THE JOURNAL OF PHYSICAL CHEMISTRY B

B: Biophysical Chemistry and Biomolecules

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J. Phys. Chem. B, **Just Accepted Manuscript** • Publication Date (Web): 18 Apr 2018

Just Accepted

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Modulation of the Formation of Aβ and Sup35NM Based Amyloids by Complex Interplay of Specific and Non-Specific Ion Effects

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ABSTRACT

In vitro formation of highly ordered protein aggregates, amyloids, is influenced by the presence of ions. Here, we have studied the effect of anions on amyloid fibril formation by two different amyloidogenic proteins, human Amyloid Beta-42 ($A\beta_{42}$), associated with Alzheimer disease and produced recombinantly with a N-terminal methionine (Met-$A\beta_{42}$), and histidine tagged NM fragment of Sup35 protein (Sup35NM-His_6), a yeast release factor controlling protein-based inheritance, at pH values above and below their isoelectric points. We demonstrate here that pH plays a critical role in determining the effect of ions on aggregation of Met-$A\beta_{42}$ and Sup35NM-His_6. Further, the electrophoretic mobilities of Met-$A\beta_{42}$ and Sup35NM-His_6 were measured in the presence of different anions at pH above and below the isoelectric points to understand how anions interact with these proteins when they bear a net positive or negative charge. We find that while ion-protein interactions generally follow expectations based on the anion positions within the Hofmeister series, there are qualitative differences in the aggregation behavior of Met-$A\beta_{42}$ and Sup35NM-His_6. These differences arise from a competition between non-specific charge neutralization and screening effects and specific ion adsorption and can be explained by the different biochemical and biophysical properties of Met-$A\beta_{42}$ and Sup35NM-His_6.

INTRODUCTION

Amyloids are highly ordered fibrous aggregates composed of proteins. The infectious versions of amyloids which can be transmitted from one organism to another are called prions (Proteinaceous Infectious Particles).[1] Amyloids and prions are involved in several neurodegenerative diseases in mammals, namely Alzheimer disease, Parkinson disease, Creutzfeldt-Jakob disease, kuru, bovine spongiform encephalopathy, scrapie, chronic wasting disease, as well as other diseases such as type-II diabetes or atherosclerosis[2]. Several prions have also been identified in yeast.[3-7] However, whether yeast prions cause disease like mammalian prions is a topic of debate in the scientific community.[8-9] In any case, yeast prions act as non-Mendelian elements of inheritance.[10-12] Amyloids or amyloid-like protein assemblies also perform biological roles such as scaffolding of covalent polymers (e.g. in melanin synthesis), formation of biological structures (e. g. spider silk), and long term memory in shellfish, *Drosophila* and mice.[13-17] Amyloid-based assemblies also possess a technological potential. Consequently, there is a lot of interest in studying the properties of this unique class of proteins.

The amyloid aggregation process is dependent on two main factors, namely, the primary sequence of the protein and environmental conditions. For the same protein sequence, changes in temperature, pH, solvent composition, or agitation have a clear impact on the aggregation kinetics and the structure of the fibrils formed.[18-21] The fibril structural patterns in turn determine strain properties which govern disease patterns in mammals. Therefore, an investigation of the effect of environmental factors on aggregation behavior and kinetics can help in understanding the root cause of differences in disease progression, and may be important for the *in vitro* assembly of amyloid for technological purposes.

Ions can be classified on the basis of their effect on protein solubility and conformation. The Hofmeister series, originally developed by the Czech pharmacist Franz Hofmeister in 1888 from observations on the ability of cations and anions to precipitate hen egg white lysozyme, serves as a guide to the effect of ions on protein stability.[22] Today, these series have been extended to other ions. The Hofmeister series of anions is shown below.

$$SO_4^{2-} > H_2PO_4^- > IO_3^- > F^- > CH_3COO^- > Cl^- > Br^- > I^- > NO_3^- > ClO_4^- > SCN^- \quad (1)$$

The ions in the series are arranged according to their ability to salt out (precipitate) and salt in (solubilize) most proteins. On one end of the series, ions like SO_4^{2-} and $H_2PO_4^-$ are strongly hydrated and are historically referred to as 'kosmotropic ions' or 'kosmotropes' because they were originally thought to promote water structure (order). Similarly, ions like ClO_4^- and SCN^-, on the other end of the series, are weakly hydrated and have a strong tendency to adsorb to hydrophobic surfaces and interfaces They are still commonly termed "chaotropic ions" or "chaotropes" in reference to the historic belief in their ability to disrupt water structure.

The presence of ions also affects the viscosity of bulk water, wherein weakly hydrated chaotropic ions result in reduction in viscosity with increase in concentration and the strongly hydrated kosmotropic ions result in a viscosity increase with increase in concentration and the position of ions in the Hofmeister series has been shown to correlate with the Jones-Dole viscosity B coefficient.[23-25]

Ions play a crucial role in many biological functions. Ion-specific effects are usually found to be more pronounced for anions than cations which are generally excluded from the

protein-water interface and exhibit less pronounced Hofmeister effects[26]. While ionic concentration and pH are tightly regulated in the body to ensure proper biological functioning, any variations in the localized ionic concentration and pH can significantly affect the stability of proteins and may promote disordered aggregation as well as amyloid formation. Therefore, an understanding of the effect of ion concentration and pH on protein stability and aggregation can shed light on the causal factors of amyloid formation in living organisms.

Previous work from our group has shown that sodium salts of different anions can alter the aggregation kinetics and the structure of the amyloid aggregates formed by the prion domain containing NM fragment of Sup35 protein from *Saccharomyces cerevisiae* at a pH of 7.4 depending upon their position in the Hofmeister series.[18-19] In a separate study on species barriers between closely related species of the *Saccharomyces sensu stricto* group, we have shown that anions can be used to form seed 'strains' or 'variants' of Sup35p with different cross-species transmissibilities.[27] The effect of ions on nucleation and elongation, and on fibril conformation has also been shown for a few other amyloidogenic proteins such as amylin, α-synuclein, β2-microglobulin, mouse prion protein, or $A\beta_{40}$.[20, 28-31] However, a clear and comprehensive understanding of these effects keeping in consideration the charge on the protein at the experimental conditions has not been presented. Moreover, a comparative study of these effects on different proteins has not been performed.

In this work, we have investigated and compared the effect of anions of the Hofmeister series on the aggregation kinetics of Sup35NM and $A\beta_{42}$ based proteins. $A\beta_{42}$ is a 42 amino acid-long peptide formed by the proteolytic cleavage of amyloid precursor protein (APP), and is considered to be the primary constituent of the fibrillar amyloid plaques associated with Alzheimer disease.[3, 32-33] Recent structural studies of $A\beta_{42}$ fibrils by two separate groups have

shown that in the fibrillar form, the peptide buries its hydrophobic residues in the core of symmetrical dimers that are perpendicular to the axis of the fibril.[34-35] The residues 15-42 were shown to form the amyloid fold with four β-strands and residues 1-14 were shown to be unstructured[34] or partially ordered[35]. Sup35NM, on the other hand, is thought to form fibrils with a single molecule per layer primarily through interactions of glutamine and asparagine residues in the N-terminal prion domain of the protein.[36] Both proteins have been shown to form aggregates composed of parallel in-register beta sheets.[34-38]

To the best of our knowledge, there has been no detailed investigation of the effect of anions on $A\beta_{42}$ till date. Here, we have studied the aggregation of Sup35NM with a 6-histidine tag attached to the C-terminal of its non-amyloid forming M-domain and $A\beta_{42}$ with an N-terminal methionine in the presence of sodium salts at pH values of 3.2, 4.5, and 7.4. The aggregation properties of the Sup35NM have been shown to be unaffected by the addition of the C-terminal 6-histidine tag. Similarly the aggregation properties of the $A\beta_{42}$ peptide were shown (and confirmed by us) to be unaffected by the additional methionine which is present at the N-terminus of the non-amyloid forming region of $A\beta_{42}$.[34, 39] Therefore, the presence of these extra amino acids at the ends of the non-amyloid forming regions of $A\beta_{42}$ and Sup35NM is expected to have no significant impact on their aggregation behavior. The biophysical properties of tagged and untagged Sup35NM and $A\beta_{42}$ with and without the N-terminal methionine and their amyloidogenic domains are compared in Table 1 below.

Table 1. Theoretical properties calculated using ProtParam[40]

	Met-Aβ$_{1-42}$	***Aβ$_{1-42}$***	***Aβ$_{15-42}$***	***Sup35NM-His$_6$***	***Sup35NM***	***Sup35N***
Total number of residues	*43*	*42*	*28*	*259*	*253*	*123*
Theoretical pI	*5.3*	*5.31*	*6.07*	*6.01*	*5.3*	*7.81*
Aliphatic Index	*95.12*	*97.38*	*132.14*	*33.94*	*34.74*	*8.05*
GRAVY (Grand average of hydropathicity)	*0.244*	*0.205*	*1.086*	*-1.633*	*-1.596*	*-1.915*

The calculated isoelectric points (pI) of Sup35NM and Aβ$_{42}$ nearly superimpose, at pH 5.30 and 5.31, respectively; the isoelectric point of Sup35NM-His$_6$ was experimentally confirmed to be 5.3.[18] Even the theoretical pI values of Sup35NM-His$_6$ and Met-Aβ$_{42}$ are relatively close to each other (see Table 1) for the purpose of selecting pH values for studying aggregation and protein-ion interactions. In the present study, we observe several similarities in the effect of ions on the fibrillation kinetics of the two proteins, pointing to the universal nature of the effect of ions, as well as some differences which can be correlated to their biophysical and biochemical properties and points to differences in the aggregation mechanisms of the two proteins.

MATERIALS AND METHODS

Expression and purification of Sup35NM-His$_6$. *E. coli* host strain HMS174 (DE3) pLysS (Novagen) was transformed with pET21b vector containing the NM domain coding region of Sup35p from *Saccharomyces cerevisiae* with an attached C-terminal His$_6$ tag.[41] Sup35NM-His$_6$ was expressed and purified as described previously.[19] Briefly, the cells were transformed with the cloning vector, protein expression was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG), and the cells were harvested after about 4 hours of induction at 37°C. The cell pellets were stored at −80°C until purification and the protein was purified by Ni-NTA His-tag affinity purification under denaturing conditions. The purified protein was precipitated using cold methanol at -20°C, the protein pellet was collected by centrifugation and washed with cold methanol, and finally stored at -80°C in 80% methanol.

Expression and purification of recombinant Met-Aβ$_{42}$. Gene encoding Aβ$_{42}$ peptide form Plasmid pcDNA3.1(+)-Aβ$_{42}$[42] was amplified using primers (5′-GCGCGCGC CC ATG GAT GCA GAA TTC CGA -3′ (forward) and 5′-GCGC CTC GAG TTA CGC TAT GAC AAC ACC-3′ (reverse)) and cloned into pET28 vector using restriction enzymes, NcoI and Xho1 (New England Biolabs Inc.). *E. coli* host strain BL21 STAR (DE3) (Invitrogen) was transformed with pET28 vector containing the gene encoding Aβ$_{42}$. Met-Aβ$_{42}$ was purified by a modified protocol adapted from Walsh et al. 2009.[39] This strategy results in a Aβ$_{42}$ peptide with an additional N-terminal methionine as the nascent peptide aggregates rapidly after being synthesized and is directed to inclusion bodies, and therefore does not undergo post-translational N-terminal methionine cleavage. The presence of the extra methionine at the N-terminus was shown to not affect the aggregation kinetics and fibril morphology.[39] Anion exchange chromatography was performed using Q-Sepharose resin (Sigma Aldrich). Fractions containing

pure peptide, determined using SDS PAGE analysis, were pooled together and concentrated using a 3 kDa centrifugal filter (EMD Millipore). The concentrated peptide sample was then buffer exchanged into 2 mM NaOH[43], lyophilized, and stored at −80°C.

Fibrillation assays using Thioflavin T. Sup35NM-His_6 protein pellet stored in 80% methanol at -80°C was collected by centrifugation. The supernatant was discarded and the protein was resuspended in 8 M urea. Sup35NM-His_6 was then concentrated by 3 kDa centrifugal filter (EMD Millipore) and diluted 100-fold into the buffer of choice to a final concentration of 20 μM. The protein concentration was determined from absorbance at 280 nm using an extinction coefficient of 1.045 (ml mg^{-1} cm^{-1}). The Sup35NM-His_6 samples were boiled for about 10 min before starting the aggregation experiments to break down any preformed aggregates.

To prepare Met-Aβ_{42} for fibrillation assays, lyophilized Met-Aβ_{42} was re-suspended in HPLC grade water. The peptide was then filtered through a 30 kDa centrifugal filter to obtain the low molecular weight fractions and diluted with 10X buffer to a final concentration of 40 μM. The peptide concentration was determined using micro BCA assay (Pierce).

A stock solution of 1 mM thioflavin T (ThT; Sigma Aldrich) was prepared fresh in the buffer. Aggregation experiments were conducted in triplicates in a clear flat-bottom 96-well plate (Greiner CELLSTAR) with final ThT, Sup35NM-His_6, and Met-Aβ_{42} concentrations of 10 μM, 10 μM, and 20 μM, respectively, containing 0.1 M, 0.3 M, and 0.5 M sodium salt. The solutions at pH 3.2, 4.5, and 7.4 contained 20 mM sodium citrate, 20 mM sodium acetate, and Phosphate Buffered Saline (PBS) with 13.7 mM NaCl respectively in addition to the sodium salt to be tested. Fibrillation assays were carried out at 37°C in a 96-well plate with orbital shaking at

307 rpm (5 mm amplitude) in a BioTek Synergy H1 Multi-Mode Microplate Reader. Fluorescence readings were recorded every 10 minutes using an excitation wavelength of 440 nm and emission wavelength of 485 nm. They are interpreted as a measure of the total amount of amyloid aggregates formed.[44-45]

Electrophoretic mobility measurements. Electrophoretic mobility values of Sup35NM-His_6 and Met-$A\beta_{42}$ at concentrations of 10 μM and 50 μM, respectively, were measured in solutions at pH of 3.2, 4.5, and 7.4 containing 0.1 M, 0.3 M, and 0.5 M additional sodium salts using a Malvern Zetasizer Nano ZS.

RESULTS

Fibril formation by Sup35NM-His$_6$ and Met-Aβ$_{42}$ above the pI. Since the isoelectric points of both Sup35NM-His$_6$ and Met-Aβ$_{42}$ are well below 7.4 (Table 1), both proteins bear a net negative charge at pH 7.4. We observed that the effect of ions on the fibrillation of Sup35NM-His$_6$ at pH 7.4 correlated with the position of the ions along the Hofmeister series, with kosmotropic ions such as SO_4^{2-}, IO_3^-, and F^- accelerating aggregation and chaotropic ions such as Cl^-, Br^- and ClO_4^- slowing down the aggregation with increasing salt concentration (

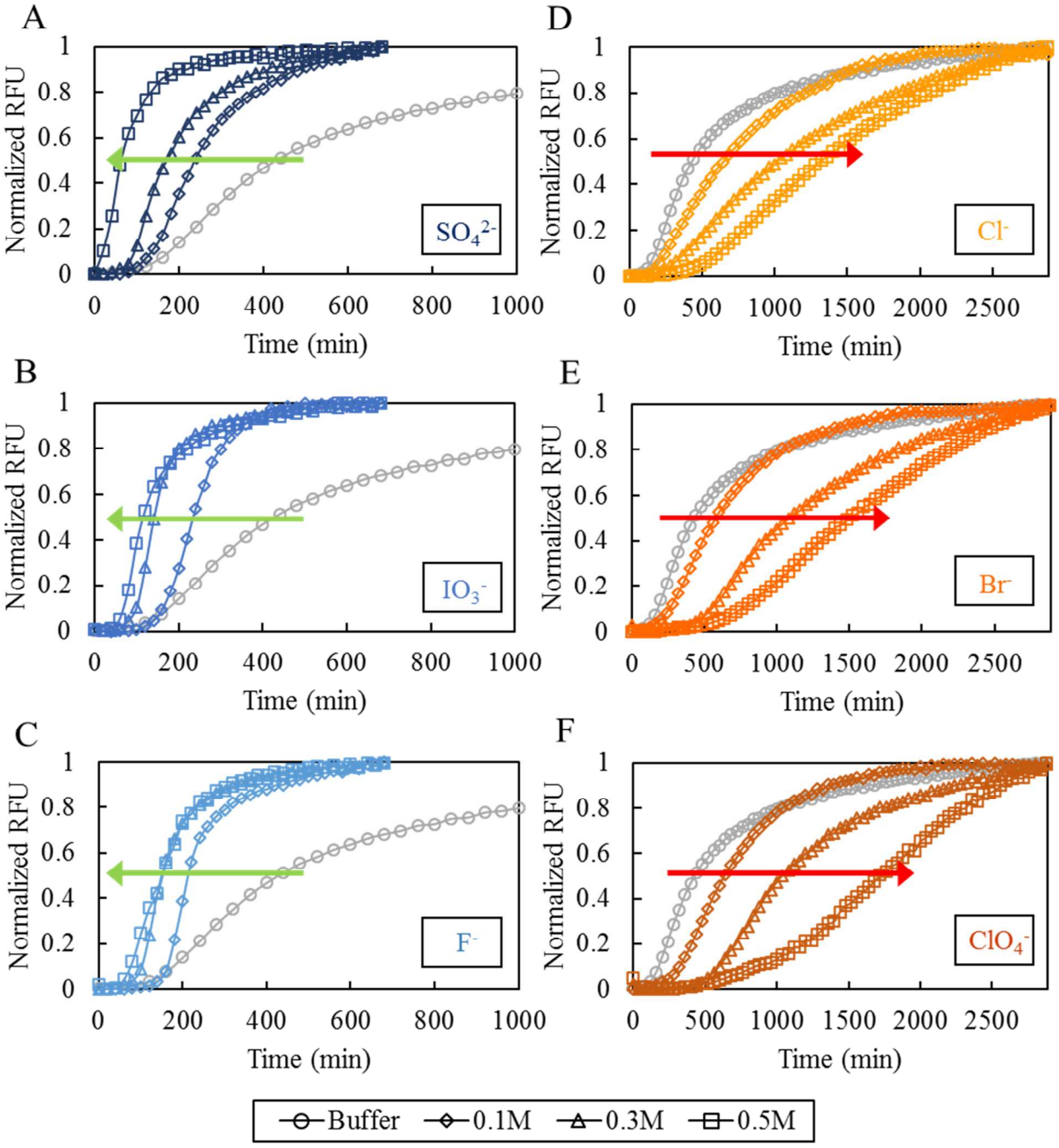

Figure 1). This finding agreed with our previous reports on the effect of ions on the aggregation of Sup35NM-His_6 under similar conditions.[18-19]

On the other hand, we saw that the presence of both kosmotropic and chaotropic anions promoted faster fibrillation of Met-Aβ_{42} at pH 7.4 (

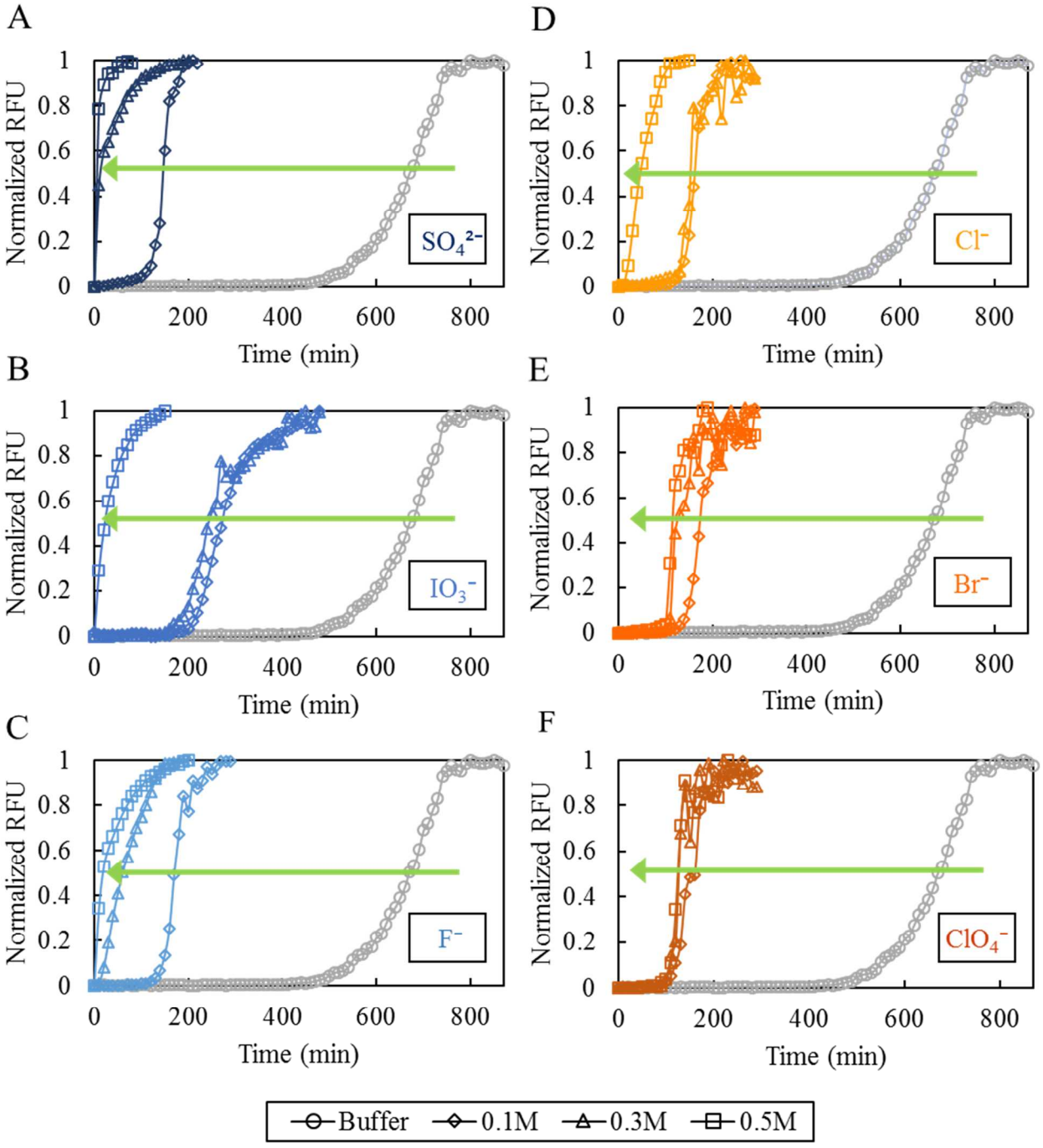

Figure **2**). Most remarkably, the chaotropic coions, which *stabilize* Sup35NM-His$_6$ against aggregation (

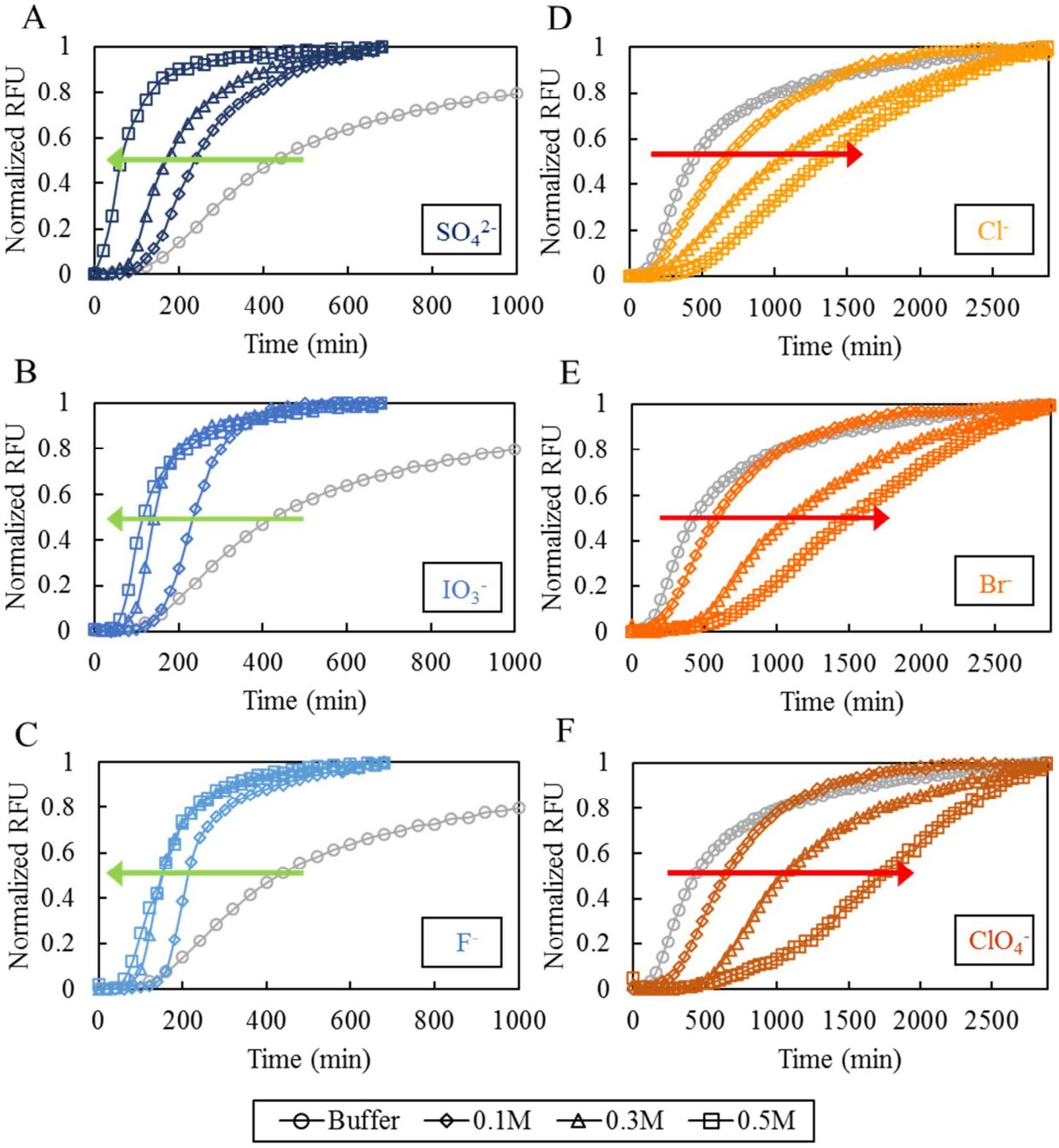

Figure 1), are seen to *promote the aggregation* of Met-Aβ_{42} (

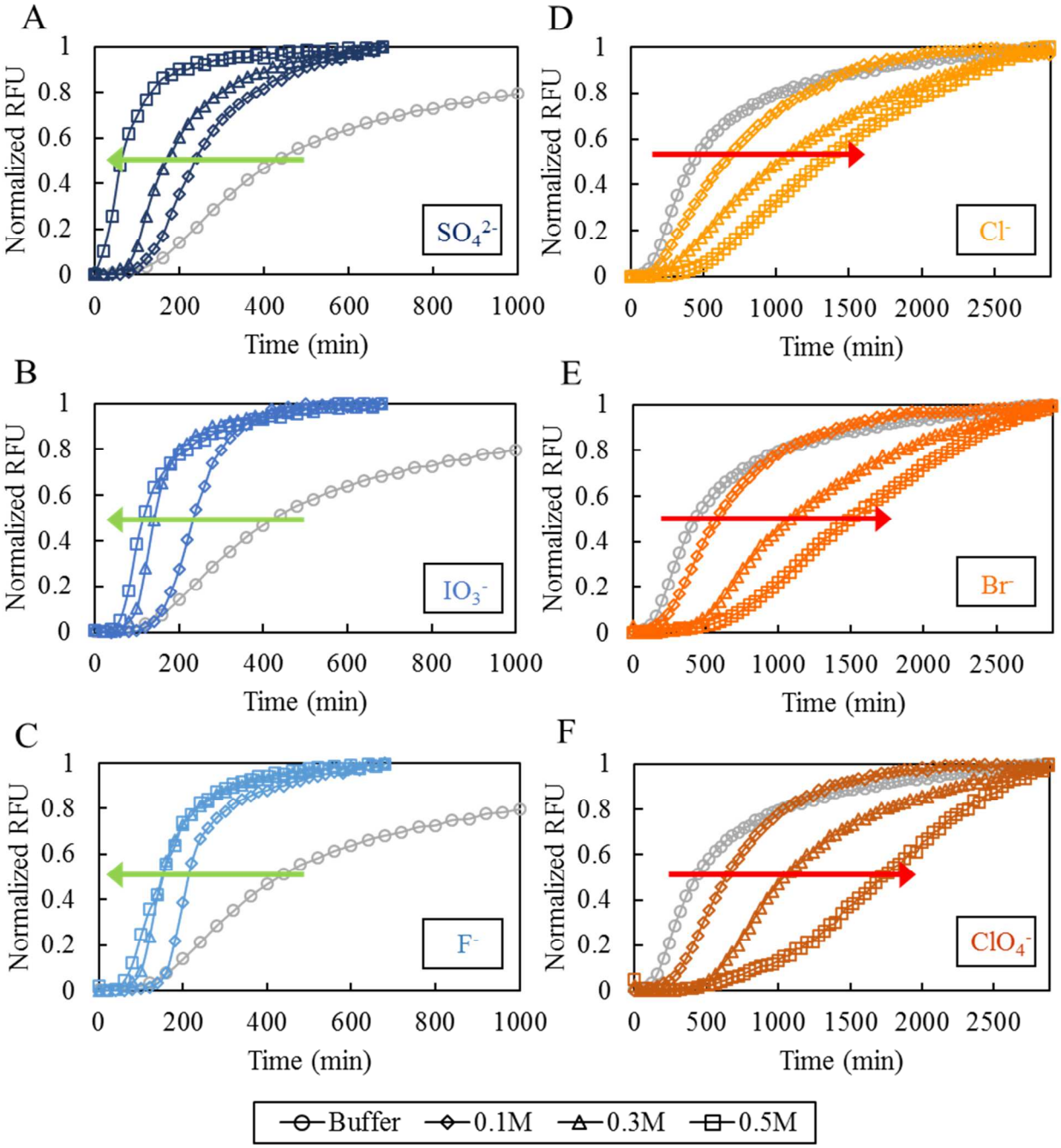
A
B
C
D
E
F
Normalized RFU
Time (min)
SO_4^{2-}
IO_3^-
F^-
Cl^-
Br^-
ClO_4^-
Buffer
0.1M
0.3M
0.5M

Figure 1).

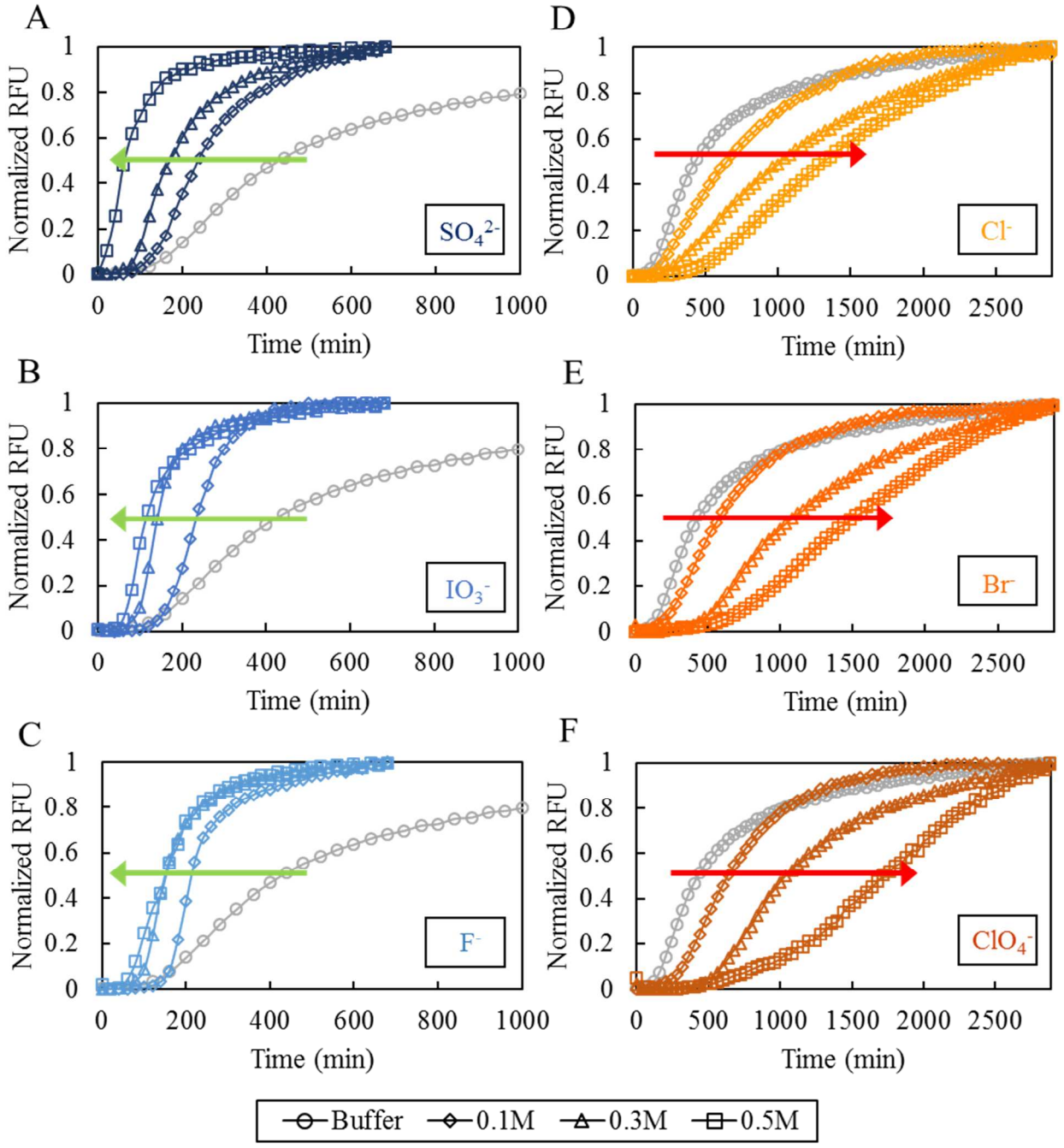

Figure 1. Fibrillation of Sup35NM-His_6 at pH 7.4 (above pI) in the presence of sodium salts of (A) SO_4^{2-}, (B) IO_3^-, (C) F^-, (D) Cl^-, (E) Br^-, and (F) ClO_4^- at 37ºC. The normalized RFU (relative fluorescence unit) was determined from fluorescence of thioflavin T (ThT) measured at an excitation wavelength of 440 nm and emission wavelength of 485 nm. The arrows in each graph point in the direction of increasing salt concentration. Green arrows indicate that aggregation rate increases with increasing salt concentration and red arrows indicate that aggregation rate decreases with increasing concentration. The X-axes of the graphs A-C have been cut off at 1000 min to clearly depict the effect of increase in ion concentration on the aggregation profiles.

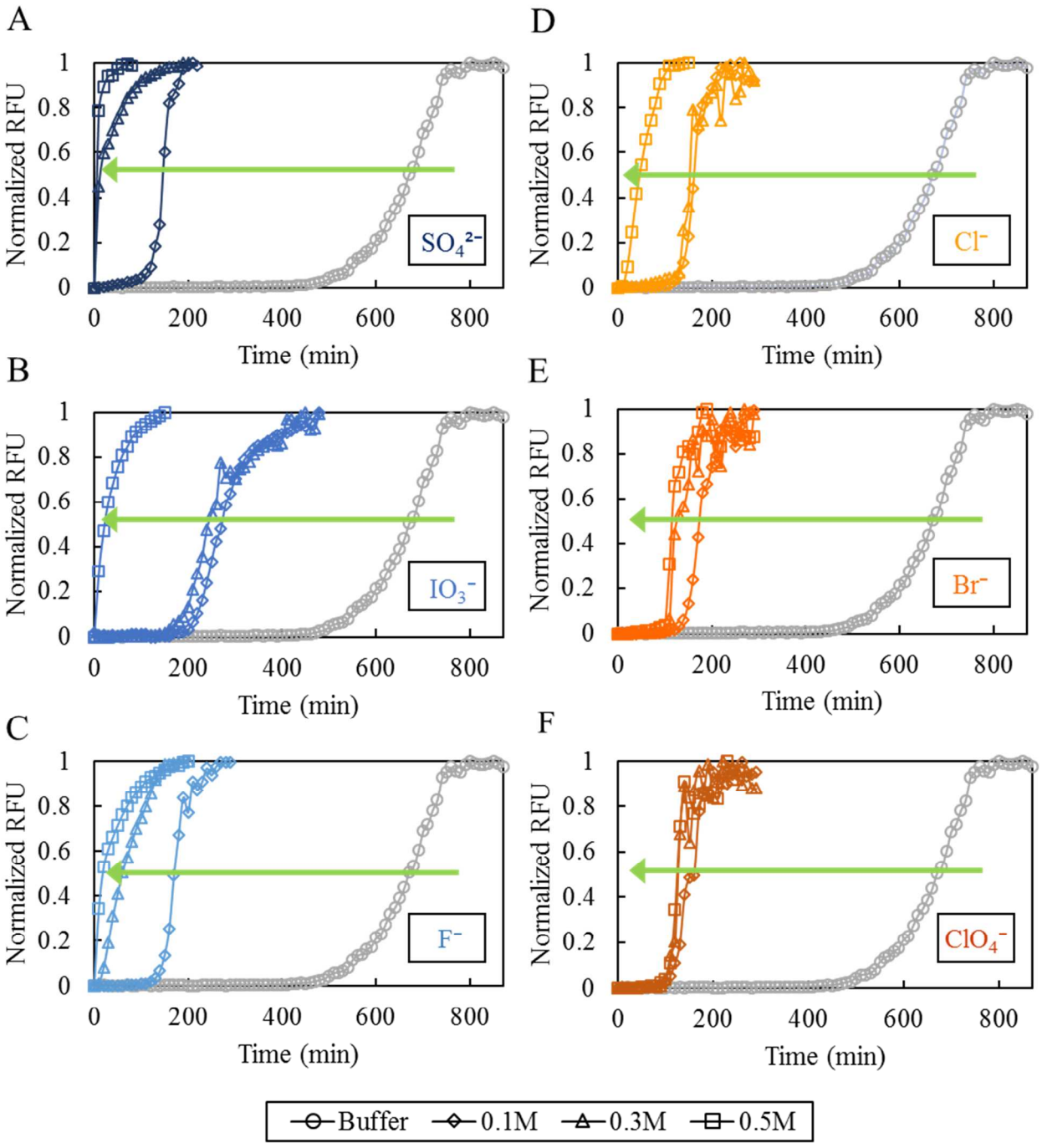

Figure 2. Fibrillation of Met-Aβ_{42} at pH 7.4 (above pI) in the presence of sodium salts of (A) SO_4^{2}, (B) IO_3^-, (C) F^-, (D) Cl^-, (E) Br^-, and (F) ClO_4^- at 37ºC. The normalized RFU (relative fluorescence unit) count was determined from fluorescence of thioflavin T (ThT) measured at an excitation wavelength of 440 nm and emission wavelength of 485 nm. The arrows in each graph point in the direction of increasing salt concentration. Green arrows indicate that aggregation rate increases with increasing salt concentration.

While increasing the concentration of chaotropic ions favored faster aggregation for Met-Aβ$_{42}$, the relative effect of anions (at the same concentration) on the kinetics of aggregation of Met-Aβ$_{42}$ still correlated with their position in the Hofmeister series, similar to Sup35NM-His$_6$ (

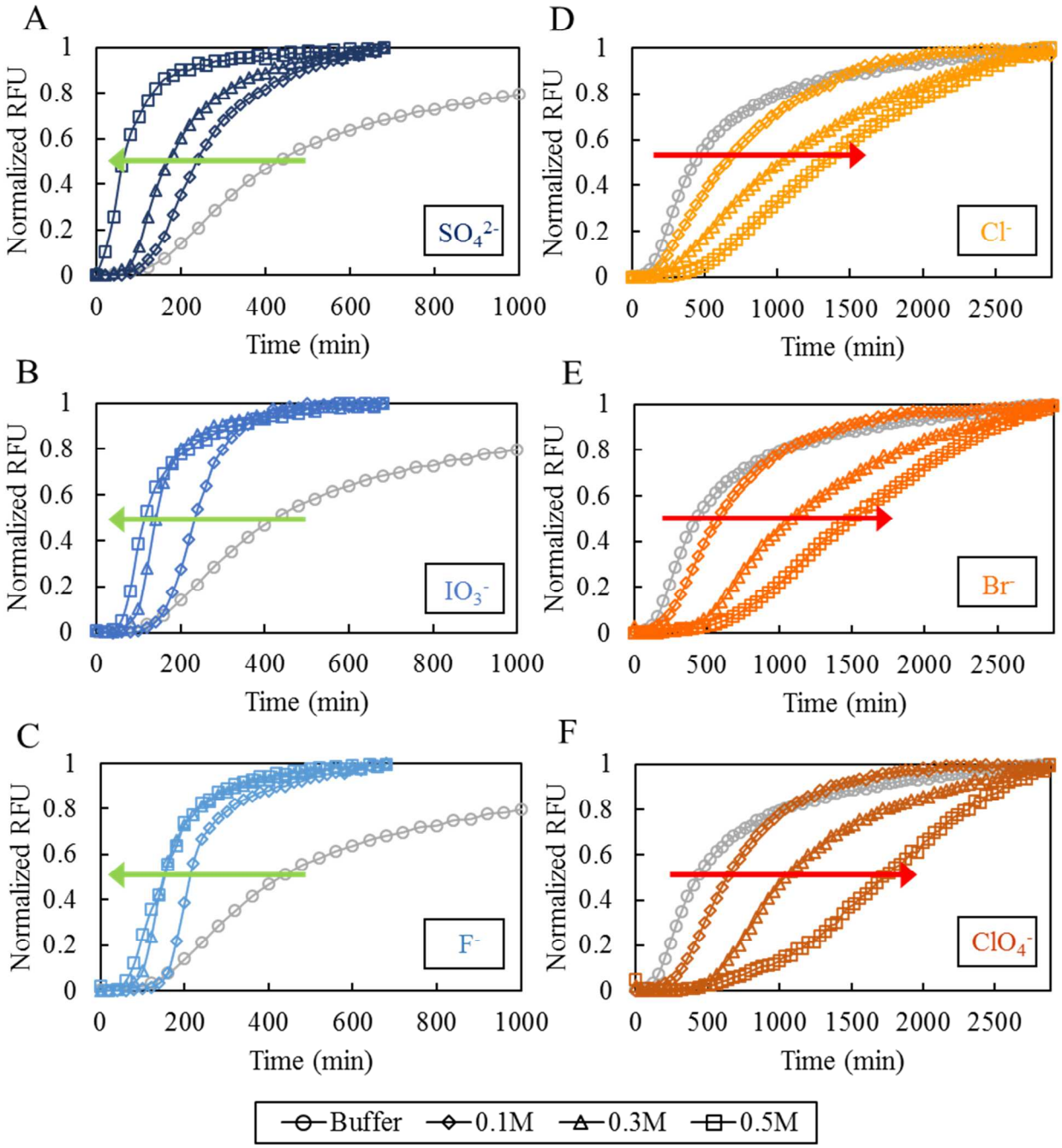

Figure 1 and

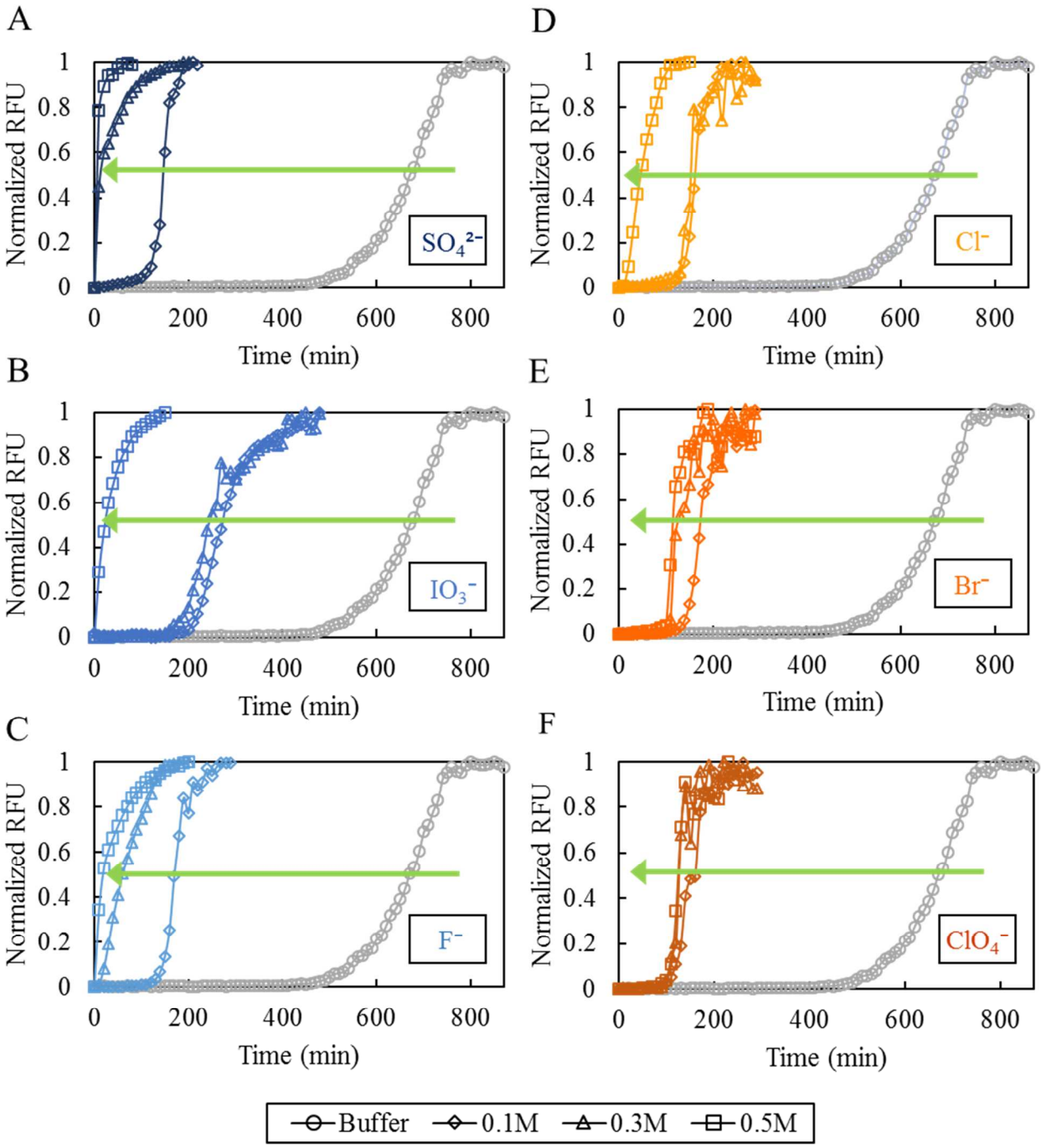

Figure **2**). For example, at a concentration of 0.5 M the most kosmotropic SO_4^{2-} was the most effective in promoting aggregation while the most chaotropic ClO_4^- was the least effective for both Sup35NM-His_6 and Met-$A\beta_{42}$ (Figure S1).

Electrophoretic mobilities of Sup35NM-His$_6$ and Met-Aβ$_{42}$ above the pI. Next, we performed electrophoretic mobility measurements in the presence of ions to understand how they interact with Sup35NM-His$_6$ and Met-Aβ$_{42}$. We acknowledge that the change in the properties of ions as we move along the Hofmeister series is gradual, and any statements we make in this article to describe the behavior of kosmotropes and chaotropes are meant to highlight respective general trends within the series, not to suggest a binary distinction between all kosmotropes and all chaotropes. We observed that, as the concentration of the ions is increased, charge screening increases, resulting in an overall reduction in electrophoretic mobility. However, chaotropes were able to adsorb strongly to both Sup35NM-His$_6$ and Met-Aβ$_{42}$ in spite of the net negative charge on both proteins at pH 7.4. This resulted in the electrophoretic protein mobility being systematically more negative (negative with larger magnitude) in the presence of chaotropes than in the presence of kosmotropes (Figure 3). We note that the only exception was sulfate, the only divalent anion in our experiments (omitted from Figure 3 for clarity), which led to intermediate protein mobilities despite being a strong kosmotrope.

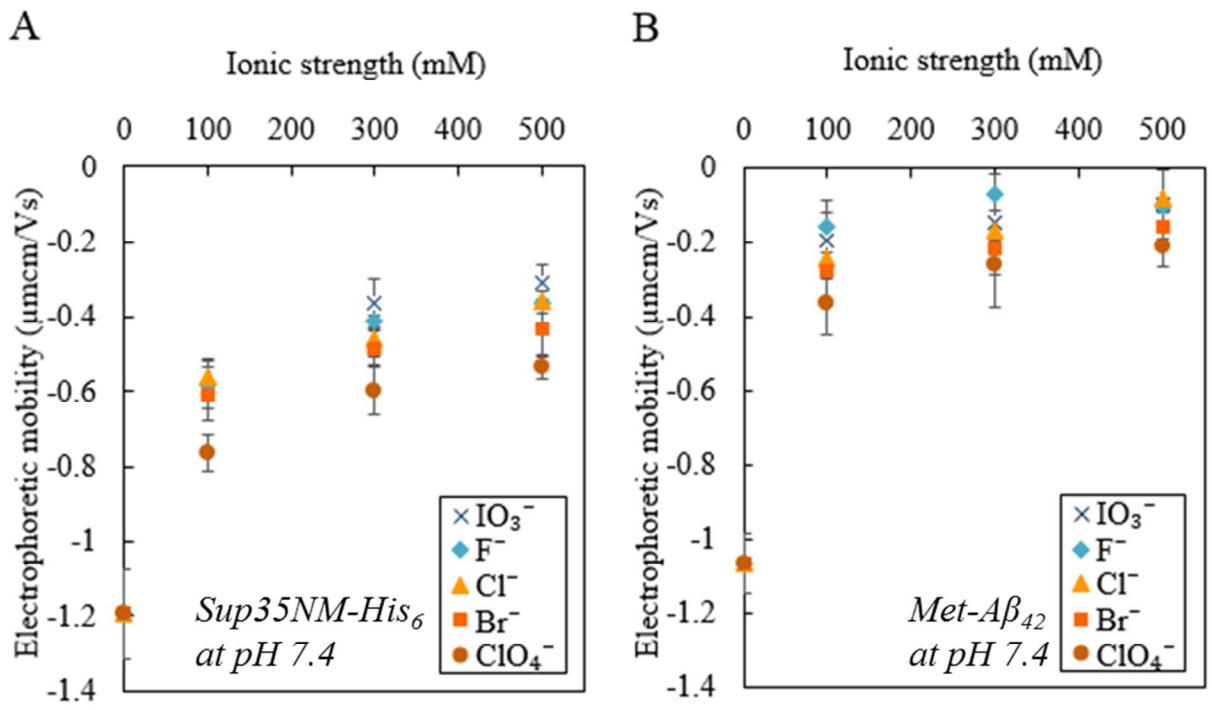

Figure 3. Electrophoretic mobilities of (A) Sup35NM-His_6 and (B) Met-$A\beta_{42}$ at pH 7.4 which is above the pI values of both proteins, in the presence of sodium salts of monovalent anions. Anion adsorption correlates with positions of anions in the Hofmeister series. Larger negative electrophoretic mobilities were observed in the presence of more chaotropic anions as compared to less chaotropic anions and kosmotropes. Highest adsorption to both Sup35NM-His_6 and Met-$A\beta_{42}$, and largest negative electrophoretic mobilities were observed in the presence of ClO_4^-.

Fibril formation by Sup35NM-His_6 and Met-$A\beta_{42}$ at pH values below the pI. In addition to testing aggregation in cases of net negative protein charge, we investigated the effect of anions on the aggregation kinetics of Met-$A\beta_{42}$ and Sup35NM-His_6 when the net charge on the proteins is reversed and the anions act as counterions to the protein charge. To that end, we performed aggregation experiments and electrophoretic mobility measurements at pH 4.5 and 3.2. While we have used the same chaotropic ions at all three pH values (7.4, 4.5 and 3.2), we were limited to different kosmotropic ions at different pH values as incomplete dissociation of most of the kosmotropic ions in the acidic pH range results in the presence of neutral species

below the pK_a (4.75 for CH_3COO^-, 3.75 for $HCOO^-$). Therefore, we were limited to just SO_4^{2-} ($pK_a \sim 2$) and IO_3^- ($pK_a \sim 0.8$) at pH 3.2, and SO_4^{2-}, IO_3^-, and $H_2PO_4^-$ ($pK_a \sim 2.15$) at pH 4.5. At pH 3.2, we observed an inversion in the effect of chaotropes on Sup35NM-His_6 (Figure 4), while the effect of kosmotropic ions, which adsorb weakly to the protein molecules, was the same as at pH 7.4. At pH 3.2, chaotropic ions which can interact with specific regions in a polypetide chain, resulted in more effective charge screening and neutralization, and promoted fast fibril formation by Sup35NM-His_6. The effect of both kosmotropes and chaotropes on fibril formation by Met-$A\beta_{42}$ at pH 3.2 was found to be similar to Sup35NM-His_6 (Figure 4 and Figure 5). This reversal of the Hofmeister effect for chatropes on inversion of the net surface charge has been reported for colloidal systems and globular proteins.[46-52]

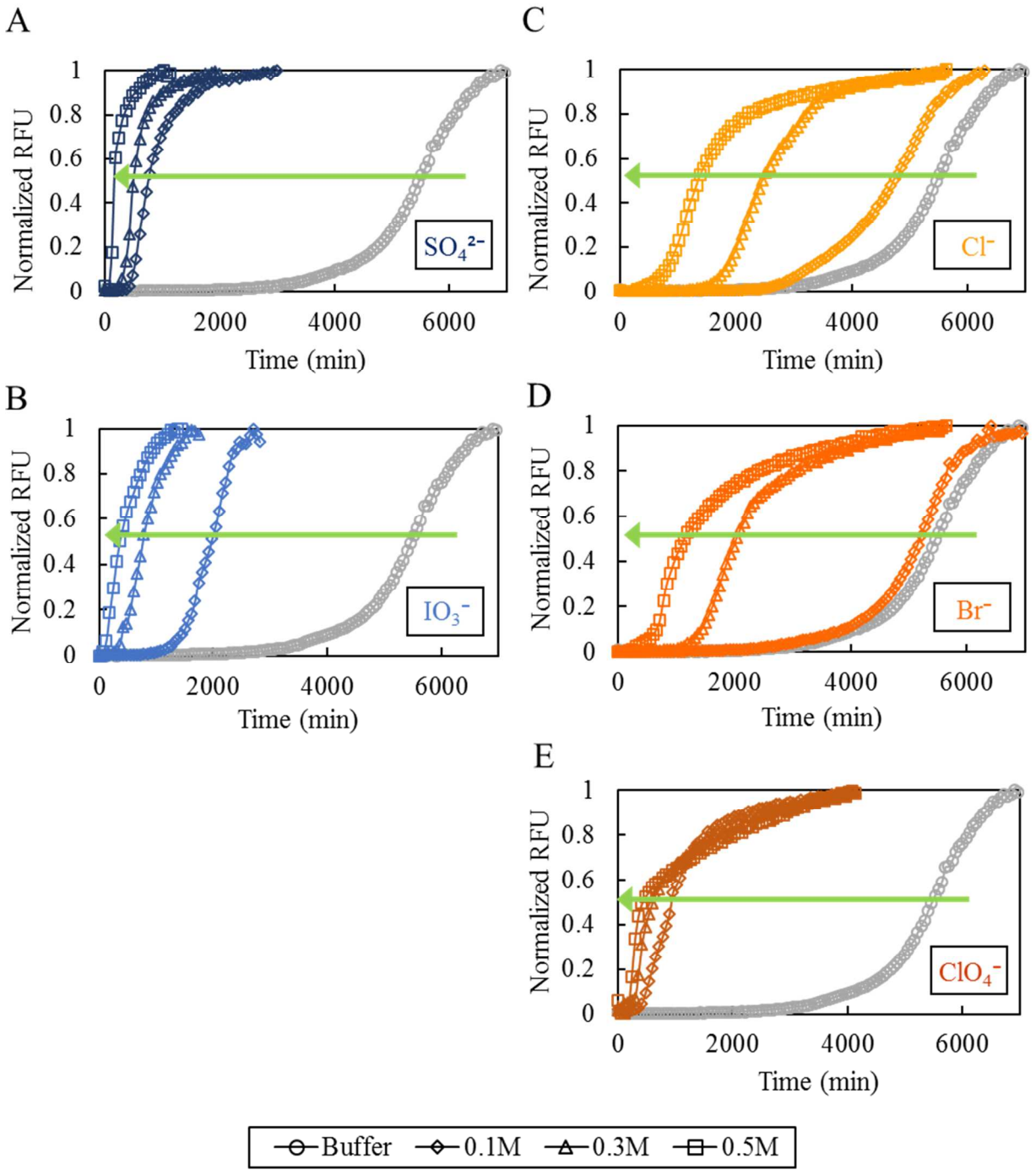

Figure 4. Fibrillation of Sup35NM-His_6 at pH 3.2 (below pI) in the presence of sodium salts of (A) SO_4^{2-}, (B) IO_3^-, (C) Cl^-, (D) Br^-, and (E) ClO_4^- at 37ºC. The normalized RFU (relative fluorescence unit) was determined from fluorescence of thioflavin T (ThT) measured at an excitation wavelength of 440 nm and emission wavelength of 485 nm. The arrows in each graph point in the direction of increasing salt concentration. Green arrows indicate that aggregation rate increases with increasing salt concentration and red arrows indicate that aggregation rate decreases with increasing concentration.

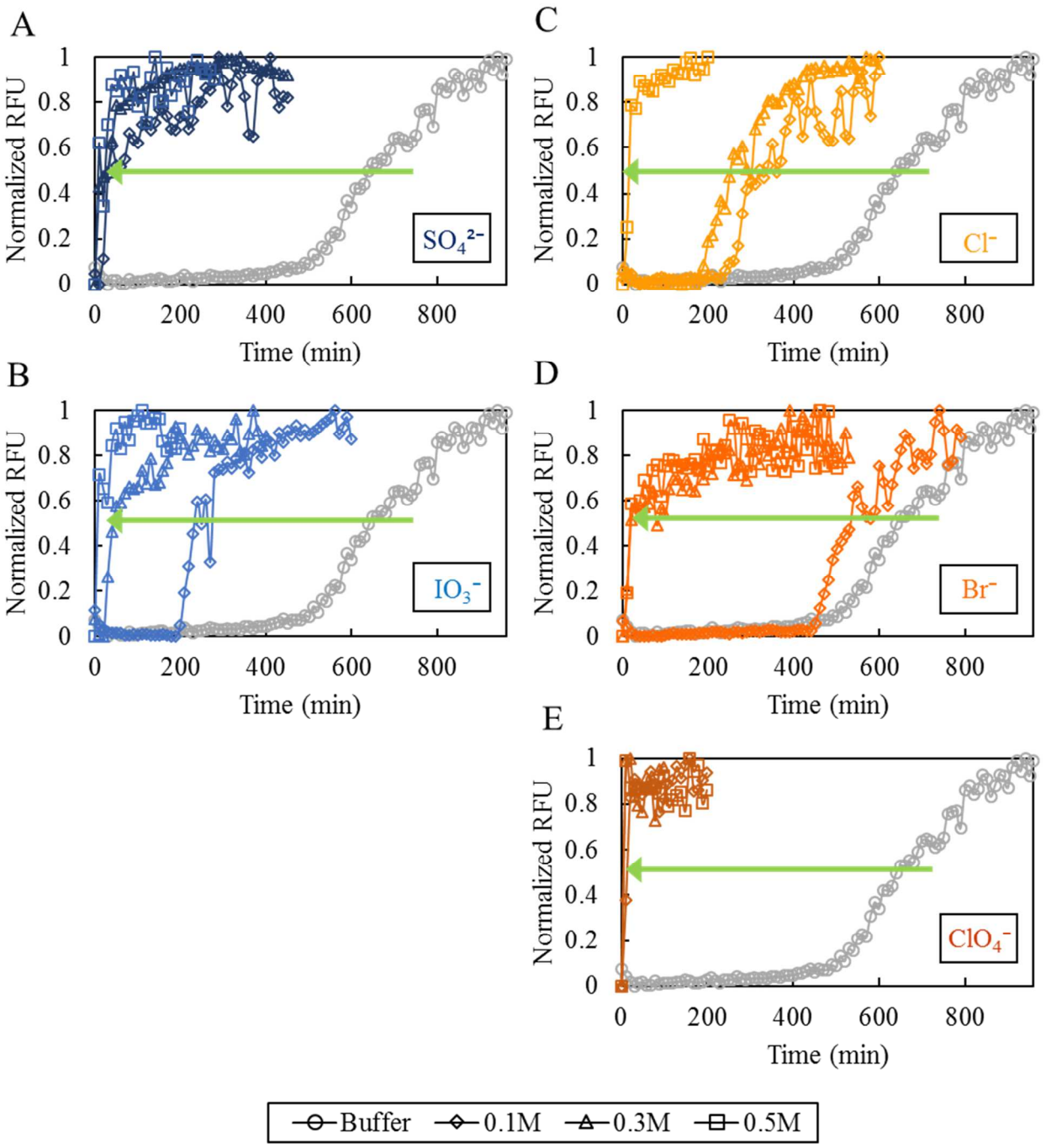

Figure 5. Fibrillation of Met-Aβ$_{42}$ at pH 3.2 (below pI) in the presence of sodium salts of (A) SO_4^{2-}, (B) IO_3^-, (C) Cl^-, (D) Br^-, and (E) ClO_4^- at 37ºC. The normalized RFU (relative fluorescence units) was determined from fluorescence of thioflavin T (ThT) measured at an excitation wavelength of 440 nm and emission wavelength of 485 nm. The arrows in each graph point in the direction of increasing salt concentration. Green arrows indicate that aggregation rate increases with increasing salt concentration.

Next, we investigated the consequence of reduction in the net charge on Sup35NM-His_6 and Met-$A\beta_{42}$ on the ion effect for aggregation. While the effect of kosmotropes on both Sup35NM-His_6 and Met-$A\beta_{42}$ remains the same at pH 4.5, the strongest chaotrope (ClO_4^-) showed a partial reversal in its fibrillation promoting effect at pH 4.5 (Figure S2 and Figure S3). Above a concentration of 0.1M $NaClO_4$ the aggregation became slower upon further increasing the concentration of the strongly adsorbing ClO_4^- ions (Figure 6). Interestingly, no such reversal was seen for Met-$A\beta_{42}$ (Figure S3).

Electrophoretic mobilities of Sup35NM-His_6 and Met-$A\beta_{42}$ below the pI. Next, we measured the electrophoretic mobilities of Sup35NM-His_6 and Met-$A\beta_{42}$ at pH 3.2 and 4.5, where both proteins bear a net positive charge and anions act as counterions. Again, we observed that an increase in the ion concentration enhanced charge screening, resulting in an overall reduction in electrophoretic mobility. Additionally, as observed for pH 7.4, the chaotropic anions were better able to adsorb to both Sup35NM-His_6 and Met-$A\beta_{42}$ than kosmotropes. This resulted in a greater reduction in protein mobility in the presence of chaotropes at pH 3.2 and 4.5 (Figure 7 and Figure S4). At pH 4.5, where the net charge on the protein was only slightly positive we observed that increasing the concentration of Br^- and ClO_4^- resulted in a reversal of the protein mobility and, hence, the sign of charge on Sup35NM-His_6, as one might expect from the strong adsorption tendency of chaotropes. The significant mobility reversal of Sup35NM-His_6 in the presence of the most chaotropic ion ClO_4^- at pH 4.5 agreed with the reversal observed in our

aggregation data (Figure 6). Qualitatively, a charge reversal was also seen in the mobilities of Met-Aβ_{42} at pH 4.5; however, this reversal was not reflected in the aggregation data, plausibly because the absolute mobility value remained very close to zero. (Figure S3 and Figure S4).

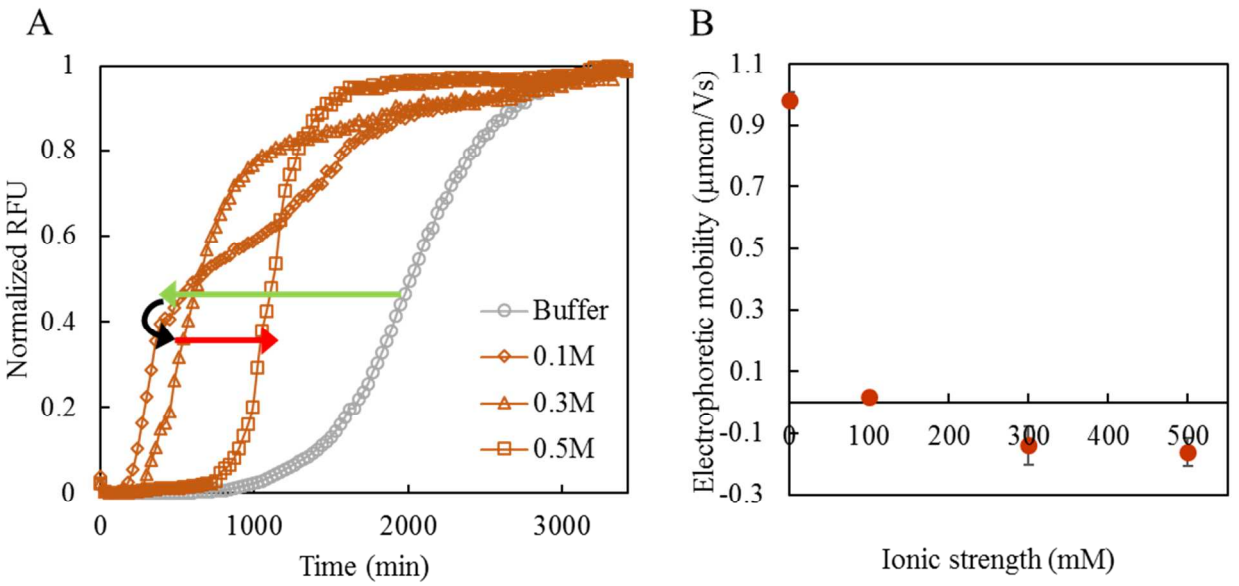

Figure 6. (A) Fibrillation of Sup35NM-His$_6$ at pH 4.5 (below pI) in the presence of $NaClO_4$ at 37ºC. The normalized RFU (relative fluorescence unit) was determined from fluorescence of thioflavin T (ThT) measured at an excitation wavelength of 440 nm and emission wavelength of 485 nm. The arrows are in the direction of increasing salt concentration. Green arrows indicate that aggregation efficiency increases with increasing salt concentration and red arrows indicate that aggregation efficiency decreases with increasing concentration. (B) Electrophoretic mobilities of Sup35NM-His$_6$ at pH 4.5, in the presence of $NaClO_4$. A reversal in the electrophoretic mobility is observed around an ionic strength of 100 mM.

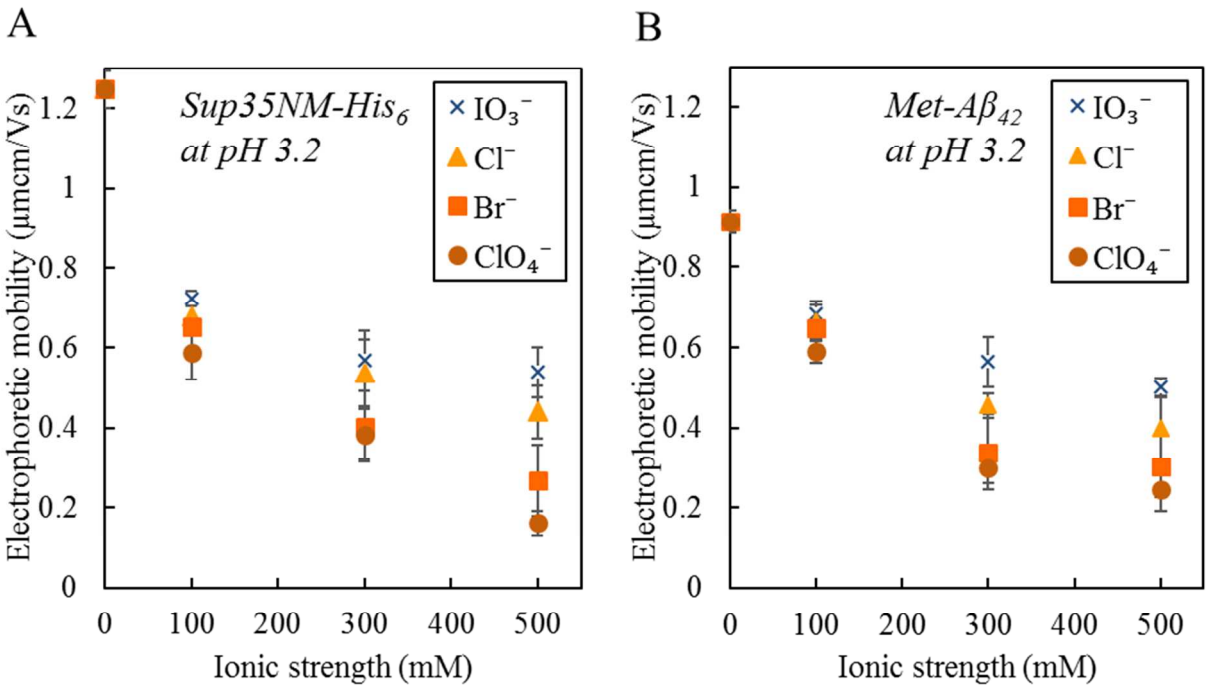

Figure 7. Electrophoretic mobilities of Sup35NM-His$_6$ (A) and Met-Aβ_{42} (B) at pH 3.2, in the presence of sodium salts of monovalent anions. Anion adsorption to both Sup35NM-His$_6$ and Met-Aβ_{42} correlates with positions of anions in the Hofmeister series. Larger negative electrophoretic mobilities were observed in the presence of more chaotropic anions as compared to less chaotropic anions and kosmotropes. Highest adsorption to both Sup35NM-His$_6$ and Met-Aβ_{42}, and largest negative electrophoretic mobilities were observed in the presence of ClO_4^-.

DISCUSSION

Role of biochemical properties of proteins in determining ion-protein interactions.

The effect of anions on the aggregation of both Sup35NM-His$_6$ and Met-Aβ_{42} is quantified in terms of half time of aggregation in table S1 and qualitatively summarized in table 2.

Table 2. Effect of increase in concentration of ions on aggregation

pH	***Sup35NM-His$_6$***		***Met-Aβ_{42}***	
	Kosmotrope	***Chaotrope***	***Kosmotrope***	***Chaotrope***
7.4	*Accelerates*	*Decelerates*	*Accelerates*	*Accelerates*
4.5	*Accelerates*	*Accelerates**	*Accelerates*	*Accelerates*
3.2	*Accelerates*	*Accelerates*	*Accelerates*	*Accelerates*

*Exception: strong chaotrope accelerates aggregation at low concentrations and decelerates aggregation at higher concentrations where the charge on the protein is reversed

The effect can be evaluated in terms of specific and non-specific effects arising from electrostatic and hydrophobic interactions, and preferential interactions or solvent (hydration) effects. The isoelectric point (pI) of both Sup35NM with His_6 tag and $A\beta_{42}$ with N-terminal methionine are close to each other (6.01 and 5.3 respectively, Table 1). Hence, both proteins carry a net positive charge at pH values of 3.2 and 4.5 and a net negative charge at pH 7.4. The N and C terminals of the proteins and the polar charged residues can play a role in electrostatic interactions with the ions in solution. These include lysine (Lys), arginine (Arg), histidine (His), aspartic acid (Asp), and glutamic acid (Glu). The number of these charged amino acids in both Sup35NM-His_6 and Met-$A\beta_{42}$, and their amyloid fold forming domains (Sup35N and $A\beta_{15-42}$) are shown in Table 3.

Table 3. Number of charged amino acid residues in Sup35NM-His_6 and Met-$A\beta_{42}$, and their amyloid forming domains

Amino acid (side chain pK_a)[53]	***Met-$A\beta_{1-42}$***	***$A\beta_{15-42}$***	***Sup35NM-His_6***	***Sup35N***
Arg (12.48)	*1*	*0*	*2*	*2*
Asp (3.65)	*3*	*1*	*9*	*2*
Glu (4.25)	*3*	*1*	*22*	*0*
His (6.0)	*3*	*0*	*7*	*0*
Lys (10.53)	*2*	*2*	*25*	*1*

At a pH of 7.4, Lys and Arg are expected to be completely protonated and will carry positive charges while Asp and Glu will be completely deprotonated and will carry negative charges. At a pH of 4.5, His will also be nearly completely protonated (~97%) in addition to Lys and Arg. Asp and Glu will lose some of their negative charge and become only partially deprotonated (~88% and 64% respectively). At pH 3.2, Asp will be almost 26% deprotonated

and Glu will become nearly completely protonated and will lose all of its negative charge. Therefore, the charge distribution on the proteins changes depending on the pH of the solution. The calculated charges on Sup35NM-His_6 and Met-$A\beta_{42}$, and their amyloid forming domains at the experimental pH values are shown in Table 4.

Table 4. Calculated net charge on Sup35NM-His_6 and Met-$A\beta_{42}$, and their amyloid forming domains

pH	***Met-Aβ$_{1-42}$***	***Aβ$_{15-42}$***	***Sup35NM-His$_6$***	***Sup35N***
3.2	*+5*	*+1.7*	*+29.8*	*+2.5*
4.5	*+1.4*	*+0.5*	*+11.8*	*+1.2*
7.4	*-2.9*	*0*	*-3.7*	*+1*

Moreover, the local environment can also affect the pK_a and the charge on individual amino acid side chains. It is important to note that most of the charged residues in Sup35NM-His_6 are present in the M domain which does not take part in the amyloid fibril formation. However, as per the proposed parallel in-register structure of Sup35NM fibrils, the M-domains of adjacent molecules are in close proximity to each other and can play a crucial role in facilitating or hindering the N-domain in forming fibrils.[36, 54]

Further, the Grand Average of Hydropathicity (GRAVY) scores calculated for Sup35NM-His_6 and Met-$A\beta_{42}$ indicate that Met-$A\beta_{42}$ with a GRAVY value of 0.244 is much more hydrophobic in nature than Sup35NM-His_6 with a calculated GRAVY value of -1.633 (Table 1).[40] Recent work on determining the structure of $A\beta_{42}$/ Met-$A\beta_{42}$ monomer in the fibrillar form has shown that $A\beta_{42}$/ Met-$A\beta_{42}$ fibrils consist of two molecules per layer forming dimers arranged in parallel-in-register orientation[34-35]. The dimer is assembled such that the hydrophobic residues are maximally buried while only the hydrophilic sides chains are exposed to the solvent.

The hydrophobic character of $A\beta_{42}$/Met-$A\beta_{42}$ and their structure in the fibrillar form suggest that aggregation of $A\beta_{42}$/Met-$A\beta_{42}$ is driven by the hydrophobic effect.

Besides electrostatic and hydrophobic interactions, competing preferential interactions between the protein, ions, and water can play a crucial role in governing fibrillation kinetics. While small, strongly hydrated kosmotropes are generally considered to act by exerting an excluded volume effect, large weakly hydrated chaotropes are thought to interact directly with hydrophobic regions on a protein[26, 55-59]. Interestingly, in a study of ion interaction with an uncharged 600-residue elastin-like polypeptide, chaotropic anions were shown to mainly interact with the polypeptide backbone while no significant binding of the ions to the hydrophobic side chains was detected[60]. In another study, chaotropes were shown to interact with the peptide backbone of a triglycine model peptide[46]. This suggests that besides the specific residues in a protein the peptide backbone or the length of the protein can have a significant impact on the overall effect of ions on protein stability and aggregation tendency.

Ions can also act by screening electrostatic forces. Screening effects are non-specific in nature and only depend on the ionic strength of the solution. While electrostatic effects resulting from ion interaction with specific charges on the protein are expected to be dominant at low ionic concentrations, the observed effects at high concentration are due to an interplay of ion-specific Hofmeister effects and non-specific screening effects.

Effect of anions on amyloid formation when anions act as counter-ions. The effect of ions on the fibrillation of Sup35NM-His_6 and Met-$A\beta_{42}$ at acidic pH values can be explained by electrostatic interactions. At a pH value below the pI, the proteins bear a net positive charge and the anions act as counterions. At pH 3.2 and 4.5, kosmotropes act in the same way as at pH 7.4,

through depletion interactions and increased screening; chaotropic anions, on the other hand, interact directly with specific regions on the proteins and neutralize the charge on the protein more effectively, reducing repulsion between molecules and promoting fibrillation. Hence, we see a reversal of the Hofmeister effect for chaotropic anions when the charge on the proteins is reversed. As we approach the pI, at pH 4.5 we observe charge inversion due to adsorption of an excess of chaotropic anions, resulting in slower fibril formation of Sup35NM-His_6.

Effect of anions on amyloid formation when anions act as co-ions. At pH 7.4, both Sup35NM-His_6 and Met-$A\beta_{42}$ bear a net negative charge and anions act as co-ions. Kosmotropes which are excluded from the protein-water interface increase the surface tension and destabilize the monomeric protein resulting in faster fibril formation similar to their effect on globular proteins. Chaotropes, on the other hand, preferentially interact with the hydrophobic regions on the proteins and the polypeptide backbone, and result in stabilization against aggregation. As a result, at a particular ionic strength the relative effects of kosmotropes and chaotropes on aggregation are qualitatively similar for Sup35NM-His_6 and Met-$A\beta_{42}$ and are correlated with the position of the ions in the Hofmeister series. In summary, at the same salt concentration aggregation is the fastest in the presence of the most kosmotropic ion, SO_4^{2-}, and slowest in the presence of the most chaotropic ion, ClO_4^-, as seen in **Error! Reference source not found.**.

The main difference in the effect of ions on the fibrillation kinetics of Sup35NM-His_6 and Met-$A\beta_{42}$ at pH of 7.4 is that an increase in the concentration of the salts always promotes the fibrillation of Met-$A\beta_{42}$, irrespective of whether the anion is a kosmotrope or a chaotrope. By contrast, an increase in the concentration of chaotropic anions hinders the fibrillation of Sup35NM-His_6 while increase in the concentration of kosmotropes promotes fibril formation. This suggests that the fibrillation of Met-$A\beta_{42}$ is dominated by screening effects which are

determined by ionic strength, and is less sensitive to specific interaction of ions with the protein. On the other hand, interaction of chaotropes with Sup35NM-His_6 is driven by specific interactions, as the strongly adsorbing chaotropes appear able to modify the 'electrostatic landscape' of the protein sufficiently to interfere with the templated growth of the amyloid aggregates and effectively hinder Sup35NM-His_6 fibril formation.

Proposed explanations for the observed differences in the effect of ions on the aggregation of Met-Aβ_{42} and Sup35NM-His_6. One plausible explanation of this observation invokes a two-step process of nucleus formation where the first step is the initial agglomeration of monomers to form a 'pre-organized' oligomeric intermediate, followed by conformational conversion or structural reorganization to an organized stable nucleus. Such a nucleation mechanism termed as "nucleated conformational conversion", has been previously described by Serio et al.[61] According to this model, molten globule-like oligomeric intermediates are formed first, followed by conformational rearrangement generating an amyloid nucleus.[61] Nuclei promote further amyloid growth through templating or induced-fit mechanism at the fibril end.

Ions can affect these steps through different mechanisms. An increase in ionic strength is likely to promote the agglomeration step through screening effects while ion-specific binding may affect the conformational conversion to form a stable nucleus. Binding of chaotropes to the protein will likely hinder conformational conversion of the oligomer to the nucleus by disrupting the recognition landscape. Since screening effects due to increase in ionic strength dominate over ion-specific effects, the first agglomeration step is expected to be the rate-limiting step in Met-Aβ_{42} fibrillation.

In the case of Met-Aβ_{42}, most of the protein is involved in a cross-beta structure and initial agglomeration is frequently followed by conformational conversion, because once the molecules are brought in close-proximity, they begin interacting and forming a cross-beta structure. On the other hand, conformational conversion to the nucleus may be the rate-limiting step in the aggregation of Sup35NM-His_6 as the process is highly sensitive to specific ion binding. Sup35NM-His_6 contains a long M-domain region which is not involved in the amyloid core but needs to be in the proper orientation and conformation that favors nucleus formation. Due to the presence of this long extra domain, initial agglomeration is highly reversible and may not always lead to conformational conversion, which requires interactions between specific residues within the amyloid core domain.

Our observations can also be explained by the previously observed dual 'salting-in' and 'salting-out' behavior of chaotropes[62]. At pH 7.4, Met-Aβ_{42} has only 3 positively charged residues and a much shorter backbone than Sup35NM-His_6. It is possible that the sites for chaotrope binding become saturated quickly in Met-Aβ_{42} and a further increase in ionic strength leads to increased screening, resulting in faster fibrillation similar to 'salting-out' of globular proteins by chaotropes at high concentrations. Nevertheless, at pH 7.4 the more chaotropic ions still manage to absorb more effectively to Met-Aβ_{42} resulting in a greater negative charge on Met-Aβ_{42} than in the presence of the less chaotropic anions and kosmotropes. This explains why the relative effect on fibrillation still correlates with the position of the ions in the Hofmeister series. On the other hand, Sup35NM-His_6 has about 27 positively charged residues and a much longer peptide backbone providing more sites for chaotropic anions to bind as compared to Met-Aβ_{42}. It is rather plausible that the potential ion binding sites on Sup35NM-His_6 are not completely occupied and an increase in chaotrope concentration results in delayed aggregation

due to further stabilization of the monomer similar to 'salting-in' of globular proteins at relatively moderate chaotrope concentrations. A proposition that follows from the above hypothesis is that the observed effect of ions on aggregation of proteins critically depends on the relative concentrations of ions and proteins, along with other factors such as the length of the polypeptide chain and the amino acid composition of the protein and suggests that a reversal in the aggregation behavior may be observable even when the ions act as co-ions. In fact, such a behavior was observed in a previous study on amyloid formation by α-synuclein, where an inversion in the effect of chaotropes was seen at a pH above its pI of 4.7 as the concentration of the chaotropes was increased[31]. Notably, α-synuclein with a size of 140 amino acids lies between Met-Aβ_{42} and Sup35NM-His$_6$.

CONCLUSION

In this work, we have performed a detailed investigation of the effect of ions on the aggregation of amyloids. We show that ions affect amyloid formation through a complex interplay of specific and non-specific effects. Through a comparison of two amyloid-forming proteins, namely Sup35NM-His_6 and Met-$A\beta_{42}$, we have shown that protein properties such as polypeptide chain length, amino acid composition, which affects hydrophobicity and charge distribution, and length of the amyloidogenic domains play a crucial role in determining the effect of ions on monomer stability and aggregation kinetics. By studying aggregation and protein mobility at pH values above and below the pI, we find that below the isoelectric point, anions promote aggregation by charge neutralization and screening effects. On the other hand, at pH values above the pI, aggregation is driven by a competition between ionic strength effects and specific ion adsorption. We have shown that at fixed ionic strength the relative effect of different co-ions on the aggregation of Sup35NM-His_6 and Met-$A\beta_{42}$ is similar. However, there are important differences in the absolute effects of increasing ion concentration, which may suggest crucial differences in the aggregation mechanism, in line with the two-step nucleation hypothesis. Despite all these differences, we show that ion-protein interactions follow expectations based on the position of the ions in the Hofmeister series, both above and below pI, and the relative effect of anions on aggregation of both Sup35NM-His_6 and Met-$A\beta_{42}$ at the same ion concentrations is generally similar and thus consistent with the universality of ion-specific effects on proteins.

ASSOCIATED CONTENT

Supporting Information

The following files are available free of charge.

Supporting aggregation data at pH 4.5 and Table of aggregation half times (PDF)

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Author Contributions

AS conducted the experiments, analyzed the results and wrote the manuscript; SHB, YOC, and ASB designed experiments, discussed results and edited the manuscript; ASB acted as a corresponding author.

Notes

The authors declare no competing financial interests.

ACKNOWLEDGMENTS

We thank Dr. Matthew Torres of Georgia Institute of Technology for the use of his BioTek Synergy H1 Multi-Mode Microplate Reader, and Dr. K. Ugen, University of South Florida for providing plasmid pcDNA3.1(+)-Aβ42 containing the human $A\beta_{1-42}$ coding sequence.

Funding Sources

AS and ASB gratefully acknowledge support through the NSF-I/UCRC Center for Pharmaceutical Development (CPD) (NSF grants IIP-1540017 and IIP-0969003). YOC gratefully acknowledges support from Emory Alzheimer's Disease Research Center (subaward on the NIH grant P50AG025688) and Russian Science Foundation (grant 14-50-00069).

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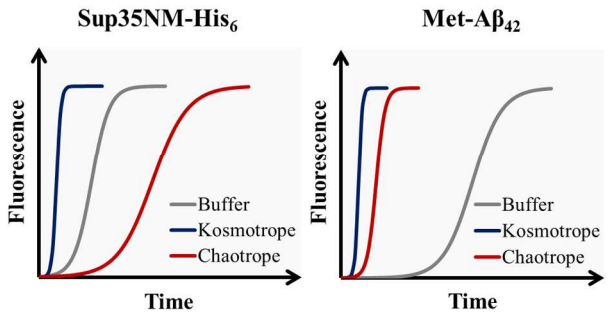
TOC Graphic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