Inhomogeneity in Distribution and Conformation of Bovine Serum Albumin in Sol–Gel: A Closer Look with a Near-Infrared Multispectral Imaging Technique

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Abstract. Concentration distributions and conformations of bovine serum albumin (BSA) entrapped in sol–gels were successfully determined for the first time by use of the recently developed NIR multispectral imaging instrument. It was found that BSA molecules were inhomogeneously distributed within the sol–gel matrix, independent of its concentration. At relatively high concentration (366 mg/mL) the encapsulation process does not seem to produce any observable changes in the conformation of BSA. However, when the concentration of BSA was decreased to 220 mg/mL, pronounced changes in the spectra of the protein were observed as a function of (sol–gel reaction) time. The observed inhomogeneity might be traced to both a non-uniform concentration distribution of BSA and changes in protein native conformation resulting from interactions between the charged protein and the silicate anions. Results obtained seem to suggest that the protein molecules might adapt themselves to accommodate evolving geometry and sites of the silica network.

Keywords: sol–gel, near-infrared, imaging, inhomogeneity, protein encapsulation

1. Introduction

Encapsulating biopolymers in sol–gel has been a subject of popular studies in recent years. The popularity stems from the fact that proteins encapsulated in sol–gel matrix are suitable for use as biocatalysts and biosensors [1–4]. Specifically, the sol–gel method is compatible with proteins, and because the pores of the sol–gel glass contain water, encapsulated protein molecules are solvated and retain their structure and reactivity. In fact, a number of proteins and enzymes, such as cytochrome-c, horseradish peroxidase, trypsin and glucose oxidase, have been successfully embedded and retained their catalytic properties [1–4].

Although many studies have been performed on the catalytic activities, relatively little is known about structure of encapsulated proteins. While sol–gel is compatible with proteins and the sol–gel matrix can be prepared by polymerizing in physiological pH solutions and at room temperature, there are possible complications which, to date have not been systematically studied and/or satisfactorily addressed. For example, because alcohol is released when alkoxysilane is used as a precursor to prepare sol–gel, structure of embedded proteins might be altered because alcohol is known to be a modifier of the enzyme structure. Additionally, the microstructure of the sol–gel matrix is known to undergo changes at various stages during the sol–gel process. It is possible that these changes can affect the properties of the embedded biomolecules. It is, therefore, of particular importance that structure of proteins embedded in sol–gel matrices be thoroughly investigated. Near-infrared spectroscopic imaging technique is particularly suited for this type of study.

Near-Infrared (NIR) spectrometry has been used extensively in recent years for chemical analysis and
characterization [5]. The technique is popular because it has high sensitivity and selectivity, wide applicability, is nondestructive and non-invasive, and requires no sample preparation [5]. NIR technique has been successfully used to determine conformation of polypeptides in aqueous solutions [6, 7], and structure of sol-gels [8, 9]. In principle, it is possible to use NIR technique to study structure and conformation of embedded proteins. Care must be taken, however, in such studies to avoid possible errors. This is due to the properties of the sol-gel and the NIR instrument used for measurements. Specifically, sol-gel samples are known to be chemically inhomogeneous. Embedded protein molecules might not be homogeneously distributed in the sol-gel matrix. Furthermore, embedded protein molecules might adopt different conformation at different positions within the sol-gel matrix. It is, therefore, important that concentration and conformation of embedded protein be simultaneously measured at different positions in a sol-gel sample. Unfortunately, because NIR spectrometers are equipped with single channel NIR detectors, it is not possible to simultaneously determine concentrations and conformation of embedded protein at different positions in a sol-gel sample. A NIR multispectral imaging instrument is needed for such a purpose.

Multispectral imaging spectrometer is an instrument that can simultaneously record spectral and spatial information of a sample [10–12]. Chemical homogeneity of the sample can be elucidated from recorded images. In this instrument, the spatial distribution of the sample is obtained by a camera, and the spectral information is gained by scanning a dispersive device [10–12]. Recently we have constructed such an instrument by use of an acousto-optic tunable filter (AOTF) for spectral scanning and an InSb focal plane array camera for recording NIR images [12–20]. With this imaging spectrometer, we were able to perform studies which to date were not possible using existing techniques. These include the authentication of documents, the determination of chemical inhomogeneity of copolymers, kinetic inhomogeneity of curing of epoxy and of sol–gel processes, the identity and sequences of peptides synthesized by combinatorial solid phase method [12–20].

The information presented is indeed provocative and clearly indicates that it is possible to use the NIR multispectral imaging instrument to determine the concentration distribution and conformation of protein molecules embedded in sol-gel. Results on sol-gel encapsulated bovine serum albumin (BSA) will be reported in this paper.

2. Experimental Section

2.1. Chemicals

Tetramethyl orthosilicate 98% (TMOS) (Alfa Aesar) and Fatty-acid free BSA (Worthington Biochemicals) were used as received. sol-gels were prepared by mixing 7.4 ml of TMOS with 1.69 ml of deionized, distilled water and 0.12 ml of 0.04 N HCl following by sonicating at 4°C for 30 min [21, 22]. 0.1 ml of the sol produced was then combined with an equal volume of buffer following to adding of the BSA solution [21, 22]. The protein solutions were prepared by dissolving 3–15 μM of BSA in 50 mM Bis-Tris buffer solution at pH 6.5. The resulting mixture was immediately transferred into a 1 mm path length quartz cell and left for solidification. Final protein concentrations after dilution with sol and buffer were between 40 and 366 mg/ml. Gelation occurred within 1 to 5 min.

2.2. Instrumentation

The NIR multispectral imaging instrument used in this study is similar to the imaging spectrometer used in our previous studies [8, 9, 12–14]. Spectral images of samples (in 1 mm pathlength cell) were recorded in the 1800–2450 nm range at 2-nm steps. Since the instrument is configured for measuring spectral images in the transmission mode, two images were recorded at each wavelength: one with a sample and the other without the sample. Absorbance of the sample at each wavelength can then be calculated from the two images at the corresponding wavelength, NIR absorption spectra at different positions within a sample were calculated from recorded images using appropriate numbers of pixels (e.g., an average of a square of either 3 × 3, 7 × 7, 15 × 15 or 50 × 50 pixels).

3. Results and Discussion

3.1. Near-Infrared Spectra of BSA in Buffer Solution and in Sol-Gel

NIR spectrum of 350 mg/mL of BSA in buffer (Fig. 1B), obtained by subtracting the background
Figure 1. (A) Spectra of Bis-Tris buffer at pH 6.5 (dotted line) and Bis-Tris buffer with 350 mg/mL of BSA (solid line); (B) spectrum of 350 mg/mL of BSA in Bis-tris buffer with background absorption of the buffer subtracted and its second derivative spectrum (B'); (C) spectrum of 366 mg/mL of BSA encapsulated in sol-gel with background absorption of sol-gel subtracted and its second derivative spectrum (C'). The spectra were calculated from an average of 15 x 15 square of pixels. See text for detailed information.

absorption of the buffer solution from that of the BSA in buffer, shows that the protein has bands at 2056, 2185 and 2306 and a shoulder at 2360 nm. The cluster of bands at around 2060 nm can be attributed to a combination of amide A and amide II [23, 24]. The 2185 nm band can be assigned to the combination of amide B and amide I [25]. This band is thought to be associated with the relative content of α-helix, β-sheet and random coil structures [26]. The broad band at 2306 nm can be resolved into three bands at 2275, 2308 and about 2360 nm. The deconvolution of this band was further confirmed by taking second derivative of the spectrum shown in Fig. 1. As illustrated in Fig. 1(B'), the second derivative spectrum clearly shows that the 2306 band is comprised of three bands at 2275, 2308 and 2360 nm. These bands are known to provide information on the α-helix, β-sheets and random coil structure of the protein [26]. However, C–H groups are known
to have combination bands in this region, namely the C–H stretching vibration coupling with CO stretch is reported to produce a band at 2306 nm [25]. The bands at 2306 and 2360 nm can, therefore, be assigned to a superposition of combination bands of C–H stretching and bending modes from the side chains and peptide backbone vibrations [26].

3.2. NIR Spectra of BSA Encapsulated in Sol–Gel

Spectrum of 366 mg/mL of BSA encapsulated in sol–gel, (following subtraction of the spectrum of the undoped gel [8, 9]) is shown in Fig. 1(C), together with its second derivative spectrum ((1′C)). It is evident that the spectrum of encapsulated BSA is different from that of BSA in solution in terms of shape and position of bands. Based on 2nd derivative spectrum and from resolution of bands in spectrum 1(C) and 1(D'), it is clear that encapsulated BSA exhibits bands which are similar but not identical to BSA in buffer: 2059, 2185, 2253, 2314 and 2370 nm compared to 2056, 2184, 2274, 2307 and 2366 nm for BSA in buffer. The most notable difference is the shift of solution band from 2274 nm to an encapsulated band at 2253 nm. Furthermore, the shape of these encapsulated bands is also somewhat different from those of solution bands. For example, the full width at half maximum (FWHM) of the 2184 nm band is 51 nm for BSA in buffer but 55 nm for encapsulated BSA. These results clearly indicate that the conformation of encapsulated BSA in sol–gel is different from that of BSA in buffer solution.

3.3. Inhomogeneity in the Concentration and Conformation of BSA Encapsulated in TMOS Sol–Gel

Different from spectrometers, the multispectral imaging instrument used in this work is based on the use of an area camera to record spectral images of a sample. The NIR area camera used in this work is equipped with 320 × 256 pixels. Due to its high sensitivity, data from a small area such as a square of either 3 × 3 pixels or even a single pixel can be used to calculate a spectrum at a single location in a sample. Consequently, the imaging spectrometer can simultaneously measure spectra at 81,920 different positions in a sample. For each position, spectra can also be calculated using data either from a single pixel or from an average of a square of pixels of various sizes such as 3 × 3, 7 × 7, 15 × 15 or 50 × 50. Alternatively, the same number of pixels can be used to calculate spectra at different positions. As described above, in this NIR spectral region, the spectra of the BSA contains not only information on the concentration but also on the conformation of the protein. As a consequence, imaging data recorded by this spectroscopic imaging instrument provide information on the concentration distribution as well as on the conformation of BSA molecules entrapped at different positions within the sol–gel sample. Accordingly, spectra of encapsulated 366 mg/mL BSA (with background contribution of the sol–gel [8, 9] subtracted) at three different positions were calculated from an average of a square of either 7 × 7, 15 × 15 or 50 × 50 pixels (denoted as A, B and C in Fig. 2). The results obtained are shown in Fig. 2 as A1–A4, B1–B4 and C1–C4, respectively. For each set of square pixels, the spectra were calculated at four different reaction times: 0, 120, 480 min and 24 hrs. As illustrated, spectra of entrapped BSA at different positions are the same within experimental error, regardless of an average of either a square of 7 × 7, 15 × 15 or 50 × 50 pixels were used for calculation. Furthermore, the spectra are not only the same at the beginning of the reaction (t = 0 min) but also during the sol–gel reaction up to 24 hrs.

Inhomogeneity among spectra begins to show up at lower BSA concentrations. This can be seen in Figs. 3 and 4 that show corresponding spectra of entrapped BSA at 220 and 40 mg/mL, respectively. As illustrated in Fig. 3 for 220 mg/mL, pronounced differences between the spectra were observed at the beginning of the reaction and continued up to 24 hrs. When smaller number of pixels were used (e.g., 7 × 7), not only that the differences among spectra are larger but also that the relative absorbance is larger than spectra calculated using larger number of pixels (e.g., 15 × 15 and 50 × 50). Further decrease in the protein concentration led to an increase in differences among the spectra as observed in Fig. 4 for 40 mg/mL of entrapped BSA. The relative differences among the spectra are larger at this concentration compared to those of 220 mg/mL, and the dependence of the inhomogeneity on reaction time differs as well. Namely, higher absorbance values and large differences among the spectra are encountered when smaller number of pixels was used especially at the early stage of the gelation (0 min) (Fig. 4(A1), (B1) and (C1)). Differences decrease concomitantly with the advance of the polycondensation process (24 hrs) (Fig. 4(A4), (B4) and (C4)).
Additional information on the concentration distribution and conformation of entrapped BSA can be obtained by comparing spectra at the same position but calculated using different number of pixels. Specifically, Fig. 5 show three sets of spectra (A, B and C) for entrapped 366 mg/mL of BSA. Each set contains four spectra at a single position. The three positions in three sets, A, B and C, correspond to the three positions used in Fig. 2. However, different from Fig. 2, for each set, four spectra were calculated for the same position using different number of pixels: 3 × 3, 7 × 7, 15 × 15 and 50 × 50. Again, for each set, the spectra were calculated at four different reaction times: 0, 240, 480 min and 24 hrs (A1–A4, B1–B4 and C1–C4). As expected, at this high concentration, spectra are the same within experimental error, regardless of number of pixels used for calculation and reaction time. When BSA concentration was reduced to 220 mg/mL,
pronounced differences were observed. For example as shown in Fig. 6, A1, at time $t = 0$, absorbance was found to be dependent on the number of pixels used to calculate the spectra, namely absorbance was found to be inversely proportional to the number of pixels used. The largest absorbance was found for the spectrum calculated using a square of $3 \times 3$ and the smallest was for $50 \times 50$. The results seem to suggest that at this concentration, entrapped BSA did not distribute evenly throughout the sol–gel sample. Consequently, a spectrum with relatively smaller absorbance will result when a larger number of pixels is used for spectrum calculation. This is because the average concentration of the BSA in a larger square of pixels is lower than
that in the smaller pixels square. Relative differences among spectra at one position (e.g., A1) are different from those at other positions (e.g., B1 and C1). This is not only as expected but also lends credence to our explanation. Specifically, because the entrapped BSA is inhomogenously distributed in the sol-gel, its average concentration is different at different positions and/or different numbers of pixels used to calculate the spectra.

At much lower concentration (40 mg/mL), encapsulated BSA exhibits different spectral behavior. Spectra at three different positions (A, B and C) calculated using different number of pixels (7 × 7, 15 × 15 and 50 × 50) are shown in Fig. 7. As illustrated, at positions A and C, compared to 15 × 15 and 50 × 50 spectra, the 7 × 7 spectra exhibits a shift from 2188 to 2200 nm compared to 15 × 15 and 50 × 50 data. Interestingly, no shift was observed for spectra at position B. Furthermore, in general, the spectra shown in Fig. 7 do not exhibit the significant pixel-dependent changes in absorbance observed for the 220 mg/mL BSA data series (Fig. 6). Additionally, different from
the 220 mg/mL sample, there are no differences in FWHM for 7 × 7, 15 × 15 and 50 × 50 spectra of the 40 mg/mL sample. Exact mechanism responsible for the differences observed for spectra of 40 mg/mL samples, and their relative differences from those of 220 mg/mL samples is still unknown at this time. Additional and detailed experiments are currently being designed and perform to understand these interesting results.

The spatial resolution of the imaging instrument was determined to be about 10 µm/pixel. Since the InSb camera is equipped with 320 × 256 pixels, this corresponds to a recording area of 3.2 mm × 2.56 mm of a sample. The exact size of inhomogeneous pocket within a sample was not accurately measured. However, based on the observations above, it can be concluded that the size of the inhomogeneous pockets can be as large as 30 µm × 30 µm.
In summary, it has been demonstrated that NIR multispectral imaging technique can be successfully used to determine concentration distribution and conformation of BSA entrapped in sol–gel. Interestingly, it was found that BSA molecules were inhomogenously distributed within the sol–gel matrix, independent of its concentration. However, when BSA was encapsulated at relatively high concentration (366 mg/mL), the encapsulation process does not seem to produce any observable changes in the conformation of the protein compared to that in aqueous solution. Conversely, when the concentration of BSA was decreased to 220 mg/mL, pronounced changes in the spectra of the protein were observed as a function of (sol–gel reaction) time. The observed inhomogeneity might be traced to both a non-uniform concentration distribution of BSA and changes...
Figure 7. Spectra of 40 mg/mL of BSA encapsulated in sol–gel. Spectra were calculated at four different reaction times, using different numbers of pixels for each of the three position (A, B and C), similar to those shown in Fig. 5 for 366 mg/mL of BSA.

in protein native conformation resulting from interactions between the charged protein and the silicate anions. Results obtained seem to suggest that the protein molecules might adapt themselves to accommodate evolving geometry and sites of the silica network. The differences observed in the BSA spectra at different concentrations may be primarily traced to the template effect of protein molecules leading to formation of networks with distinctly different kinetic history, structure and pore-size distribution. It is possible that these processes led to inhomogenous distribution of the protein molecules and their conformation in the sol–gel samples as well as to different gel architectures, which in turn affect the conformational stability of the entrapped BSA.

References