Chapter 25

Analysis of S100 Oligomers and Amyloids

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Abstract

The S100 proteins are a large family of 10–12 kDa EF-hand signaling proteins that bind calcium, and in some cases zinc and copper, functioning as central regulators in a diversity of cellular processes. These proteins have tissue, cell, and subcellular-specific expression patterns, and many have an extracellular function. Altogether, these properties underlie their functional diversity and involvement in several pathological conditions including cancer, inflammation, and neurodegeneration. S100 proteins exhibit considerable structural plasticity, being able to exist as monomers or assemble into dimers, higher oligomers, and amyloids, frequently in a metal-dependent manner. Many of these oligomers are functionally relevant, and S100 amyloids have been recently found in prostatic inclusions. Here, we report experimental procedures for the isolation and quantitation of S100 oligomers from tissues, purification of recombinant human S100 protein for assays and use as standards, and an amyloidogenesis assay that allows monitoring the formation of S100 β-oligomers and amyloids in apo- and metal-bound S100 proteins.

Key words: S100 proteins, Brain tissue, Oligomer analysis, Recombinant S100, Thioflavin T, FT-IR, DLS, Amyloid kinetics, Metal ions, Calcium, Zinc

Abbreviations

ATR FT-IR	Attenuated total reflectance Fourier transform infrared spectroscopy
DLS	Dynamic light scattering
KPi	Potassium phosphate
OD	Optical density
ThT	Thioflavin T
SEC	Size exclusion chromatography

1. Introduction

The S100 proteins constitute a protein family involved in Ca^{2+} signal buffering and/or transduction in vertebrates, with an intra- and extracellular action. The relevance of their functions is highlighted by the fact that S100s are the largest subgroup of EF-hand

Einar M. Sigurdsson et al. (eds.), *Amyloid Proteins: Methods and Protocols*, Methods in Molecular Biology, vol. 849,
DOI 10.1007/978-1-61779-551-0_25,

Ca^{2+}-binding proteins, engaged in cell cycle regulation, growth, differentiation, and motility. Ca^{2+} binding (K_d in the micromolar range) typically mediates the exposure of hydrophobic surfaces to which downstream signaling partners bind to (1). Many S100 proteins are also structurally and functionally regulated by binding of other divalent metal ions such as Zn^{2+} and Cu^{2+} at distinct binding sites. These properties and the tissue and cell expression of the S100 proteins results in their involvement in processes related to inflammation, cancer, neurodegenerative disorders, and autoimmune diseases.

Oligomerization is determinant to S100 function. With the exception of monomeric S100G, all S100 proteins are homo- or heterodimers. S100 heterodimers exhibit distinct signaling properties and the functional and structural diversity of S100 proteins is expanded by a number of functional oligomers (2), including tetramers (S100B (3), S100A2 (4), and S100A8/A9 (5)), hexamers (S100B (3), S100A12 (6, 7)), and octamers (S100B (3)). The formation of these species is, in some cases, promoted by metal ion binding, and for S100B, Ca^{2+} promotes tetramerization and tighter interaction with RAGE (3).

Adding to the range of functional oligomers, S100A8/A9 have been recently shown to assemble into amyloid structures *in vivo*, associated with chronic prostate inflammation and cancer (8) in a Ca^{2+}- or Zn^{2+}-dependent manner. This finding has broadened the interest in S100 biology and pathology, as S100 amyloids may have yet unknown roles in human disease, possibly by interfering with physiological S100 functions (2). Indeed, a variety of human S100 proteins have since then been observed to be amyloidogenic (Botelho et al., unpublished observations).

Having in mind the relationship between the functional properties and the S100 oligomeric states, as well as the putative biomedical implications of amyloidogenic processes involving S100 proteins, this chapter details a series of protocols that define the experimental procedures for (a) the isolation and quantitation of S100 oligomers from tissues, (b) the purification of recombinant human S100 protein for assays and use as standards, and (c) an amyloidogenesis assay which allows monitoring the formation of S100 β-oligomers and amyloids in apo- and metal-bound S100 proteins.

2. Materials

Molar protein concentrations refer to the S100 subunit.

2.1. Analysis of S100B Multimers from Brain Tissue

1. 50 mM Tris–Cl, pH 8.0.
2. 20 mM Tris–Cl, 150 mM NaCl, pH 8.0.
3. C0mplete, EDTA free protease inhibitor cocktail (Roche).

4. Potter-Elvehjem homogenizer.
5. Superdex 75 prep grade (GE Healthcare) column (1.6 cm × 60 cm).
6. LIAISON Sangtec 100 assay (AB Sangtec Medical).

2.2. S100 Protein Expression

1. DYT medium supplemented with 0.2% glucose and 100 μg/ml ampicillin.
2. 1.0 M IPTG.

2.3. S100 Protein Purification

1. 50 mM Tris–Cl, pH 7.6.
2. 1.0 M $CaCl_2$.
3. 200 mM EDTA–NaOH, pH 7.6.
4. 20 mM Tris–Cl, 150 mM NaCl, pH 7.6.
5. DNAse I.
6. 1.0 M $MgCl_2$.
7. C0mplete, EDTA-free protease inhibitor cocktail (Roche).
8. Ultracentrifuge.
9. Phenylsepharose High Sub (GE Healthcare) column (1.6 cm × 15 cm).
10. Superdex 75 prep grade (GE Healthcare) column.

2.4. Preparation of Protein Solutions

1. 50 mM Glycine pH 2.5.
2. 100 mM $CaCl_2$.
3. 100 mM $ZnCl_2$.
4. 100 mM $CuSO_4$.
5. 1 M DTT, freshly prepared.
6. 50 mM EDTA (see Note 1).
7. Chelex 100 resin (Sigma).
8. Bradford's reagent.
9. HiTrap Desalting Column (GE Healthcare).
10. Amicon Ultra-4 Centrifugal Filters, cutoff 3 kDa (Millipore).
11. Centrifuge with swinging bucket rotor.
12. Benchtop centrifuge.
13. Water bath or dry bath.
14. UV–visible spectrophotometer.

2.5. Thioflavin T Fluorescence

1. Thioflavin T (ThT) solution: 65 μM ThT (Sigma) in 10 mM potassium phosphate (KPi) pH 7.0, 150 mM NaCl (see Note 2).
2. 10 mg/ml Lysozyme (Fluka) in 50 mM glycine pH 2.5 (see Note 3).

3. 0.22 μm Syringe filters, acetate membrane (e.g., Carl Roth A061.1).
4. 100 μl Multiply PCR tubes (e.g., Sarstedt Safecup, 72.733.200) (see Note 4).
5. Fluorescence cell with stirring (Hellma 119.004F-QS) and magnetic followers (e.g., Carl Roth 0973.1).
6. Spectrofluorimeter with magnetic stirring cell holder.

2.6. Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy

1. FT-IR spectrometer (e.g., Bruker IFS 66).
2. Thermostatized ATR cell suitable for liquid samples (e.g., Harrick BioATR II cell).

2.7. Dynamic Light Scattering

1. Syringe filters (0.22 μm), acetate membrane (Carl Roth).
2. Low volume cuvette (e.g., Hellma 105.215-QS).
3. Mineral oil, for PCR (Sigma).
4. DLS instrument equipped with cell thermostatization (e.g., Malvern Zetasizer Nano ZS).

3. Methods

3.1. Analysis of S100B Multimers from Brain Tissue

S100B forms oligomers in the human brain. To detect such oligomers, a method was designed that does not include any step with non-physiological pH or salt conditions.

1. Thaw brain tissue on ice.
2. Add ice cold 50 mM Tris–Cl, pH 8.0 containing protease inhibitors (C0mplete, Roche) at a 1:2 (v/w) ratio.
3. Homogenize the tissue on ice using a Potter-Elvehjem homogenizer.
4. Ultracentrifuge the brain homogenate at 100,000 × *g* for 60 min.
5. Filter the supernatant through a 0.2 μm membrane.
6. Load filtered supernatant to a size exclusion chromatography column Superdex 75 (GE Healthcare) and elute with 20 mM Tris–Cl, 150 mM NaCl, pH 8.0.
7. Eluted fractions are analyzed for S100B content using a commercial ELISA-based assay kit (LIAISON Sangtec 100 assay, AB Sangtec Medical) that uses a pair of monoclonal S100B antibodies for capture and detection of S100B (Fig. 1).

3.2. S100 Protein Expression

S100 proteins are expressed using *Escherichia coli* as expression host.

1. S100 protein cDNA is cloned into the pGEMEX plasmid by standard cloning techniques.

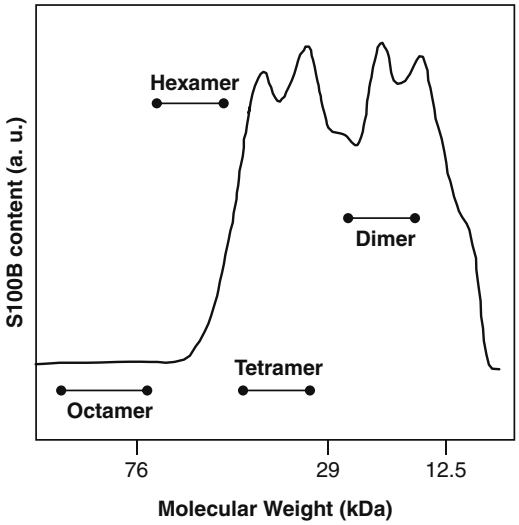

Fig. 1. S100B multimeric species are detected and analyzed by SEC following tissue extraction. The figure shows a typical elution profile of S100B from human brain extract in the presence of EDTA. Redrawn from ref. 3.

2. *E. coli* expression strain BL21(DE3) is transformed with the corresponding plasmid.
3. Select the transformants using solid media plates containing 100 μg/ml ampicillin.
4. Inoculate a single colony of the corresponding expression clone in 10 ml DYT containing 100 μg/ml ampicillin and 0.2% glucose.
5. The culture is grown on a shaker at 37°C overnight.
6. The overnight culture is used to inoculate the expression culture incubated at 37°C. Growth of *E. coli* is followed by OD at 600 nm.
7. As soon as $OD_{600\ nm} = 0.6$ is reached, expression is induced by the addition of 1 mM IPTG. The expression culture is harvested by centrifugation 3–4 h after induction. Cell pellets are frozen at −80°C and stored until further use.

3.3. S100 Proteins Purification

S100 proteins undergo a conformational change upon Ca^{2+} binding which results in exposing a hydrophobic patch for interaction with target proteins. This property is utilized for Ca^{2+}-dependent affinity chromatography on hydrophobic matrices, such as phenyl sepharose. This conformational change is fully reversible and bound protein can be eluted by complexation of the bound Ca^{2+} ions.

1. Frozen cell pellets are thawed on ice.
2. The cells are suspended 50 mM Tris–Cl pH 7.6.
3. A small amount of DNAse I is added to the cell suspension.
4. $MgCl_2$ is added to final concentration of 2 mM.

5. Cells are broken by one passage through a French pressure cell.
6. Crude extracts are subjected to ultracentrifugation at 100,000 × *g* for 1 h.
7. 2 mM $CaCl_2$ is added to the supernatant and subsequently applied to a phenylsepharose column equilibrated in 50 mM Tris–Cl, 2 mM $CaCl_2$, pH 7.6.
8. The column is washed with the same buffer until absorption at 280 nm reaches baseline again.
9. Bound S100 protein is eluted with 50 mM Tris–Cl, 4 mM EDTA, pH 7.6.
10. Fractions containing S100 protein are combined and concentrated by ultrafiltration.
11. Concentrated S100 is applied to a Superdex 75 column equilibrated in 20 mM Tris–Cl, 150 mM NaCl, pH 7.6.
12. Fractions containing S100 protein are combined, concentrated by ultrafiltration, shock-frozen in liquid nitrogen and stored at −80°C until further use.

3.3.1. Obtaining Reduced S100 Proteins

Most S100 proteins have cysteine residues which spontaneously oxidize to the disulfide form (9–11), a process which also occurs *in vivo* for some S100 proteins (12). This step is not required for characterizing S100 amyloidogenesis but must be used to infer the role of disulfides in the process (see Note 5). Since S100 solutions to be used in amyloidogenesis assays are prepared at pH 2.5, they should be manipulated at 4°C at all times to avoid aggregation.

1. Add 300-fold excess of DTT and 0.5 mM EDTA to the as-purified S100 stock.
2. Incubate 2 h at 37°C.
3. Desalt using a HiTrap desalting column equilibrated in oxygen-free 50 mM glycine pH 2.5 at 1 ml/min flow rate (see Note 6). Sample volume should not exceed 1.2 ml. Monitor the elution through the absorption at 280 nm and conductivity. The S100 protein will elute in the void volume of the column; DTT, EDTA and components of the initial buffer will elute at a later volume, as indicated by the perturbation of both the absorbance and conductivity traces.
4. Pool the fractions containing S100 protein, having caution not to include any DTT or EDTA (see Note 7).
5. Reconcentrate the S100 fraction by ultrafiltration using Amicon filters.
6. Centrifuge the concentrate at 12,000 × *g* for 10 min in a benchtop centrifuge at 4°C to pellet any aggregates which formed during concentration.
7. Remove the solution to a new tube, assuring that no pellet is collected.

Table 1
Absorption extinction coefficients (280 nm) for selected human S100 proteins, according to the oxidation state of the cysteine residues

		$\varepsilon^{280\ nm}$ ($M^{-1}.cm^{-1}$)	
Protein	**MW (Da)[a]**	**All cysteines**	**All cysteines**
S100A1	10,546	8,480	8,480
S100A2	10,986	3,230	2,980
S100A3	11,713	15,065	14,440
S100A4	11,729	3,230	2,980
S100A5	10,744	4,595	4,470
S100A6	10,180	4,470	4,470
S100A7	11,457	4,595	4,470
S100A8	10,835	11,460	11,460
S100A9	13,242	6,990	6,990
S100A10	11,187	3,105	2,980
S100A11	11,740	4,595	4,470
S100A12	10,575	2,980[b]	
S100A13	11,340	6,990[b]	
S100A14	11,662	7,115	6,990
S100A15	11,305	4,595	4,470
S100A16	11,801	12,950	12,950
S100B	10,713	1,615	1,490
S100G	9,016	1,490[b]	
S100P	10,400	2,980	2,980
S100Z	11,619	3,105	2,980

Calculated using Expasy (15)
[a] Subunit molecular weight
[b] Proteins containing no cysteines

8. Quantify the S100 solution spectrophotometrically (molar extinction coefficients are supplied in Table 1) or using Bradford's assay.
9. S100 stock solution for amyloidogenesis: amyloidogenesis is favored at higher protein concentrations. The final protein concentration should be at least 3.5 mg/ml (see Note 8). If needed, repeat steps 5 and 6 until the required concentration

is achieved. This is the stock protein solution which is used for preparing all samples in the amyloidogenesis assays.

10. If the S100 proteins are to be studied in the cysteine oxidized state, steps 1 and 2 are omitted (see Note 9).

3.4. Amyloidogenesis Assay of apo- and Metal-Bound S100

Many S100 proteins promptly form amyloids at pH 2.5 (50 mM glycine) at 3 mg/ml or higher concentration. This concentration is adequate for amyloid detection using a variety of biophysical techniques. Tenfold molar excess of metal ions over the S100 subunit (2.3–3.3 mM) is adequate for examining metal binding.

1. All buffers should be passed through a Chelex column to remove trace metal ions. The same applies to the water used in metal ion solutions.
2. Flasks should be previously rinsed in chelex-treated water before storing the trace metal ion-free solutions.

3.4.1. Assessing Amyloid Formation by Extrinsic ThT Fluorescence

The enhancement of ThT fluorescence is one of the hallmarks of amyloid species. Additionally, this assay provides a simple, inexpensive, and quantitative way of detecting amyloids in real-time and deriving kinetic data. Our fluorescence measurements are recorded using a Varian Cary Eclipse spectrofluorimeter.

1. Dilute the S100 protein stock to 100 µl at 3 mg/ml using 50 mM glycine pH 2.5 (see Note 10). Metal ions (Ca^{2+}, Zn^{2+}, Cu^{2+}) may be added as tenfold molar equivalents over the S100 subunit (2.3–3.3 mM, depending on the specific protein). Alternatively, 1 µl 50 mM EDTA is added to obtain apo protein (0.5 mM final concentration).
2. Dilute 5 µl of the S100 solution in 995 µl of ThT solution.
3. Homogenize the solution by inversion.
4. Incubate for at least 2 min at room temperature and in the dark. At pH 7, amyloidogenesis is inhibited and ThT fluorescence is stable for at least 3 h, allowing time for fluorescence measurements.
5. Measure ThT fluorescence at 482 nm: excite the solution at 440 nm using 10 nm excitation and emission slits, 600 V photomultiplier voltage and 0.5 s integration time while maintaining stirring (see Note 11).
6. This is the ThT fluorescence before amyloid formation is promoted. It should be identical to the fluorescence of a blank sample (see Note 12).
7. Incubate the S100 solution at 57°C under quiescent conditions.
8. After 48 h amyloidogenesis is mostly complete. At this time, homogenize the S100 solution by inversion and repeat

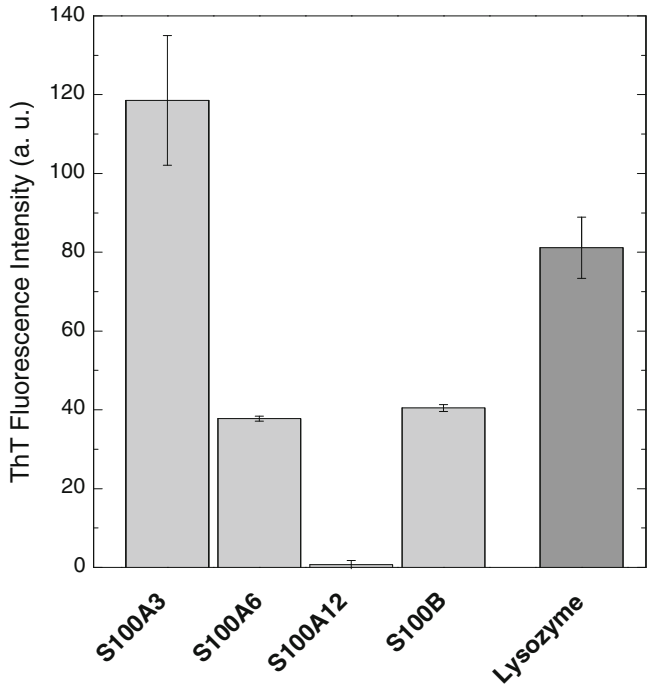

Fig. 2. S100 amyloidogenic species are identifiable by ThT fluorescence after incubation at 3 mg/ml, pH 2.5 and 57°C. For most S100 proteins, the aggregation process is complete after 2 days. AFM analysis further indicates that S100A3 forms amyloid fibrils, S100A6 and S100B assemble into β-oligomers and S100A12 forms non-amyloid oligomers (not binding ThT). Lysozyme (10 mg/ml) serves as a positive control for amyloid formation.

steps 2–5 in triplicate for measuring ThT fluorescence and assessing amyloid/β-oligomer formation.

9. Amyloid species are recognizable by ThT fluorescence emission at 482 nm (Fig. 2 and see Note 13).
10. To calibrate the fluorescence measurement, perform steps 2–9 with a 10 mg/ml lysozyme solution, a positive control for amyloid formation (13). After 10 days, the amyloidogenesis process is complete (Fig. 2).
11. This method can be adapted for obtaining kinetic data by repeating sampling as a function of time and representing ThT fluorescence intensity as a function of time (Fig. 3a, d).

3.4.2. Time Resolved Detection of S100 Oligomers and Amyloids with Attenuated Total Reflectance FT-IR

β-Sheet structures give rise to infrared absorption at specific wavenumbers in the amide I region according to the specific structure, rendering FT-IR an extremely sensitive technique for continuous monitoring of amyloid formation. Our FT-IR measurements are acquired using a Bruker IFS 66/S spectrometer equipped with a nitrogen-cooled MCT detector and a thermostatized Harrick BioATR II cell.

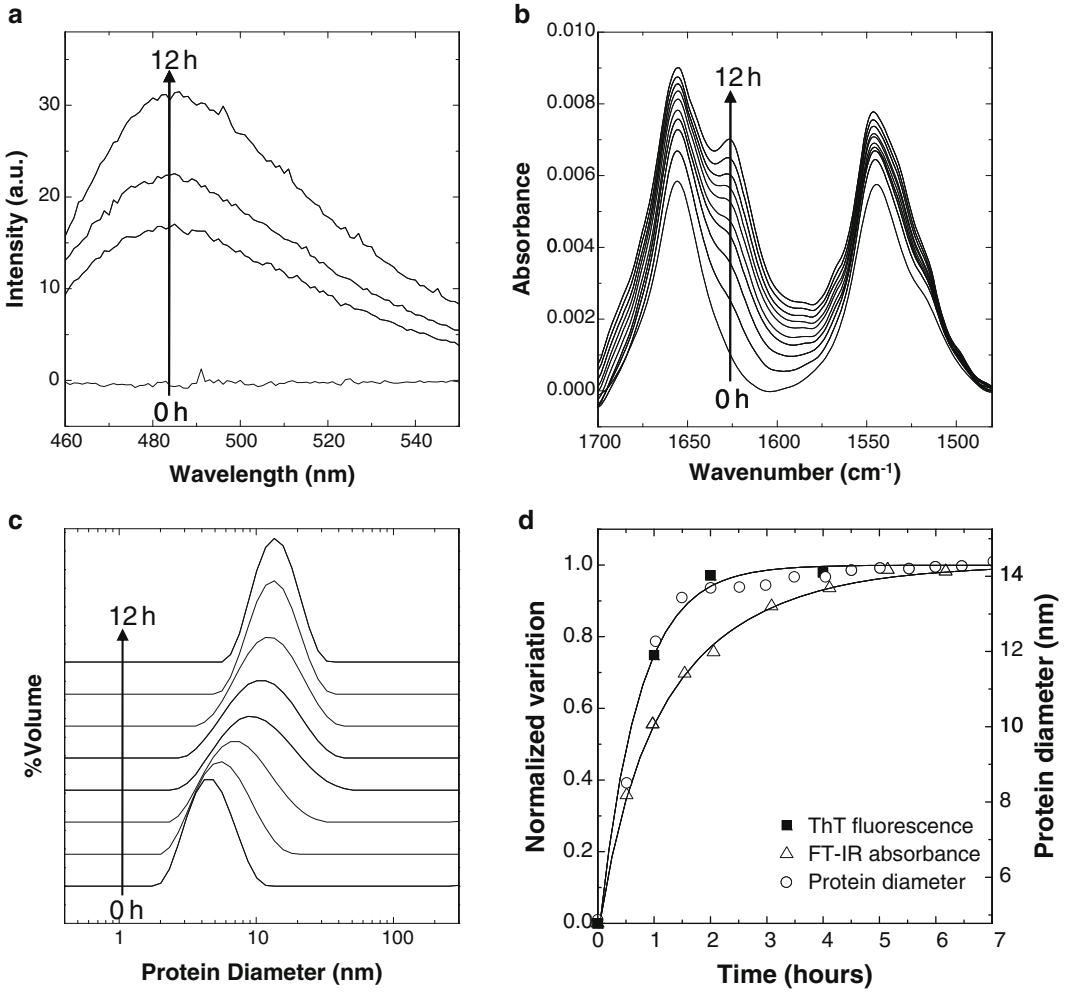

Fig. 3. Kinetic analysis of S100 amyloidogenic pathways. As an example, all data were acquired during incubation of apo S100A6 at pH 2.5, 57°C and 3 mg/ml (0.3 mM) protein concentration. (**a**) ThT fluorescence spectra acquired during the amyloidogenesis assay. The fluorescence band with maximum at 482 nm arises due to intercalation of ThT in β-sheet regions, which are absent in S100A6 in the absence of the thermal challenge. (**b**) Amide I/amide II ATR FT-IR spectra acquired during 12 h of incubation. The buildup of β-oligomers is indicated by the appearance of the band at 1,627 cm^{-1}. (**c**) Time-resolved size distribution as determined from a dynamic light scattering experiment. The distributions indicate the conversion of dimeric S100A6 (4.5 nm diameter) to β-oligomers (14 nm diameter). (**d**) Kinetics of apo S100A6 amyloidogenesis extracted from ThT fluorescence at 482 nm, the ration between infrared absorption at 1,627 cm^{-1} and the spectral maximum (1,655 cm^{-1}) and the DLS-determined protein diameter. Data are fitted with single exponential decay curves.

1. Setup the FT-IR spectrometer: turn it on for enough time to stabilize the light source, cool the MCT detector with liquid nitrogen, align the ATR cell and set the temperature to 57°C according to the manufacturer's instructions.
2. Set acquisition parameters as follows: 1,000–3,000 cm^{-1} spectral range, 4 cm^{-1} resolution, 2 min scan, 20 kHz scanner velocity, 12 mm aperture. Fourier transform may be done using a Blackman–Harris 3 Term apodization function, 5,687 phase

interferogram points, Mertz phase correction mode, and second-order zerofilling factor. Set the acquisition mode to continuous spectra acquisition.

3. Record the baseline spectrum: transfer the buffer solution (containing metal ions or EDTA at the same concentration as the S100 solution) to the ATR cell surface. Close the ATR and sample compartments. Record the spectrum.
4. Remove the blank solution and replace it by the S100 solution at 3 mg/ml (in 50 mM glycine pH 2.5).
5. Immediately start recording spectra.
6. Amyloid formation is assessed by the absorption ratio between the β-sheet band at 1,627 cm^{-1} and the amide I absorption maximum, around 1,655 cm^{-1} (Fig. 3b, d) (14).

3.4.3. Dynamic Light Scattering

Due to the intrinsic sensitivity of DLS to small amounts of aggregated protein material or dust particles, samples should be cleared of such contaminants. We use a Malvern Instruments Zetasizer Nano ZS instrument for DLS measurements. It is equipped with a Peltier-controlled thermostatized cell support. The manufacturer's software automates the autocorrelation analysis of time-resolved light scattering as well as the Mie Theory analysis of the data to extract multimodal particle size distributions.

1. Prepare 200 µl of a 3 mg/ml S100 solution in 50 mM glycine pH 2.5.
2. Filter the S100 solution with a 0.22 µm pore filter (see Note 14).
3. Pipet 50 µl of S100 solution to the DLS cuvette.
4. Carefully place 20 µl mineral oil on top of the solution. This will form an inert seal to prevent evaporation.
5. Setup the DLS instrument to average 15 10 s accumulations in the backscattering mode, using quadratic weighting and 0.01 regularizer. Set the Peltier temperature at 25°C.
6. Make a size measurement. This is the size of the S100 protein in the native-like state at pH 2.5, which should be around 4–5 nm (diameter).
7. Remove the cuvette and keep it at room temperature (see Note 15).
8. Quickly set the Peltier temperature to 57°C and set the instrument for continuous repeated measurements.
9. Replace the cuvette and start the measurement.
10. Retrieve the size distributions (by volume) and plot the distribution maxima as a function of time to obtain kinetic information (Fig. 3c, d). Scattered light intensity can also serve as a kinetic reporter.

4. Notes

1. EDTA is an acid and a 50 mM solution requires pH ~7.0. The pH should be raised by adding NaOH.
2. The 65 μM ThT solution is prepared by diluting a 2.5 mM stock solution which had been filtered though a 0.22 μm filter to remove undisolved particles. ThT should be stored in the dark to avoid photobleaching.
3. Diluting lyophilized lysozyme into 50 mM glycine pH 2.5 will yield a final solution with pH around 4.5. The solution pH should be adjusted to 2.5 with a concentrated HCl solution or lysozyme will not form amyloid under the conditions described. This is better achieved using a micro-pH electrode.
4. These tubes seal the sample, avoiding sample loss through evaporation.
5. S100A3 is an exception. The fully oxidized protein is highly disulfide cross-linked, forms macroscopic insoluble aggregates and requires resolubilization to be amenable to most spectroscopic analyses.
6. After desalting, the column should be washed with 1 M NaCl to remove S100 protein bound to the matrix and equilibrated in 20% ethanol for storage.
7. Both DTT and EDTA chelate metal ions and interfere in the metal binding assays.
8. The working concentration of S100 proteins in amyloidogenesis assays is 3 mg/ml. Preparing a S100 stock solution of at least 3.5 mg/ml allows diluting the solution with additives such as metal ion solutions.
9. For obtaining small amounts (<1 mg) of oxidized S100 proteins, an alternative protocol has been devised: dilute the S100 protein stock 50- to 100-fold in 50 mM glycine pH 2.5, reconcentrate using centrifugal filters and quantify the protein solution.
10. The assay may be upscaled provided that S100 solutions are incubated in tubes appropriate for their volume. Using tubes with capacity much larger than the S100 solution volume will promote sample evaporation at 57°C in amyloidogenesis assays.
11. Report the fluorescence value after subtracting a blank.
12. Most S100 proteins keep a native-like fold at pH 2.5 if kept at 4°C and do not bind ThT, not giving rise to any fluorescence. The α-helical native-like conformation can be assessed by far UV CD spectroscopy.

13. ThT fluorescence identifies both amyloid oligomers and amyloid fibrils. For knowing which type of amyloid structure is formed, the ThT results should be confirmed by Atomic Force or Electron Microscopy imaging.
14. To ensure dust-free conditions, purge the syringe filter with double-distilled water before filtering the S100 solution. Also, all tubes, pipette tips, and other material that comes into contact with the solution after filtration should be rinsed with double-distilled water and dried using a compressed air blower.
15. The cuvette with the S100 sample should be kept at room temperature just for the time required for the Peltier to reach 57°C. It should not be temporarily kept at a cooler temperature (e.g., 4°C) because temperature cycling may produce bubbles in the solution which interfere with DLS measurements.

Acknowledgments

This work was supported by grants POCTI/QUI/45758 and PTDC/QUI/70101 (to CMG) from the Fundação para a Ciência e a Tecnologia (FCT/MCTES, Portugal), by grants FR 1488/3-1 and FR 1488/5-1 from the Deutsche Forschungsgemeinschaft (DFG) (to GF). CMG and GF are recipients of a CRUP/DAAD collaborative grant A-15/08. HMB was a recipient of a Ph. D. fellowship (SFRH/BD/31126/2006) from Fundação para a Ciência e a Tecnologia (FCT/MCTES, Portugal). Ludmilla Morozova-Roche (Umeå University) is gratefully acknowledged for AFM imaging.

References

1. Santamaria-Kisiel, L., Rintala-Dempsey, A. C., and Shaw, G. S. (2006) Calcium-dependent and -independent interactions of the S100 protein family, *Biochem J 396*, 201–214.
2. Fritz, G., Botelho, H. M., Morozova-Roche, L. A., and Gomes, C. M. (2010) Natural and amyloid self-assembly of S100 proteins: structural basis of functional diversity, *FEBS J 277*, 4578–4590.
3. Ostendorp, T., Leclerc, E., Galichet, A., Koch, M., Demling, N., Weigle, B., Heizmann, C. W., Kroneck, P. M., and Fritz, G. (2007) Structural and functional insights into RAGE activation by multimeric S100B, *EMBO J 26*, 3868–3878.
4. Koch, M., Bhattacharya, S., Kehl, T., Gimona, M., Vasak, M., Chazin, W., Heizmann, C. W., Kroneck, P. M., and Fritz, G. (2007) Implications on zinc binding to S100A2, *Biochim Biophys Acta 1773*, 457–470.
5. Korndorfer, I. P., Brueckner, F., and Skerra, A. (2007) The crystal structure of the human (S100A8/S100A9)2 heterotetramer, calprotectin, illustrates how conformational changes of interacting alpha-helices can determine specific association of two EF-hand proteins, *J Mol Biol 370*, 887–898.
6. Moroz, O. V., Blagova, E. V., Wilkinson, A. J., Wilson, K. S., and Bronstein, I. B. (2009) The crystal structures of human S100A12 in apo form and in complex with zinc: new insights into S100A12 oligomerisation, *J Mol Biol 391*, 536–551.
7. Moroz, O. V., Antson, A. A., Dodson, E. J., Burrell, H. J., Grist, S. J., Lloyd, R. M., Maitland, N. J., Dodson, G. G., Wilson, K. S.,

Lukanidin, E., and Bronstein, I. B. (2002) The structure of S100A12 in a hexameric form and its proposed role in receptor signalling, *Acta Crystallogr D Biol Crystallogr* ***58***, 407–413.

8. Yanamandra, K., Alexeyev, O., Zamotin, V., Srivastava, V., Shchukarev, A., Brorsson, A. C., Tartaglia, G. G., Vogl, T., Kayed, R., Wingsle, G., Olsson, J., Dobson, C. M., Bergh, A., Elgh, F., and Morozova-Roche, L. A. (2009) Amyloid formation by the pro-inflammatory S100A8/A9 proteins in the ageing prostate, *PLoS One* ***4***, e5562.
9. Kordowska, J., Stafford, W. F., and Wang, C. L. (1998) Ca2+ and Zn2+ bind to different sites and induce different conformational changes in human calcyclin, *Eur J Biochem* ***253***, 57–66.
10. Raftery, M. J., Collinson, L., and Geczy, C. L. (1999) Overexpression, oxidative refolding, and zinc binding of recombinant forms of the murine S100 protein MRP14 (S100A9), *Protein Expr Purif* ***15***, 228–235.
11. Scotto, C., Mely, Y., Ohshima, H., Garin, J., Cochet, C., Chambaz, E., and Baudier, J. (1998) Cysteine oxidation in the mitogenic S100B protein leads to changes in phosphorylation by catalytic CKII-alpha subunit, *J Biol Chem* ***273***, 3901–3908.
12. Winningham-Major, F., Staecker, J. L., Barger, S. W., Coats, S., and Van Eldik, L. J. (1989) Neurite extension and neuronal survival activities of recombinant S100 beta proteins that differ in the content and position of cysteine residues, *J Cell Biol* ***109***, 3063–3071.
13. Morozova-Roche, L. A., Zurdo, J., Spencer, A., Noppe, W., Receveur, V., Archer, D. B., Joniau, M., and Dobson, C. M. (2000) Amyloid fibril formation and seeding by wild-type human lysozyme and its disease-related mutational variants, *J Struct Biol* ***130***, 339–351.
14. Calero, M., and Gasset, M. (2005) Fourier transform infrared and circular dichroism spectroscopies for amyloid studies. *Methods Mol. Biol.* ***299***, 129–151.
15. Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D., Bairoch, A. (2005) Protein identification and analysis tools on the ExPASy server, in *The proteomics protocols handbook* (Walker, J. M., Ed.), pp. 571–607, Humana Press.