Direct and Indirect Salt Effects on Homotypic Phase Separation

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Abstract

The low-complexity domain of hnRNPA1 (A1-LCD) phase separates in a salt-dependent manner. Unlike many intrinsically disordered proteins (IDPs) whose phase separation is suppressed by increasing salt concentrations, the phase separation of A1-LCD is promoted by > 100 mM NaCl. To investigate the atypical salt effect on A1-LCD phase separation, we carried out all-atom molecular dynamics simulations of systems comprising multiple A1-LCD chains at NaCl concentrations from 50 to 1000 mM NaCl. The ions occupy first-shell as well as more distant sites around the IDP chains, with Arg sidechains and backbone carbonyls the favored partners of Cl^- and Na^+, respectively. They play two direct roles in driving A1-LCD condensation. The first is to neutralize the high net charge of the protein (+9) by an excess of bound Cl^- over Na^+; the second is to bridge between A1-LCD chains, thereby fortifying the intermolecular interaction networks in the dense phase. At high concentrations, NaCl also indirectly strengthens π-π, cation-π, and amino-π interactions, by drawing water away from the interaction partners. Therefore, at low salt, A1-LCD is prevented from phase separation by net charge repulsion; at intermediate concentrations, NaCl neutralizes enough of the net charge while also bridging IDP chains to drive phase separation. This drive becomes even stronger at high salt. Based on this understanding, four salt effect types on the phase separation of IDPs can be predicted based on their amino-acid compositions.

Introduction

Biomolecular condensates formed via liquid-liquid phase separation (LLPS) mediate a variety of cellular functions such as biogenesis of the ribosome and stress response. The driver for phase separation or condensation is intermolecular interactions, including electrostatic, hydrogen bonding, π-π, cation-π, amino-π, and hydrophobic.[1,2] Salt can tune all these interactions and thus exert significant effects on phase separation. While the screening effect of salt on electrostatic interactions is well-known, its effects on other types of interactions may be indirect and perhaps are less appreciated. In particular, high salt strengthens hydrophobic interactions by increasing the surface tension of water.[3,4] Because salt can exert disparate effects on different types of interactions, it can be used as a perturbation to dissect the relative importance of these interactions in phase separation.[5,6] Inside cells, proteins can encounter varying salt conditions at different locations or at different times, and therefore the drive for their phase separation can span a wide range.

Numerous studies of salt effects on protein phase separation have been reported. The most typical effect is the suppression of phase separation by screening electrostatic attraction, both for homotypic systems[7-14] and for heterotypic systems.[5,15] However, salt can also promote phase separation, although the mechanism is not always clear.[7,16-24] In particular, Krainer et. al.[16] observed a "reentrant" salt effect on the phase separation of five intrinsically disordered proteins (IDPs): phase separation occurs without salt, is prohibited by medium salt, and reemerges at high salt. They attributed the reemergence of phase separation to strengthened π-type and hydrophobic interactions that overcompensate weakened electrostatic attraction. A unifying understanding of how salt affects the phase separation of IDPs is still lacking. For example, it is an open question whether salt effects on phase separation can be predicted from the protein sequence. Deep knowledge, in particular at the atomic level, of how salt affects intermolecular interactions and, ultimately, phase separation is required.

The low-complexity domain of hnRNPA1 (A1-LCD) represents another IDP where an atypical salt effect was reported.[7] The full-length protein, comprising folded domains along with the LCD, phase separates in the absence of salt and the tendency to phase separate is reduced upon adding salt, thus exhibiting the typical salt effect. A screening mechanism, specifically of electrostatic attraction between the LCD and the folded domains, is supported by small-angle X-

ray scattering and coarse-grained molecular dynamics (MD) simulations. In contrast, A1-LCD does not phase separate without salt and starts to do so only after 100 mM NaCl is added. An earlier study revealed that π-types of interactions, mediated by aromatic residues, drive the phase separation of A1-LCD.[25] A follow-up study, based on charge mutations, further showed that the net charge plays a strong suppressive role.[26] The saturation concentration (C_{sat}) for phase separation is minimum at a net charge near 0, and increases by two orders of magnitude, signifying an enormous weakening of the drive for phase separation, when the net charge moves away from neutrality in either direction. In a more recent study, salt promoted the homotypic phase separation of both A1-LCD and FUS-LCD, but suppressed the heterotypic phase separation of their mixture.[5] The salt effect on the phase separation of another IDP, Ddx4, has been analyzed using the random phase approximation based on a coarse-grained representation.[27]

All-atom MD simulations can uniquely provide mechanistic insight into the driving force and properties of biomolecular condensates.[2] For example, these simulations showed that ATP, a small molecule with a -4 charge, bridges between positively charged IDP chains in driving phase separation.[28] The intermolecular interactions quickly break and reform, explaining why the condensates can rapidly fuse despite very high macroscopic viscosity. Similarly, quick breakup and reformation of salt bridges in a heterotypic condensate allow the protein molecules to be extremely dynamic in a highly viscous environment.[15] Recently, all-atom MD simulations provided explanations for wide variations in phase equilibrium and material properties among condensates of tetrapeptides with different amino-acid compositions.[29]

Here we study salt effects on A1-LCD condensation by all-atom MD simulations. The simulations reveal two direct effects and one indirect effect of NaCl: neutralization of net charge and bridging between protein chains as well as strengthening of π-type interactions by drawing water away from the interaction partners. We also present a unifying picture of salt effects on phase separation by classifying them into four distinct types, and predict these types based on amino-acid composition.

Results

Salt condenses A1-LCD and increases inter-chain interactions

The 131-residue A1-LCD is comprised mostly of Gly and Ser (51 and 22, respectively), followed by 18 aromatic residues (11 Phe and 7 Tyr), 17 residues with sidechain amides (13 Asn and 4 Gln), and 15 charged residues (10 Arg, 2 Lys, and 3 Asp), with a large net charge of +9 (Figure 1A). Using initial conformations of A1-LCD from the previous single-copy simulations,[30] we built an 8-copy model for the dense phase (with a concentration of 3.5 mM) at five NaCl concentrations ranging from 50 to 1000 mM (Figure 1B). Each system was simulated in four replicates for 1 μs each. All the results reported here are averages over the four replicate simulations. From the same initial configuration with very few inter-chain contacts, the systems expand slightly at 50 mM NaCl (Figure 1C) but condense noticeably at 1000 mM NaCl (Figure 1D). At low salt, the protein chains fill the simulation box in two of the three orthogonal directions, with chain configurations rearranging frequently. In contrast, at high salt, the chains aggregate into a sphere with slower chain reconfiguration.

We also quantified the contrast between low and high salt by calculating D_{max}, the maximum side length of the rectangular box that parallels the simulation box and circumscribes the multi-chain system. The D_{max} values are above or close to the initial value (~150 Å) at 50 and 300 mM NaCl, but show a clear decrease (to ~125 Å) at 500 and 1000 mM NaCl (Figure 2A). The D_{max} results at 150 mM NaCl are mixed, falling between those at 50 and 300 mM NaCl as expected for the first 400 ns and the last 100 ns but dipping below those at 300 mM for the other half of the simulation time. The latter out-of-order behavior reflects the fact that the multi-chain system can sometimes get stuck in an overly condensed state. Overall, the systems show growing condensation with increasing salt. A small part of the reason for the growing condensation is the compaction of the individual chains. The average radius of gyration (R_g) shows a decreasing trend with increasing salt (Figure 2B), which matches the experimental data.[7] Correspondingly, the average number of intrachain contacts per residue increases slightly, by 4%, when the salt concentration is increased from 50 mM to 1000 mM (Figure 2C). However, the main driver of the condensation is inter-chain interactions, with inter-chain contacts per residue increasing by 22% over the same salt range. Clearly, high salt induces A1-LCD condensation, with an increased number of interactions between protein chains. Below we present the molecular mechanisms for these effects.

Ions selectively bind to backbone and sidechain sites to neutralize the net charge of A1-LCD

The large net charge of A1-LCD implies significant electrostatic repulsion between chains. Potentially salt ions can neutralize the net charge. To investigate ion-protein binding, we calculated radial distribution functions (RDFs) of Cl^- and Na^+ around polar groups of A1-LCD. At 1000 mM NaCl, the RDFs of Cl^- show a strong 1st peak at 3.2 Å around Arg and Lys sidechain nitrogens and a moderate 1st peak around the Gln and Asn sidechain nitrogens and the Ser sidechain oxygen (Supplementary Figures 1A). Each Arg sidechain often coordinates two Cl^- ions simultaneously, but each Lys sidechain coordinates only one Cl^- ion. A 2nd peak, at ~5 Å, is also observed around Arg and Lys sidechain nitrogens. Of the remaining polar groups, only the Cl^- RDFs around the Tyr sidechain oxygen and the backbone nitrogen show a weak 1st peak (Supplementary Figures 1B). We used cutoffs of 4 and 6.4 Å, respectively, to define 1st-shell and 2nd-shell Cl^- binding. On a per-residue basis, Arg and Lys sidechains coordinate the most 1st-shell Cl^- ions, reaching ~0.25 ions per residue (Figure 3A, left panel). In comparison, each Gln, Asn, or Ser residue, on average, coordinates ~0.05 Cl^- ions. Given the large numbers of Arg and Ser residues in the A1-LCD sequence, these two residue types coordinate the most 1st-shell Cl^- ions, 21.6 and 8.9, respectively, in the 8-chain system at 1000 mM NaCl. Although the RDF around backbone nitrogens has only a weak 1st peak, given their large number (131 per chain), they actually coordinate 14.1, or the second most 1st-shell Cl^- ions. Among these 14.1 Cl^- ions, 57% are coordinated with Gly residues. A large part of this high percentage is due to the enrichment of Gly (39% of all residues) in the A1-LCD sequence, but Gly is additionally favored for its lack of sidechain, which allows the close approach of ions to the backbone.

Na^+ shows a very strong preference for the Asp carboxyl in 1st-shell coordination, with a peak RDF value of 12.5 at 2.2 Å (Supplementary Figure 2A). Each Asp sidechain carboxyl typically coordinates a single Na^+ ion, usually in a bifurcated geometry. In addition, Na^+ shows a strong preference in 1st-shell coordination with Asn and Gln sidechain amide oxygens and the backbone carbonyl oxygen as well as a moderate preference with the Ser sidechain oxygen. A 2nd peak, at ~4.2 Å, is also observed in the Na^+ RDF around the Asp carboxyl. Of the remaining polar groups, only a weak 1st peak is seen in the RDF around the Tyr sidechain oxygen (Supplementary Figure 2B). We used cutoffs of 3 and 5.4 Å, respectively, to define 1st-shell and 2nd-shell Na^+ binding. On a per-residue basis, the Asp sidechain coordinates the most 1st-shell Na^+ ions, reaching 0.22 ions per residue (Figure 3A, right panel). The per-residue Na^+ ion number reduces to ~0.06 for Asn and Glu sidechain oxygens and further to 0.03 for the backbone oxygen and Ser sidechain

oxygen. Again, due to their large number, backbone oxygens coordinate a very large number, 30.5, of 1st-shell Na^+ in the 8-chain system at 1000 mM NaCl. This number dwarfs the counterparts for sidechains, the largest of which are 7.8, 5.3, and 4.6, respectively, for Asn, Asp, and Ser. For coordination with the backbone, similar to the case with Cl^-, 55% of the 30.5 Na^+ ions involve Gly residues, again reflecting the fact that this amino acid allows the close approach of ions to the backbone. On the other hand, the largest number of 1st-shell Cl^- ions are coordinated with Arg sidechains but the largest number of 1st-shell Na^+ ions are coordinated with backbone carbonyls. Whereas each Arg sidechain often coordinates two Cl^- ions, multiple backbone carbonyls often coordinate a single Na^+ ion (see below).

In Figure 3B, we display the total numbers of 1st-shell Cl^- and Na^+ ions in the 8-chain systems as a function of NaCl concentration. Both the 1st-shell Cl^- and Na^+ ions increase with increasing NaCl concentration, and Cl^- ions always outnumber Na^+ ions. The difference remains almost constant, with around 6 more 1st-shell Cl^- ions than 1st-shell Na^+ ions. This difference is not enough to neutralize the total charge, +72, on the protein chains. However, when 2nd-shell ions are also included, the excess of bound Cl^- ions over bound Na^+ ions grows quickly with increasing NaCl concentration. Correspondingly, the net charge of the system reduces to only +8 at 1000 mM NaCl (Figure 3C), which may be completely neutralized by ions beyond the second shell. Net charge repulsion explains the expansion of the chains in the simulations at 50 mM NaCl (Figure 1C) and the absence of phase separation at this low salt concentration.[7] The protein net charge is largely neutralized by 1st- and 2nd-shell ions at high salt; hence the protein chains condense (Figure 1D) and phase separation is readily observed.[7]

Ions act as bridges between protein chains to drive condensation

In addition to charge neutralization, we suspected that ions could also fortify intermolecular interactions by bridging between proteins, similar to the role played by ATP molecules in driving phase separation of positively charged IDPs.[28] Indeed, we found that Cl^- has a tendency to bind with Arg and other sidechains from multiple chains and likewise, Na^+ has a tendency to bind with backbone carbonyls and sidechain oxygens from multiple chains (Figure 4A).

To quantify this tendency, we calculated the number of bridging Cl^- or Na^+ ions, i.e., those that bind (both 1st- and 2nd-shell) to residues on more than one A1-LCD chain (Figure 4B). Close to 20% of all 1st- and 2nd-shell ions bridge between A1-LCD chains. For both Cl^- and Na^+, the average number of bridging ions increases with increasing salt, but the pace of increase is much greater for Na^+ than for Cl^-, commensurate with the trend shown by the total number of bound ions of each type (Figure 3B). The number of bridging ions is 4.1 for Cl^- and only 0.7 for Na^+ in the 8-chain system at 50 mM NaCl, and increases to 24.4 and 12.0, respectively, at 1000 mM NaCl. The greater fold change of bridging Na^+ ions (18-fold, vs 6-fold for Cl^-) is also apparent when we break the bridging ions according to the number of A1-LCD chains being bridged (Figure 4C). For Cl^-, the curves plotting the ion count against the number of bridged chains are close to each other among the different NaCl concentrations, but the counterparts for Na^+ are more spread out.

These disparate effects of salt concentration on chain bridging by Cl^- and Na^+ can be explained by the difference in coordination properties between the two ion types presented above. Cl^- strongly prefers Arg sidechains and, even at low salt, occupies a large number of sites lined by them, of which ~20% are bridging sites. However, there is a relatively limited supply of Arg sidechains (a total of 80 in the 8-chain system) and hence the fold change in bridging Cl^- ions upon salt increase is somewhat tempered. In contrast, although Na^+ prefers Asp sidechains, there are very few of those in the system; instead, Na^+ predominantly binds to backbone carbonyls. As the latter binding is relatively weak, the number of bridging Na^+ ions is very small at low salt. However, since there is a large supply of backbone carbonyls (a total of 1048 in the 8-chain system), at high salt, Na^+ ions bind to a portion of these backbone carbonyls and bridge A1-LCD chains. Consequently, at low salt, chain bridging is dominated by Cl^- (Na^+ only 14% of bridging ions), but at high salt, Na^+ becomes more even (33% of bridging ions) with Cl^- in bridging A1-LCD chains. An important reason for the latter is Na^+ coordination by backbone carbonyls, especially those of Gly residues (Figure 4A).

Salt also contributes to condensation indirectly by strengthening π-type interactions

As noted above, the number of inter-chain interactions increases by 22% when the NaCl concentration increases from 50 mM to 1000 mM. While this result could be accounted for by the

two direct salt effects presented so far, i.e., charge neutralization and chain bridging, which act to condense A1-LCD, there are additional factors. A breakdown of inter-chain sidechain interactions into different types reveals that when the salt concentration is increased from 50 to 1000 mM, the number of salt bridges per chain actually decreases by 7%, while the numbers of π-π, cation-π, and amino-π interactions increase by 37%, 27%, and 21%, respectively (Figure 5A). That is, as the A1-LCD is condensed at high salt, there is an overall increase in inter-chain interactions, but there is also a redistribution of these interactions among the different types. At increasing salt, more π-types of interactions are formed while fewer salt bridges are formed, suggesting a strengthening of the former interactions but a weakening of the latter.

The weakening of salt bridges by salt can be understood as ions can compete with the salt-bridge partners for coordination (Supplementary Figures 1A and 2A). Indeed, at 1000 mM NaCl, cationic and anionic partners in a salt bridge are often found to also coordinate Cl^- and Na^+, respectively (Figure 5B). By contrast, π-π interactions are hardly affected by ion competition (Figure 5C), as Tyr has only a weak ability to coordinate Cl^- and Na^+ (Supplementary Figures 1B and 2B) and Phe completely lacks this ability. In comparison, one partner in both cation-π and amino-π interactions can potentially suffer from ion competition (Supplementary Figures 1A and 2A), which may explain why the increases in cation-π and amino-π interactions at high salt are not as large as the counterpart in π-π interactions.

For π-types of interactions, instead of a weakening mechanism through competition, salt may exert a strengthening mechanism by drawing water away from the interaction partners (Figure 5D). We calculated the RDFs of water around Tyr sidechains that form π-types of interactions and found decreases in water density when the salt concentration is increased from 50 to 1000 mM (Figure 5E and Supplementary Figure 3A-C). As a control, no decrease in water density is seen around Asp sidechains that form salt bridges (Supplementary Figure 3D). Water can interfere with and weaken π-types of interactions; by drawing water away from the interaction partners, high salt strengthens these interactions and thereby indirectly contributes to condensation.

Discussion

We have shown in atomistic detail the actions of ions in the condensation of A1-LCD over a wide range of NaCl concentrations. The MD simulations reveal that NaCl has both direct and indirect effects in driving phase separation. The first direct effect is to neutralize the net charge of A1-LCD and thereby attenuate net charge repulsion. The second direct effect is to bridge between protein chains and thereby fortify intermolecular interaction networks. In addition, high salt strengthens π-types of interactions by drawing water away from the interaction partners, thereby also indirectly driving phase separation. The net result is that, while phase separation of A1-LCD is prevented by net charge repulsion at low salt, it is enabled at intermediate salt through charge neutralization and chain bridging by ions. The drive for phase separation becomes even stronger at high salt, where π-types of interactions are strengthened.

These findings broaden our understanding of the roles of charges and ions in phase separation. That high net charge is a suppressive factor is highlighted by the strong effect of A1-LCD charge mutations on C_{sat}.[26] Here we have shown that this suppressive effect can be tuned out by the addition of salt. The released ions not only neutralize the net charge but also bridge between protein chains. Krainer et al.[16] observed a reentrant salt effect on the phase separation of IDPs, and attributed the reemergence of phase separation at high salt to strengthened π-type and hydrophobic interactions that overcompensate weakened electrostatic attraction. This conclusion was based on the salt dependences of the potentials of mean force calculated for pairs of sidechains of various types. Instead of a single pair of sidechains, our simulations are on multiple copies of protein chains. We show that, at high salt, the number of salt bridges is reduced while the numbers of π-types of interactions are elevated in the multi-chain system. The simulations further reveal that high salt achieves the latter effect by drawing water away from π-interaction partners.

The present work puts us in a position to paint a comprehensive picture of salt effects (Figure 6A, B and Table 1). The salt effect that is most often reported is screening, where salts weaken the electrostatic attraction between protein chains and thereby suppress phase separation.[7-14] The reentrant salt effect reported by Krainer et al.[16] can be seen as an extension of the screening scenario, whereby high salt overcompensates the screening effect by strengthening π-type and hydrophobic interactions and leads to reemergence of phase separation. The high net charge scenario represented by A1-LCD is similar to the reentrant scenario at high salt but differs from it at low salt, where phase separation is prevented by net charge repulsion.[7,17-22,24] In this salt effect

type, a certain amount of salt is required to start phase separation. For A1-LCD, this minimum salt is ~100 mM NaCl.[7] The recombinant mussel foot protein-1 (RMFP-1) presents an extreme example, which has a net charge of +24 over a sequence length of 121 and requires up to 700 mM NaCl to begin phase separation.[18] The fourth salt effect type is a variation of the preceding one; here the net charge is low and no significant net charge repulsion is expected, so the protein can phase separate even without salt. For example, FUS-LCD has a low net charge of -2 and phase separates without salt.[23] The difference between the high and low net charge scenarios is well captured by the phase-separation behaviors of TDP43-LCD at two pHs.[24] At pH 7, this protein has a relatively low net charge of +5 and phase separates without salt; the salt effect is thus of the low net charge type. When pH is lowered to 4, the six His residues in the purification tag become positively charged, raising the net charge to +11; now phase separation requires 300 mM NaCl and salt effect switches to the high net charge type.

We can not only rationalize the four distinct sat effect types but also start to predict them based on the amino-acid composition of the protein (Figure 6C and Table 1). The foregoing physical understanding suggests three determinants of salt effect type: (1) the total number of charged residues, which determines the contribution of electrostatic interactions to the drive for phase separation and the importance of salt screening; (2) the net charge, which determines the magnitude of net charge repulsion; and (3) the total number of aromatic residues, which determines whether phase separation reemerges at high salt. We predict that the screening type occurs when the charged content is high but the net charge and aromatic content are low (Figure 6C). The other three salt effect types all require a high aromatic content. For the reentrant type, the increase in aromatic content is the only difference from the screening type. For the high net charge type, the net charge obviously has to be high, but that could also mean at least a moderately high charged content. Finally the low net charge type is predicted when the net charge is low but no bias is required of the charged content. The amino-acid compositional data in Table 1 validate these predictions. For example, of the 16 proteins where the salt effect types call for a high aromatic content, 11 (or 68%) actually have this feature (aromatic content > an 8% threshold). In contrast, of the 9 proteins where the salt effect type (i.e., "screening") calls for a low aromatic content, only 2 (or 22%) have an aromatic content above the threshold. Likewise, of the 9 proteins where the salt effect type (i.e., "high net charge") calls for a high net charge, 6 (or 67%) actually have this feature (net charge > a 6% threshold). In contrast, of the 16 proteins where the salt effect types call

for a low net charge, none has a net charge above the threshold. Lastly, of the 14 proteins where the salt effect types call for a high charge content, 10 (or 71%) actually have this feature (charged content > a 20% threshold). In comparison, of the 11 proteins where the salt effect types do not require a high charge content, only 2 (or 18%) have a charge content above the threshold.

To conclude, salts regulate intermolecular interactions in biomolecular condensates in a variety of ways, but the net effect on phase separation can be placed into four distinct types (Figure 6A, B). For homotypic condensates, salt effect types are predictable from the protein amino-acid composition. Salts also exert significant effects on heterotypic condensates;[5,15] the conclusions drawn here on homotypic condensates may prove instructive for heterotypic condensates. The fact that different IDPs respond to salts differently raises interesting questions on phase separation inside cells. For example, a fluctuation in intracellular salt concentration may promote the phase separation of some IDPs and suppress the phase separation of other IDPs. If two IDPs co-phase separate or form heterotypic condensates, salt fluctuations may change the protein composition of condensates and the relative contributions of the individual proteins to phase separation. Lastly, we note that the atomistic details of ion coordination to A1-LCD backbone or sidechain groups are reminiscent of those observed in other systems, in particular channels and transporters for Na^+ and Cl^-.[31-33]

Computational Methods

Molecular dynamics simulations. MD simulations were performed using AMBER 18[34] with ff14SB force field[35] for the protein and TIP4P-D for water.[36] The initial configuration of the 8-chain system was constructed using A1-LCD conformations from previous single-copy simulations.[30] Specifically, 4 copies with different conformations were placed in a rectangular box, positioned with minimal inter-chain contacts within 3.5 Å. This 4-copy subsystem was duplicated to form the initial configuration of the 8-chain system (Figure 1B). The protein chains were solvated in a box with dimensions of 182 Å × 146 Å × 164 Å. For each desired salt concentration (50, 150, 300, 500, or 1000 mM), an appropriate number of water molecules were randomly selected and replaced with Cl^- and Na^+ ions; excess Cl^- was added to neutralize the system. The total number of atoms was ~500,000 for the 8-chain system at each salt concentration.

After energy minimization in sander (2000 steps of steepest descent and 3000 steps of conjugate gradient), each system was heated to 300 K over 100 ps with a 1 fs timestep, under constant NVT using the Langevin thermostat[37] with a 3.0 ps^{-1} damping constant. The simulation was then continued in four replicates at constant NPT for 1 μs with a 2 fs timestep. Pressure was regulated using the Berendsen barostat[38] with a coupling constant of 2.0 ps. All simulations were run on GPUs using *pmemd.cuda*.[39] Bond lengths involving hydrogens were constrained using the SHAKE algorithm.[40] Long-range electrostatic interactions were treated by the particle mesh Ewald method.[41] A cutoff distance of 10 Å was used for the nonbonded interactions. Frames were saved every 20 ps and the last 700 ns was used for analysis.

Data analysis. D_{max} was calculated using the minmax function in VMD.[42] The differences between the maximum and minimum values of *x*, *y*, and *z* coordinates of all protein atoms were obtained; the largest of the three difference values was taken as D_{max}. Radius of gyration (R_g) was calculated using the radgyr function in CPPTRAJ.[43] For inter- and intrachain contacts per residue, the number of other residues in contact with a given residue was found using the distance mask function in CPPTRAJ with a cutoff of 6 Å between heavy atoms. Inter-chain contacts per chain were found in a similar way, and were broken into interaction types based on the residue types of the interaction partners.

Radial distribution functions (RDFs) for ions around protein atoms were calculated in CPPTRAJ using the radial command. These RDF plots were used to select cutoffs for 1st- and 2nd-shell ion binding; these cutoffs were 4.0 and 6.4 Å, respectively, for Cl^- and 3.0 and 5.4 Å, respectively, for Na^+. The number of 1st-shell or 1st- and 2nd-shell ions interacting with N and O atoms in a given sidechain type, the backbone, or the 8 chains together was calculated using CPPTRAJ. Ions were considered bridging if they interacted with two or more chains (based on 2nd-shell cutoffs) at the same time. Those interacting with an exact number of chains (e.g., 3) were identified when the same ion was found in the interaction lists of that many chains.

RDFs for water molecules around Tyr residues were calculated using VMD. Only Tyr residues that interact with specific residue types (within 6 Å of the 6-carbon ring) were selected: the partner residues were Phe (6-carbon ring; for π-π), Arg and Lys (sidechain N atoms; for cation-π), or Gln and Asn (sidechain N atoms; for amino-π). As a control, PRDFs for water were also calculated around Asp sidechain oxygens that interact with Arg and Lys sidechain nitrogens.

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Table 1. Correlation between type of salt effect and amino-acid composition

Protein	Length	Charges (+/-/net)[b]	Aromatic[d]	Ref
		Screening		
FMRP-LCD	188	**37 / 28**[e] / 9	6	8
hnRNPA1	314	**42 / 34** / 8	**33**	7
PolyQ	729	38 / 34 / 4	35	13
Oleo30G	139	15 / 12 / 3	7	9
LAF-1	708	**86 / 88** / -2	55	10
LAF-1 RGG	191	**28 / 22** / 6	14	10
Ddx4	236	**32 / 36** / -4	**22**	12
HP1α	206	**33 / 41** / -8	14	11
FIB-1	352	**55 / 34** / 21	24	14
		Reentrant		
FUS	526	51 / 37 / 14	**52**	16
TDP-43	414	**40 / 44** / -4	**36**	16
Brd4	1362	**175 / 150** / 25	48	16
Sox2	317	34 / 21 / 13	19	16
A11	505	**51 / 50** / 1	**41**	16
		High net change		
A1-LCD	131	12 / 3 / **9**	**18**	7
TDP43-LCD (pH 4)	148	14[c] / 3 / **11**	**12**	24
Prp-LCD	122	11 / 1 / **10**	**11**	21
$RLP3_8$	120	12 / 8 / 4	8	22
HBP-2 (pH 5.5)	193	**30**[c] **/ 13** / **17**	**20**	20
Lysozyme[a]	129	**17 / 9** / **8**	**12**	17
UBQLN2	624	31 / 40 / -9	22	19
UBQLN2 (450-624)	175	4 / 8 / -4	5	19
RMFP-1	121	24 / 0 / **24**	**24**	18
		Low net charge		
FUS-LCD	136	0 / 2 / -2	**20**	23
TDP43-LCD (pH 7)	148	8 / 3 / 5	**12**	24

[a]Single-domain folded protein.

[b]Charges are listed as the number of positively (R and K, including H when specifically indicated) or negatively (D and E) charged residues or net charge.

[c]H is treated as positively charged at the low pH condition.

[d]Aromatic residues are W, Y, and F.

[e]High contents of charged residues, net charges, and aromatic residues are indicated by bold letters, with thresholds at 20%, 6%, and 8%, respectively, when measured as percentages of the sequence length.

Figure Captions

Figure 1. Amino-acid sequence of A1-LCD and molecular dynamics simulations of its condensation. (A) Amino-acid sequence. (B) First and (C, D) last frames from 1-μs simulations of the 8-chain systems at low and high salt. In all figures, Cl^- or Na^+ ions are represented by green and magenta spheres, respectively.

Figure 2. Salt effects on A1-LCD condensation and inter-chain interactions. (A) D_{max} values, averaged over four replicates, as a function of simulation time. (B) Radii of gyration (R_g) from small-angle X-ray scattering[7] and from MD simulations. Dashed curves are drawn to guide the eye. (C) Average number of inter- or intra-chain contacts per residue at each salt concentration. Dashed lines are drawn to show trends.

Figure 3. Levels of ion binding at different NaCl concentrations. (A) Number of 1st-shell ions, with (darker colors; tick marks on the left vertical axis) and without (lighter colors; tick marks on the right vertical axis) normalization by the number of residues of a given amino-acid type, at 1000 mM NaCl. (B) Total number of 1st-shell only or 1st- and 2nd-shell ions. (C) Net charge of the system with 1st- and 2nd-shell ions included.

Figure 4. Bridging ions. (A) Examples of chain bridging by Cl^- and Na^+; Cl^- ions are coordinated to Arg and other sidechains whereas Na^+ ions are coordinated to both backbone carbonyls (including from Gly) and sidechain oxygens. (B) Average number of Cl^- or Na^+ ions engaged in bridging between A1-LCD chains. (C) Average number of ions bound in 1st- and 2nd-shell sites lined by a given number of A1-LCD chains.

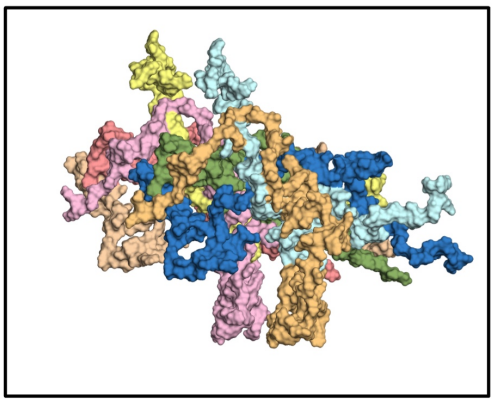
Figure 5. Indirect effects of ions on different types of interactions. (A) Number of inter-chain contacts per chain for each interaction type. Bars from left to right correspond to increasing salt concentrations (50, 150, 300, 500, and 1000 mM). Dashed lines are drawn to show trends. (B) An inter-chain salt bridge, with ion coordination by the partner sidechains. (C) An inter-chain π-π interaction, free of ion coordination. (D) Schematic showing a π-π interaction facilitated by high salt, via drawing water away from the interaction partners. (E) Radial distribution functions of water around Tyr residues that interact with Phe, Arg, Lys, Gln, and Asn. Lower values at high salt demonstrate water withdrawal.

Figure 6. Four salt effect types and their prediction based on amino-acid composition. (A) Salt effect on the tendency of liquid-liquid phase separation (LLPS). (B) Charge-charge interactions and their regulation by salt. (a) Significant Charge-charge attraction. (b) Screening of charge-charge attraction by salt. (c) Strengthening of π-type interactions by high salt. (d) Repulsion due to high net charge. (C) Distinctions of the four salt effect types by three determinants: charged content ("Chg"), net charge ("Net"), and aromatic content ("Aro").

A

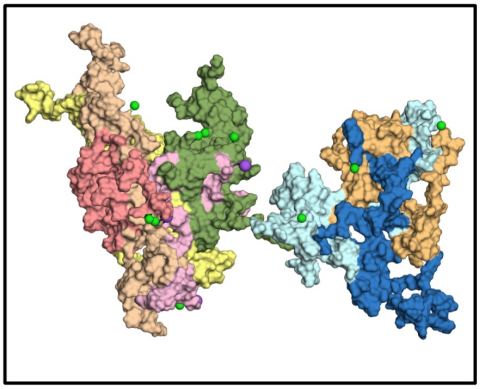
```
1    GSMASASSSQ RGRSGSGNFG GGRGGGFGGN DNFGRGGNFS  40
41   GRGGFGGSRG GGGYGGSGDG YNGFGNDGSN FGGGGNYNNQ  80
81   SSNFGPMKGG NFGGRSSGPY GGGGQYFAKP RNQGGYGGSS  120
121  SSSSYGSGRR F                                 131
```

B



+ 50 mM NaCl

C



+ 1 M NaCl

D

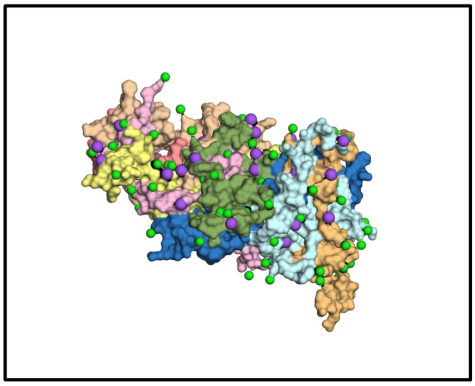


Figure 1

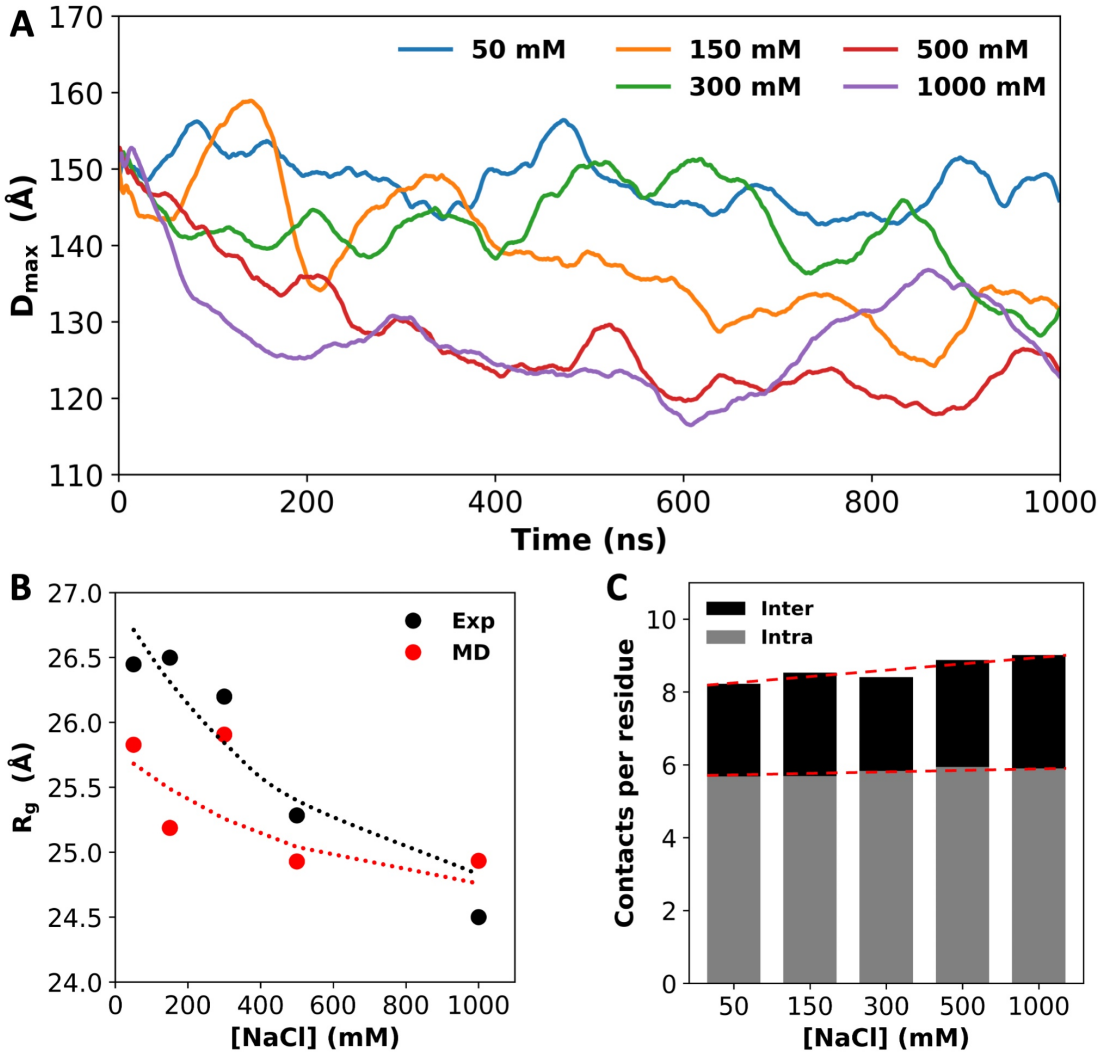

A
50 mM
150 mM
300 mM
500 mM
1000 mM
D_{max} (Å)
Time (ns)
B
Exp
MD
R_g (Å)
[NaCl] (mM)
C
Inter
Intra
Contacts per residue
[NaCl] (mM)

Figure 2

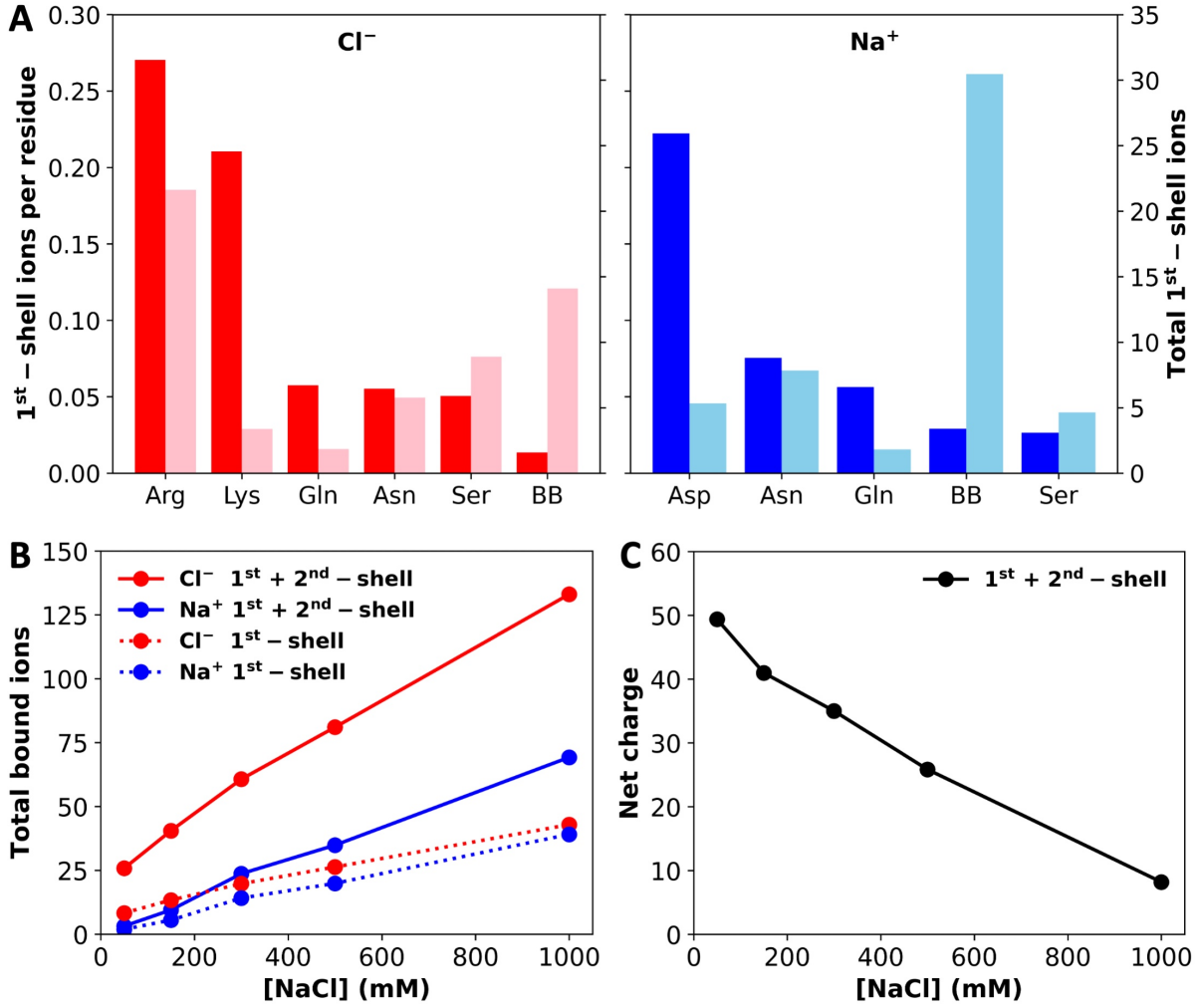

A
Cl⁻
Na⁺
1st – shell ions per residue
Total 1st – shell ions
Arg
Lys
Gln
Asn
Ser
BB
Asp
Asn
Gln
BB
Ser
B
Cl⁻ 1st + 2nd – shell
Na⁺ 1st + 2nd – shell
Cl⁻ 1st – shell
Na⁺ 1st – shell
Total bound ions
[NaCl] (mM)
C
1st + 2nd – shell
Net charge
[NaCl] (mM)

Figure 3

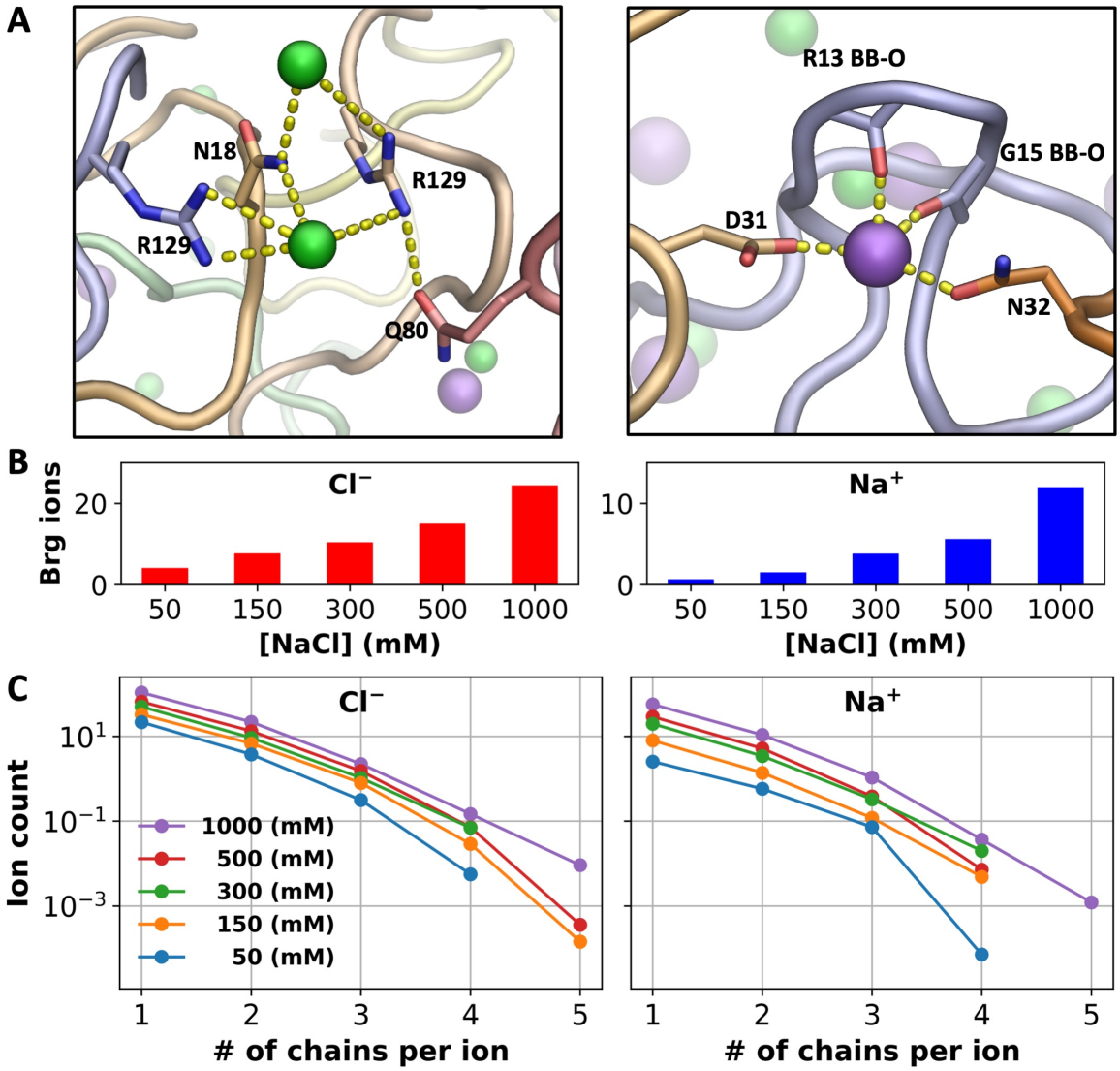

A
N18
R129
R129
Q80
R13 BB-O
G15 BB-O
D31
N32
B
Brg ions
Cl⁻
Na⁺
[NaCl] (mM)
[NaCl] (mM)
C
Ion count
Cl⁻
Na⁺
1000 (mM)
500 (mM)
300 (mM)
150 (mM)
50 (mM)
# of chains per ion
# of chains per ion

Figure 4

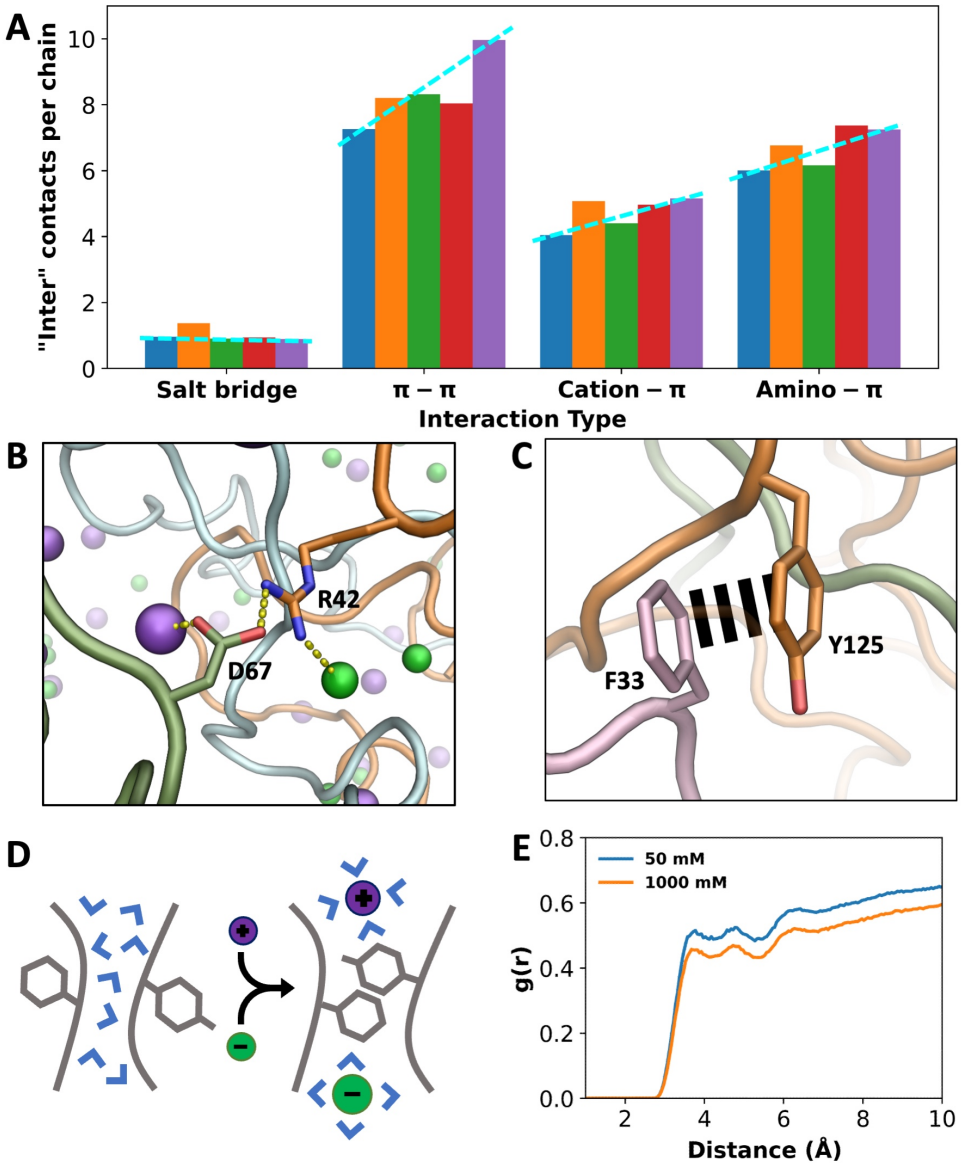

A
"Inter" contacts per chain
0
2
4
6
8
10
Salt bridge
π – π
Cation – π
Amino – π
Interaction Type
B
R42
D67
C
Y125
F33
D
E
50 mM
1000 mM
g(r)
0.0
0.2
0.4
0.6
0.8
2
4
6
8
10
Distance (Å)

Figure 5

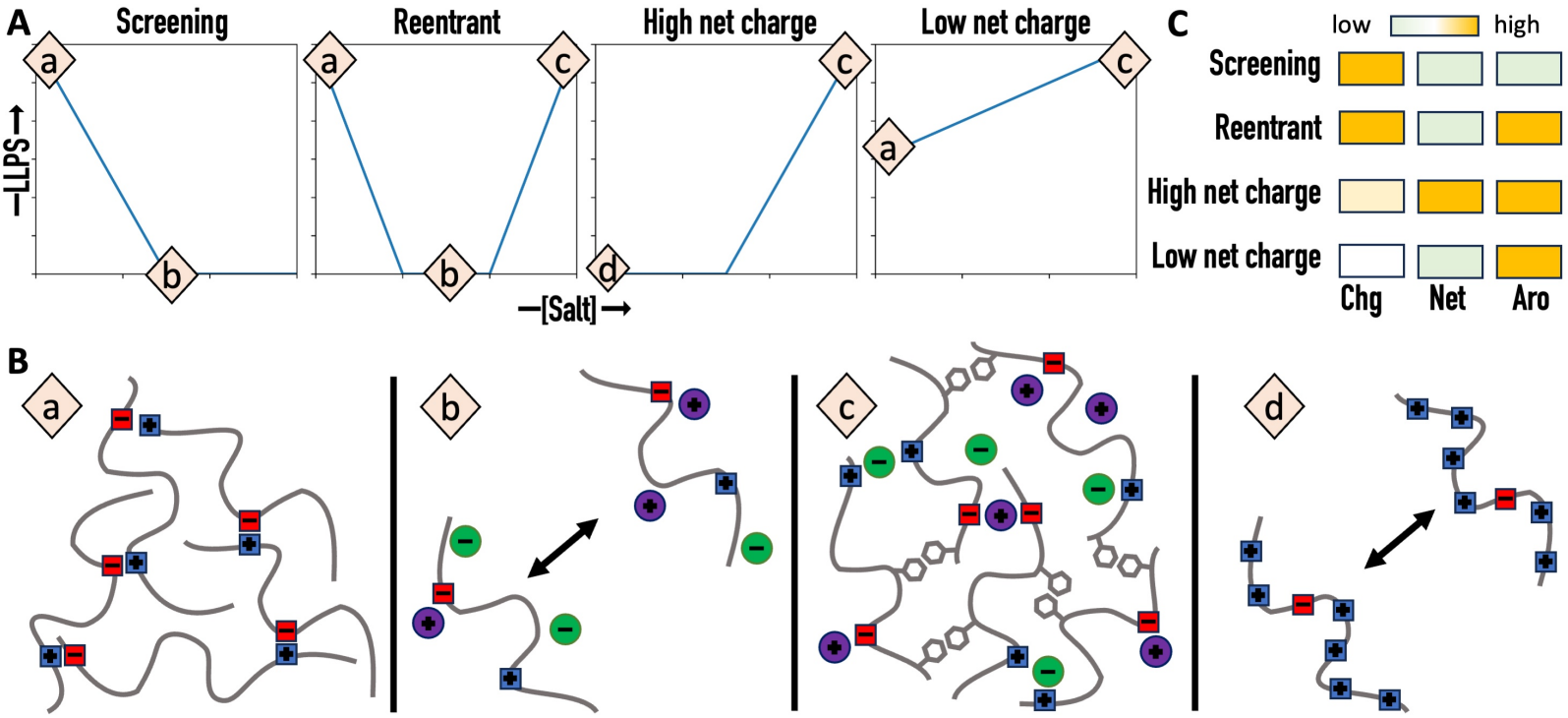

A
Screening
Reentrant
High net charge
Low net charge
a
b
c
d
LLPS
[Salt]
C
low
high
Screening
Reentrant
High net charge
Low net charge
Chg
Net
Aro
B

Figure 6