Ion-activated attractive patches as a mechanism for controlled protein interactions

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The understanding of protein interactions to control phase and nucleation behavior of protein solutions is an important challenge for soft matter, biological and medical research. Here, we present ion bridges of multivalent cations between proteins as an ion-activated mechanism for patchy interaction that is directly supported by experimental findings in protein crystals. A deep understanding of experimentally observed phenomena in protein solutions—including charge reversal, reentrant condensation, metastable liquid-liquid phase separation, cluster formation and different pathways of crystallization—is gained by an analytic model that directly displays parameter dependencies and physical connections. The direct connection between experiment and theory provides a conceptual framework for future experimental, computational and theoretical research on topics such as rational design of phase behavior and crystallization pathways on the basis of the statistical physics of patchy particles.

Patchy particles represent elegant models to explore the statistical physics of soft matter systems with directional interactions, in particular via the Wertheim theory for associating fluids1–6. Exploiting different choices of the patch-patch interaction, novel phenomena such as empty liquids7, reentrant network formation8 and control of crystallization pathways9,10 have been found in calculations and simulations. In several theoretical studies, patchy particles have been suggested to be a model system for proteins9–15. So far the connection between patchy particles and proteins has been based on general considerations concerning the nonspherical shape and inhomogeneous surface patterns of charge and hydrophobicity, as well as on indirect indications such as low density crystals and low critical volume fractions16. Furthermore, single point mutations in the protein sequence have been found to induce significant differences in the phase behavior, suggesting that local variations at the protein surface can change the interaction strongly17–19. Recently, a computational study used crystal structures of four mutants of one protein to parametrize a patchy model that successfully reproduced the experimental variation of the phase behavior of the mutants19. These findings demonstrate that the framework of patchy particles is appropriate for proteins. Nevertheless, exploiting the full power of the conceptual framework of patchy particles for control of experimental phase behavior of protein solutions depends on identifying experimental ways to control interaction patches at the protein surface.

Here we present a mechanism for ion-activated attractive patches between proteins that is directly supported by experimental evidence. Our model introduces a novel analytical concept how to account for ion-induced protein interactions by explicitly modeling the role of the cations. This concept can be seamlessly employed in both theory and simulations of patchy particles. Here we use the well-established analytical theory due to Wertheim1–6 in order to highlight the essential physics that drives the interesting and rich phenomenology in protein solutions in the presence of multivalent cations.

Multivalent metal cations such as Yttrium(III) form multidentate coordinative bonds with solvent-exposed carboxylic side chains on the protein surface20,21. For globular proteins with negative charge, ion binding is experimentally reflected in a charge inversion of the protein net charge upon the addition of YCl3 (Fig. 1A)22,23. Importantly, multivalent cations cross-link protein molecules in crystals (Fig. 1B)24. These ion bridges represent attractive patches that are activated by cations, and can be employed to tune the interactions and the associated phase behavior of globular proteins. Since solvent-exposed carboxylic side chains are ubiquitous in globular proteins, the presented mechanism applies to a large part of the protein family25. Thus, while the control of other interaction patches through e.g. surface patterns of hydrophobicity or charge requires rather involved
biotechnological methods such as genetic engineering and structure prediction, the addition of cations allows for activation of patches via a comparatively simple physicochemical experimental procedure.

This mechanism for ion-activated protein interaction is of special interest in the context of phase behavior of protein solutions. In general, additives such as NaCl or PEG are known to induce liquid-liquid phase separation (LLPS) and solvent-controlled crystallization pathways. Equilibrium clusters have been found in lysozyme solutions as expected for charged and attractive colloids and attractive fluids in general. In these cases, the results are usually discussed invoking unspecific effects such as screened Coulomb interaction or depletion effects. Here, we focus on the addition of trivalent cations that allows via a specific mechanism to control the protein interactions. Trivalent salts such as YCl₃ have been found to induce a rich experimental phenomenology in solutions of globular proteins, including reentrant condensation, LLPS (Fig. 2A), cluster phases, and one-step as well as two-step pathways of protein crystallization. Using our analytical model of ion-activated attractive patches by bridges of multivalent cations, the rich phenomenology is explained and understood in a very natural way (Fig. 2B), demonstrating that the concept of ion-activated patches provides a novel and very powerful framework to control the experimental phase behavior of protein solutions. This study introduces the basic mechanism using comparisons to experimental results in protein solutions with YCl₃. We emphasize that for a quantitative modeling further effects such as pH variation and electrostatics have to be accounted for explicitly. Although Y³⁺ is not biologically active cation, the study presents relevant results for the understanding of protein solutions. First, similar results are obtained for several tri- and divalent cations such as the biologically active cations Fe²⁺ and Al³⁺, implying that ion bridges indeed present a general mechanism. Second, the possibility to activate attractive protein interactions by multivalent cations provides control on phase behavior and pathways of nucleation and crystallization, which is of importance for structural biology as well as an understanding of self-assembly in protein solutions.

**Analytical Model for the Activation of Attractive Patches**

Within our analytical approach proteins are modeled as particles with m patches per particle. Multivalent cations are modeled as bridge particles, which can bind to a patch and thereby activate it. Other ions are not considered here. The interaction between proteins is given by the hard sphere repulsion $V_{\text{HSS}}$ between the particles and square-well attractions between the patches:

$$V(1,2) = V_{\text{HSS}}(R_{12}) + \sum_{i=1}^{m} \sum_{j=1}^{m} V_{pp}(r_{ij}^{*})$$

$$V_{pp}(r_{ij}) = \begin{cases} -\epsilon_{pp} & \text{for } r_{ij}^{*} < r_{c} \\ 0 & \text{for } r_{c} \leq r_{ij}^{*} \end{cases}$$

$R_{12}$ denotes the center-to-center distance of the particles, $r_{ij}^{*}$ is the distance between the centers of patch $i$ of particle 1 and patch $j$ of particle 2.

The key point of our model is the fact that ion binding controls the attraction strength $\epsilon_{pp}$ thereby representing the activation of interaction patches. The binding sites are considered as independent from each other with binding energy $\epsilon_{b}$. Since a binding site can either be
unoccupied \((u)\), or occupied \((o)\) by at most one cation, the average occupancy \(\Theta\) of each binding site is given by the statistics of a two-state system in the grand canonical ensemble:

\[
\Theta = \frac{1}{\exp(\beta (\mu_o - \mu_u)) + 1},
\]

with \(\beta = (k_B T)^{-1}\). The chemical potential \(\mu_s\) of the salt \((s)\) cations sets the reservoir concentration \(c'_s\). For the concentrations used in the experiments we can approximate this relation via \(c'_s(\mu_s) = \rho_s \exp(\beta \mu_s)\). Note that possible non-idealities of the salt bulk solution do not change the resulting phase behavior qualitatively, but only slightly rescale the relation \(c'_s(\mu_s)\). We have obtained a comparable behavior for numerical calculations of charged hard spheres, but prefer to use the analytic form of Eq. (3) to allow for an analytic and understandable model focusing on the mechanism of ion-bridging.

The patch-patch interaction energy \(\varepsilon_{pp}\) is determined from three contributions (see Fig. 1C,D) depending on the average occupancy \(\Theta\) of two interacting patches:

\[
\varepsilon_{pp} = \varepsilon_{uu} (1 - \Theta)^2 + 2\varepsilon_{uo} (1 - \Theta) + \varepsilon_{oo} \Theta^2.
\]

The first term accounts for the interaction between two unoccupied patches, which meet with a probability of \((1 - \Theta)^2\). The second term represents the ion-bridge attraction, when an unoccupied and an occupied patch become cross-linked, with a probability of \(2\Theta(1 - \Theta)\). The third term describes the interaction between two occupied patches, with a probability of \(\Theta^2\). In order to recover hard-sphere interaction for fully occupied or unoccupied patches, we choose \(\varepsilon_{uu} = \varepsilon_{oo} = 0\). Then \(\varepsilon_{pp}\) reads

\[
\varepsilon_{pp} = 2\mu_o \exp(\beta\mu_s - \beta\mu_u) \exp(\beta(\mu_s - \mu_u) + 1)^{-1}.
\]

Eq. (5) contains the essential physics of our model: the binding energy \(\varepsilon_s\) relates to the effective patch-patch attraction strength \(\varepsilon_{pp}\), thereby expressing the activation of patches with fixed attraction \(\varepsilon_{uu}\). While the model suggests a clear physical picture of cation binding at specific surface sites and cation bridges, the physical origin of \(\varepsilon_b\) and \(\varepsilon_{uo}\) is of minor importance within this framework. In particular, electrostatic contributions and ion-ion correlations between the valent cations as well as coordinative binding of metal ions to surface groups can contribute to the energies.

The experimental control parameter is the total salt concentration \(c_s\) in the system, which is the sum of the ions bound at the surfaces and the free ions in solution. In the model it is more convenient to use the salt concentration in the reservoir \(c'_s\) as control variable. The connection between these two quantities is given by

\[
c_s = m \Theta \rho + c'_s(\mu_s) \left(1 - \eta (1 + R_s/R_p)^3\right),
\]

where \(R_s\) and \(R_p\) are the (effective) radii of salt ions and proteins, respectively. The first term accounts for the ions bound to proteins. The second term originates from the free ions in the solution, corrected for the volume excluded to ions\(^{47}\). The number density \(\rho\) is related to the protein volume fraction \(\eta = 4\pi R_p^3\rho\).

The outlined novel concept to account for the attractive interactions due to ion-bridges can be embedded seamlessly into the Wertheim theory of patchy particles (see Methods). The liquid-liquid and solid-liquid phase coexistence at temperature \(T\) follows from chemical and mechanical equilibrium, \(\mu = \mu_1 = \mu_2\) and \(P = P_1 = P_2\), respectively, between phases 1 and 2. The chemical potential \(\mu = \rho f(\rho, T, V)\) pressure \(P = \rho \mu - f\), and isothermal compressibility \(\chi_T = 1/(\rho c_P c_P^{-1})\) are calculated analytically from the free energy densities \(f\) of the solid and fluid phase (for further details, see Methods).

As an alternative approach within the Wertheim theory, the proteins could be modeled as particles with two dissimilar types of patches – patch type \(A\), if the binding site is unoccupied, and patch type \(B\), if it is occupied. In this case, the average occupancy \(\Theta\), Eq. (3), would control the numbers of \(A\) and \(B\) patches. The protein-protein attraction \(\varepsilon_{pp}\), Eq. (5), would be generated by the Wertheim theory, for a given interaction between \(A\) and \(B\) patches of \(\varepsilon_{AB} = \varepsilon_{uu}\). We have verified that, while this approach is numerically and theoretically somewhat more involved, it results in equivalent behavior for our system. By presenting the model more explicitly in Eqs. (5)–(6) we wish to highlight the underlying physics of the ion-activated protein interactions.

**Model Analysis and Discussion**

The main feature of our model is the formation of ion bridges: If an occupied patch interacts with an unoccupied patch, an ion bridge forms and links the participating proteins (Fig. 1D). Based on the attractive interaction induced by the ion bridges, several phenomena in the system become intuitively clear. At low ion concentrations \((\Theta \to 0)\) the proteins repel each other – in the model by the hard-sphere interaction and in the experimental system through electrostatic repulsion. As more ions are added to the system more binding sites become occupied, \(\Theta\) increases, which in the experimental system results in a reduced net charge (Fig. 1A,C). For \(\Theta < 0.5\) the addition of ions increases the attraction between proteins, since the probability for an occupied and an unoccupied patch to meet increases. Further increasing the ion concentration \((\Theta > 0.5)\) decreases the attraction since too many binding sites are already occupied, thereby reflecting the charge reversal (Fig. 1A,C). At high enough ion concentration, the probability for ion-bridge formation is low and proteins mainly repel each other, corresponding to the reentrant homogeneous phase.

If the attraction is sufficiently strong and the protein volume fraction \(\eta\) is in the right range, a LLPS occurs in a closed area (green line in Fig. 2). Importantly, the low volume fractions of the coexisting liquid phases can be understood with patchy particles, which is not possible using only isotropic potentials\(^{42}\). As expected for protein solutions, the LLPS is found to be metastable with respect to a crystal phase (blue line in Fig. 2B). We find excellent agreement between theory and experiment (Fig. 2).

A natural consequence of the ion bridges is the formation of clusters throughout the entire phase diagram (Fig. 2B). Using the Flory-Stckemeyer theory\(^{35,40}\) the number density \(n_{\text{cl}}\) of \(n\)-clusters and the number fraction \(\Phi\) of proteins in clusters are given by

\[
\rho_n = \rho (1 - \rho_b)^m [\rho_b (1 - \rho_b)^{-2}]^{n-1} \frac{m(mn-n)!}{(mn-2n+2)!n!},
\]

\[
\Phi = \frac{\rho - \rho_1}{\rho} = 1 - (1 - \rho_b)^m.
\]

With increasing average ion-bridge probability \(\rho_b\) which is provided by the Wertheim theory\(^4\) (see Methods), larger cluster become more frequent (Fig. 3A). Thus, with increasing \(\rho_b\) protein diffusion is expected to be reduced substantially due to cluster formation, as indeed observed in recent experiments\(^45\). Once \(\rho_b\) exceeds the percolation value \(\rho_b^* = 1/(m-1)\), a system-spanning cluster can form, implying that dynamics in the solution is severely slowed down or even arrested.

The formation of clusters provides an explanation for the experimentally observed turbidity in the condensed regime in the concentration range between the boundaries \(c^*\) and \(c^{**}\) (red lines in Fig. 2A). Using the approximation of Rayleigh scattering, the scattering of \(n\)-clusters grows with \(V^2 \propto n^2\), which causes a steep increase of the integrated scattering power \(I_\theta = \sum_{n=1}^{\infty} n^2 \rho_n\) with increasing \(\rho_b\) in particular when approaching the percolation threshold \(\rho_b^*\) (Fig. 3B). In addition, as the liquid-liquid phase boundary is approached, opalescence causes turbidity in the solution, being man-
nified in the increase of the isothermal compressibility of the patchy particle solution (Fig. 3C).

In protein solutions with multivalent cations, crystals are found experimentally to grow from the dilute phase after LLPS\textsuperscript{99,100,101}, whereas crystal nucleation should be more favorable in the dense phase according to classical nucleation theory. Although crystallization pathways of patchy particles depend also on dynamical aspects\textsuperscript{11}, two thermodynamic features of our model help to rationalize the experimental observations. First, the dense phase occurs beyond the percolation line and, thus, might be an arrested phase, implying that motions necessary to rearrange from disordered clusters to ordered crystals might be hindered. Second, a considerable amount of clusters is present around the dilute coexistence boundary (Fig. 2B). These clusters might act as precursors in two-step nucleation pathways that occur much faster than the classical one-step nucleation from homogeneous solution\textsuperscript{11}. The presented framework focuses on the basic understanding of the phenomenology of the ion-bridging mechanism, and therefore models the complex experimental system by its essential features in an analytical and accessible description using the Wertheim theory. The analytic approach allows not only to describe but also to understand the phenomena observed in solutions of protein and salt. The mechanism of cation-activated attractive patches can be seamlessly embedded in more detailed theoretical studies and simulations to address multiple questions beyond the present study. First, electrostatics are accounted for in our framework only implicitly by the choice of the effective interaction. Including electrostatics explicitly, the effect of long-ranged repulsion on cluster formation and gelation\textsuperscript{15,16} as well as crystallization pathways could be studied. Second, cation–cation correlations as well as dynamical aspects affecting the patch occupation are neglected in the present mean-field equilibrium picture, since the large binding energy of the cations dominates the cation-protein interaction, and thus the ion bridging\textsuperscript{22}. However, cation–cation correlations could provide relevant information on e.g. the dynamics and the detailed pathways of protein crystallization\textsuperscript{10,11}. Third, the effect of the geometry of the patch arrangement is not accounted for by the Wertheim theory and is only included implicitly by the choice of the crystal volume fraction (see Methods). An analysis of the ion-bridge mechanism extended towards inhomogeneous patch occupations would be very interesting for a detailed description of the dense and solid phase as well as crystal polymorphs\textsuperscript{10,11}. The presented mechanism thus is the basis for detailed theoretical predictions, and presents promising opportunities not only for the study of the protein systems, but also for the general understanding of charged soft matter.

**Conclusions**

We have presented an experimentally supported mechanism of cation bridges between neighboring molecules as a method to activate attractive patches at the protein surface. This mechanism provides a comprehensive understanding of the experimental observations in protein solutions, and allows validation and application of patchy particle models in real protein solutions. The cation concentration represents an independent control parameter, promising rational design and control of phase behavior of protein solutions using multivalent cations and the statistical physics of particles with ion-activated patches. Our model provides a natural analytical connection between experimental results of protein biophysics and the study of patchy particles within the framework of theory and simulations. A natural next step is to extend our model to inhomogeneous density distributions such as adsorption profiles at walls or the free interface between a protein-rich and a protein-poor phase. This can be done either in the framework of classical density functional theory, which allows one to determine density distributions and thermodynamic quantities on equal footing, or with the help of computer simulations.

**Methods**

**Bonding probability from Wertheim theory for patchy particles.** The basic result of the Wertheim theory is the bonding probability \( p_b \), from which the free energy and other properties can be calculated. The bonding probability for \( m \) similar patches follows from the mass-action equation:\textsuperscript{11}

\[
\frac{p_b}{1-p_b} = \rho_m \Delta_{ij}.
\]

\[(9)\]

\[
\Delta_{ij} = 4\pi \int_{0}^{\infty} g_{HS}(R_{ij}) (f_j(1,2)) R_{ij}^2 \, dR_{12}.
\]

\[(10)\]

\( f_j(1,2) \) denotes the average of the Mayer function \( f_j(1, 2) = \exp(-\beta V_j(1, 2)) - 1 \) over all relative orientations of the two particles at fixed distance \( R_{ij} \). For very short-ranged square-well attraction one can employ the Carnahan-Starling contact value for the pair correlation function \( g_{HS}(r) \) of the hard sphere reference system, in order to approximate \( \Delta_{ij} \) analytically\textsuperscript{4}.

**Choice of model parameters.** For our calculations we set the number of patches \( m = 4 \), according to the number of ion bridges per monomer in the protein crystal. The centers of the patches are located within the particle at a distance \( d = 0.9 R_p \) from the particle center. \( R_p = 3.6 \) nm corresponds to the effective sphere radius\textsuperscript{4}. For the range of the attractive interaction we choose \( r_c = 0.33 R_p \), which is sufficiently small than the maximum attraction range for our parameter choices

\[
r_c^{(\text{max})} = \sqrt{d^2 - 2 \sqrt{5} R_p + 4 R_p^2} = d = 0.408 R_p,
\]

(11)

for which hard core repulsion still ensures the condition of only one bond per patch as required for the Wertheim approach. The ratio between the ion and protein radii is \( R_p/R_i = 1/18 \). The bridge energy \( \beta \mu_{\text{ion}} = 14 \) and binding energy \( \beta \mu_{\text{prot}} = -5 \) are chosen inspired by the experimental behavior of human serum albumin (HSA) and YCl\textsubscript{3} (cf. the Langmuir isotherm \( Q = Q_b + 3N/\Theta \) with \( N = 6.8, Q_b = -4.4 \) and \( \beta \Theta_0 = -5.9 \) in Fig. 1A).

**Free energy of liquid phase.** The free energy density of the patch bonds between the particles is given by:\textsuperscript{15,16}
with the particle number density $\rho = N/V$. Both the chemical potential $\mu$ and the pressure $P$ of the patchy particles consist of the reference part from the hard sphere system and a part arising from the patch interaction:

$$\beta\mu = \frac{n(3(\eta - 1))\eta}{(1-\eta)^2} + \ln(\eta) + \beta\frac{\Delta_{\text{bond}}}{\rho},$$

$$\beta P = \frac{1 + \eta + \eta^2 - \eta^3}{(1-\eta)^2} + \beta\frac{\Delta_{\text{bond}}}{\rho}. \quad (14)$$

**Free energy of solid phase.** For the solid phase, we used a cell model assuming that all m bonds are formed in a crystal. The free energy density $f_S(\eta)$ is calculated from the restricted volume for free motion $V_{\text{free}}$ in radial and angular direction at particle distance $r$:

$$f_S(\eta) = -\frac{1}{\rho} \log \left[ V_{\text{free}} \left( \frac{R_b}{\eta} \right)^{1/3} \right] - \frac{m}{2} \eta^2 \rho,$$

$$V_{\text{free}}(r) = 4 \frac{(r - R_b)(r - d - r_b)}{(d + r - R_b)R_b^2}. \quad (16)$$

The density $\rho$ is given by the volume fraction of a simple cubic crystal $\eta = \pi/6 = \rho R_b^{3/2}$.  

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**Author contributions**

F.R.-R., F.S. and R.R. performed the calculations and analyzed data. F.R.-R. prepared all figures. F.R.-R., F.S. and R.R. wrote the paper. F.R.-R., F.S. and R.R. reviewed the manuscript.

**Additional information**

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