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Detection of high molecular weight amyloid serum protein complexes using Biological On-Line Tracer Sedimentation (BOLTS)

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Abstract

The systemic amyloidoses are a rare, but deadly class of protein folding disorders with significant unmet diagnostic and therapeutic needs. The current model for symptomatic amyloid progression includes a causative role for soluble toxic aggregates as well as for the fibrillar tissue deposits. Although much research is focused on elucidating the potential mechanism of aggregate toxicity, evidence to support their existence *in vivo* has been limited. We report the use of a technique we have termed Biological On-Line Tracer Sedimentation (BOLTS) to detect abnormal high molecular weight complexes (HMWCs) in serum samples from individuals with systemic amyloidosis due to aggregation and deposition of wild-type transthyretin (senile systemic amyloidosis, SSA) or monoclonal immunoglobulin light chain (AL amyloidosis). In this proof-of-concept study, HMWCs were observed in 31 out of 77 amyloid samples (40.3%). HMWCs were not detected in any of the 17 non-amyloid control samples subjected to BOLTS analyses. These findings support the existence of potentially toxic amyloid aggregates, and suggest that BOLTS may be a useful analytic and diagnostic platform in the study of the amyloidoses or other diseases where abnormal molecular complexes are formed in serum.

Keywords

Analytical ultracentrifugation; Amyloidosis; Serum aggregates; Sedimentation; Transthyretin

Amyloidosis is a broad term used to describe a group of fatal disorders that are attributed to the misfolding, aggregation, and organ deposition of various soluble precursor proteins [1].

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[†]Susan F. Chase passed away on November 24, 2011. This work is dedicated to her memory.

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Although the biophysical properties of the soluble forms vary considerably, once aggregation has occurred, all precursor proteins adopt an identical and unique fibril ultrastructure referred to as amyloid [2]. Some precursor proteins such as transthyretin (TTR) in familial transthyretin amyloidosis (ATTR) contain destabilizing amino acid substitutions arising from single inherited genetic mutations [3]. In other cases, no apparent destabilizing structural feature of the circulating precursor has been identified. This latter situation is the case in senile systemic amyloidosis (SSA) where TTR-containing fibrils are the hallmark of disease. Unlike ATTR, SSA does not feature an inherited mutation in the TTR gene and the deposited fibril in SSA consists of C-terminal fragments and disulfide linked dimers of wild-type TTR [4; 5]. Another systemic amyloidosis which is more frequent and clinically the most severe, immunoglobulin light chain amyloidosis (AL), represents a more complicated precursor-deposit relationship because the involved protein, the immunoglobulin (Ig) light chain, is highly polymorphic, and it is unclear which amino acid substitutions confer the propensity to aggregate and form fibrils. In both SSA and AL amyloidosis, cardiac-related clinical features are common and furthermore, in patients with AL amyloidosis, cardiomyopathy is associated with a poor prognosis [6].

Historically, the onset of clinical symptoms and eventual failure of organ systems has been attributed to the deposition of amyloid fibrils, which can infiltrate tissues and disrupt cellular architecture. In addition to the amyloid protein, the deposits have been shown to include a variety of associated biomolecules including amyloid P component [7], glycosaminoglycans [8], and clusterin (also known as apolipoprotein J) which we have recently reported [9]. More recently, the concept that non-fibrillar toxic aggregates of the precursor protein could contribute to pathology has gained considerable favor [10]. This “toxic aggregate hypothesis” is supported by the clinical observation that the extent of fibril deposition does not always directly correlate with disease severity. We have shown that non-fibrillar Ig light chains purified from AL amyloidosis patient urine cause cardiac dysfunction in isolated murine hearts [11] and provoke oxidative stress and reduced contractility in cultured rat cardiomyocytes [12] through activation of p38 mitogen-activated protein kinase (MAPK) [13]. Others have found that small aggregates of recombinant wild-type and variant TTR are cytotoxic to neuroblastoma cells in culture [14; 15; 16]. In addition, annular or ring-like oligomeric structures of Ig light chains can be induced under fibrillogenic conditions [17; 18]. The “toxic aggregate hypothesis” has recently been reviewed [19].

Despite the growing evidence to support the existence of toxic aggregates in the systemic amyloidoses, such structures have never been observed in or purified from patient sera. The lack of biochemical data in the literature suggests that the structures are not readily amenable to purification and characterization. Such an observation could be attributed to metastability, where oligomerization is favored in the complex milieu of circulating blood, but is lost during the physical manipulation that may occur during purification such as dilution or removal of associated components. Furthermore, well established proteomic strategies that can detect trace quantities of disease-related serum components are frequently conducted under denaturing conditions or involve albumin depletion. Such conditions may disrupt assembly of the aggregated species by abrogating direct or indirect molecular interaction. Thus, there are currently no reported analytic strategies by which metastable molecular assemblies can be observed in order to provide direct support for the toxic amyloid aggregate hypothesis.

The analytical ultracentrifuge (AUC) provides a robust analytical platform for evaluating interacting molecular systems. AUC is a solution-based technique that monitors solute redistribution in a centrifugal field and requires no sample preparation or separation matrix. The sedimentation velocity method is routinely used in biotechnology to monitor aggregates in protein therapeutics [20; 21; 22; 23; 24] and offers a valuable alternative to more

frequently employed strategies such as size exclusion chromatography [20]. A key advantage of sedimentation velocity is the use of minimally diluted samples (where dilutions are only due to the sector shape of the sample holder) and the elimination of stationary chromatographic phase which may affect data interpretation due to matrix effects. These properties afford a unique niche for AUC in the investigation of reversible or metastable molecular assemblies. Until recently, AUC analyses have been restricted to simple solutions due to selectivity limitations of the available absorbance and interference optical systems. The recent availability of fluorescence optics for the AUC [25] allows the monitoring of target molecules within a high concentration of background species (for review see [26]). Quantitative analyses yielding information about molecular size and shape are still limited to simple solutions because the evolution of sedimenting boundaries with time in complex solutions cannot yet be modeled from first principles. However, previous analyses have suggested the utility of the technique for qualitative analyses of fluorescent proteins in complex solutions such as blood serum [27; 28] and *E. coli* cell lysates [29].

Here, we report the first use of AUC in the evaluation of high molecular weight complexes (HMWCs) in the serum of patients with amyloidosis. We have designed this study as a proof-of-concept for the technique, termed Biological On-Line Tracer Sedimentation (BOLTS), as well to test for the presence of amyloid protein aggregates in serum samples obtained from patients with two distinct forms of systemic amyloidosis: SSA and AL.

Materials and methods

Reagents and serum samples

Fluorescein reference standard was purchased from Invitrogen (Carlsbad, CA, USA). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human wild-type transthyretin (rTTR) was produced as described [30]. All other chemicals were of analytical grade and purchased from scientific supply houses. Patient serum was obtained with consent, in a protocol approved by the Institutional Review Board for human studies at Boston University Medical Campus and stored at -20 °C. Deidentified patient sera used in this study were collected between 1996 and 2006. Samples were grouped according to clinical information that was available from a password-protected database. Patients included in the SSA and AL amyloidosis groups were diagnosed with disease based on established clinical and laboratory testing criteria [31; 32]. The non-amyloid control (NA) group consisted of individuals, referred to the Amyloid Treatment and Research Program for evaluation, in which a diagnosis of amyloid disease was ruled out by negative histopathological and biochemical testing. As cardiomyopathy (CMP) is the major clinical feature in patients with SSA and a frequent finding in AL, the AL cohort was subdivided into two groups: patients with CMP as the predominant clinical manifestation of AL disease (AL-CMP) and patients with major extracardiac manifestations and no CMP (AL). Serum samples included in this study were obtained from patients at initial visit and prior to treatment for amyloidosis. Total protein concentration in the serum samples was determined by the BCA protein assay (ThermoFisher Scientific, Waltham, MA, USA).

BOLTS using fluorescein probe

A 100 µg/mL stock solution of fluorescein reference standard was prepared in DMSO. Three µL of this stock solution were added to 297 µL of serum or BSA at either 100 mg/mL or 0.1 mg/mL in 150 mM sodium chloride, 20 mM sodium phosphate, pH 7, and the mixtures were incubated at room temperature for ~1 hr before loading into ultracentrifuge cells assembled with either charcoal-filled epon velocity centerpieces (Beckman Coulter, Brea, CA, USA) or SedVel60K velocity centerpieces (Spin Analytical, Durham, NH, USA). For serum samples co-sedimented with rTTR, freshly-thawed 5 mg/mL rTTR stock was

diluted in 150 mM sodium chloride, 20 mM sodium phosphate, pH 7 to yield the appropriate 10 $\times$ concentration. Next, 30 μ L of this solution was spiked into a mixture of 267 μ L of serum and 3 μ L fluorescein. For all analyses, both sectors of the centerpieces were routinely filled with sample, as reference sectors were not required for these experiments. The cells were inserted into an An50Ti rotor (Beckman Instruments) and brought to experimental temperature (25 $^{\circ}$ C) in a Beckman Optima XLA analytical ultracentrifuge retrofitted with a prototype fluorescence optical system (commercial version available from Aviv Biomedical). Sedimentation velocity was conducted at 50,000 rpm and data were acquired with continuous scanning at 20- μ m radial increments, averaging 5 revolutions per scan. For all data sets, the programmable gain amplifier was set to 1 $\times$ and the photomultiplier was set at 73% gain. The centrifuge was controlled with the Advanced Operating System (available from the RASMB software archive).

Analysis of sedimentation data

For BOLTS experiments using fluorescein as a probe, the observed sedimentation patterns were categorized as either type I or type II based on the number of sedimenting boundaries. Type I patterns consisted of a single boundary that was consistent with that observed in 100 mg/mL albumin. Type II patterns consisted of either more than one boundary or a single boundary where the rate of sedimentation was at least two standard deviations greater than that of a corresponding 100 mg/mL albumin boundary. For all data sets, the apparent sedimentation coefficient (s) was determined qualitatively for each observable boundary from a plot of the natural log of the boundary midpoint (half-height of plateau signal) referenced to the meniscus position (r/r_m) as a function of ω^2t . Where indicated, data were analyzed with the continuous $c(s)$ size distribution model implemented in SEDFIT V9.4c [33]. Solvent parameters for dilute solutions were calculated from composition as implemented in the program SEDNTERP V1.09 [34].

Results and discussion

Fluorescein co-sediments with albumin in concentrated solutions

The binding of fluorescein by serum proteins, particularly albumin, is well established [35; 36]. Here, we investigated the potential of fluorescein as a general non-specific probe in BOLTS analyses of serum samples. Fluorescein is an attractive candidate for this purpose for several reasons. First, the absorbance band of the molecule overlaps the spectral output of the solid-state cyan laser used in the ultracentrifuge optical system. In addition, as a small molecule, the sedimentation of free fluorescein would be clearly differentiated from most fluorescein-protein complexes. As proof-of-concept, fluorescein was sedimented both in 0.1 mg/mL and 100 mg/mL solutions of BSA. In the dilute solution, the fluorescence trace (Fig. 1A) and $c(s)$ distribution (Fig. 1C) indicated the predominance of a small sedimenting component that was not clearly resolved in the size-distribution. These data were consistent with the behavior of free fluorescein. The corresponding experiment in concentrated albumin (Fig. 1B and D) indicated a single boundary sedimenting at $s = 2.5$ S. The poor residuals of the $c(s)$ fit in concentrated solution (Fig. 1D) are a result of the inability of the model to recapitulate the evolution of the complex, hydrodynamically non-ideal boundary. Despite the poor fit, the sedimentation coefficient returned from the superposition of Lamm equation solutions demonstrated a reasonable approximation to that obtained by boundary midpoint migration (*data not shown*). This observation is consistent with previous findings [27; 29] suggesting that ideal transport models can reliably capture the sedimentation coefficient of a non-ideal boundary, while the time-dependent spreading of the boundary is not reliably modeled. Taken together, these data indicate that fluorescein binds to (and sediments with) albumin, although the association is of low affinity.

Some amyloidosis serum samples contain high molecular weight complexes

To test the hypothesis that fluorescein-probed BOLTS could be used to detect abnormal high molecular weight complexes (HMWCs) in serum, clinical samples from amyloid groups (SSA, AL, and AL-CMP) and no amyloid (NA) controls were tested. In total, 94 sera were analyzed. A variety of BOLTS patterns were observed; however, there were shared common features and samples could be categorized based on the observed patterns. In general, all samples demonstrated between one and three sedimenting boundaries (Fig. 2). In some cases an additional inverted, slow moving boundary was apparent (Fig. 2, *right panel*). The reason for this peculiar feature is not immediately obvious, but is consistent with the sedimentation of a serum component that quenches the signal of unbound fluorescein and may result from the Johnston-Ogston effect, a sedimentation artifact arising from hydrodynamically non-ideal boundaries [37]. The two most divergent patterns are indicated in Fig. 2. Many samples demonstrated a pattern termed type I (*middle panel*), which resembled that of fluorescein-probed albumin (*left panel*). Boundary midpoint migration analyses confirmed the comparability of the sedimentation coefficients (2.3 S for albumin, 2.1–3.3 S for type I serum boundaries). This pattern was observed in samples from all four clinical groups. Conversely, some samples demonstrated a more complex boundary trace (type II, *right panel*) that indicated a rapidly sedimenting boundary at >10 S (s_3). Moreover, type II serum profiles frequently contained boundaries that migrated between the ~ 10 S boundary and the ~ 2.5 S albumin-like boundary (s_2). In many type II cases, the ~ 2.5 S boundary was either not observed or represented only a small fraction of the total signal. Significantly, a type II profile was not observed in any of the sera from the NA group or in the high concentration albumin controls. Thus, these boundaries appeared to be a characteristic of the amyloidosis groups and were attributed to the sedimentation of abnormal HMWCs present in the amyloid patient sera. Since these boundaries could not be fitted with ideal transport models, the size of these larger components could not be accurately determined. In dilute solution, a sedimentation coefficient of 10 S for a typical globular protein may indicate a molecular weight on the order of 200,000 Da. In a corresponding solution containing a high background concentration of smaller molecules (such as that of albumin in serum), a 10 S boundary would correspond to a much larger molecular species due to the transport of the target molecule within an increasingly dense concentration gradient of the background molecule.

The apparent sedimentation coefficients for each boundary in the BOLTS profiles were determined by boundary midpoint migration and tabulated for the four clinical groups. HMWC boundaries were seen in 31/77 (40.3%) of the amyloid sera. These boundaries were most common in AL-CMP serum, where over half of the samples subjected to BOLTS demonstrated type II sedimentation patterns (Table 1). As indicated in Figure 3, Type II patterns were less frequently observed in the AL and SSA samples and were not observed in any of the 17 NA samples that were tested. Since cardiac involvement in AL represents the shortest median survival from diagnosis among all amyloidoses [1], it is interesting to speculate if HMWC detection may correlate with severity of disease. Further studies are necessary to test this hypothesis.

High molecular weight complexes in SSA sera incorporate exogenous rTTR

In the above studies, fluorescein is a non-specific probe and it is unclear if detection is provided by binding to HMWC-associated albumin or to other HMWC components. Lending to this uncertainty, the biochemical composition of the observed HMWCs is unknown and cannot be determined from the above studies. Since fractionation is not possible with this technique, compositional analyses must be performed *in situ*. To investigate the composition of HMWCs, we evaluated whether exogenous rTTR could bind to HMWCs after spiking the protein into a type II SSA serum sample. We then monitored

the relative HMWC boundary fraction using fluorescein as a probe. This approach relies on the principle of mass action, in which it is assumed that an observed increase in boundary fraction corresponding to HMWCs is due to binding of the exogenous protein promoted through re-equilibration into the higher order structures. The serum concentration of endogenous TTR is approximately 0.2–0.4 mg/mL under normal conditions[38]. This concentration was supplemented with a range of up to four-fold higher concentration of rTTR. This exogenous protein was not expected to be amyloidogenic, since it requires partial denaturation in order to adopt the amyloid fold [39; 40]. However, the resulting BOLTS patterns (Fig. 4) clearly indicate a higher boundary fraction of HMWCs when rTTR was supplemented at higher concentrations. Assuming this effect was due to mass action, the results suggest that TTR is a component of the HMWCs in SSA sera and that the purported complexes are reversible (or at least able to adsorb exogenous rTTR) under the conditions employed.

Conclusions

We have tested the recently developed BOLTS platform as a methodology to detect HMWCs in serum samples from 77 patients with systemic amyloidosis and 17 controls. In this proof-of-concept study, we report that rapidly sedimenting boundaries (4–6 S and 9–13 S) occurred in 40.3% of the patient samples, but in none of the 17 controls. These findings suggest that the observed complexes may be involved with amyloid pathology. However, analyses of a greater number of control sera are necessary to conclude that HMWCs are unequivocally absent in the serum of healthy individuals. Complicating this issue is the fact that the sensitivity of BOLTS used in this manner is not known and is difficult to ascertain due to the apparent metastable nature of the HMWCs and the challenges involved with quantitative fluorescence-detected sedimentation [26]. It is possible that the observed HMWCs are ubiquitous, but are present at concentrations below the limit of detection except for in some cases. Strategies for determining the sensitivity limits and other quantitative attributes of this technology must be developed in order to realize the diagnostic potential of this analytic platform. Finally, this study provides evidence that in patients with TTR-associated amyloidosis, i.e. SSA, these complexes likely contain TTR since exogenous rTTR appeared to incorporate into the HMWCs, presumably due to mass action. Corresponding analyses using other amyloid precursor proteins are warranted in order to investigate the composition of HMWCs observed in the non-SSA amyloidoses, especially considering the prevalence of HMWCs in the AL-CMP group. Perhaps preferable to this mass action approach would be the development of more specific fluorescent probes to identify HMWC components *in situ*. These probes may be based on fluorescent derivatives of monoclonal antibodies, ligands, or inhibitors, which can bind to and sediment with the target proteins.

To our knowledge, this is the first report of abnormal complexes observed during direct examination of serum from patients with amyloidosis. These findings suggest that BOLTS may be useful as a diagnostic tool in the context of the amyloid diseases or other disorders where abnormal molecular complexes are formed. Further validation and additional studies will be required to characterize the attributes of BOLTS as a diagnostic assay, to determine the precise composition of the HMWCs detected by BOLTS, and to determine how these complexes correlate with the pathophysiology of amyloid disease.

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Fig. 1.

Fluorescein binds albumin with low affinity. Sedimentation traces for fluorescein in 0.1 mg/mL albumin (*A*) and 100 mg/mL albumin (*B*). Continuous $c(s)$ distributions for fluorescein in 0.1 mg/mL albumin (*C*) and 100 mg/mL albumin (*D*) with residuals bitmaps (above distributions). Distributions were obtained with a resolution of 80 sedimentation coefficients between 0.1 and 10 S and the resulting best-fit curves were not regularized.

Fig. 2.

Fluorescein binds to and sediments with various serum components. A solution of 100 mg/mL albumin (*left panel*) and two different serum samples (*center and right panels*) were analyzed by BOLT. The fluorescence traces (*top*) and determination of boundary midpoint sedimentation (*bottom*) are indicated. To facilitate visual comparison, the traces in each panel represent an identical window of acquisition time.

Fig. 3.

Serum samples from patients with amyloidosis contain HMWCs. BOLTS analysis was conducted on 77 serum samples from patients with amyloidosis and 17 with no evidence of amyloid disease. The sedimentation coefficients of observed boundaries were determined by midpoint migration. *Circles* indicate the value from the albumin-like position in type I (TI) boundaries and the slowest moving boundary in type II (TII) traces where the *s*-value fell within two standard deviations of the mean from the NA data set (2.6 S). For type II traces, *squares* indicate the *s*-value for the fastest sedimenting boundary and *triangles* indicate the *s*-value for the intermediate boundary. *Lines* indicate the mean sedimentation coefficient for each of the three boundary positions within each clinical group

Fig. 4. HMWCs in patient sera incorporate exogenous rTTR. A serum sample exhibiting a type II sedimentation pattern from a patient with SSA was spiked with either 17 µg (*left panel*) or 500 µg (*right panel*) rTTR and analyzed by BOLTS using fluorescein as a probe. The relative proportion of slower and faster moving boundary components, determined as the fraction of total fluorescence in early scans, is indicated.

Table 1Descriptive statistics and BOLTS results for control and amyloid sera^a.

	NA	SSA	AL	AL-CMP
<i>n</i>	17	24	25	28
Sex (M/F)	8/9	24/0 ^b	15/10	19/9
Age (y), mean ± SD (range)	52 ± 2 (32–72)	74 ± 1 ^c (63–84)	65 ± 2 ^c (43–79)	62 ± 2 ^c (41–82)
Total Protein	99 ± 4	111 ± 4	83 ± 4 ^d	84 ± 3 ^d
Concentration (g/L) % with HMWCs	0.0%	29.2%	36.0%	53.6%

^aVariables are listed as mean ± standard error of the mean.^bNo female patients were in this SSA group; this disease has been identified predominantly in men.^cStatistically higher than the NA group (*t*-test) with *p* < 0.005^dStatistically lower than the NA group (*t*-test) with *p* < 0.01