# Nanometer Silica Particles Encapsulating Active Compounds: A Novel Ceramic Drug Carrier

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**Abstract:** Novel, injectable, and sprayable nanometer size hydrated silica particles encapsulating high molecular weight compounds such as [$^{125}$I]tyraminylinulin (mol wt 5 kD), FITC-dextran (mol wt 19.6 kD), and horse radish peroxidase (mol wt 40 kD) have been prepared and characterized. The size of these particles is always below 100 nm in diameter and the entrapment efficiency was found to be as high as 80%. The entrapped compounds show practically zero leachability for more than 45 days. Enzymes entrapped in these particles show Michaelis−Menten kinetics and the catalytic reaction takes place only after the diffusion of substrate molecules into the particles through the pores of the silica matrix. Peroxidase entrapped into silica nanoparticles shows higher stability toward temperature and pH changes compared to free enzyme molecules.

## Introduction

The newly emerging area of inorganic particles entrapping biomolecules has already exhibited its diversity and potential applications in many frontiers of modern material science including sensors, biosensors, optical materials, biocatalysts, electrochemistry, immunochemistry, and materials for use in environmental sciences.[1] Nanoencapsulation of enzymes by inorganic materials for the *in vivo* use of sustained drug release as well as drug targeting is also expected to have potentiality in enzyme therapeutics.[2] Particulate carriers of colloidal dimensions and below such as liposomes, polymeric nanoparticles, microemulsion droplets, etc. have already attracted considerable attention as drug carriers for achieving controlled delivery of drugs at specific body sites.[3,4] The inorganic particles have a number of advantages over organic ones *in vivo* applications in the sense that (i) these are not subjected to microbial attack,[5] (ii) there is no swelling or porosity change occurring in these particles with the change of pH,[5] (iii) these materials can be prepared at low temperature[6] so that the enzyme activity can be retained, (iv) some of these inorganic materials are nontoxic and highly biocompatible, and (v) these particles containing enzymes exhibit excellent storage stability of enzymes.[7]

Entrapment of bioactive materials by ceramic gels was mostly performed by using sol-gel methods.[1,8−12] The glassy mass obtained by this method is dried to obtain xerogels and is grinded to powder before application.[13] This leads to not only the formation of particles of uncontrolled sizes but also far from uniform distribution of entrapped molecules in the ceramic matrix. The enzyme entrapped in these dry as well as wet gel monoliths does not follow Michaelis−Menten kinetics and, therefore, quantitative assay, sometimes, becomes unreliable.[14−16] Moreover, photometric monitoring of absorbencies of the transparent glass of ceramic materials is always associated with scattering of light and consequent loss of light intensity.[17]

Aqueous dispersion of ceramic particles of nanometer size containing enzymes (or other active molecules) entrapped in them could solve most of the above problems. We present in this paper a method to prepare nanometer size (below 100 nm in diameter) ceramic particles with uniform size distribution and encapsulating active molecules that display, to a large extent, the characteristics which may overcome the above problems.

Most of the works reported so far are on the entrapment of enzymes by ceramic materials made of silica gels.[1] Reverse micelle mediated preparations of nanometer size void silica particles without having any encapsulated materials have also been reported in the literature[18,19] by controlled hydrolysis of tetraalkylorthosilicate in reverse micellar droplets. In this paper we are reporting a novel method of preparing enzyme doped

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(1) Avnir, D.; Braun, S.; Lev, O.; Ottolenghi, O. *Chem. Mater.* **1994**, *6*, 1605.
(2) Wiseman, A. *Handbook of Enzyme Biotechnology*; Horwood: Chichester, 1985.
(3) Kreuter, J., Ed. *Colloidal Drug Delivery Systems*; Marcel Dekker: New York, 1994.
(4) Yokoyoma, M.; Okano, T. *Adv. Drug Delivery Rev.* **1996**, *21*, 77.
(5) Weetal, H. H. *Biochim. Biophys. Acta* **1970**, *212*, 1.
(6) Brinker, C. J.; Schrer, G. *Sol-Gel Science: The Physics and Chemistry of Sol-Gel Processing*; Academic Press: San Diego, 1990; p 601.
(7) Akbarian, F.; Lin, A.; Dunn, B. S.; Valentine, J. S.; Zink, J. I. *J. Sol-Gel Sci. Technol.* 1997, *8*, 1067.
(8) Garcia, M.; Bello, F.; Gonzalez, F.; Ibrahim, N.; Cachaza, J. M. *Biochem. Educ.* 1986, *14*, 180.
(9) Avnir, D.; Braunn, S., Eds. *Biochemical Aspects of Sol-Gel Science and Technology*; Kluwer Academic Publisher: Boston, MA 1996.
(10) Livage, R. *C. R. Acad. Sci., Paris II* 1996, *322*, 417.
(11) Bronshtein, A.; Aharonson, N.; Avnir, D.; Turniansky, A.; Altstein, M. *Chem. Mater.* 1997, *9*, 2632 and references therein.
(12) Pope, E. J.; Braun, K.; vanHirtum, M.; Peterson, C. M. *J. Sol-Gel Sci. Technol.* 1997, *8*, 635.
(13) *Better Ceramics Through Chemistry, VI*; Sanchez, C., Mecartney, M. L., Brinker, C. J., Cheetham, A., Eds., Mater. Res. Soc. Sympos. Proc. 1994; p 346.
(14) Braun, S.; Rappaport, S.; Zusmann, R.; Avnir, D.; Ottolenghi, M. *Mater. Letts.* 1990, *10*, 1.
(15) Yamanaka, S. A.; Nishida, F.; Ellerby, L. M.; Nishida, C. R.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Chem. Mater.* 1992, *4*, 495.
(16) Shtelzer, S.; Braun, S. *Biotech. Appl. Biochem.* 1994, *19*, 293.
(17) Lovett, S. *Anal. Biochem.* 1975, *64*(*1*), 110.
(18) Arriagada, F. J.; Osseo-Asare, K. In *The colloid Chemistry of Silica*; Bergna, H., Ed.; Advances in Chemistry Series, No. 234; American Chemical Society: Washington, DC, 1994; p 113.
(19) Arriagada, F. J.; Osseo-Assare, K. *J. Colloid Interface Sci.* 1995, *170*, 8.

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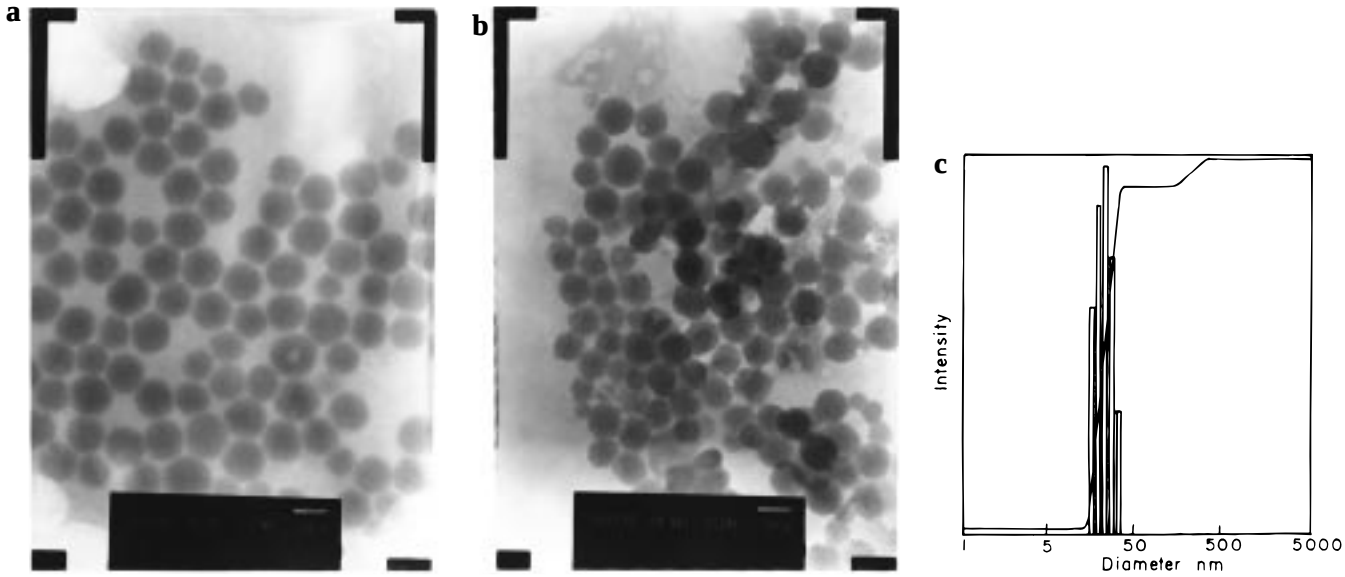


**Figure 1.** TEM pictures of (a) empty and (b) FITC-dextran loaded silica nanoparticles showing highly monodispersed nonsticking particles. (c) Quassielastic laser light scattering (QELS) spectrum of encapsulating nanoparticles showing unimodal size distribution (mean particle diameter ~31 nm).

silica nanoparticles using the aqueous core of reverse micellar droplets as host reactors.[20]

## Experimental Section

**Materials.** Triton X-100 (*t*-octyl-$C_6H_4$-$(OCH_2CH_2)_x$OH, where $x = 9-10$), *n*-hexanol, cyclohexane, and horse radish peroxidase ($R_z = 3.0$) were purchased from SRL, India, and were used without any further treatment. Fluorescein Isothiocyanate-Dextran (FITC-Dextran) and tetramethyl orthosilicate (TMOS) were obtained from Sigma (USA). [$^{125}$I]Tyraminylinulin was a gift from Prof P. C. Ghosh (Department of Biochemistry, Delhi University).

To form the core of the particles, we used hydrated silica as the material is known to be safe in the human body within the range of administered dose. By adjustment of the size of the aqueous core of the reverse micellar droplets, the size of the silica nanoparticles can be controlled. Silica nanoparticles encapsulating FITC-Dextran of average molecular weight of 19.6 kD, as an example, were prepared as follows: a 18.4 mL sample of 10% w/w of a mixture of Triton X-100 and *n*-hexanol (in the molar ratio 1:5) in cyclohexane was taken to which 18.0 $\mu$L of FITC-Dx solution (16 mg/mL) and 212 $\mu$L of 1.05 M ammonia solution (in case of void nanoparticles 230 $\mu$L of 1.05 M ammonia only and no active compound) were added. The final mixture was a clear microemulsion solution. Since the temperature has a strong influence on the size of the particles formed in micellar media,[21] the hydrolysis reaction was allowed to take place at ice-cold temperature to have the minimum possible size of the particles. Hence, to an ice-cold solution of this microemulsion was added 290 $\mu$L of neat TMOS and the mixture was shaken continuously for 10−15 min. TMOS goes slowly into the aqueous core of reverse micellar droplets where it is hydrolyzed in ammonia solution to form hydrated silica. The insoluble hydrated silica took the shape of particles encapsulating active molecules present in the host aqueous core. The mixture was kept in a refrigerator for 72 h. The solution was turbid. It was then subjected to vacuum evaporation at room temperature to completely remove cyclohexane. The viscous glassy mass was then extracted with 5 mL of aqueous ammonia buffer (pH ~9.0) in instalments and the aqueous dispersion was subjected to dialysis through a 12 kD cut-off cellulose membrane for 24 h. The unentrapped large molecules like enzymes or FITC-Dx, as in the present case, that did not escape through dialysis were separated by gel filtration with sephadex G-100. The silica nanoparticles containing encapsulated materials are eluted at the void volume, whereas free compounds which escape encapsulation elute at later times.

Quasielastic laser light scattering (QELS) spectra of encapsulating nanoparticles were taken with a Brookhaven BI9000 fitted with a BI200SM goniometer. An argon ion air-cooled laser was operated at 488 nm as a light source and the measurements were done at a scattering angle of 90°. The time-dependent autocorrelation function was derived by using a 136 channel digital photon correlator. TEM pictures were taken in a JEOL JEM2000 Ex200 model electron microscope.

## Results and Discussion

TEM and QELS results show that the particles have spherical shape and are highly monodispersed (Figure 1a,b,c). The QELS spectrum shows unimodal size distribution (mean diameter ~31 nm for the particles prepared with the above protocol).

To determine the encapsulation properties of hydrated silica particles, loaded nanoparticles were prepared in the same one-step procedure in reverse micelles and were extracted with aqueous buffer. After dialysis, the entire solution was diluted to 10 mL by aqueous buffer and a part of it was filtered through millipore UFP2THK24 (100 kD cut-off) filter to separate the unentrapped compound. Entrapment efficiency (*E*, %) of silica nanoparticles was calculated from the concentrations of unentrapped and total added material. The *E* of the particles containing [$^{125}$I]tyraminylinulin (mol wt 5 kD) at its different added concentrations in the micellar medium is shown in Figure 2. The *E* of the compounds like FITC-Dx and Horse Radish Peroxidase (HRP) (mol wt 40 kD) are also very high and in the range of 80−90%.

*In vitro* leaching of FITC-Dx and HRP from hydrated silica particles were studied. The compounds are practically unleachable even in aqueous dispersion for a prolonged period of 45 days or more. The encapsulated HRP in silica nanoparticles shows enzymatic activity on pyrogallol oxidation by hydrogen peroxide. The time-dependent formation of purpurogallin (OD measured at 420 nm) in the presence of HRP entrapped in silica

(20) Note Added in Proof: A reviewer has kindly referred to a paper on encapsulation of lipase by silica nanoparticles in w/o microemulsions. The detailed reference of the paper is: Kusunoki, K.; Kawakami, K. Immobilization of Lipase into silica fine particles by sol-gel transition in w/o microemulsions. In *Bull. Fac. Eng. Kyushu Sangyo Univ.* 1996, *33*, 48. The paper was intentionally omitted from the reference list because it appeared that the paper is of little interest to the present work.

(21) Munshi, N.; De, T. K.; Maitra, A. N. *J. Colloid Interface Sci.* 1997, *190*, 387.

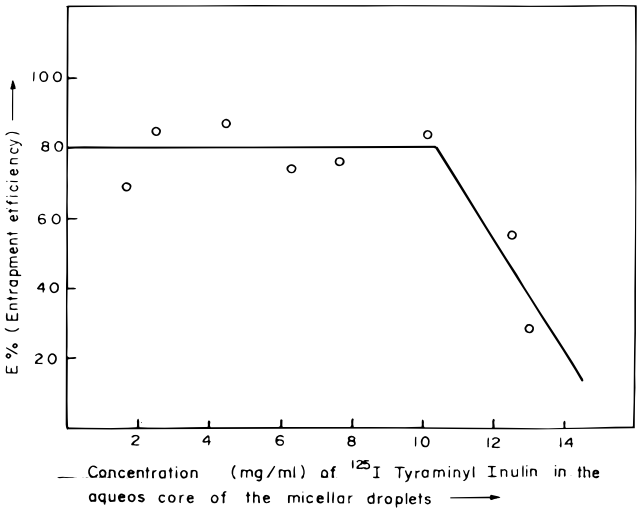


**Figure 2.** Entrapment efficiency (*E*, %) of silica nanoparticles containing different concentrations of [$^{125}$I]tyraminylinulin in the micellar medium.

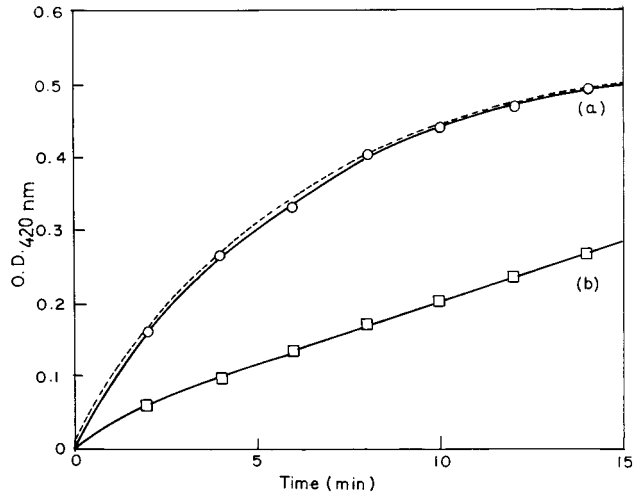


**Figure 3.** Time-dependent formation of purpurogallin by and HRP catalyzed oxidation of pyrogallol by hydrogen peroxide at pH 7.5 (0.05 M phosphate buffer) and 20 °C. OD was measured at 420 nm. Curve a: free enzyme in aqueous buffer with (dotted) and without dispersed void nanoparticles. Curve b: Enzyme entrapped in silica nanoparticles and dispersed in aqueous buffer. Concentrations of enzyme and substrates are same in all the solutions. [HRP] = $9.6 \times 10^{-7}$ mg/mL; [$H_2O_2$] = 0.44 mM; [pyrogallol] = 60 mM.

nanoparticles, as shown in Figure 3, is slower than that in the presence of exactly the same amount of free HRP in aqueous buffer. Void silica nanoparticles incubated overnight with the same amount of HRP in solution at room temperature (∼20 °C) produce HRP activity more or less identical with that of free enzyme and higher than that of entrapped enzyme in nanoparticles under similar conditions. This indicates that HRP, indeed, is encapsulated inside the matrix of silica nanoparticles and is not adsorbed on the particle surface. It is apparent that the reasons for the low activity of the entrapped enzyme may be either (i) due to the conformational change of the enzyme in the silica matrices, which caused partial denaturation of enzyme, or (ii) the diffusional constraint of the substrate through the pores of the nanoparticles to reach the enzyme surface entrapped inside, or (iii) both the factors simultaneously. Temperature-dependent initial slopes of rate of formation of purpurogallin in the presence of free enzyme, free enzyme adsorbed on the void particle surface, and the presence of entrapped enzyme

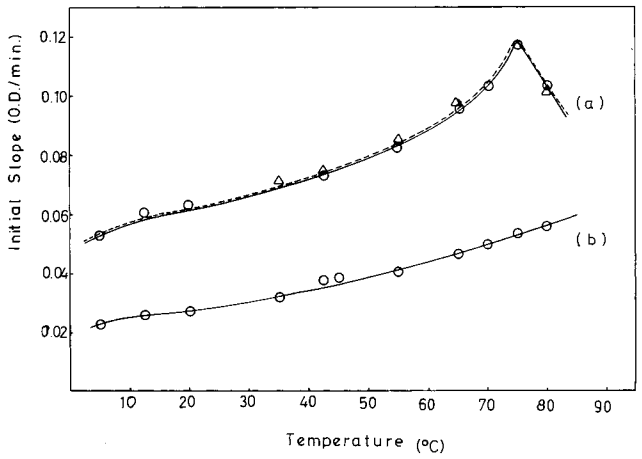


**Figure 4.** Temperature-dependent initial slopes of the formation of purpurogallin (OD at 420 nm) by HRP catalyzed oxidation of pyrogallol by hydrogen peroxide at pH 7.0 (0.05 M phosphate buffer). Curve a: Free enzyme in aqueous buffer with (dotted) and without dispersed void nanoparticles. Curve b: Enzyme entrapped in nanoparticles. Concentrations of the reactants are the same in all solutions and as mentioned in Figure 3.

show that the reaction rate is directly proportional to temperature in all three cases although the increase in rate with temperature is less for the system containing enzyme entrapped in silica nanoparticles (Figure 4). The free enzyme is stable up to 75 °C, above which it loses its activity, while the entrapped enzyme is stable even at higher temperature. Measurements beyond 80 °C did not give reliable results due to formation of bubbles in the solutions.

The analysis of the Michaelis−Menten kinetics of the entrapped as well as free HRP from the Lineweaver Burk plots revealed signs of diffusion-dependent kinetics. The high affinity of the free enzyme is depicted from the Michaelis constant, $K_m$, of 2.53 $\mu$mol/mL while the entrapped enzyme has an average $K_m$ of 5.40 $\mu$mol/mL. The reduced affinity for the substrate can be accounted for by such factors as constrained diffusion of the substrates, conformational distortion of the entrapped enzyme, and, perhaps, silylation of some protein surface groups by TMOS. Actually it is remarkable that despite all these negative factors, the entrapped enzyme shows significant catalytic activity while inside the matrix of the silica nanoparticles.

The pH-dependent turnover numbers of HRP in encapsulated as well as in free form are shown in Figure 5. While the free enzyme shows a maximum of its activity at pH ∼7.2, the entrapped enzyme exhibits a gradual rise of activity even at pH 8.0. No measurement beyond this pH has been made because of autoxidation of pyrogallol in alkaline solution. Silica nanoparticles are negatively charged particles.[22] Therefore, the polyionic matrices of the charged silica particles should have the general effect of causing a partitioning of protons between the bulk phase and the enzyme microenvironment. Such proton partitioning in the encapsulated enzyme system and the consequent shift of pH maximum are observed in the case of charged polymeric nanoparticles also.[23]

In all these experiments it has been observed that the entrapped enzyme induces a lower rate of catalytic reaction than that of free enzyme. If the enzyme has a high intrinsic specific activity, the substrate concentration gradient between the bulk

(22) Iler, R. K. *The Chemistry of Silica*; Wiley: New York, 1979.
(23) Trevan, M. D. In *Immobilised Enzymes—An Introduction and Applications in Biotechnology*; John Wiley & Sons: New York, 1980; Chapter 2, p 11.

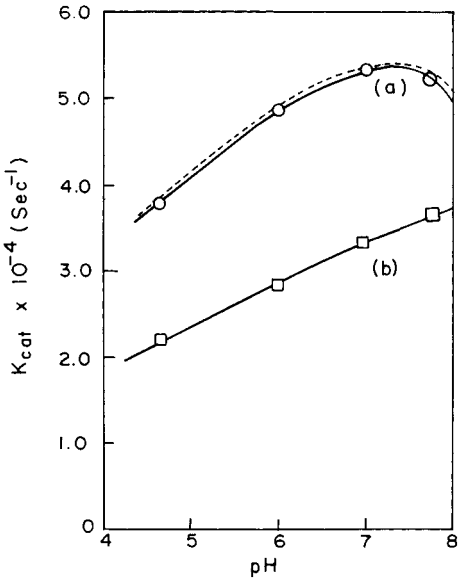


**Figure 5.** pH-dependent turnover number ($k_{cat}$) for HRP catalyzed oxidation of pyrogallol by hydrogen peroxide at 20 °C. Curve a: Free enzyme with (dotted) and without dispersed void nanoparticles in aqueous buffers, Curve b: Entrapped enzyme in silica nanoparticles and dispersed in various aqueous buffers. [$H_2O_2$] was varied from 2.2 to 0.44 mM.

of solution and inside the nanoparticles will be steep. There will be a time when the enzyme may starve for the substrate and its activity will remain under-utilized and thus the effective activity of the enzyme will be formally reduced. Since the reaction rate is also dependent, among other factors, on the substrate diffusion through the pores of the nanoparticles, the rate is also related to the diffusion path length (i.e. particle size) as well as the rate of diffusion of the substrate toward the enzyme surface.[24] At the steady state of reaction, the rate of internal substrate diffusion inside the polymer matrix at any point must equal its rate of removal by the enzyme. Let us assume that (i) the enzyme is completely entrapped within the matrix of nanoparticles, (ii) the Michaelis−Menten model describes the enzyme kinetic reaction, (iii) the system is operating under steady state condition and is isothermal, (iv) the diffusion of the substrate and the product obeys Fick's law and the effective diffusivity is constant throughout the nanoparticles, (v) there is no external diffusion resistance, and (vi) neither partition nor inhibition occurs. Under such circumstances, the reaction velocity is related to the substrate as well as the diffusional path length (i.e. particle size).[25] The reaction velocity is related to the substrate diffusion in a very complicated way. In the limit $R \rightarrow 0$, there will be no difflusional resistance to mass transfer and the reaction rate to be very high and approaching that of the free enzyme. The relatively higher reaction rate of free HRP as well as HRP adsorbed on the particle surface compared to entrapped enzyme is, therefore, direct evidence of the fact that the diffusional constraint of the substrate has a strong influence on the reaction rate.

(24) O'Driscoll, K. F.; Hingberg, I.; Korus, R.; Kapoulas, A. *J. Polym. Sci.* 1974, *46*, 227.

## Conclusions

Finally it can be concluded that ultrafine silica nanoparticles doped with enzyme can be prepared by using an aqueous core of reverse micellar droplets as the nanoreactor. These particles are highly monodispersed. Although the leachability of enzyme molecules from these particles is practically zero over a long period of time, the entrapped enzyme shows activity. Encapsulated silica nanoparticles reported in this paper could be used in various ways. Encapsulation of enzyme by these silica nanoparticles and their administration in the animal system to replenish enzyme deficiency in the body as well as use of the enzyme as medicine would be possible without having any risk of allergic or proteolytic reactions of these enzymes due to their practically zero leachability. The specific antigen−antibody reactions can be performed in the body with soluble haptens.[26] These small molecules can diffuse easily through the pores of the silica nanoparticles and can react specifically with the encapsulated antibodies. Antibody encapsulation by these particles can be used as a sensor for specific antigen detection.[27] With further study, these encapsulating ceramic particles can be used as improved biosensor devices and for vaccine formulation.[28] Some work in these directions is already in progress and will be communicated in due course.

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(25) Carr, P. W.; Bowers, L. D. In *Immobilised Enzymes in Analytical and Clinical Chemistry*; John Wiley & Sons: New York, 1980; p 379.

(26) Jordan, J. D.; Dunbar, R. A.; Bright, F. V. *Anal. Chim. Acta* 1966, *332*, 83.

(27) Roux, C.; Livage, J.; Farhati, K.; Monjour, L. *J. Sol-Gel Sci. Technol.* 1997, *8*, 663.

(28) Kreuter, J. Nanoparticles as Adjuvants for Vaccines. In *Vaccine Design: The Subunit and Adjuvant Approach*; Powell, M. F., Newman, M. J., Eds.; Plenum Press: New York, 1995.