Biofriendly Sol–Gel Processing for the Entrapment of Soluble and Membrane-Bound Proteins: Toward Novel Solid-Phase Assays for High-Throughput Screening

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ABSTRACT

The last decade has seen a revolution in the area of sol–gel-derived biomaterials since the demonstration that these materials can be used to encapsulate biological species such as enzymes, antibodies, and other proteins in a functional state. In particular, recent years have seen tremendous progress in the development of more "protein-friendly" sol–gel processing methods and their use for immobilization of delicate proteins, including key drug targets such as kinases and membrane-bound receptors. The latter example is particularly impressive, given the inherently low stability of membrane receptors and the need to stabilize an amphiphilic bilayer lipid membrane to maintain receptor function. In this Account, we provide an overview of the advances in biofriendly sol–gel processing methods developed in our research group and others and highlight recent accomplishments in the immobilization of both soluble and membrane-bound proteins, with particular emphasis on enzymes and membrane receptors that are drug targets. Emerging applications of sol–gel-entrapped proteins, focusing on the development platforms for high-throughput screening of small molecules, are also described.

Introduction

Technological advancements in the immobilization of biological macromolecules over several decades have resulted in a revolution in the use of biomolecules for the selective extraction, delivery, separation, conversion, and detection of a wide range of chemical and biochemical reagents. The use of biological species such as proteins, peptides, nucleic acids, and even whole cells in these applications relies largely on their successful immobilization in a physiologically active form. Traditional methods for immobilizing biomolecules onto inorganic, organic, or polymeric surfaces have been based on physical adsorption,[1] covalent binding to surfaces,[2] entrapment in semipermeable membranes,[3] and microencapsulation into polymer microspheres and hydrogels.[4,5]

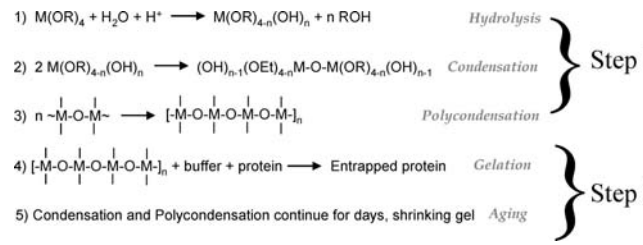

FIGURE 1. Two-step process for entrapping proteins within silica materials using metal alkoxide precursors. M = Si, Ti, or another suitable metal center. R = Me or Et.

A more recent advance in protein immobilization is entrapment of proteins in inorganic matrixes formed by the sol–gel method. The nanometer-scale pores allow small molecules to diffuse freely in and out of the matrix while retaining the entrapped protein. While the earliest reports of protein entrapment in sol–gel-derived glasses appeared in the 1950s,[6] it was not until Avnir and co-workers published a seminal paper in 1990, describing the entrapment of proteins in alkoxysilane-derived glasses, that the field began to bloom.[7] This report was followed by the work of Dunn's and Zink's groups in 1992, which demonstrated that other proteins, such as cytochrome *c* and myoglobin, could be entrapped in tetramethylorthosilicate (TMOS)-derived silicates with retention of O_2 binding ability.[8] The key advance that these groups demonstrated was the ability to process alkoxysilanes in aqueous solvents without added alcohol, instead using the alcohol liberated from the silane hydrolysis reaction to solubilize the silica precursor. In addition, a two-step processing method (Figure 1) was developed to separate the relatively harsh hydrolysis reaction, which uses highly acidic or basic pH conditions, from the condensation reaction, which could be performed at physiological pH values in the presence of the protein.

By 2000, a large body of work emerged describing: the entrapment of a wide variety of biological species, including enzymes, antibodies, regulatory proteins, transport proteins, etc., into an assortment of sol–gel-derived composite materials; the development of composite bio-doped materials containing organically modified silanes (ormosils) and polymers; fundamental studies of proteins within sol–gel materials; and the use of sol–gel-entrapped proteins for affinity chromatography, biosensing, bioreactors, and other applications.[9–14] However, issues related to the presence of alcohol liberated during hydrolysis and the need for large shifts in pH during processing still resulted in problems with the initial and long-term stability of entrapped proteins and prevented advances in key areas such as the entrapment of viable membrane-bound receptors.

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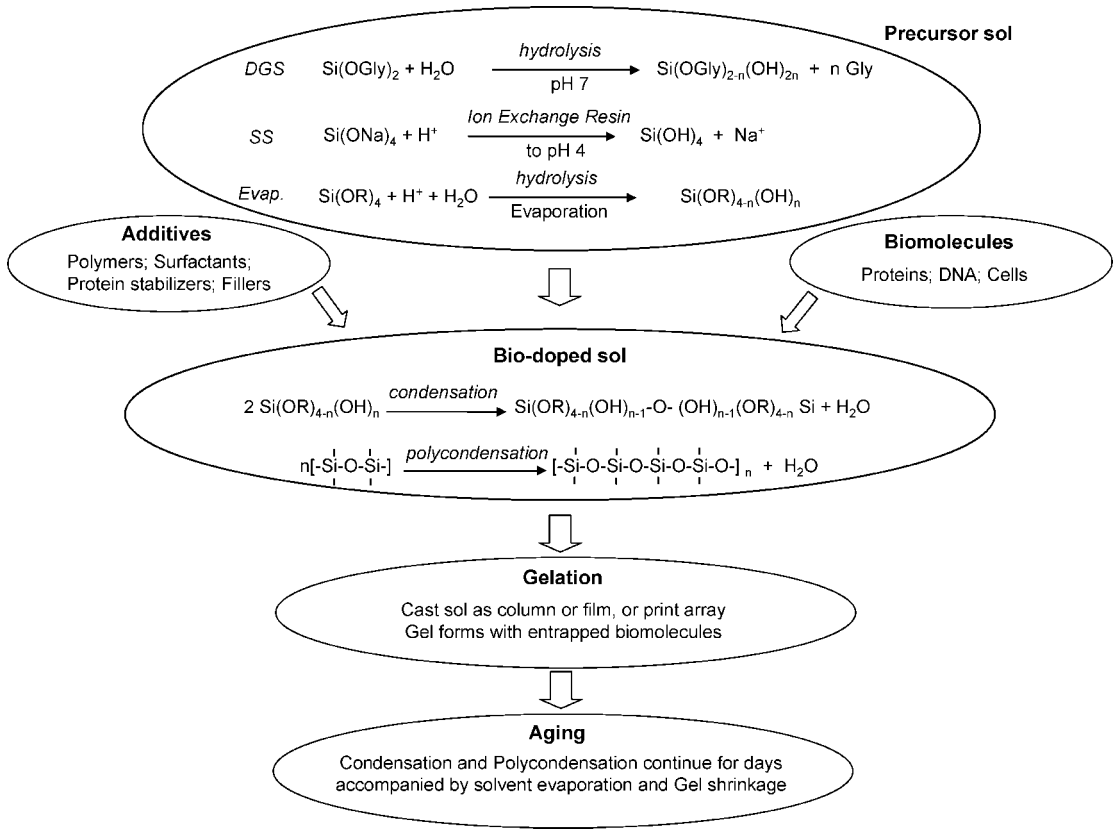

FIGURE 2. Schemes for fabrication of protein-friendly sol–gel-derived materials.

Since 2000, there have been a number of advances in the field, spurred in part by the development of new biocompatible precursors and processing methods, which have largely overcome the issues noted above. Such methods have ultimately allowed several new classes of biomolecules to be immobilized and used for a variety of new applications. In this Account, the work that led to the development of improved sol–gel precursors and processing conditions is described, as well as how these materials ultimately led to the successful immobilization of a range of delicate proteins, including membrane-bound receptors. A discussion of emerging uses of these materials for medically oriented applications is also provided, with emphasis on how the unique properties of sol–gel-derived materials can be exploited for bioassay development geared toward high-throughput screening of small molecule–protein interactions.

Biocompatible Sol–Gel Processing

During the 1990s, groups headed by Bright, Friedman, and Kostic, as well as our group, examined fundamental issues such as the thermodynamic stability, dynamics, accessibility, binding constants, and reaction kinetics for proteins entrapped within alkoxysilane-derived materials, as well as the effects of material aging on the evolution of protein properties.[9,15–18] Together, these studies revealed that while entrapped proteins often displayed improved thermal stability, the proteins also (1) exhibited hindered dynamics due to interactions of proteins with the anionic silica surfaces, (2) were often distributed between various environments, some of which were not accessible to external reagents, and (3) generally showed a decrease in function with time caused by the presence of relatively high levels of alcohol byproducts from the hydrolysis reaction and continued evolution and shrinkage of the sol–gel material with time.[19] While the addition of organically modified silanes (ormosils) and polymers could improve some of the properties of entrapped proteins, the materials were still not ideal for entrapment of labile proteins. This highlighted the need for more protein-compatible processing methods and more "protein-friendly" materials.

Early studies examined the addition of glycerol or other small molecule "osmolytes", such as sugars and amino acids, during sol–gel processing. These compounds alter protein hydration and were observed to increase entrapped protein stability.[20,21] However, such approaches do not address the presence of alcohol during processing, and furthermore, the stabilizing effect is easily removed since the additives can be washed out of the material. More recently, new biocompatible silane precursors and processing methods have been reported on the basis of glycerated silanes such as polyglycerylsilicate (PGS) or diglycerylsilane (DGS),[22–24] sodium silicate (SS),[25] or aqueous processing methods that involve the removal of alcohol byproducts by evaporation before addition of proteins[26] (see Figure 2). Each of these methods resulted in "alcohol-free" sol–gel processing and often produced significant improvements in the activity of entrapped proteins. Methods based on glycerated silanes also had

an advantage in that the solid precursors could be dissolved directly in water at physiological pH, removing the need for altering the pH during sol–gel processing. Furthermore, transesterification of a wide range of alkoxysilanes with glycerol[22,27] or ethylene glycol[28–30] is possible, which results in the ability to produce a range of biocomposites with different properties. However, the polyols that are present during fabrication of the material can be easily removed upon washing, and this can affect the long-term stability of the materials during repeated use.

Sugar-Modified Silica Materials

Given the fact that small molecules such as sugars can stabilize a wide range of proteins,[20,31] it was reasoned that covalent attachment of a sugar to the silica surface should provide a means for permanently altering the protein compatibility of silica materials. Silica precursors bearing a covalently attached glucose [gluconamidyltriethoxysilane (GLS)] or maltose [maltonamidyltriethoxysilane (MLS)] moiety were prepared and doped into DGS- or SS-derived materials. It was determined that such species could not leach from the material, as expected, but also that these species modified the cross-linking and charge within the silica matrix and retained water, which weakened the effects of aging (i.e., loss of solvent and shrinkage of the material).[32] DGS-derived materials containing 10–20 mol % GLS or MLS were used to entrap delicate proteins such as firefly luciferase or Src kinase[33,34] and provided an environment that retained the activity of the entrapped enzymes during repeated use over several weeks, even with multiple washing steps. The use of DGS alone or the addition of the sugar-modified silanes to alcohol evolving sols such as those derived from tetraethylorthosilicate (TEOS) did not provide good enzyme activity.[35,36] Detailed examination of the properties of the model protein human serum albumin within GLS-modified DGS materials revealed that several factors contributed to the improvement in protein stability, including the retention of the native protein conformation, higher accessibility to external analytes, better thermal stability, and an improvement in the ligand binding affinity.[35] It was also noted that the entrapped protein was significantly restricted in its global and segmental motions, suggesting preferential interactions of the protein with the sugar-coated surfaces. Together, these factors lead to significantly improved long-term stability for the entrapped protein and highlighted the potential of sugar-modified materials for entrapment of soluble proteins.

Overview of Membrane Protein Entrapment via the Sol–Gel Method

While soluble proteins such as enzymes, antibodies, and regulatory proteins have been utilized extensively for applications such as biosensor development, the desire to utilize sol–gel materials to develop solid-phase small molecule screening platforms for drug discovery required the extension of this technology to membrane-bound proteins.[37] Membrane receptors serve several key roles in transduction and amplification of signals across the cell membrane and allow cells to communicate with each other; thus, it is not surprising that they are desirable targets for the formulation of novel therapeutics.[38,39]

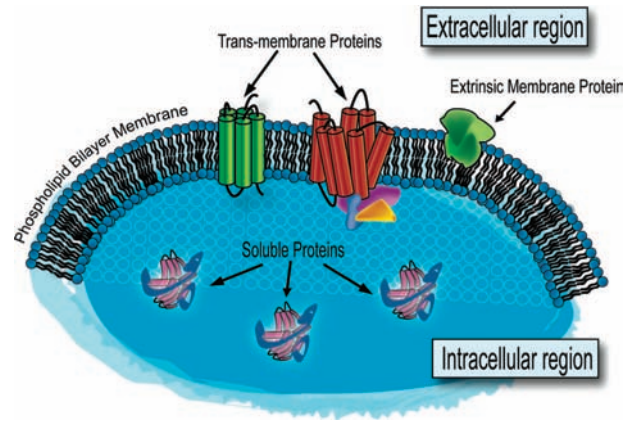

FIGURE 3. Different types of proteins that are utilized for sol–gel entrapment. Soluble proteins are located within the hydrophilic intracellular compartment of the cell. Intrinsic transmembrane proteins span the cellular phospholipid bilayer membrane, whereas extrinsic membrane proteins are partially embedded within the membrane and are exposed to either the intracellular or extracellular regions. [Reproduced from ref 37. Copyright 2006, with kind permission of Springer Science and Business Media]

Soluble proteins reside in an aqueous environment and thus are generally amenable to aqueous sol–gel processing methods for immobilization. Membrane-associated proteins, on the other hand, are either completely (intrinsic membrane proteins) or partially (extrinsic membrane proteins) embedded within an amphiphilic bilayer lipid membrane (BLM), as shown in Figure 3, and thus it is important to retain at least the essential bound lipids, and often the entire BLM, to keep the membrane-associated protein properly folded and functional. This makes such species particularly difficult to immobilize by conventional methods and highlights the need for protein-compatible immobilization methods.

Entrapment of Liposomes

The first report of immobilization of BLMs was in 1997, when Yamanaka et al. reported on the immobilization of pyrene-labeled liposomes in TEOS-derived silica,[40] and their use for optical sensing of heavy metal ions. This early work utilized conventional alcohol-based sol–gel processing, and it was not clear that the bilayers were entrapped as intact vesicles. A later study by Nguyen et al. in 1999, using a similar processing method, described the entrapment of dye-encapsulating liposomes in TMOS-derived silica to prevent leakage of the pH sensitive fluorescent dye carboxyfluorescein.[41] However, the study indicated that only 10% of the dye remained within the liposomes, confirming that a significant fraction of the liposomes ruptured during entrapment as a result of the alcohol byproduct.

In an effort to understand the nature of the entrapped liposomes, and to examine the effect of protein-friendly processing on liposomal properties, our group investigated

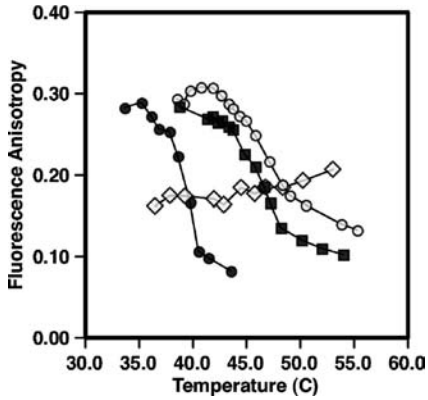

FIGURE 4. Comparison of phase transition behavior measured by the steady-state anisotropy of DPH in phospholipid vesicles composed of DPPC following entrapment in various sol–gel-derived materials: (●) solution, (■) sodium silicate, (○) DGS, and (◇) TEOS. Decreases in the fluorescence anisotropy indicate increased mobility of the probe within the membrane. [Reproduced from ref 42. Copyright 2002 American Chemical Society]

aspects such as the stability, dynamics, and phase transition behavior of entrapped vesicular BLMs in TEOS-, DGS-, and SS-derived materials.[42] Figure 4 shows the changes in the steady-state fluorescence anisotropy of the probe, diphenylhexatriene (DPH), embedded in unilamellar liposomes consisting of pure dipalmitoylphosphatidylcholine (DPPC) as a function of increasing temperature. It was observed that liposomes retained the ability to undergo gel-to-liquid phase transitions in DGS and SS materials but were not able to undergo this transition after entrapment in TEOS-derived materials because of vesicle rupture, providing key evidence for the benefits of biocompatible processing. These results were later corroborated by Bhattacharyya et al., who used time-resolved fluorescence methods to show that liposomes entrapped in TEOS-derived materials ruptured and adsorbed the silica surface[43] while liposomes entrapped in sodium silicate-derived materials remained intact upon entrapment.[44]

An important role for lipid membranes is the ability to allow translocation of ions from the exterior to the interior of the liposome in response to a specific perturbation. To demonstrate this process for entrapped liposomes, we used fluorescently doped liposomes containing gramicidin A (gA), a small peptide that spontaneously inserts itself into a lipid membrane causing passive influx of ions across and into a cell, as shown in Figure 5.[45] Upon addition of potassium ions, the fluorescence of the dye in the internal aqueous compartment changed in a manner that was dependent on the concentration of gA embedded in the membrane. This work showed that ion flux across entrapped membranes was possible, verifying that both an interior and exterior aqueous environment existed for the entrapped liposomes. As noted below, this demonstration led to more advanced studies wherein a proton pump was used to activate ATP synthesis from proteoliposomes entrapped in sol–gel materials, a first step toward light-harvesting biomaterials that can store light energy in chemical form.[46]

Entrapment of Photoactive Membrane Proteins

The first membrane-associated protein to be entrapped in silica materials was the small (26 kDa) photoactive proton pump bacteriorhodopsin (bR). This protein has seven membrane-spanning α-helices[47] that enclose an all-*trans*-retinal chromophore which undergoes an isomerization process upon light absorption, resulting in the translocation of a proton from the cytoplasmic side to the extracellular side of the membrane.[48] Several groups have reported on the entrapment of bR in alkoxysilane-derived silica materials and have characterized the structure of the entrapped protein using circular dichroism and Raman spectroscopy[49] and the photocycle intermediates of bR through time-resolved absorbance and fluorescence measurements.[49–51] Overall, these measurements showed that the kinetics governing the formation of photocycle intermediates in the entrapped bR were very close to those of bR in aqueous suspension; however, the decay of the intermediates was much slower for entrapped bR because of a reduced water content and a decreased level of proton transport through the dense silica framework.

Recently, Dunn, Montemagno, and colleagues used the sol–gel method to entrap bR that had been reconstituted into an intact phospholipid vesicle.[46] Through a combination of alcohol removal via evaporation and inclusion of the water soluble polymer poly(ethylene glycol), they were able to entrap bR such that it retained its proton pumping capability, as demonstrated using liposomes with a pH sensitive fluorophore in the internal aqueous compartment. They also showed that the photoinduced ion gradient could be coupled to the production of adenosine triphosphate using a second entrapped membrane protein, F_0F_1-ATP synthase. Hence, such materials could serve as biosolar cells or solar-based biofuel cells.

Very recently, O'Neill and Greenbaum reported on the immobilization of the large transmembrane Photosystem I (PSI) protein complex in glycerol-doped TMOS-derived silica.[52] Consistent with the results observed for liposomes and bR, PSI could be successfully immobilized using a low-alcohol route involving evaporated TMOS, although large amounts of glycerol were needed to optimize the immobilization of the active transmembrane protein complex. By following the absorbance of PSI upon stimulation with light, it was demonstrated that the intermolecular electron-transfer function was intact, and this was further evidenced by PSI-mediated hydrogen production.

Entrapment of Membrane Receptors

While several groups had demonstrated the entrapment of liposomes and photoactive proteins, the extension to membrane-bound receptors remained a challenge. In 2004, we reported on a range of mesoporous and macroporous materials that were able to entrap two ligand-binding receptors, the nicotinic acetylcholine receptor (*n*AChR) from *Torpedo californica* and the dopamine D2 receptor ($D2_{short}$, D2R), with retention of ligand binding ability.[53] A significant and unexpected finding was that low-alcohol and otherwise "protein-compatible" silica processing methods using DGS

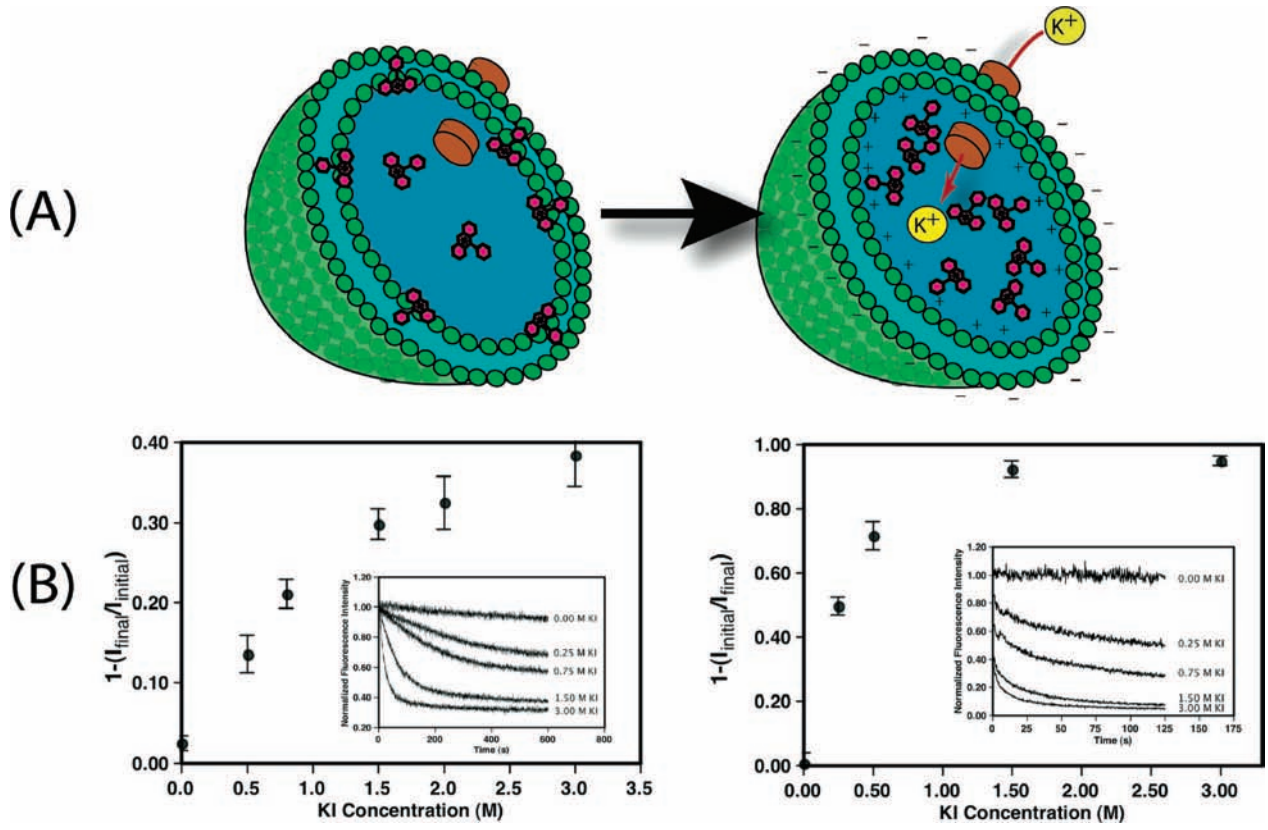

FIGURE 5. (A) Schematic of the assay used to monitor ion flux through the membrane-spanning peptide gramicidin A. Passage of monovalent cations through the gA ion channel induces membrane polarization that repels the positively charged dye, safranine O, from the membrane with a concurrent decrease in fluorescence intensity. (B) Changes in fluorescence intensity of safranine O as a function of potassium iodide concentration in solution (right) and following entrapment in DGS-derived silica (left). The insets show the time-dependent intensity response of safranine O at a given KI concentration. [Panel A reproduced from ref 37. Copyright 2006, with kind permission of Springer Science and Business Media. Panel B reproduced from ref 45. Copyright 2003 American Chemical Society]

and sodium silicate precursors did not maintain the activity of the entrapped receptors. In all cases, the water soluble polymer polyethylene glycol (PEG) was required as an additive to maintain the receptors in an active state (ca. 40–80% activity relative to solution). Low-molecular weight PEG (MW 600) could be used to produce optically clear mesoporous materials suitable for spectroscopic studies, while higher-molecular weight PEG (MW 10000) could be used to produce bimodal meso/macroporous materials with entrapped receptors that were amenable to the formation of monolithic chromatographic columns[54] and thus could serve as a platform for chromatographic screening methods. Most importantly, as shown in Figure 6, these experiments provided the first demonstration of ligand binding and extraction of IC_{50} values for some prototypical agonists to the entrapped receptors, which were in good agreement with those obtained in solution. This highlighted the potential of the entrapped receptors as a platform for high-throughput screening. Furthermore, both proteins retained significant activity upon storage, and in the case of *n*AChR, the receptor could be reused over several assay cycles. Even with the presence of large macropores, no receptor was observed to leach from the materials, indicating that the proteoliposome assembly was intact and sufficiently large to remain entrapped within macroporous materials.

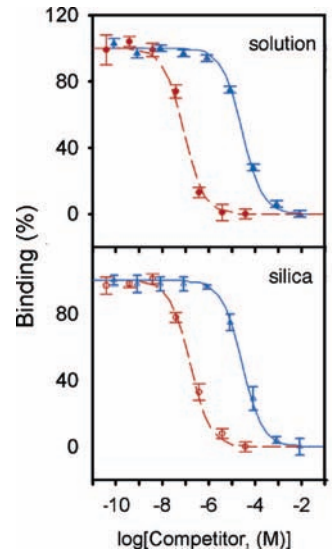

FIGURE 6. Binding isotherms generated for solution-phase and entrapped nicotinic acetylcholine receptor for epibatadine (red, dashed line) and nicotine (blue, solid line). Solution-phase experiments (top) were performed using standard radioligand ultrafiltration measurements. Measurements for entrapped receptors were performed via radioligand equilibrium dialysis, where the silica matrix served as a pseudodialysis membrane for ligand partitioning. [Reproduced from ref 53. Copyright 2004 American Chemical Society]

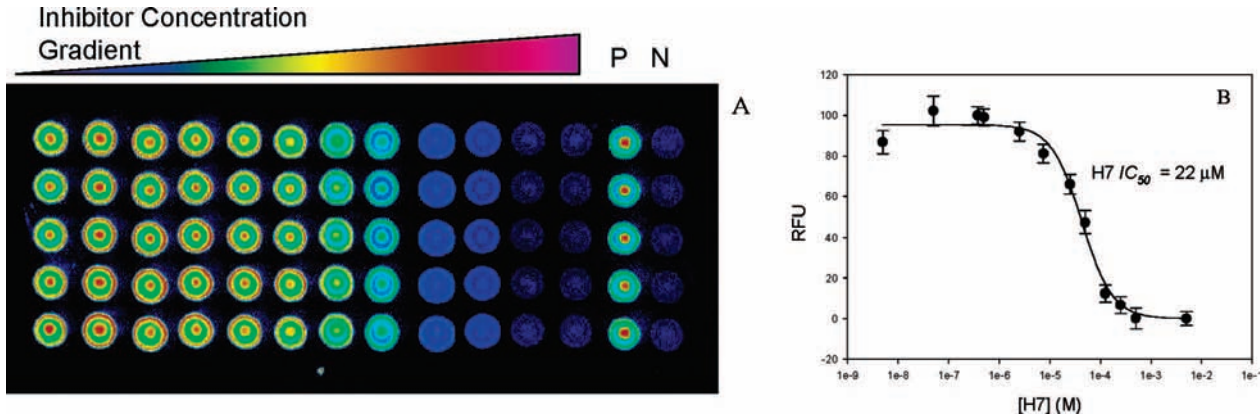

FIGURE 7. (A) Inhibition assay performed on a PKA–kemptide microarray. Inhibitor concentration increases from left to right, resulting in decreased fluorescence intensity due to inhibition of the phosphorylation reaction. N is the BSA negative control, and P is the β-casein positive control. (B) An IC_{50} curve generated from the inhibition assay. The background signals from the negative control sample have been subtracted, and the data have been normalized to the maximum intensity obtained in the absence of inhibitor. [Reproduced from ref 63. Copyright 2005 American Chemical Society]

Emerging Applications

Since the initial reports on the immobilization of proteins by the sol–gel route, there has been a significant body of work on the use of this technology for the development of devices and new applications. As noted in several reviews, initial applications focused primarily on development of biosensors,[9,10] supported biocatalysts for biosynthesis,[9,11] and the development of immunoextraction columns for sample cleanup.[9,55] More recent applications include bio-doped columns for protein digestion, which are geared toward proteomic analysis, and sol–gel-based microfluidic chips for sample preparation and cleanup, as reviewed by Kato et al.[56]

Sol–gel materials are also emerging as important tools in the medical area, and in particular in the area of high-throughput screening (HTS) of small molecules that can modulate protein function. As a starting point for the development of sol–gel-based solid-phase HTS assays, we examined the catalytic properties and inhibition of a series of clinically relevant enzymes when entrapped in DGS materials within 96-well plates.[57] While both the catalytic constant and Michaelis constant for entrapped enzymes differed significantly from the solution values (mostly because of mass transfer limitations and variations in partitioning of substrates between solution and the solid phase), it was determined that inhibition constants were very similar to those obtained in solution (usually within a factor of 2), indicating that inhibitor screening with sol–gel-entrapped enzymes should be feasible.

The success in determining inhibition constants for entrapped enzymes provided an impetus for the extension of the sol–gel-based solid-phase screening platform toward both microarray and column-based screening formats. In the case of microarrays, our group and others have shown that protein-doped sol–gel-derived materials can be pin-printed onto surfaces as a microarray and used for biosensing purposes.[58,61] Furthermore, our group demonstrated the potential of using microarrays of sol–gel-entrapped enzymes for detection of inhibitors.[62] However, it was not until 2005 that we were able to show the true potential of microarrays for HTS. Using a microarray with individual elements containing the α-catalytic subunit of cAMP-dependent protein kinase (PKA) co-entrapped with the peptide substrate kemptide, we demonstrated the ability to monitor both phosphorylation and inhibition of the kinase using overprinting of subnanoliter volumes of inhibitors onto individual array elements.[63] The co-immobilized kinase–substrate microarray system was shown to be suitable for quantitative inhibition assays and was able to provide accurate IC_{50} values for PKA inhibitors (see Figure 7).

Microarray-based screening has recently been extended by Clark and co-workers, who reported on the fabrication of cytochrome P450 (CYP450) microarrays based on pin-spotting of methyltrimethoxysilane (MTMS)-derived materials containing P450-doped microsomes.[64] Clark's study demonstrated the ability of entrapped CYP450s to metabolize prodrugs to active compounds, as demonstrated by a microarray-based cytotoxicity screen, and also showed the effects of CYP450 inhibitors on the enzyme function on the array.[64] Interestingly, this work was the first to demonstrate entrapment of a viable membrane-bound protein within an organically modified silica material and highlights the need for further investigation of suitable sol–gel processing methods for entrapment of membrane proteins.

As noted above, part of our motivation for entrapping membrane-bound proteins was to develop a chromatographic assay platform for screening of small molecule mixtures against membrane receptors. As part of this goal, we developed a protein-compatible method that allowed us to fabricate protein-doped monolithic silica materials that contained both mesopores (~3 nm diameter) to entrap proteins and macropores (500 nm diameter) to allow high rates of flow through the material with low backpressure. These materials were initially utilized for the fabrication of monolithic bioaffinity columns containing entrapped enzymes and for frontal affinity chromatographic (FAC) analysis of ligand–protein interactions.[54] In this method, ligand mixtures are continuously infused into the capillary-scale column, and the compounds break through in rank order

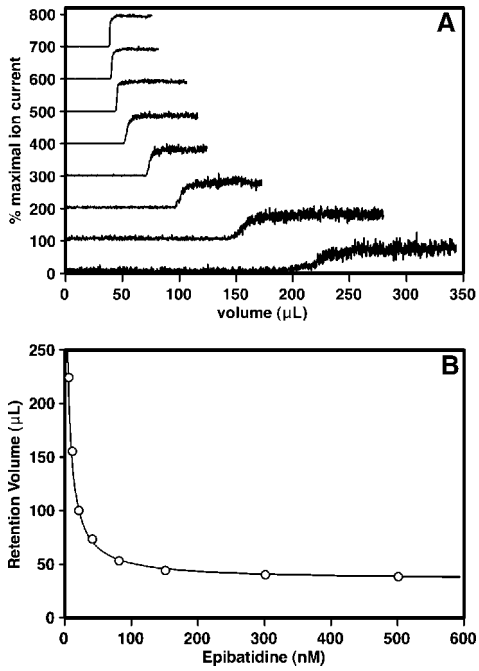

FIGURE 8. Determination of the number of binding sites (B_t) and dissociation constant (K_D) values for binding of epibatadine to *n*AChR columns based on the effect of ligand concentration ([A]) on the breakthrough volume. (A) FAC-MS elution profiles of different epibatadine concentrations normalized as a percentage of the maximum ion current and offset every 100% for clarity. Concentrations of epibatadine from top to bottom were 500, 300, 150, 80, 40, 20, 10, and 5 nM. (B) Breakthrough volumes of epibatadine as a function of epibatadine concentration fit to the equation $V = V_0 + B_t / K_D + [A]$, where V is the retention volume and V_0 is the void volume. The B_t and K_D values for epibatadine were 1.59 pmol and 3.4 nM, respectively. [Reproduced from ref 67. Copyright 2006, with permission from Elsevier]

of their affinity for the entrapped protein. Several compounds can be monitored simultaneously using mass spectrometry in multiple-reaction monitoring mode, allowing for rapid screening of mixtures.[65] Importantly, our initial studies showed that dissociation constants obtained for interactions between ligands and proteins entrapped in monolithic columns matched the values obtained in solution, validating the use of such columns for mixture screening. Such columns could also be used for functional screening of enzyme inhibition by monitoring changes in product/substrate ratios with a continuous flow immobilized enzyme reactor format and allowed for mixture screening and deconvolution as well as accurate determination of inhibition constants.[66]

Very recently, we have extended this work to the entrapment of *n*AChR for screening small molecule–protein interactions by FAC-MS. [67] Using an optimized macroporous sol–gel material, the monolithic bioaffinity columns retained 100% of the initially loaded receptor and exhibited very good chromatographic performance. Figure 8 shows the frontal chromatograms and the associated binding curve for the *n*AChR-containing bioaffinity columns. Such columns provided frontal breakthroughs with excellent resolution and a receptor–ligand binding constant that was very close to that predicted from solution-based experiments.

Conclusions and Future Directions

While the entrapment of proteins via the sol–gel process has been an active field of research since 1990, recent advances in biocompatible processing have proven to be important for the extension of this technology to delicate proteins and critical for the entrapment of membrane proteins. In particular, the use of polyol silane precursors such as DGS and the development of sugar-modified silica materials have led to significant improvements in material properties. Over the past 5 years, a number of labile proteins, including a range of key drug targets, have been successfully entrapped in protein-friendly silica materials, and this has allowed the development of a series of new formats for screening small molecule–protein interactions. However, at this point, only a limited number of receptors have been entrapped, and further efforts are needed to extend the range of membrane proteins that have been entrapped and to diversify into other families of receptors, including receptor tyrosine kinases and membrane transport proteins.

Besides extending to different proteins, other fundamental questions should also be addressed. For example, there is a need to better understand the nature of the interactions among proteins, lipids, and silica and how the silica materials and sol–gel processing method might be modified to alter these interactions in a rational manner. There is also a need to address practical issues such as how to increase the level of membrane protein loading, improve protein accessibility, and control factors that affect catalytic and/or binding constants. It is expected that fundamental and technological advances will expedite the uses of these membrane protein-doped materials for HTS and drug discovery applications based on microarrays and bioaffinity chromatography techniques.

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