag could still be recognized by the Ab, the sol–gel based immunoelectrode could be used in a competitive assay to detect Abs in solution, and the measurement range and detection limits of these electrodes compared favorably with those of other electrochemical immunosensors and of ELISAs.

Another example of a thick-film electrochemical electrode where the Ag was entrapped in a sol–gel matrix is described by Du et al. [41]. An electrochemical graphite electrode was developed, onto which a carbohydrate Ag (CA) 19-9 (a carbohydrate tumor marker) was immobilized with titanium sol–gel membranes. Detection of CA19-9 in unknown samples was determined on the basis of competition between the free and the entrapped Ags on an HRP-labeled CA19-9 Ab present in the sample solution. The immunosensor demonstrated good accuracy and acceptable selectivity, sensitivity, reproducibility, storage stability, and precision.

Another example of an electrochemical electrode is described by Zhong and Liu [42]. An electrochemical biosensor based on sol–gel was used to entrap an Ag of a human parasite, *Schistosoma japonicum*, for the detection of anti-Schistosoma *japonicum* Abs. Detection of Abs in unknown samples was determined on the basis of competition between an HRP-labeled anti-*Schistosoma japonicum* Ab and the Ab present in the sample solution. As in the entrapped Ab IA, the sensor exhibited excellent physical and electrochemical stability with a renewable external surface, low background, a wide range of working potentials, and a relatively long lifetime. The method also proved successful in providing a useful sensing device for directly monitoring the concentration of *Schistosoma japonicum* Abs in serum samples.

Sol–gel entrapped peptides were also used to make peptide arrays for highly sensitive and specific Ab-binding, high-throughput screening (HTS) IAs. Currently, the miniaturization of biological assays is gaining a lot of attention for advances in biological and medical research. The design of miniaturized devices for highly sensitive, specific, and simultaneous detection of multiple Abs from complex biological samples is of high importance. Over the past decade, the trends toward the development of miniaturized HTS assays have generated a great deal of interest in the scientific community. Microarrays have been employed for a variety of applications, especially in genomic studies, but their application to diagnostics has not yet been fully explored. In a study by Melnyk et al. [39], two hepatitis C virus (HCV)-derived peptides and an Epstein-Barr virus (EBV)-derived peptide were printed on a semicarbazide sol–gel layer for the detection of HCV and EBV Abs in human sera, and the amount of serum Ab was determined with a fluorescent secondary anti-human Ab. The assay displayed very high sensitivities and specificities for Abs directed toward several peptidic epitopes; it enabled detection in very small blood samples from infected individuals, and a comparison with standard ELISA demonstrated large gains in sensitivity and specificity.

15.2.3 Immunoaffinity Chromatography—General Aspects

Immunoaffinity chromatography is one of the most powerful techniques for single-step isolation and purification of individual compounds or classes of compounds from liquid matrices. IAC is based on the highly selective interaction between Abs and their Ags. Because of the high affinity and high selectivity of the Ag–Ab interaction, the method provides a high degree of molecular selectivity. IAC involves three steps: (a) preparation of the Ab matrix, followed by packing the

sorbent as a column where the adsorption/desorption chromatography will be carried out; (b) binding the Ag to the Ab matrix; and (c) elution of the Ag. In the first step, Abs are immobilized onto a solid-phase matrix. After the preparation of the Abs-containing matrix, the Ag is bound and the contaminating macromolecules are removed by washing. In the last step, the Ab–Ag interaction is dissociated, and the Ag is released into the eluate. IAC can be performed off-line or on-line, and it can be coupled to a wide variety of separation techniques and analytical methods such as liquid chromatography (LC), gas chromatography (GC), and immunochemical analysis (e.g., ELISA).

IAC has been widely applied for over four decades for pharmaceutical and biomedical trace analysis and, in the more recent decades, for analysis of environmental contaminants and pesticide residues in occupational and environmental health monitoring, in forensic examinations, and in food safety analysis. The varied and complex matrices that serve as sources for analyte monitoring, the low concentrations of the analytes within the matrix, and the presence of compounds that interfere with the analytical method raised the need for a highly specific, quick, and cost-effective method of clean-up and concentration of the tested materials. This, together with the intensive research in the area of SPE that has led to the development of new formats and new sorbents and the drastic decrease in the use of traditional liquid–liquid extractions because of restrictions on some of the solvents (e.g., chlorinated organic compounds), has resulted in the emergence of IAC as a preferred method in trace analysis. To date, IAC has been successfully utilized for monitoring pesticides and other trace organics in environmental and food samples as well as for detecting drug metabolites and endogenous compounds in biological fluids in occupational exposure and clinical trials. Interestingly, the applications where the high potential of IAC for class-selective extractions, has been clearly shown, belong to the environmental field of analysis (see below item Section 15.2.4.1). The basic principles of IAC approaches, the new developments in this field, and their applications in clinical and environmental analysis have recently been reviewed in detail [21,22,48–51].

Successful employment of IAC requires special supports for immobilization of Abs. The supports must be (a) porous—to allow Ag penetration and to provide high-capacity support; (b) chemically and biologically inert—to minimize non-specific adsorption; (c) stable—to allow the use of denaturing reagents in the elution process; and (d) easily activated. To date, the usual approach to the production of bioaffinity chromatography devices has been based on covalent or affinity coupling of Abs (via streptavidin, protein A, or protein *G)* to solid supports, and the supports traditionally used in IAC include agarose, silica, cellulose, and synthetic polymers. This approach suffers from limitations such as loss of Ab activity upon coupling (because of poor control over protein orientation and conformation), low surface loading, potentially low mechanical stability (that prevents the on-line coupling of IAC columns with separation methods), difficulties in the loading of beads into narrow columns, difficulties in miniaturizing to very narrow columns, poor flexibility with certain proteins, long preparation time, low regenerability, and—most importantly—high cost. The recent advances in the development of monolithic columns have not been widely adapted to IAC as the method involves harsh chemical processes that are not compatible with biomolecules. The successful application of the sol–gel doping methodology to a wide variety of biomolecules, the ability to tailor the porosity of the sol–gel matrix, as well as all of the other above-mentioned long list of potential advantages of the sol–gel technique have stimulated the extension of the sol–gel studies to IAC applications for clinical, environmental, forensic, and food safety residue monitoring. A summary of the sol–gel based IAC applications of the past decade is presented below and in Table 15.3.

15.2.4 Sol–Gel Based Immunoaffinity Chromatography

Although entrapment of Abs in sol–gels has been widely studied (see above), only a few IAC applications (less than 20) have been reported in the literature, and even fewer studies have been

TABLE 15.3
Sol–Gel Entrapped Antibodies Applied for Immunoaffinity Chromatography

Entrapped Antibody	Immobilization Matrix	Sol-Gel Form	Method of Detection	Application	Tested Parameters	References
Anti-dinitro-benzene (environmental-contaminant)	TMOS TMOSPEG	Slurry column	ELISA (colorimetric assay)	IAC-POC	Proof of binding, non-specific binding. comparison of binding capacity of whole serum vs. IgGs, optimization of sol–gel formats and additives, binding capacity, elution, reproducibility. leaching, comparison with other IAC methods	[52,58]
Anti-atrazine-(herbicide)	TMOSPEG	Slurry column	ELISA (colorimetric assay)	IAC-POC	Binding, dose response, optimization of sol gel formats, capacity, elution, leaching, stability in storage, reproducibility	[54,57]
Anti-TNT	TMOS/PEG	Slurry column	ELISA (colorimetric assay)	IAC-POC	Binding, non-specific binding, dose response, comparison with other Ag–Ab binding assays (i.e., immunoprecipitation). leaching, stability, tolerance toward organic solvents, elution	[59]
Anti-1-nitro-pyrene-(environmental contaminant)	TMOS	Slurry column	ELISA or HPLC-(fluorescence detector)	IAC-POC	hoof of binding, non-specific binding, capacity, leaching, elution. reusability, aging and storage	[53]
Anti-pyrene (environmental contaminant)	TMOS	Slurry column	ELISA or HPLC (fluorescence detector)	IAC-POC	Proof of binding. non-specific binding, effects of surfactants, PEG and high-MW blockers on non-specific binding, capacity. cross reactivity, leaching, elution, reusability, aging and storage	[55,56]

(continued)

TABLE 15.3 *(Continued)*

Entrapped Antibody	Immobilization Matrix	Sol–Gel Form	Method of Detection	Application	Tested Parameters	References
Anti-2.4-D (herbicide)	TEOS	Slurry column	HPLC (UV detector)	IAC-POC	Binding capacity of wet gels vs. semi-dry gels and xerogels, flow rates, physical properties of doped vs. non doped gels; optimization of elution conditions, reusability, comparison with Ab–Ag binding in solution	[60]
Anti-1-nitro-pyrene (environmental contaminant)	TMOS	Slurry column	HPLC (fluorescence detector)	IAC	Mechanical stability, capacity, reusability, reproducibility, sample preparation compatible with sol–gel IAC, matrix interference and analyte recovery from spiked herb samples	[63]
Anti-pyrene (environmental contaminant)	TMOS	Slurry column inserted into an outdoor sample (rainwater) collector	HPLC (UV and fluorescence detector)	IAC	Binding capacity, effect of pH on binding. stability in storage at high temperatures, cross reactivity, analyte recovery from rain water	[62]
Anti-pyrene (environmental contaminant)	TMOS	Slurry column coupled on line to HPLC	HPLC (fluorescence detector)	IAC	Pressure stability of column, kinetics of Ag–Ab binding, column capacity, elution conditions, reusability, analysis of river samples	[64]
Anti-pyrene (environmental contaminant)	TMOS	Slurry column	HPLC (fluorescence detector)	IAC	Binding capacity, leaching, elution, specificity, matrix interference, comparison of sol–gel IAC with SPE, reusability, recovery from spiked and real urine samples	[65]
Anti-*s*-triazine(herbicide)	TMOS/ glycerol	Slurry column	GC (NPD detector)	IAC	Ab immobilization efficiency, leaching, binding capacity, selectivity, non-specific binding, binding and elution conditions, reusability. recovery from spiked water and real soil samples, comparison with other SPE methods	[66]
Anti-bioallethrin-(insecticide)	TMOS/PEG	Slurry column	ELISA (colorimetric assay)	1AC	Determination of the analyte in spiked fruit and vegetable extracts	[69]

Anti-bisphenol A(environmental contaminant)	TMOS	Slurry column	HPLC (fluorescence detector)	IAC	Loading and elution conditions, binding capacity, recovery, regeneration. cross reactivity, determination of analyte in spiked and real samples (canned beverages and food)	[68]
Anti-morphine Anti-M3G Anti-M6G	TMOS	Slurry column	HPLC (fluorescence detector and laser induced fluorescence)	IAC	Comparison of sol–gel IAC with SPE extraction, capacity, recovery, determination of analytes in real blood samples	[67]
Anti-tumor IgG	TMOSIPEGI PVP/3-aminopropyl-trimethoxy-silane	Columns composed of glass fiber coated sol–gel membranes	HPLC (UV detector)	IAC	Non-specific binding, leaching, capacity, determination of tumor-associated antigens from patients' sera	[61]

Experiments are divided into two groups: (A) experiments that proved the concept (POC) that sol–gel entrapped Abs can be used as immunoaffinity chromatography (IAC) devices (such assays are termed IAC-POC and involve studies in which the Abs were used to purify standard analytes); (B) Experiments where the entrapped Abs were used to monitor analytes from spiked or real samples (such assays are termed IAC). Abbreviations: GC: Gas chromatography; HPLC: High pressure liquid chromatography; IgG: immunoglobulin; M3G: anti-morphine-3-β-D-glucuronide; M6G: anti-morphine-6-β-D-glucuronide; NPD: Nitrogen phosphorus detector; PEG: Polyethylene glycol; PVP: polyvinylpyrrolidone; SPE: solid phase extraction; TEOS: Tetraethylorthosilicate; TMOS: Tetramethoxysilane; TNT: 2.4.6-trinitrotoluene.

reported that describe the use of sol–gel based IAC for group-selective enrichment and recovery of small analytes from real samples (Table 15.3). As with sol–gel based IAs, some of the studies were designed as IAC-POC experiments, i.e., studies where the ability to use sol–gel entrapped Abs for IAC has been proven by using standard analytes [52–60]; other studies [61–69] employed the devices for clean-up and concentration of real samples. Most of the IAC experiments differed from those of the sol–gel based IAs described above by being focused on environmental analytes; only two [61,67] were concerned with compounds of clinical interest.

Several approaches have been used so far in the development of sol–gel based IAC devices. Most of the studies used crushed Ab-doped silica monoliths that were loaded into a column in the form of a slurry. Only one study [61] used a different format where glass fibers covered with Ab-doped sol–gel, as a support, were used for the affinity separation. Almost all studies used silica based composites (TEOS or TMOS) with or without PEG or glycine as the entrapping matrix; one study [61] used a mixture of TMOS, PEG, 3-aminopropyl trimethoxysilane, and polyvinylpyrrolidone (PVP). Almost all the columns were used as off-line devices. Only one study [64] used a slurry IAC column coupled to on-line HPLC via a reverse-phase pre-column. Eluted analytes from the on-line and off-line columns were monitored by ELISA, HPLC, or GC. A general scheme of a sol–gel based IAC process is depicted in Figure 15.3.

The development of a sol–gel based robust analytical device for IAC relies on having the entrapped Ab mimic or even be enhanced relative to its behavior in solution (with respect to its activity or stability to denaturation). In order to achieve this goal, it is important to optimize entrapping conditions, check binding, capacity and, most important, to compare the activity of the entrapped Abs with those in solution and with other IAC methods. These topics were indeed the main objective of most of the IAC-POC studies as indicated below. A quick overview of the main issues that were addressed by these studies reveals that they focused on: (a) optimization of gel formats and additives (wet gel, xerogels, presence of PEG, etc.) and mechanical stability of gel; (b)

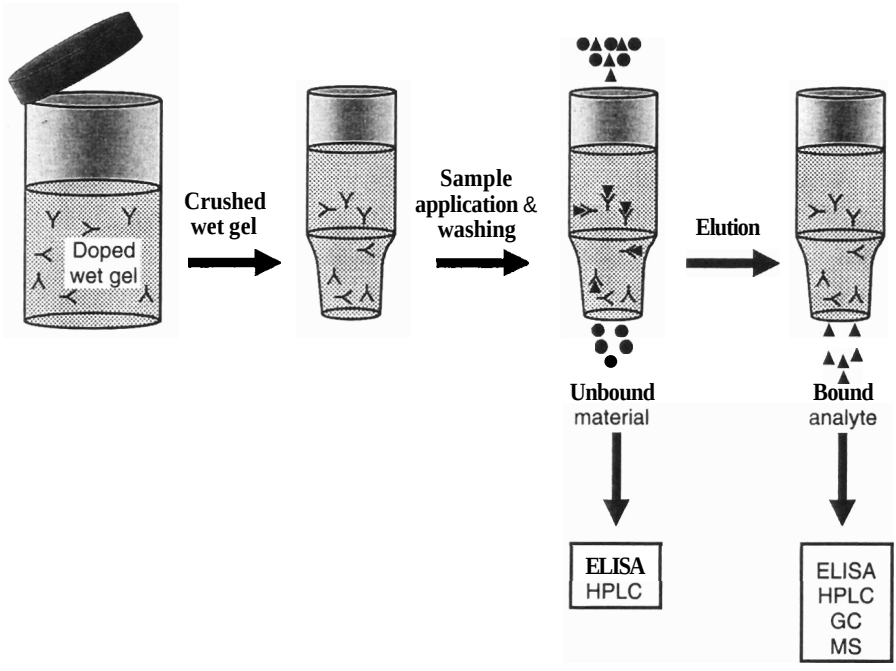

FIGURE 15.3 General scheme of sol–gel based IAC process. ELISA: Enzyme linked immunosorbent assay; GC: Gas chromatography; HPLC: High pressure liquid chromatography; MS: Mass spectrometry.

proof of binding; (c) optimal loading conditions; (d) non-specific binding and effect of surfactants on its reduction; (e) binding capacity; (f) cross reactivity/specificity (mainly in comparison with ELISA); (g) tolerance of entrapped Abs toward organic solvents; (h) elution conditions; (i) recovery; (j) leaching; (k) stability during storage and aging; (l) reproducibility; (m) reusability; and (n) comparison with other IAC methods and SPE. The applied studies extended their focus beyond the above and examined the ability of the IAC columns to clean up and concentrate analytes from complex food, environmental, and clinical samples. All of the studies proved, without doubt, that sol–gel entrapped Abs could serve as highly efficient, reproducible, stable, and reusable IAC devices as detailed below.

15.2.4.1 Sol–Gel Immunoaffinity Chromatography for Environmental, Forensic, and Occupational Monitoring

The first use of sol–gel entrapped Abs for IAC purposes was reported over a decade ago in 1994 by the group of Altstein et al. [52,58] who entrapped anti-dinitrobenzene (DNB) IgGs in a TMOS based sol–gel matrix. The study revealed that the entrapped Abs, in the form of a slurry column, retain their ability to bind analytes from solution. That study was followed by entrapment of anti-DNB antiserum in a TMOS/PEG sol–gel matrix [58], entrapment of anti-atrazine monoclonal Abs (MAbs) [54,57], and later of anti-trinitrotoluene (TNT) MAbs [59] and anti-pyrethroid MAbs [69] with the latter study focusing on employment of the sol–gel columns for IAC of the pyrethroid bioallethrin from food (crude acetonic extracts of tomatoes, cucumbers, and strawberries). Regardless of the entrapped Ab or tested analyte, the above studies revealed that the hydrophilic wet gel (in the form of a slurry column) with a TMOS:aqueous ratio of 1:8, enriched with 10% PEG, was the preferred working format, the entrapped Abs retained their ability to bind analytes from solution in a dose-dependent manner within a time frame that did not greatly differ from that in solution, the analytes could be eluted at high recoveries (86%–100%) with either organic solvents (ethanol, acetone, acetonitrile) or highly basic or acidic buffers, and the analytes did not adhere non-specifically to the matrix. The studies also revealed that the Abs did not leach from the column even under extreme elution conditions, the column could be stored for several months without losing activity, and the assays were highly reproducible. The assays were equally effective with either polyclonal Abs (PAbs) or protein A-purified IgGs (that eliminated the need to purify IgGs from the whole antiserum), and they could be carried out with PAbs, MAbs, or even hybridoma culture fluid (without the need to purify the MAbs from the tissue culture medium). Interestingly, the sol–gel IAC columns exhibited binding capacities that were either significantly higher than or equal to those obtained with protein A-agarose coupled Abs.

Almost in parallel to the above studies, Zhulke et al. [53] reported in 1995 the successful entrapment of anti-1-nitropyrene Abs in a TMOS-based sol–gel matrix. The study addressed similar questions to those listed above and further proved the feasibility of the concept, namely, that sol–gel entrapped Abs could serve as successful IAC devices. Zhulke's study was followed by additional reports from the same group who examined the properties of sol–gel entrapped anti-pyrene Abs [55,56]. These studies revealed, once again, that entrapped Abs bound analytes, that the Abs did not leach from the matrix, the columns could be regenerated and reused, aging did not affect the binding properties, and the method was highly efficient and yielded excellent analyte recoveries. Unlike the analytes that were tested by Altstein et al. (e.g., [57,59]) that did not adsorb non-specifically to the matrix, members of the polycyclic aromatic hydrocarbons (e.g., pyrene and related compounds [56]) did adsorb to the matrix and non-specific binding to the columns slowly increased during storage. A variety of approaches (e.g., modification of the sol–gel composite with PEG, addition of nonionic surfactants, or combination of a high-molecular-weight blocking agent with a surfactant) were found to markedly reduce non-specific adsorption and reduce cross reactivity [56].

From the late 1990s, the POC-IAC reports showed increasing attention to applied studies where the method was employed for monitoring real environmental, occupational, and food samples.

Several such reports were published, most of them from the group of Niessner and coworkers [62–66,681. Application of the sol–gel based IAC method for recovery of analytes from real samples included entrapment of the following Abs: (a) anti-*s*-triazine for detection of triazine herbicides in water and soil samples [66]; (b) anti-1-nitropyrene for detection of the analyte from herb samples [63]; (c) anti-bisphenol A for clean-up of the analyte from canned beverages, fruits, and vegetables [68]; and (d) anti-pyrene for detection of polycyclic aromatic hydrocarbons (e.g., pyrene) and their metabolites in spiked and real urine [65], river water [64], and rain water [62] samples. The study described by Scharnweber, et al. [62] employed a device that was used outdoors and was intended to calculate the penetration of chemical compounds deposited from the atmosphere into the soil. Beyond the successful application of monitoring analytes from real samples, the study demonstrated the ability of the IAC device to withstand the harsh conditions that are met outdoors in on-site field experiments. All of the above studies revealed that the columns could effectively cleanup analytes from complex, spiked samples, and they could significantly reduce the matrix interference that could affect downstream chemical or immunochemical analyses.

Although most of the above studies confirmed what had already been reported and proven with regard to the successful entrapment of Abs in sol–gel matrices, several of them revealed drawbacks and problems with the method. For example, in the study of Braunrath and Cichna [68], the cross-reactivity pattern of the IAC columns was significantly higher than that obtained in ELISA. In another study [70], a few compounds that could be detected by Abs in solution could not interact with the sol–gel entrapped Abs. Furthermore, in a study by Cichna et al. [64], it was shown that the selectivity of the sol–gel IAC column was comparable with that of a conventional reverse-phase (RP-8) column, i.e., the sol–gel IAC approach did not show any advantage over the RP-8. In most cases, however, sol–gel IAC resulted in higher analyte recovery compared with that obtained with other SPE methods (e.g., [65–67]). Also, in some studies, the IAC column did not remove all interfering matrix components. This was obvious, especially when the samples were monitored by standard chemical analytical methods (rather than ELISA or electrochemical methods), and there was a need for further optimization of the purification procedure [63]. In some studies, the binding capacity of the entrapped Ab was lower than that obtained in solution (e.g., [66]), but in most cases, it was high enough'to retain the analyte on the IAC support for further analysis.

15.2.4.2 Sol–Gel-Based Immunoaffinity Chromatography for Clinical Monitoring

Although immunological assays and IAC have been in regular use for many years in pharmaceutical, biomedical, and clinical research, sol–gel based IAC has not been widely implemented, and the method has been employed in only two cases: for monitoring morphine and its phase II metabolites in blood samples taken from heroin victims and heroin consumers [67] and for monitoring anti-tumor IgGs from patient sera [61]. In the first study, anti-morphine, anti-morphine-3-β-D-glucuronide (M3G), and anti-morphine-6-P-D-glucuronide (M6G) Abs were entrapped in TMOS and were used in combination with laser-induced fluorescence coupled to HPLC to monitor the above compounds in blood samples. The method enabled the detection of low analyte concentrations, and it could be applied without interference to complex matrices such as post-mortem blood samples.

The second study, by Zusman and Zusman [61], introduced a new IAC format, i.e., gel fiberglass membranes. The membranes were comprised of glass fibers covered with oxysilane to provide a sol–gel-glass matrix. A thin-layer of Abs trapped in the gel glass during its preparation was deposited on the surface of a glass lattice to form a sol–gel membrane with the entrapped Abs. The gel-fiberglass (GFG) membranes were used to form GFG columns that were assembled from a series of 20–30 membranes. The columns, containing anti-tumor IgGs, were used to isolate a variety of proteins. The IAC application involved the isolation of tumor-associated Ags from sera of cancer patients. As in all other cases, the sol–gel entrapped Abs were very stable and could be stored for several months at room temperature, and the columns were found to be highly effective in

enabling the isolation of large quantities of proteins, mainly as a result of the large active area of the membranes.

15.3 FUTURE PROSPECTS AND CONCLUDING REMARKS

As described above, sol–gel derived biocomposites offer a series of significant advantages over other immobilization matrices or methods for the development of advanced analytical devices. Sol–gel derived materials can be based on a wide range of compositions and can be used to entrap a large number of different biomolecules. No other immobilization method provides such a generic and flexible approach where both the nature of the matrix and its interactions with the entrapped molecule can be so well controlled. The physical and chemical properties of the sol–gel, its amenability to modifications of the properties of the composite, the high biomolecule content that can be loaded onto the gel, and the improved properties of the entrapped biomolecules (e.g., high stability) present a unique combination with an immense application potential. The ability to use sol–gel biocomposites for the applications discussed above has emerged from a huge number of studies in this field, most of which were performed in the past 10–15 years. Overall, these studied have yielded useful insights into the fundamental factors that control the behavior of entrapped biomolecules and have thereby provided guidance in the development of improved materials and processing methods that enable the activity of entrapped Abs to be maintained for the above-mentioned applications.

Although sol–gel derived biocomposites have been shown to be useful in many analytical applications, some issues are still unresolved and need to be further studied. For example, material properties still need to be improved to reduce cracking, age-linked shrinkage, pore collapse, and phase separation. There is still ample space for significant improvement in the physical and chemical parameters of precursors and reaction conditions (e.g., nature of precursors and of additives hydrolysis ratio, nature and presence of solvents, condensation kinetics, etc.), and there is a need to improve organic–inorganic composite materials to gain a better bioactivity of the entrapped molecules. There is a need for better understanding of the protein (or any other entrapped molecule)-silica and analyte-silica interactions (caused by electrostatic, hydrogen, or hydrophobic interactions). Studies clearly indicate that polymer-protein interactions can be used advantageously to maximize the stability and function of some proteins. However, at present, there is very limited information available on the mechanisms by which other proteins may be stabilized. Studies along these lines should be extended, aiming at the improvement of bioactivity and stability of the entrapped molecules. Such interactions are also disadvantageous because they cause analytes to adsorb non-specifically to the matrix. The nature of such interactions should be further studied in order to find simple ways to minimize or overcome the problem. Furthermore, there is still a need to be able to optimize the physical properties of entrapped sol–gels (with respect to size, shape, pore size, etc.) so as to achieve better performance (e.g., faster diffusion of analyte) without losing activity, especially in the case of on-line biosensors. In addition, it is necessary to find ways to scale up the entrapment process and, most important, to find entrapment conditions under which the biomolecules will be compatible with prolonged use.

Some aspects of these needs are currently being addressed. Measures being examined to improve the bioactivity of entrapped molecules include changes in the physical and chemical parameters of precursors and reaction conditions (e.g., the nature of the precursor and of additives, hydrolysis ratio, the presence or absence of various solvents, condensation kinetics, etc.) and the use of different combinations of these factors and an introduction of improved organic–inorganic composites. Development of novel advanced materials is also underway. Adoption of combinatorial approaches in combination with high-throughput material characterization may enable the discovery of optimal biocomposites in a rapid screening process to select the most suitable matrices for a given application. Integration of molecular imprinting methods, controlled pore architectures,

micro fabrication, nanotechnology and other novel methods (for review see [4]) may also lead to the development of new sol–gel based tools applicable for IAC, immunosensing, and a wide variety of other applications.

In the current review, we focused on two applications of sol–gel entrapped biomolecules: solid phase IAs and IAC. The entrapment of Abs in a sol–gel matrix in a simple one-step procedure that maintains their binding capacity, enhances their stability, enables dissociation of the analyte from the Ab at high recoveries, and allows no leaching, offers many advantages and opens the way for the development of highly selective biosensors/immunosensors for applications in immunochemical detection methods and in IAC. It is envisioned that the next decade will see much faster progress in sol–gel applications in the above and many other fields of research, and this will lead to the emergence of new and improved devices that will integrate the above-mentioned new approaches and will help to facilitate simple, highly reproducible, environmentally friendly, and cost-effective environmental, forensic, agricultural, and clinical monitoring.

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