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Monovalent cations have different effects on the assembly kinetics and morphology of α-synuclein amyloid fibrils

Fritjof Havemeister, Marziyeh Ghaeidamini, Elin K. Esbjörner *

Division of Chemical Biology, Department of Life Sciences, Chalmers University of Technology, Kemivägen 10, S-41296, Gothenburg, Sweden

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ABSTRACT

Formation of α-synuclein amyloid fibrils is a pathological hallmark of Parkinson's disease and a phenomenon that is strongly modulated by environmental factors. Here, we compared effects of different monovalent cations (Li^+, Na^+, K^+) on the formation and properties of α-synuclein amyloid fibrils. $Na^+ > Li^+$ were found to have concentration-dependent catalytic effects on primary nucleation whereas K^+ ions acted inhibitory. We discuss this discrepancy in terms of a superior affinity of Na^+ and Li^+ to carboxylic protein groups, resulting in reduced Columbic repulsion and by considering K^+ as an ion with poor protein binding and slight chaotropic character, which could promote random coil protein structure. K^+ ions, furthermore, appeared to lower the β-sheet content of the fibrils and increase their persistence lengths, the latter we interpret as a consequence of lesser ion binding and hence higher line charge of the fibrils. The finding that Na^+ and K^+ have opposite effects on α-synuclein aggregation is intriguing in relation to the significant transient gradients of these ions across axonal membranes, but also important for the design and interpretation of biophysical assays where buffers containing these monovalent cations have been intermixedly used.

1. Introduction

α-synuclein (α-syn) is a 14 kDa, intrinsically disordered protein that forms amyloid fibril inclusions as part of the pathology of several neurodegenerative disorders including Parkinson's disease [1], multiple system atrophy [2], and Lewy body dementia [3]. The physiological functions of α-syn are not entirely known, nor are the underlying reasons for its aggregation in disease. α-syn is normally localized mostly to nerve terminals [4] where it appears involved in the regulation of synaptic vesicles [5]. The nerve terminal experiences significant ion fluxes, including action potential driven transmembrane regulations of Na^+/K^+, and influx of Ca^{2+} that triggers synaptic vesicle fusion and neurotransmitter release [6]. Whilst Ca^{2+} ions have been explored as modulators of α-syn interactions and aggregation [7], and ionic strength affects its *in vitro* aggregation rate [8,9], possible differences in the effects of Na^+ and K^+ ions have not been explored. In fact, chloride salts of these monovalent cations have been used seemingly intermixedly in many *in vitro* studies [10], even though it is widely recognized that amyloid formation is highly sensitive to environmental conditions. For example, recent cryo-EM studies have identified disease-specific α-syn fibril polymorphs from post-mortem brain samples [11]. Different *in vitro* prepared α-syn fibrils can furthermore have distinct biological effects with respect to, for example, seeding [12], prion-like propagation [13], and pathological effect [14]. This underscores the importance of understanding how different environmental conditions modulate α-syn aggregation.

This biophysical study explores the effects of different monovalent cations on α-syn amyloid formation. α-syn is a highly charged protein (15 basic and 24 acidic residues, net charge −9 at neutral pH). The charge is unevenly distributed rendering the N-terminus (residues 1–60), positive, the central region (residues 61–95) largely hydrophobic and the C-terminus (residues 96–140) negatively charged. The C-terminus contributes to monomer solubility [15], modulates aggregation [16], and can coordinate various metal ions [7], including alkali metals [17]. Electrostatic interactions are generally important for amyloid fibril formation [16]. For highly charged, intrinsically disordered proteins, like α-syn, Coulombic charge-charge repulsion within the polypeptide chain can disfavour both folding and aggregation [8]. Salt ions can neutralize this electrostatic repulsion and favour monomer-monomer as well as monomer-fibril interactions, either indirectly by Debye-Hückel screening or via charge-altering specific and non-specific ion interactions with the polypeptide chain [8]. The rate of α-syn aggregation

* Corresponding author.
E-mail address: eline@chalmers.se (E.K. Esbjörner).

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[8], and other amyloidogenic proteins [18] typically increases with increasing salt. The magnitudes of electrostatic effects appear to depend on salt type and not merely ionic strength; Debye-Hückel screening has therefore been ruled out as a major effector of protein aggregation [8, 18]. Salt can also modulate protein aggregation by indirect influence on protein solubility. Low salt typically keeps proteins soluble by creating a hydration shell [19], whereas high salt reduces protein solubility by breaking this hydration shell by a 'salting out' effect [20]. The relative effect of different salts on the solubility of proteins has been described by the Hofmeister series [21]. Both protein-ion interactions (which reduce Columbic repulsion) and Hofmeister effects have been suggested to modulate amyloid formation [8,18]. However, comparative studies with different salts have largely focused on the effect of different anions [8, 18] or higher valency cations [16].

We systematically studied the salt dependence of α-syn fibril formation. We compared effects of equimolar concentrations sodium (Na^+), potassium (K^+), and lithium (Li^+) chloride salts on the aggregation kinetics and monomer-fibril equilibrium at the aggregation end-point, as well as on the morphology, clustering propensity, and persistence lengths of the resulting fibrils. We report significant differences in the aggregation modulatory effect of the three ions, primarily related to the kinetics of the *de novo* formation of aggregates from monomers (primary nucleation). Notably, Na^+ (and to a lesser extent Li^+) ions accelerate primary nucleation whereas K^+ ions retard this process and hence appear to be aggregation protective. Moreover, K^+ ions decreased the β-sheet content and increased the persistence length of fibrils compared to Li^+ and Na^+. We discuss our results in terms of binding affinity differences of the cations to α-syn and to chaotropic behaviours of K^+. Our study contributes to current understanding of α-syn aggregation biophysics, pinpoints differential modulation of physiologically relevant cations and highlights the importance of carefully selecting experimental conditions for biophysical studies.

2. Methodology

2.1. Expression and purification of monomeric α-syn

α-syn was recombinantly expressed in BL21(DE3)pLysS *E.coli* and purified by acid precipitation, ion exchange and size exclusion chromatography (SEC) [22]. Monomeric α-syn fractions (Fig. S1) were aliquoted and stored at −80 °C.

2.2. Kinetic analysis of amyloid fibril formation

Aggregation kinetics was monitored by thioflavin-T (ThT) fluorescence in a BMG Labtech FLUOstar® Omega microplate reader with bottom optics and appropriate filters. SEC (Superdex®75 10/300 column) monomerized α-syn (50 μM, 20 mM Tris-HCl, pH 7.4) was distributed in Corning® 96-well microplates (#3881), together with LiCl, NaCl, or KCl and 20 μM ThT. One 2-mm glass bead was added per well to facilitate fibril formation [23]. The plates were incubated at 37 °C, using 200 rpm orbital shaking. Reaction half times, lag times (time taken to reach 10% of ThT_{max}), and growth times (time taken to reach from 10 to 90% of ThT_{max}) were extracted from the kinetic curves.

2.3. Residual monomer concentration

End-point samples from the plates were centrifuged to pellet fibrils (30 min, 14,000 rpm, Eppendorf® 5430R centrifuge). The residual α-syn concentrations in the supernatants were determined by absorption (ε_{280} = 5960 M^{-1} cm^{-1}, Cary 4000 UV–Vis spectrophotometer). The supernatants were confirmed free of amyloid fibrils by absence of ThT emission, using a Cary Eclipse fluorimeter (440 nm excitation).

2.4. Circular dichroism (CD) spectroscopy

CD spectra were recorded on aggregation end-point samples (diluted 1:9 in 20 mM Tris-HCl, pH 7.4 buffer without salt to reduce background) using an Applied Photophysics Chirascan instrument. Three spectra were recorded and averaged prior to subtraction of appropriate blanks.

2.5. Atomic force microscopy (AFM)

The end-point CD samples were diluted 1:2 with MQ water, deposited onto freshly cleaved mica and allowed to settle (10 min). The mica was rinsed (10X, MQ water) and dried. AFM images (10×10 μm, 256x256-pixel) were recorded on an NT-MDT NTEGRA Prima instrument using tapping mode (0.5 Hz scan frequency) and a NSG01 gold-coated single crystal silicon probe (Resonant frequency ~150 kHz, force constant ~5.1 N/m). Images were processed in Gwyddion [24] and analysed using Easyworm [25].

3. Results

3.1. Aggregation kinetics of α-synuclein as function of salt type and concentration

The aggregation kinetics of monomeric α-syn (Fig. S1) were monitored by ThT fluorescence in buffers with different chloride salts (LiCl, NaCl, KCl). Except for KCl, the kinetics increased with ionic strength, in accord with previous reports [16] and as further depicted in Fig. S2 (where the kinetic curves are grouped by salt type). The three different cations have distinct modulatory effects on α-syn aggregation despite having the same charge. At low salt (50 mM) KCl promotes the fastest aggregation and NaCl the slowest (Fig. 1a). With increasing salt concentration this trend is gradually reversed (Fig. 1b–f). For LiCl, the kinetic curves are generally intermediate, and the ionic strength dependence is low. The observed effects do not follow the Hofmeister series for cations or differences in their atomic radii.

3.2. Analysis of the aggregation kinetic curves

The kinetic curves in Fig. 1 and for α-syn aggregation without salt (20 mM Tris-HCl buffer) (Fig. S3) were further analysed by extracting lag times, half times, and growth times (Fig. 2). This depicts more clearly the effect of the cations across the different phases of α-syn aggregation. The major differences arise in the lag phase (Fig. 2a), which is dominated by primary nucleation (formation of new aggregates from monomers). Statistical testing (one-way ANOVA) showed that both cation type and concentration had significant effects (Tables S4–7). The lag time decreased with increasing NaCl or LiCl concentration, whereas the opposite trend was observed with KCl (Fig. 2a). By contrast, there was no significant effect of cation type or salt concentration on the growth phase (Fig. 2c, Tables S8–11), which is dominated by fibril elongation and secondary nucleation.

3.3. Effect of salt on the monomer-fibril equilibrium

Residual monomers and fibrils at the aggregation reaction end-point were separated by centrifugation and the monomer content in the supernatant was determined by absorption (Fig. 3a, Fig. S12). Absence of amyloid fibrils in the supernatant was confirmed by lack of ThT fluorescence (Fig. 3b). The residual monomer concentration was appreciable (20–40% of the initially added monomer) (Fig. 3c) suggesting that the conversion into fibrils (Fig. 3d) was ineffective compared to for example amyloid-β where fibril yields are typically >90% [26]. The fibril yields were independent of cation type, but increased significantly with increasing salt, indicating that the monomer-fibril equilibrium depends on ionic strength. The fibril yield largely correlated with measured β-sheet content in the aggregation end-point samples(Fig. 3e, Fig. S12).

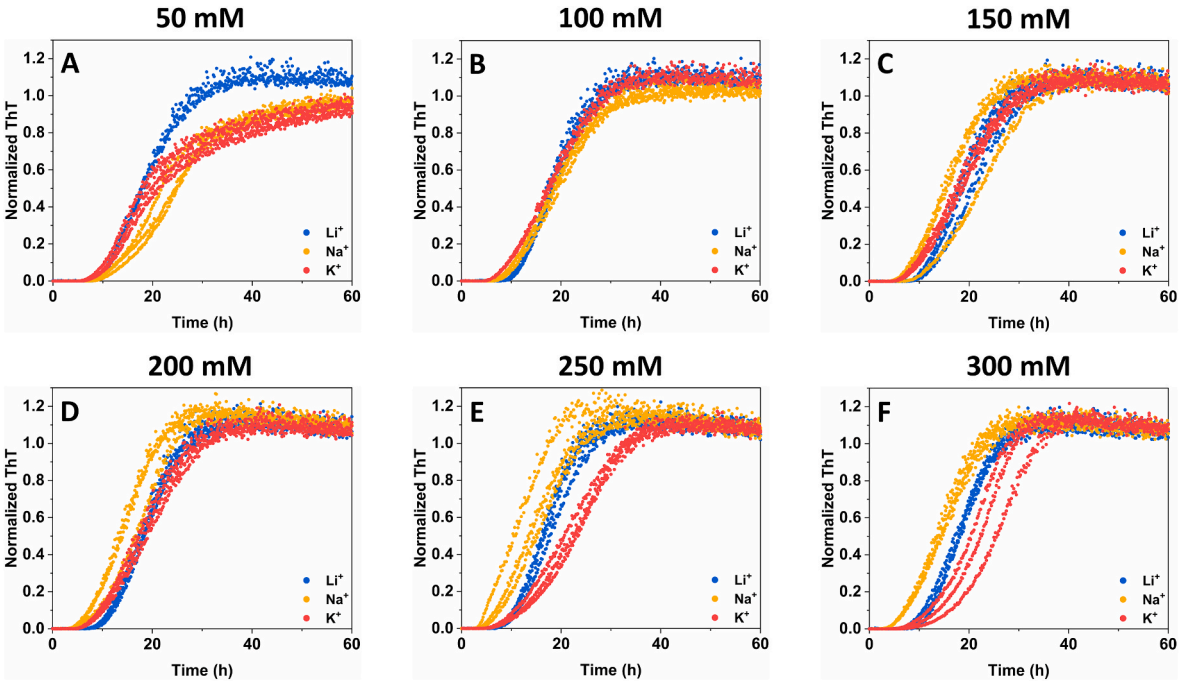

Fig. 1. α-syn aggregation kinetics in different salt solutions. (A–F) ThT monitored aggregation kinetics of 50 μM α-syn (37 °C, pH 7.4, 20 mM Tris-HCl, 200 rpm orbital shaking) with different chloride salts (LiCl, NaCl, KCl) and salt concentrations (n = 3).

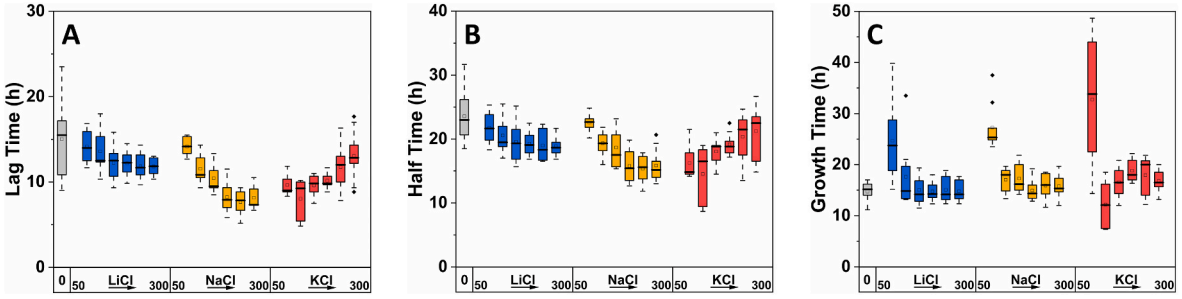

Fig. 2. (A) Lag times (B) half times, and (C) growth times of α-syn aggregation under the conditions assayed in Fig. 1 and defined as described in Materials and Methods. Data for 0 mM salt was included as comparison. Error bars represent SD (N = 2; n = 3).

The β-sheet content was, however, slightly higher in presence of LiCl and NaCl compared to KCl, in accord with reports of secondary structure stabilizing versus destabilizing effects of kosmotropic ($Li^+ > Na^+$) and chaotropic (K^+) ions [27].

3.4. Morphology of α-syn fibrils

Fibril morphology was analysed by AFM (Fig. 4a–c, Fig. S13). Cation-specific differences in fibril clustering (lateral association) were observed; NaCl induced more clustering than KCl. The cation type and salt concentration did not affect α-syn fibril length (Fig. 4d), presumably because of shaking which promotes fibril fragmentation in proportion to the applied force [28]. However, α-syn fibrils formed in KCl buffer had longer persistence lengths (Fig. 4e), as also apparent from comparison of the degree of horizontal alignment of the analysed fibrils along their initial tangents (Fig. 4d–f).

4. Discussion

Protein aggregation is highly sensitive to a variety of environmental conditions, whose fine-tuning may control the great diversity and disease-specificity of amyloid fibril structures that have been identified in post-mortem brain samples. Salt is a simplistic factor that can have substantial and rather complex effects on amyloid formation. Whilst it is widely recognized that different anions [8] and multivalent cations [16] can have distinct modulatory power and that electrostatic effects on protein aggregation are thus more complex than predicted by Debye--Hückel screening [8,18], differences between monovalent cations have so far not been well explored or assumed to be marginal.

We report that Na^+ and Li^+ speed up α-syn aggregation in a salt concentration dependent manner (Figs. 1 and 2b), consistent with previously reported effects of different anions on α-syn aggregation [8], and of salt on other amyloidogenic proteins such as amyloid-β [18,29], glucagon [30], and β2-microglobulin [31]. This agrees with the idea that charge neutralisation and reduction of Columbic repulsion promotes aggregation [8]. However, K^+ ions had opposite effect, retarding α-syn fibrillation. This has been previously observed for α-syn at low pH [9], Sup35 [32], and amyloidogenic proteins that contain specific coordination sites for multivalent transition metal ions such as amyloid-β [33,34] and the prion protein [35].

Our data suggest that monovalent cations primarily differ in their modulation of the lag time of α-syn aggregation (Fig. 2a). Hence, they appear to act differently on primary nucleation. Ions may either interact directly with a polypeptide or affect its solubility and surrounding water

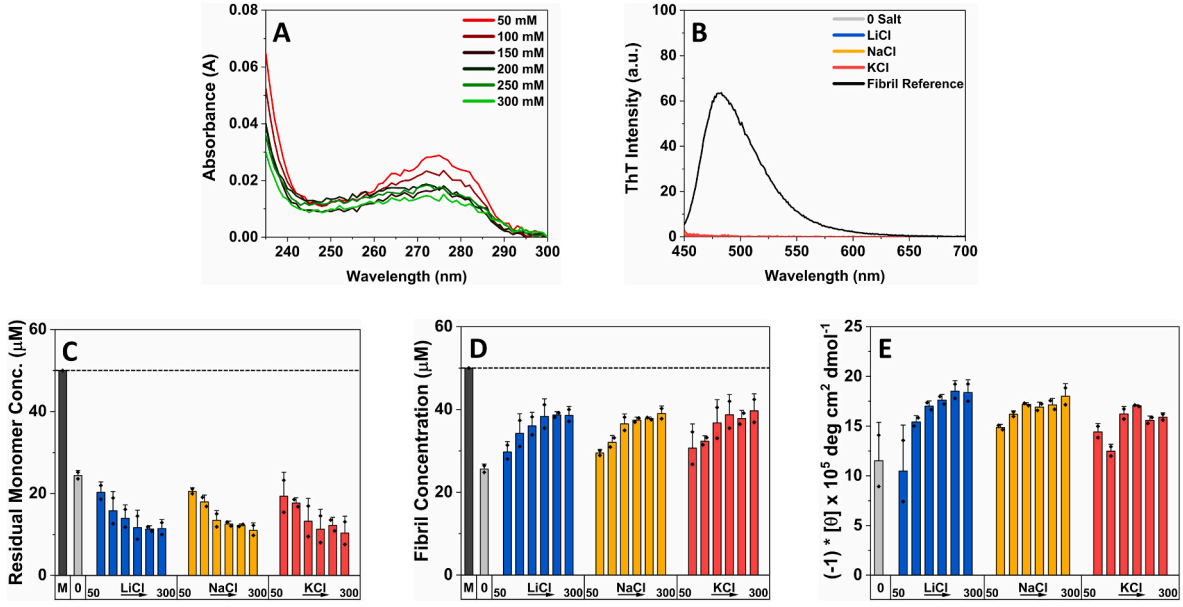

Fig. 3. Effect of salt on fibril yield and secondary structure. (A) Representative absorption spectra of α-syn in NaCl supernatants following centrifugal fibril sedimentation. (B) Representative ThT spectra of different supernatants (0 mM or 50 mM of the indicated salt) relative to a fibril reference sample (C,D) Residual monomer (C) and fibril (D) concentration measured based on supernatant absorption. The starting monomer concentration (M) was 50 μM. (E) Circular dichroism (CD) at 218 nm as indication of β-sheet content in non-centrifuged end-point samples. (C–E) Data shown for 0 mM or 50–300 mM (50 mM increments) of the different salts. Error bars represent SD (N = 2, n = 3).

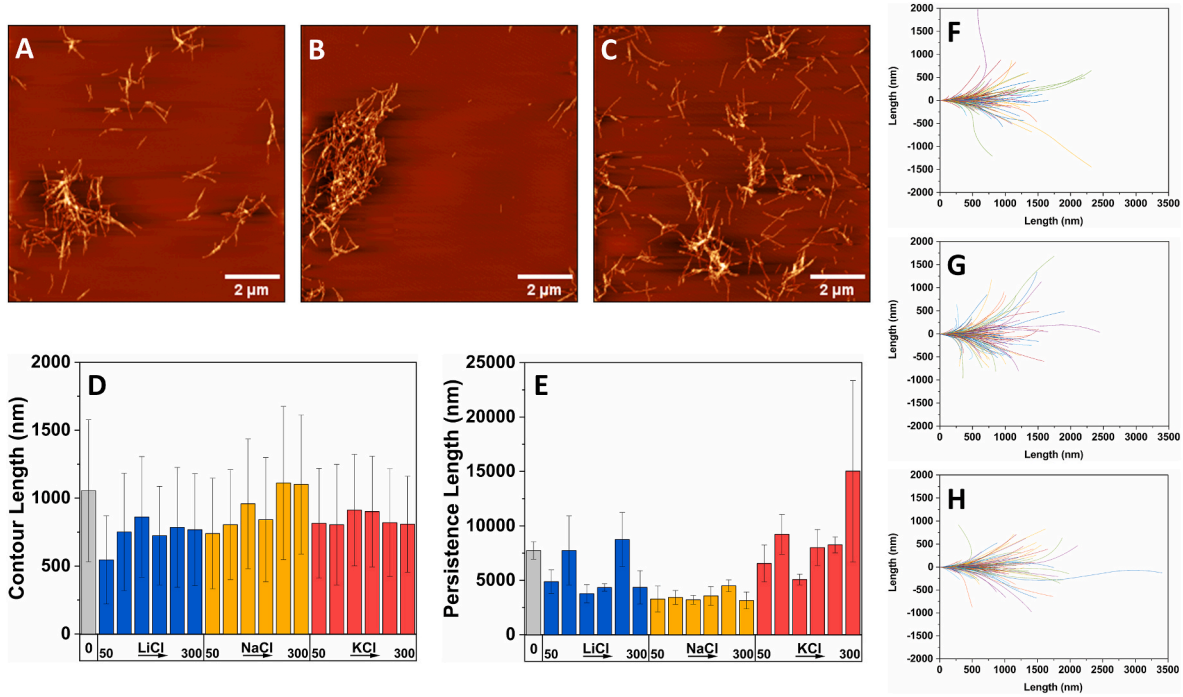

Fig. 4. Effect of salt on the morphology of α-syn amyloid fibrils. (A–C) Representative AFM images of fibrils formed in 100 mM (A) LiCl, (B) NaCl, (C) KCl. (D–E) Contour lengths (D) and persistence lengths (E) of α-syn fibrils formed in 0 mM or 50–300 mM (50 mM increments) of the different salts. Error bars represent SD (n = 200). (F–H) Horizontal alignment of the analysed fibrils along their initial tangent.

structure (so-called Hofmeister effects [36]). α-syn, is expected to bind cations due to its net negative charge. Interestingly, Aziz et al. have reported that the affinity between monovalent cations and carboxylate to decrease in the sequence $Na^+ > Li^+ \gg K^+$ [37], with K^+ ions having a much weaker affinity [37,38]. The differences have been ascribed to size matching between the cation and carboxylate, but also to the empirical law of matching water affinities, which states that ions have a tendency to associate with counterions or ionic groups that possess similar hydration enthalpies [39]. In our case, this suggests that $Na^+ > Li^+$ binds to monomeric α-syn and catalyses oligomerisation by reducing Columbic repulsion, whereas the affinity of K^+ may be too weak to exhibit this effect. Further, intrinsically disordered proteins have been reported to adopt more condensed and structured conformational states in presence of salts, due to the apparent increase in hydrophobicity that

accompanies charge reduction [8,16,40]. It has even been suggested that α-syn must adopt a compact state to aggregate [40]. Furthermore, kosmotropic ions can decrease random coil and increase β-sheet content of proteins [41], which could contribute to explain why Li^+ and Na^+ facilitate amyloid formation. The nucleation inhibitory salt dependence with K^+ may instead be understood if one considers the suggestion of Yeh et al. that ions with chaotropic behaviour thermodynamically stabilize unfolded states, possibly by hydration effects [32]. Notably, Gaspar et al. observed a similar anomalous salt dependence of α-syn aggregation with NaCl at pH 5.5 [9]. This could be due to reduced affinity between Na^+ and carboxylate at low pH [38]. The discrepancy relative to our work furthermore illustrates the high sensitivity of ion-dependent effects. In conclusion, we suggest that the differences between Na^+/Li^+ and K^+ relate to differences in binding affinity to carboxylic groups, presumably in the C-terminus of α-syn where acidic residues are abundant, but that kosmotropic and chaotropic effects also contribute and become particularly significant for K^+ due to its low affinity. We could not observe any effects of cation type or salt concentration on the α-syn aggregation growth time (Fig. 2c), even though other studies report that both fibril elongation and secondary nucleation may depend on salt [8,18]. This could be explained by the use of shaking [23], which introduces fibril fragmentation in proportion to the applied force [28]. However, we did observe a major increase in growth time (reduction in growth rate) at low (50 mM) salt (Fig. 2c). Similar effects were observed with β2-microglobulin and different Na-salts [31,42], whereas the opposite was observed for glucagon [30]. The aggregation-reducing effect has been described to depend critically on the balance between hydrophobic and hydrophilic forces and a threshold between low salt and high salt regimes. Notably, the nature of the cation (Li^+, Na^+ or K^+) did not affect fibril yields (Fig. 3d) and hence the thermodynamic equilibrium between monomers and fibrils. Most previous studies on the effect of salt, have focused on kinetic effects [18], or used ThT intensity as an indicator of fibril yield [43]. The latter is inherently unreliable due to fibril structure dependent photophysics and binding affinity of the dye [44], which is, additionally, charged and therefore affected by electrostatics. Our observations suggest that Debye-Hückel screening may contribute to the colloidal stability of fibrils, even though it is unimportant for the kinetics of fibril assembly. We also note that K^+ ions have some specific effects on the resulting α-syn fibrils, including a minor decrease in β-sheet content (Fig. 3e), an increase in persistence length (Fig. 4e–h) and reduced clustering (Fig. 4c) compared to Li^+ and Na^+. Salt specific effects on fibril structure have previously been observed for a variety of proteins including Aβ40 [18], glucagon [30] and β_2-microglobulin [31]. Furthermore, high charge density generally increases the persistence length of polymers. Along these lines, a study conducted on various succinylated and methylated ovalbumin fibrils [45] confirmed that high protein net charge resulted in rigid fibrils and that these have lesser tendency to form clusters. These effects agrees well with our data on α-syn fibrils in presence of K^+, and supports the idea of a low binding affinity of this cation.

In conclusion, this study shows that physiologically abundant cations (Na^+ and K^+) have distinct and opposing effects on the kinetics of α-syn aggregation, and on the rigidity and clustering potential of the resulting fibrils. This demonstrates the importance of carefully considering buffer and salt choices for *in vitro* studies. Our results may also have implications for the current understanding of α-syn's *in vivo* aggregation, which occurs in intracellular locations where K^+ is the dominant monovalent cation, but where neuronal activity may expose α-syn to transient Na^+ gradients which may, according to our results, trigger nucleation. Moreover, the finding that different monovalent cations have distinct effects on fibril clustering may be relevant for our understanding of how α-syn fibrils associate into Lewy bodies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.08.061.

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