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Two new sealed sample cells for small angle x-ray scattering from macromolecules in solution and complex fluids using synchrotron radiation

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Two different vacuum tight sample cells for *in situ* temperature dependent small angle scattering from liquids are presented in this article. In the first one, the sample fills a 1 mm thickness gap sealed on both sides by two thin parallel mica windows (volume 300 μl). In the second one, the liquid is injected into a 1 mm cylindrical capillary tube (volume 130 μl). The cells are lodged into temperature controlled chambers directly connected to the beamline vacuum path. Several important improvements with respect to similar instrumentation previously reported are: (1) versatile application of the mica cell, that can be used for all types of samples (gels, liquid crystals, and dispersions in organic solvents) and (2) the design of the chamber for the capillary cell allows registration of wider angle data and a convenient replacement of the capillary tube after each experiment. Signal to background ratio and data reproducibility were tested using protein solutions. We give a brief report of scattering experiments performed with different protein samples and two-dimensional data collection. © *2004 American Institute of Physics.* [DOI: 10.1063/1.1804956]

I. INTRODUCTION

A very important step in the analysis of the experimental small angle x-ray scattering (SAXS) data is the subtraction of the background scattering. A correction routine that takes into account all the possible sources of parasitic scattering originated in the instrumentation (slits, windows, and air path sections) has been generally used applying a data treatment program available for most of the users of the SAXS beamline at the Laboratório Nacional de Luz Síncrotron (LNLS).[1] In some cases, though, this task proved to be somewhat difficult. Several protocols for subtraction of the intensity with and without the sample did not give a satisfactory corrected intensity at the very low angles, especially in cases in which the samples scattered weakly (e.g., polymer solutions and biological macromolecules). A large number of experimental tests were performed and the conclusion was that three important factors, (1) nonuniform sample thickness, (2) contribution to the scattering that came from the sealing windows of the evacuated beam paths, and (3) scattering from the air gaps on both sides of the sample holder, were responsible for nonreproducibility and low signal to background ratio of the measured intensities. Consequently, we concentrated our efforts in the construction of two vacuum tight cells that would eliminate these instrumental sources of error.

The construction of the instrumentation here presented was based on the description by Dubuisson *et al.* of a capillary cell built for the D24 SAXS facility at LURE-DCI, Orsay, France.[2] In order to be able to study not only liquid samples but also gel phases or liquid crystals two sample holders were designed to perform different types of experiments. For those cases in which the amount of sample available is not a limiting factor and when the viscosity of the liquid is somewhat high, the sample is placed into a 1 mm thickness chamber sealed by two very thin and parallel mica windows. The sample can be injected into this chamber or introduced into the sample space before sealing the mica windows. In the second cell, the sample is injected into a 1.0 or 1.5 mm cylindrical capillary glass tube (Borokapillarem Mark-Röhrchen für röntgenographische Aufnahmen, GLAS Co., Germany) and is more appropriate for experiments in which there is a small amount of sample available, as is generally the case with protein solutions.

In the tests described herein we show the reduction of the parasitic scattering in the spectra taken with the sealed vacuum cells. A considerable improvement of the SAXS intensity is observed when compared with experiments performed with disposable sample cells with Mylar windows. The reproducibility of the sample cell position (which re-

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mains in place and is not modified in each sample change), is an important factor, responsible for these very positive results.

Furthermore, given the importance of obtaining high-q data, one of the sample chambers was designed in such a way that a large angular range could be detected. It has been demonstrated that important information on the secondary structure of proteins can be obtained from scattering data when the measuring range is sufficiently large. By increasing the maximum accessible momentum transfer q, the resolution of the experiment will furnish information on the shape, folding and secondary structure of the protein.[3] The success of a series of experiments at the LNLS in which the high-q data was essential to determine the three-dimensional low resolution protein structure as well as to study the stability and conformational changes induced by denaturants were due to the use of the instrumentation described here.[4,5]

II. INSTRUMENTATION DESIGN AND DESCRIPTION

The main characteristics of the two sample holders are (1) the liquid sample is injected into a 1-mm-thick sealed cell or glass capillary tube, (2) the cells are installed in chambers directly connected to the vacuum path of the primary x-ray beam, avoiding any additional windows, (3) typical liquid sample volume required is between 130 and 300 μl, (4) the temperature of the samples can be controlled by a thermocouple linked to a thermal bath, and (5) for volatile liquid samples, the exit of the cells can be sealed to avoid evaporation. Moreover the sample can be changed without breaking the vacuum of the x-ray path and the cells can be flushed clean with a suitable solution and dried with nitrogen gas.

A. Special characteristics of the mica cell assembly

Figures 1(a), 1(b), and 1(c) show details of the mica chamber and the cylindrical stainless steel sample cell. The chamber (a) consists mainly of a cubic shaped aluminum piece (1) with a view port (2) on the top face. The cylindrical (40 mm in diameter) stainless steel cell containing the sample (b) is inserted into a cavity of the aluminum block lined with a hollow copper shell (4) that is connected to a thermal bath and allows the control of the sample temperature. The cylindrical stainless steel cell has a small sample space (1 mm thickness $\times$ 4 mm height $\times$ 8 mm horizontal length) with rounded corners sealed on both sides by two thin mica windows (25 μm). Liquid samples are injected into the cell using a syringe that fits into a 0.6 mm connecting hole, coincident with the cylinder axis [see detail (5) in Fig. 1(a)]. The syringe can stay in place during the exposure, sealing the entrance, and allowing the recovery of the sample for control after irradiation. A Teflon tube at the other end allows the passage of the liquid, can be capped to avoid evaporation or open to flush cleaning liquids and nitrogen gas to dry the sample chamber before injecting a new sample. Two flexible bellows connect the chamber to the beamline vacuum paths. An $X-Y$ translation stage driven by a pair of stepping motors are used to align the chamber with the x-ray beam. The cylindrical stainless steel cell can be

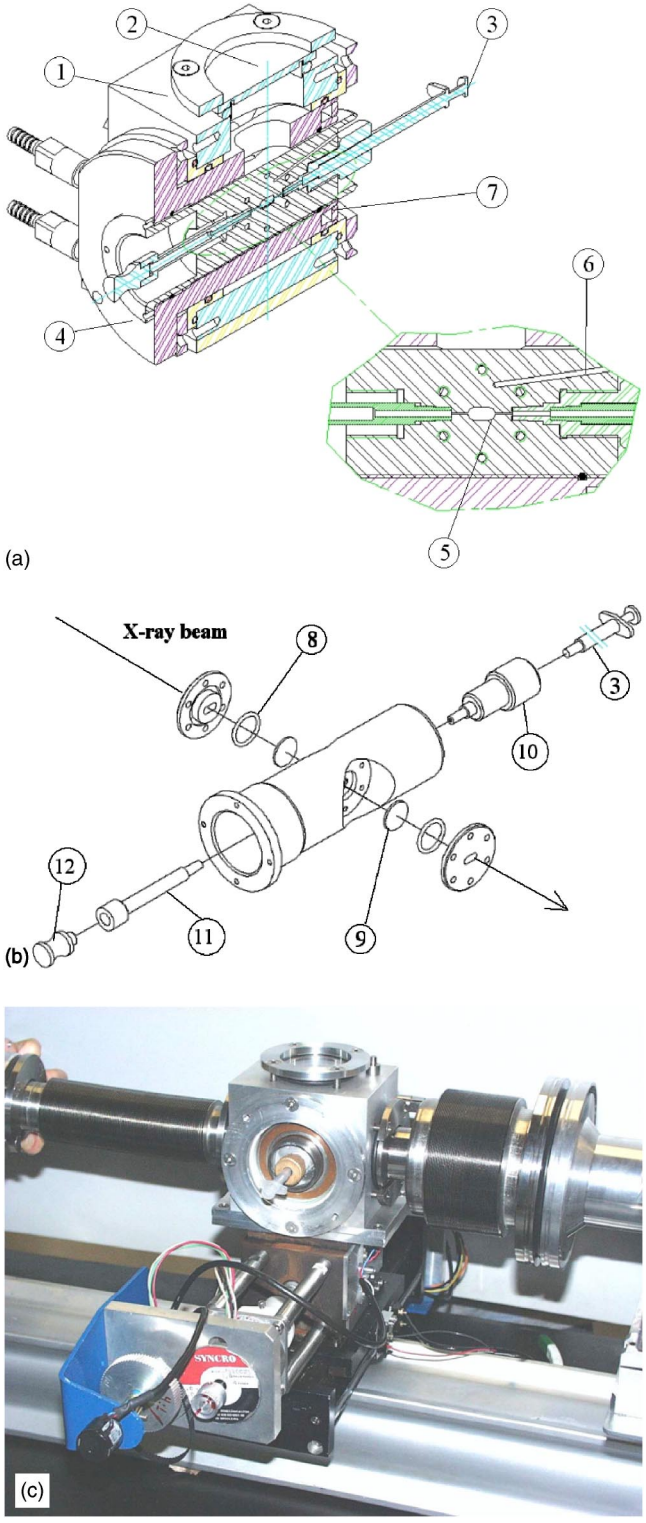

FIG. 1. (Color online) (a) Section view of the mica cell chamber: (1) aluminum block, (2) view port, (3) syringe, (4) hollow copper shell, (5) sample space, (6) thermocouple path; and (7) O-rings. (b) Details of the stainless steel sample cell: (8) O-ring, (9) mica windows, (10) Teflon adapter for syringe, (11) Teflon connector for drain hose or sealing cap, and (12) sealing cap. (c) Photograph of the sample holder installed at the SAXS beamline of the LNLS.

rotated 90 deg from the position in which the windows are perpendicular to the primary x-ray beam. In this new position the mica windows will be parallel to the view port on top of the chamber and the sample can be observed. After

checking if the chamber is completely filled and contains no bubbles, it is returned to the original position. The temperature of the sample can be preset between −6 and 90 °C or between 25 and 170 °C using different liquids in the thermal bath. The maximum scattering angle (2θ) accessible in this chamber is 14.3 deg. This mica cell has been used to study aqueous solutions and dispersions containing certain organic solvents (e.g., isopropanol, ethanol, toluene). For other organic solvents, preliminary tests of the O-rings, syringes, and other common sealing parts found in the market are advisable.

B. Special characteristics of the capillary cell assembly

Figures 2(a), 2(b), and 2(c) shows the schematic drawings of the vacuum tight capillary cell assembly. The capillary tube is mounted in a brass cylindrical core cell (5). When inserted into the cube-shaped aluminum chamber (1), this cell is surrounded by a hollow copper shell (3) connected to a circulation bath for sample temperature control. The chamber is mounted on an $X-Y$ translation stage for proper alignment and connected to the beamline vacuum path by simple contact with an O-ring (11) located in the supporting frame that is attached to the two flanges (12). Larger scattering angles are accessible with this cell (2θ–22 deg). A view port (13) on the upper side of the aluminum chamber is used to monitor the filling of the capillary tube when the liquid sample is being injected. The sample fills a 1.0 or 1.5 mm in diameter borosilicate glass capillary tube (4). Thin silicon tubes (8) are used to connect the syringe (2) to the capillary tube previously cut to size. When the capillary tube is mounted in the brass core, it is sealed by the pressure exerted by the O-ring (9) inserted over the silicon tube when the conical Teflon cap (7) is squeezed by the screwed metal cap [see details in Fig. 2(a)]. This capillary cell requires no sealing glue but demands a certain amount of patience to mount. For easier capillary tube replacement, an alternative capsule was designed, in which the capillary tube is glued with vacuum tight cement at both ends. Just as in the case of the mica cell, the successive samples are injected and can be recovered after the exposure. The capillary tube can be flushed with solvent, dried with nitrogen, and refilled. The geometry and smoothness of the capillary walls make this cell less susceptible to bubble formation usually occurring when injecting the sample or when liquid samples are submitted to higher temperatures. This fact was observed in a number of experiments in which the two different cells (mica and capillary) were used.

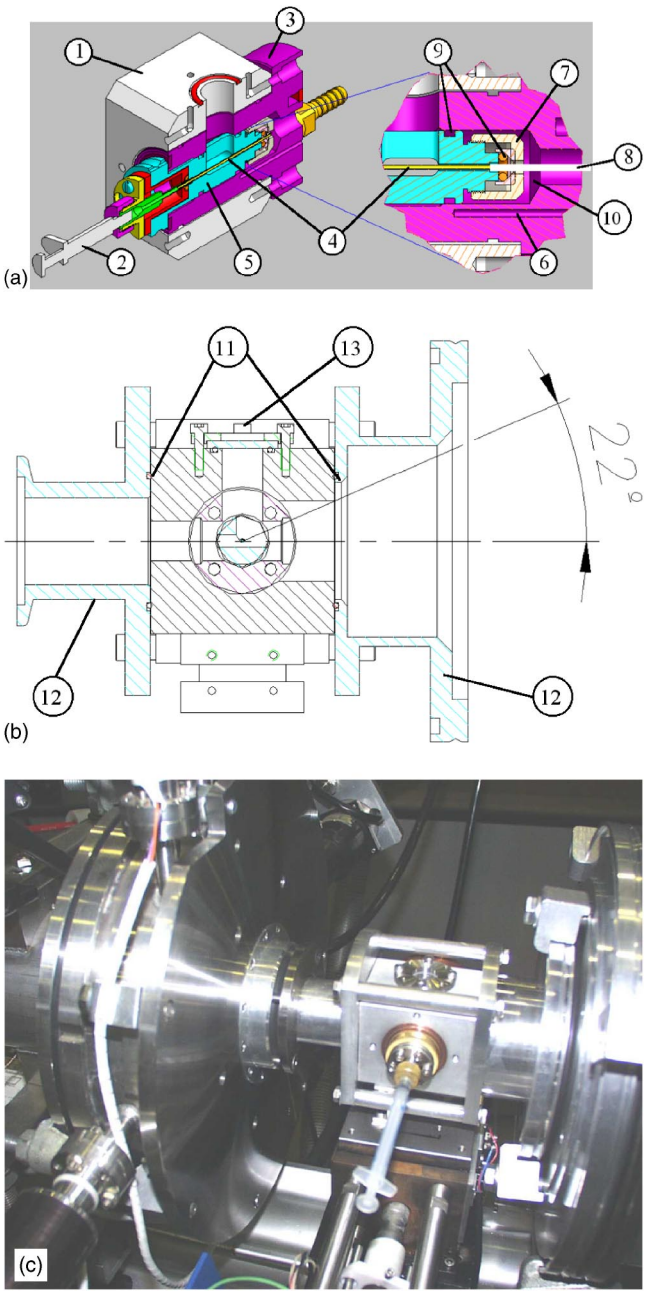

FIG. 2. (Color online) (a) Section view of the capillary cell chamber: (1) aluminum block, (2) syringe, (3) hollow copper shell, (4) capillary, (5) brass cylindrical core cell, (6) thermocouple path, (7) conical Teflon cap, (8) silicon tube, (9) O-rings, and (10) metal cap. (b) Details of the sample cell: (11) O-rings, (12) flanges, and (13) view port. (c) Complete sample holder installed at the SAXS beamline of the LNLS.

III. EXPERIMENTAL TESTS

In order to compare the performance of the new sample holders with the previously used setup, measurements of the parasitic intensity and reproducibility of the SAXS data of very well known systems were performed using the instruments here described and the previously used disposable acrylic cell of identical (1 mm) thickness sealed by two Mylar windows. This cell used to be placed in an $X-Y$ translation stage in an air gap of the beam path. Consequently, this setup had the disadvantage of the absorption and scattering from the air and, more important, the scattering contribution from the two Kapton windows of the vacuum path.

The experiments were performed at the D11A-SAXS beamline of the LNLS, Campinas, Brazil.[6] The scattering curves were recorded using a one-dimensional gas detector. Scintillation x-ray monitors placed before and after the sample measured the intensity of the incoming and transmitted x-ray beam intensity in order to determine the sample absorption. SAXS spectra were normalized by the integrated intensity of the direct beam. The SAXS curve were plotted as

a function of the modulus of the scattering vector $q = 4\pi \sin\theta/\lambda$, λ being the wavelength of the x-ray beam and θ half the scattering angle.

Two protein solutions of Albumin and Lysozyme (Sigma Co.) were prepared in three different concentrations (20, 10, and 5 mg/ml) to perform the first tests. The solvents were 0.01 M sodium acetate buffer and 0.05 M ammonium formiate buffer, respectively. Since these samples have well known scattering curves reported in the literature we considered them very appropriate to check the characteristics of the new sample holders. In addition, to test the measuring performance of the capillary cell in the high q range, scattering patterns of Lysozyme in solution (Tris buffer, 50 mM) were obtained using a two-dimensional (2D) multiwire gas detector. In all cases the samples were prepared by dilution from concentration controlled stock solution.

IV. RESULTS AND DISCUSSION

A. Low parasitic scattering contribution

In the experiments in which we compared quantitatively the intensity data obtained for the different sample cells we used Lysozyme in solution as a test sample and a linear position sensitive detector. For these experiments a monochromatic beam of wavelength $\lambda = 1.7433$ Åwas used. The signal-to-background ratio (S/B) defined as in Dubuisson *et al.*,[2] was used as a reference parameter

$$S/B = \frac{I(q)_{\mathrm{Lys}} - I(q)_{\mathrm{Buf}}}{I(q)_{\mathrm{Buf}}}, \tag{1}$$

where $I(q)_{\mathrm{Lys}}$ is the intensity from the Lysozyme solution and $I(q)_{\mathrm{Buf}}$ is the intensity from the buffer.

The results are shown in Fig. 3. The reduction of the camera background when the mica and the capillary cells are used is noticeable. This is attributed to the decrease of the parasitic intensity, which is a consequence of the elimination of the Kapton windows and air gap at the sample position. The lower parasitic scattering with the use of the new cells is responsible for a considerable increase in signal-to-background ratio at the very small angles. A (S/B) equal to 3.7 and 4.5 was obtained for the mica and capillary cells, respectively at $q = 0.07$ Å^{-1}. These values can be compared with a value $S/B = 1.7$ for the disposable Mylar cell.

B. Reproducibility of the SAXS intensity data

The test of reproducibility of the data when a sequence of identical samples is studied proved the good performance of the new sample holders. A set of three 10 min exposures filling the cell each time with the same Lysozyme solution (5 mg/ml) can be superimposed presenting a perfect coincidence. Usual corrections for the raw data and error propagation were done using the program TRAT1D.[1] The scattering curves for three samples prepared in the acrylic disposable cells with Mylar windows present an error of about 15%, which can be attributed to variable thickness of the irradiated volume as well as differences in cell position.

Other tests were performed with three different protein concentrations (20, 10, and 5 mg/ml) of Albumin solution. Since the scattering intensity for diluted samples is expected to be proportional to the concentration, intensities with ratios (4:2:1) were to be registered. The results were almost perfect for the new sample holders, but failed to match these ratios for the Mylar disposable cells (the same protein solutions were used in both tests; see Fig. 4). The advantages of having rigid windows and not having to reposition the sample holder for every exposure were then considered very important. Since the sample thickness is only 1 mm, a small deformation of the window material leads to large errors.

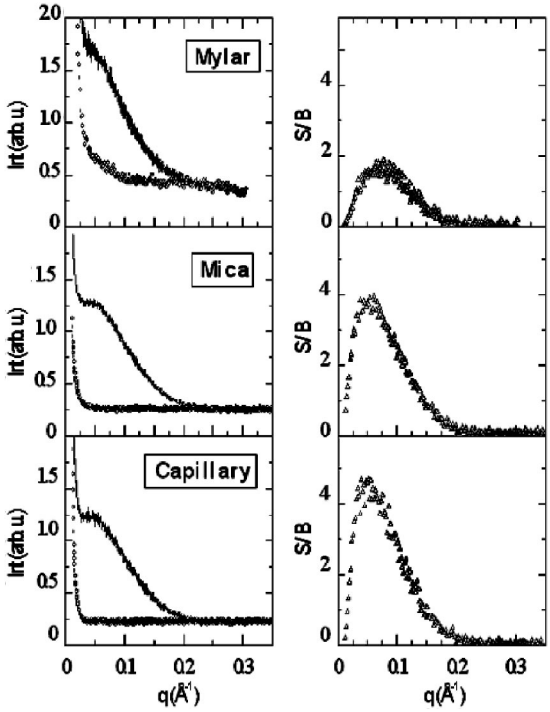

FIG. 3. Measured scattering intensity from a Lysozyme buffer solution (20 mg/ml) (full line) and from the buffer solution (open circles). The signal-to-background S/B ratio ($=[I_{\mathrm{Lys}} - I_{\mathrm{Buf}}]/I_{\mathrm{Buf}}$) is plotted for each sample holder: (a) disposable Mylar cell, (b) mica cell, and (c) capillary cell.

C. Testing the quality of the data obtained with a 2D detector

The samples used in this experiment were buffer solutions of three concentrations of Lysozyme (40, 20, and 10 mg/ml) to control possible concentration effects. Two-dimensional patterns were obtained using the 1.5 mm capillary tube sample holder, taking 15 min exposures at two different sample-detector distances (567 and 381 mm) and a wavelength of 1.488 Å. The data were recorded using a 2D multiwire gas detector (Gabriel type). The covered q range was $0.037 < q < 0.875$ Å^{-1}. Data treatment was performed using the software package TRAT2D.[7] The output of this software provides the corrected 2D intensity and error values. One-dimensional curves were obtained by radial integration. A fitting of these data was done using the program GNOM[8] and the results are shown in Fig. 5(a). A comparison was made between the observed scattering data and the theoretical curve calculated using the crystallographic coordinates for Lysozyme (6LYZ.PDB) by means of the program CRYSOL[9] for the same q range. The results can be seen in Fig. 5(b). An almost perfect coincidence is obtained for q values

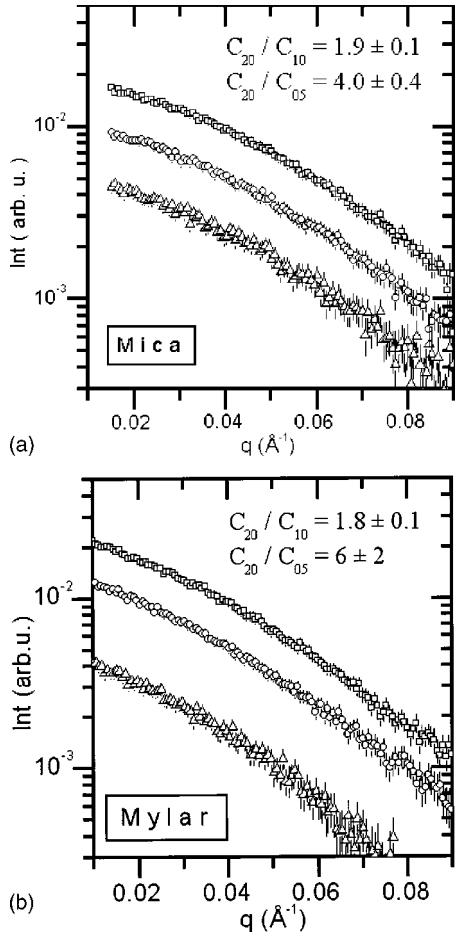

FIG. 4. SAXS intensity curves from three different concentrations of albumin in buffer solution for (a) Mica cell and (b) Mylar cell. □ 20, ○ 10, and △ 5 mg/ml. Intensity ratios should match concentration ratios. A good approximation to the 4:2:1 ratios is found for the mica cell.

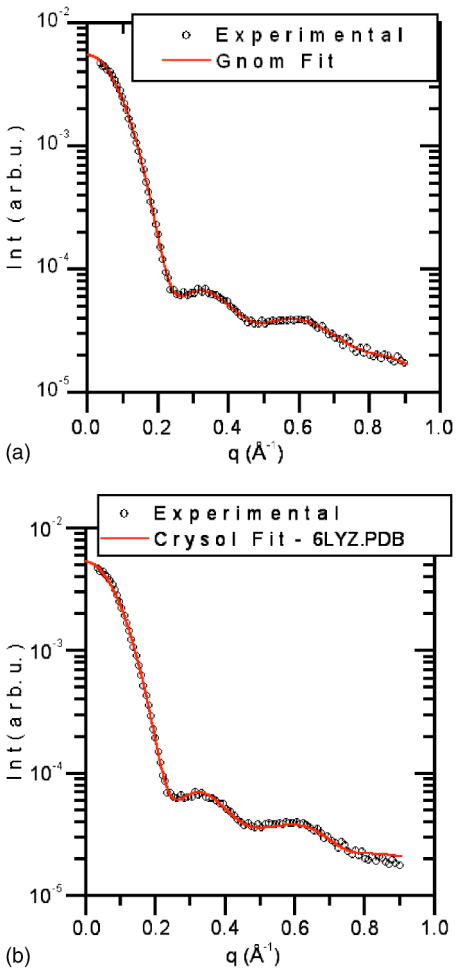

FIG. 5. (Color online) (a) One dimensional scattering curve obtained by radial integration of 2D scattering data from a Lysozyme buffer solution. The experimental points have been fitted using the GNOM program. (b) Comparison of the experimental data and the calculated scattering curve obtained from the crystallographic coordinates (6LYZ.pdb) using the CRYSOL program.

up to 0.8 $Å^{-1}$ with $\chi^2=2.29$. This remarkable coincidence between the experimental and calculated curves is due to the reliable background subtraction and high signal to background ratio obtained.

The use of the capillary cell combined with 2D data and radial integration was also very efficient in experiments designed to study the effects of chemical denaturants on the global compactness of proteins. For particles with compact shape and sharp interface, the so called Kratky plots (Iq^2) vs q show a well-defined curve with an initial upward portion followed by a descending curve. On the other hand, the curves for a polymer in an extended or random coil conformation show a characteristic plateau and rise for higher q values. These plots have been frequently used to monitor the unfolding of proteins. In Fig. 6 we show results obtained for the soluble fraction of the Amyloid Precursor Protein ($sAPP_{695}$) (taken from Botelho *et al.*[5]).

The capillary sample holder also allowed the recording of the SAXS intensity from large multilamellar vesicles of dipalmitoylphosphatidylcholine (DPPC) reaching the wide angle region. Using a wavelength $\lambda=1.3804$ Å and a sample-to-detector distance of 214.7 mm, data extended up to $q_{max}=1.45$ $Å^{-1}$ could be registered with a linear position-sensitive detector. The scattering included lamellar peaks

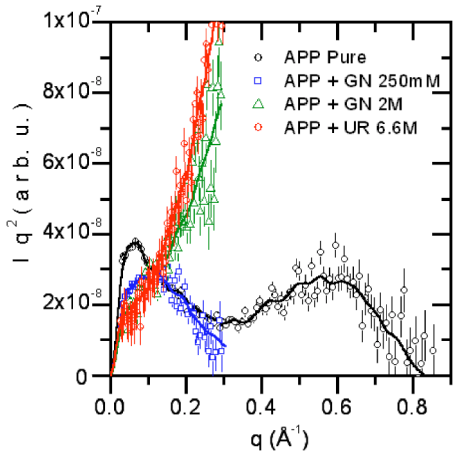

FIG. 6. (Color online) Kratky plot of a 12.5 mg/ml solution of APP in 50 mM TrisHCl buffer. Data obtained using the capillary cell. The plots show the effect of the denaturant agents (GdnHCl 0.25, 2.0, and urea 6.6 M) on the protein structure for a protein concentration of 3.6 mg/ml (from Ref. 5).

from the DPPC multilayers as well as the correlation peak from the ordered hydrocarbon chains in the system corresponding to a real space distance of 4.97 Å.

The versatility and reliability of these sample holders was also tested in other experiments with polymers and complex fluids performed with temperature control and *in situ* measurements.[10,11] Further details on the construction of the cells as well as the project designs can be obtained from the authors upon request.

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