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Ferricytochrome c Encapsulated in Silica Nanoparticles: Structural Stability and Functional Properties

Abstract: *Using a modified sol–gel technique, we have succeeded in encapsulating ferric cytochrome c in silica nanoparticles obtained from hydrolysis and polycondensation of tetramethylorthosilicate. Particles dimensions have been determined with dynamic light scattering; this technique yields an hydrodynamic radius of about 100 nm, each nanoparticle containing about 10^2–10^3 proteins. If stored in the cold at low ionic strength, nanoparticles are stable for more than one week, even if a slow radius increase with time is observed. CD measurements show that encapsulated proteins exhibit substantially increased stability against guanidinium hydrochloride induced denaturation. Reduction kinetics of encapsulated ferric cytochrome c by sodium dithionite, measured with standard stopped flow techniques, are slower by a factor of ten with respect to those measured in solution. Analogous experiments with myoglobin suggest that this slowing down is due to the diffusion time of dithionite within the silica matrix. Indeed, if a smaller ligand like CO is used, the intrinsic kinetic properties of encapsulated proteins are found to be unaltered even in the millisecond time range. The reported data show that our nanoparticles are extremely useful both for basic research, to study the stability and functions of encapsulated proteins, and for their potential biotechnological applications.* © 2004 Wiley Periodicals, Inc. Biopolymers 74: 55–59, 2004

Keywords: *sol–gel encapsulation; protein denaturation; dynamic light scattering; kinetic properties; biosensors*

INTRODUCTION

Protocols for encapsulating proteins within macroscopic silica hydrogels produced with the sol–gel approach are nowadays well established and widely used.[1–5] Encapsulated proteins exhibit increased resistance against denaturation[6,7] and retain almost intact functional properties[3–5]: these features make the sol–gel approach particularly interesting in the field of biosensors.[8] Functional studies on proteins encapsulated within standard macroscopic hydrogels are, however, limited by the diffusion time (usually from minutes to hours, depending on gel thickness) of ligands through the macroscopic silica matrix. To circumvent this difficulty, we have recently developed a new sol–gel protocol that enables to obtain clear
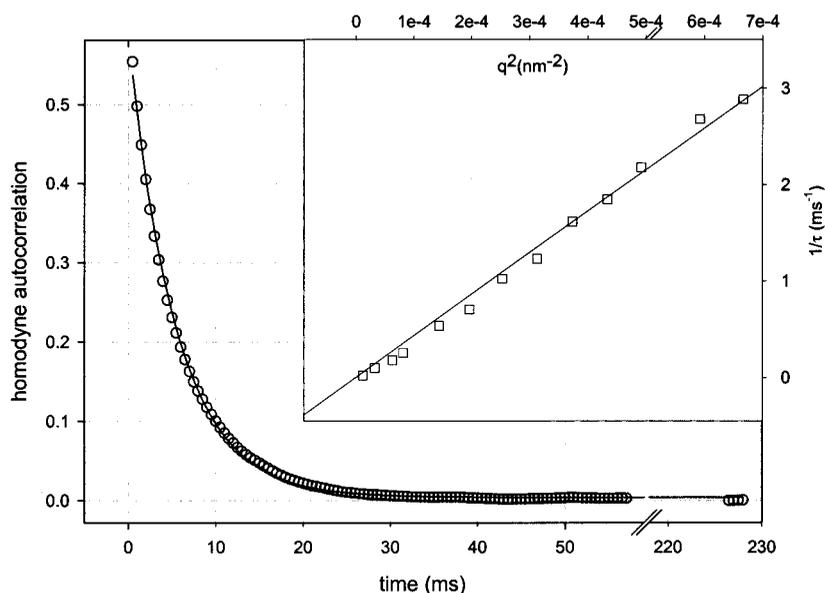
FIGURE 1 Scattered light correlogram relative to a sample of silica + ferric cytochrome c aggregates. ○: experimental points; line: fit in terms of a single exponential. The inset reports the inverse of the decay time as a function of q^2.

suspensions of nanometer-sized silica aggregates (silica nanoparticles) in which proteins are embedded. Encapsulated proteins exhibit substantially increased stability and intrinsic kinetic properties essentially unaltered even in the millisecond time range. Silica nanoparticles appear therefore as a very interesting new tool for spectroscopic, kinetic, and functional studies on encapsulated proteins, both for basic and applied research.

MATERIALS AND METHODS

Samples

Horse heart ferricytochrome c (Sigma) was used without further purification. To prepare silica + cytochrome aggregates, a water solution of $8 \times 10^{-2} M$ tetramethylorthosilicate (TMOS) and $6 \times 10^{-4} M$ HCl, after sonication, is mixed with an equal quantity of a $8 \times 10^{-5} M$ protein solution in phosphate buffer $4 \times 10^{-2} M$, pH 7, and stored at 7°C. In the first 30 min after mixing the sample becomes turbid, indicating formation of micron-sized aggregates; as time elapses these aggregates precipitate and a transparent colored "solution" is obtained, in which the remaining protein concentration is about 1/3 of the initial one. After two days this solution is centrifuged at 2500 rpm for about 20 min and filtered through a 1.2 μm filter. If the final suspension is again filtered through a 0.2 μm filter, a totally colorless filtrate is obtained: this indicates that the remaining protein is not free in solution but encapsulated within silica aggregates.

Myoglobin from horse heart (Sigma) was used after prolonged dialysis against Millipore-distilled water. Methods for myoglobin encapsulation were similar to those relative to ferricytochrome c.

Instrumentation

Dynamic light scattering measurements were performed at various scattering angles using a photomultiplier tube and a Brookhaven goniometer and correlator. The light source was a He–Ne laser, $\lambda = 633$ nm; temperature was controlled within ±0.5°C.

CD measurements in the wavelength interval 210–500 nm were performed at 20°C with a Jasco J-715 spectropolarimeter. All samples were equilibrated with the desired denaturant concentration for about 15 min prior to the measurements. A baseline measured in separate experiments was subtracted from each measured spectrum.

Kinetics of ferricytochrome c reduction by sodium dithionite and of carbonmonoxide (CO) binding to deoxymyoglobin were measured at 418 nm and at 439 nm respectively with a Biologic Science SMF-300/S stopped flow accessory used in conjunction with a Jasco J-715 spectropolarimeter.

RESULTS AND DISCUSSION

Structural properties of silica–cytochrome c aggregates have been investigated with dynamic light scattering. Figure 1 reports a typical correlogram together
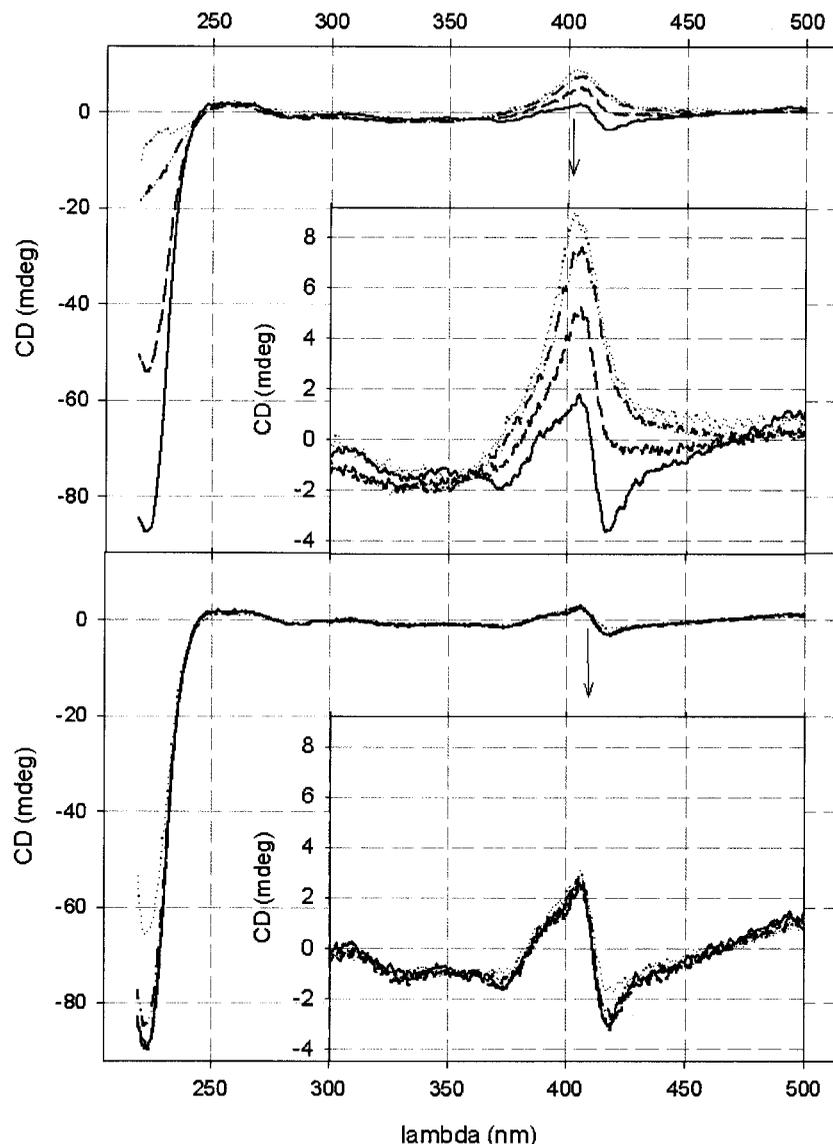
FIGURE 2 CD spectra of free (upper panel) and encapsulated (lower panel) ferricytochrome c at various GuHCl concentrations. Continuous line: 0*M;* dashed line: 2.5*M;* dashed-dotted line: 3*M;* dotted line: 6*M.* The insets show the Soret region, on an expanded scale.

with a fitting in terms of a single exponential. From the decay time, using Stokes law, we obtain the hydrodynamic radius of the particles $R_H \approx 100$ nm. The inverse of the decay time exhibits a linear dependence upon q^2, indicating that the motion of silica nanoparticles in solution is almost purely diffusive and that particle–particle interactions can be neglected. The picture emerging is that of nanometer-sized silica particles in which protein molecules are embedded and which diffuse almost freely in solution; from the protein concentration measured spectrophotometrically we estimate about 10^2–10^3 protein molecules per nanoparticle. If the sample is stored in the cold

and at low ionic strength, such a behavior is conserved for more than one week, although a slow radius increase of the aggregates is observed.

The resistance of encapsulated ferricytochrome c against denaturation induced by the chemical denaturant guanidinium hydrochloride (GuHCl) has been investigated with CD in the Soret and near-uv region. CD spectra at various GuHCl concentrations and fractions of "denatured" protein as a function of denaturant concentration, calculated from the percentual signal variation of the near-uv and Soret regions of the spectra, are reported in Figures 2 and 3, respectively. Protein conformational stability is substantially en-
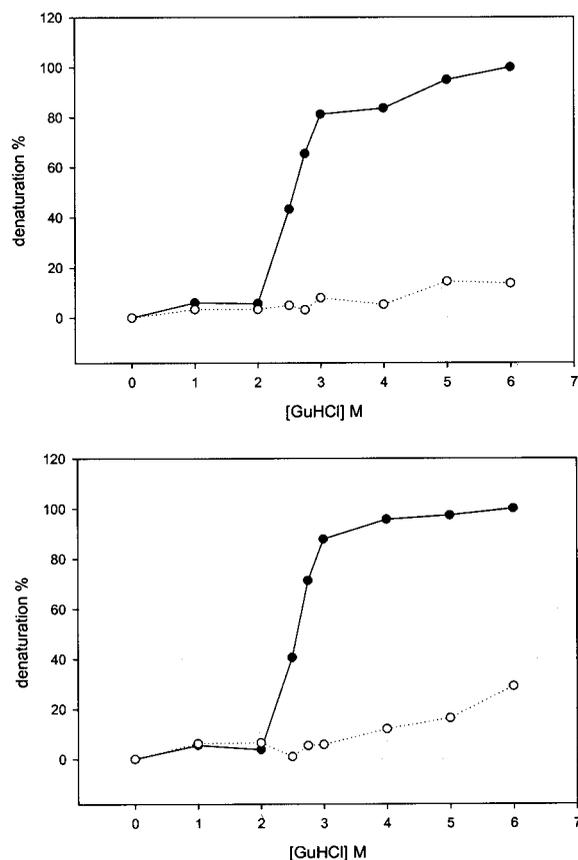
FIGURE 3 Fraction of denatured protein as a function of GuHCl concentration. ●: Protein free in solution; ○: encapsulated protein. Upper panel: data calculated from the near-uv region; lower panel: data calculated from the Soret region.

hanced by encapsulation: in fact, while the protein in solution is almost fully denatured already at 3*M* Gu-HCl, more than 90% of the nanoparticle-encapsulated proteins are found to have native heme pocket conformation even at 6*M* GuHCl. Interestingly, however, for the encapsulated protein a larger variation (about 30% at 6*M* GuHCl) is observed in the secondary structure marker band at about 220 nm; this shows that at high denaturant concentrations some local loosening of the α-helical secondary structure may occur, although the heme pocket structure is largely maintained.

Figure 4a shows the kinetics of reduction by sodium dithionite of encapsulated cytochrome c together with those relative to the protein free in solution. Data in Figure 4a show that it is possible to reduce ferric cytochrome c encapsulated within silica nanoparticles; the reduction kinetics, however, is slowed down by a factor of 10 with respect to the solution. Such an effect could be attributed either to

an intrinsic slower kinetics of the encapsulated proteins, or to the diffusion time of dithionite within the silica matrix. To further investigate this point, we made some experiments with horse myoglobin. The kinetics of ferric myoglobin reduction by dithionite and of CO binding to deoxy myoglobin are reported in Figure 4b. These data indicate that when the reduction kinetics is in the time scale of seconds, almost no difference is observed, thus suggesting that the slowing down observed for encapsulated ferric cytochrome c is likely due to the diffusion time of dithionite within the silica matrix; moreover, if a smaller ligand like CO is used, protein encapsulation within our silica nanoparticles leaves unaltered the intrinsic protein kinetic properties even in the millisecond time range.

CONCLUSIONS

Protein–silica aggregates obtained with our modified sol–gel protocol appear from light scattering experiments as nanometer-sized particles having hydrodynamic radius ~100 nm and diffusing almost freely in solution. Proteins encapsulated within these nanoparticles exhibit enhanced stability against chemically induced denaturation. The intrinsic protein ligand binding kinetics is not altered by encapsulation and appears limited only by ligand diffusion; for small ligands the binding kinetics are unaltered even in the millisecond time scale.

In conclusion, the reported data show that silica nanoparticles are extremely interesting systems from several points of view: (a) for biophysical studies on proteins, since they may be useful to stabilize functionally relevant protein conformational intermediates[9] otherwise barely detectable and to study their conformational and dynamic properties even with standard stopped flow techniques; (b) for applied research in the field of biosensors, since encapsulation within silica nanoparticles is effective in increasing the conformational stability of proteins, and does not affect the intrinsic functional properties; moreover, protein reactions with ligands are not limited by the slow ligand diffusion within a macroscopic silica matrix, thus allowing full equilibration in much shorter times.
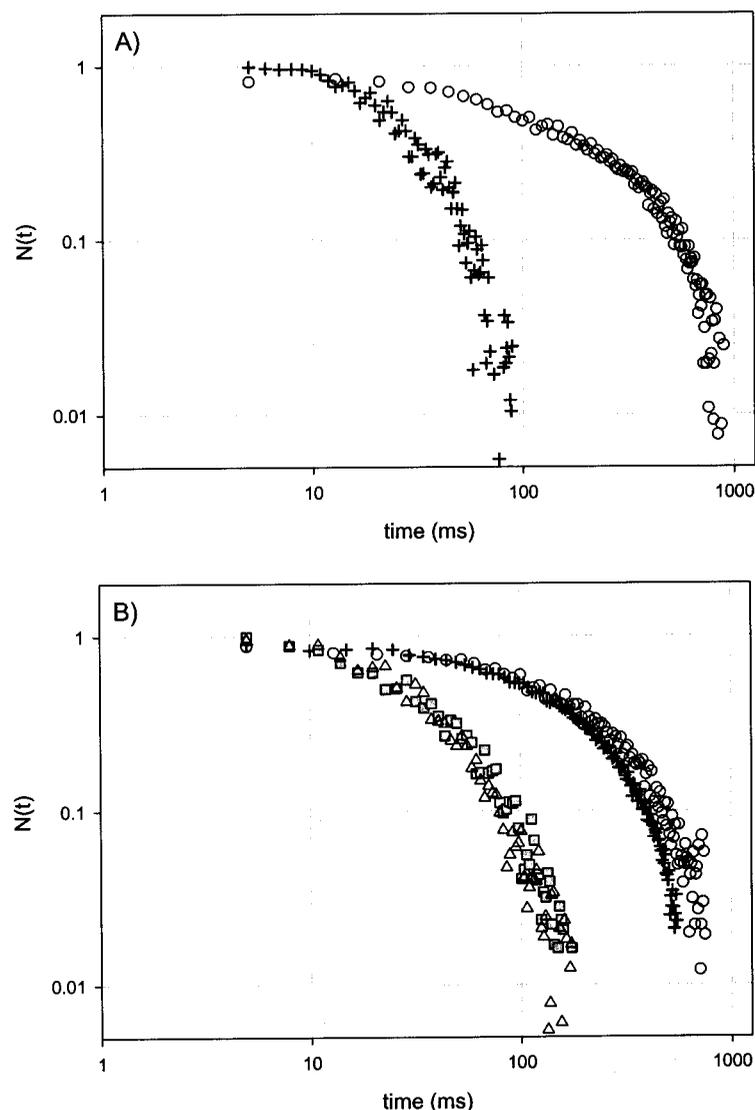
FIGURE 4 (A) Kinetics of reduction of ferric cytochrome c by Na-dithionite; +: protein free in solution; ○: encapsulated protein; experimental conditions: $5\times10^{-6}M$ protein in $2\times10^{-2}M$ phosphate buffer pH 7; $4.7\times10^{-3}M$ Na-dithionite; $T = 20°C$. (B) Same as panel (A), for horse myoglobin; experimental conditions: $4\times10^{-6}M$ protein in $2\times10^{-2}M$ phosphate buffer pH 7; $9.5\times10^{-3}M$ Na-dithionite; $T = 20°C$. Kinetics of CO binding to deoxy myoglobin are also reported; Δ: protein free in solution; □: encapsulated protein; experimental conditions: $4\times10^{-6}M$ protein in $2\times10^{-2}M$ phosphate buffer, pH 7; $8.0\times10^{-3}M$ Na-dithionite; $8\times10^{-5}M$ CO; $T = 20°C$.

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