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Conditional Reactivation of Lysozyme Nanosystems via Hydrophobic Ion Pairing

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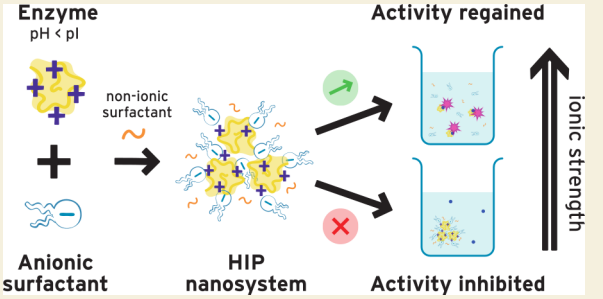
ABSTRACT: Enzyme-catalyzed reactions rely on the precise regulation of enzymatic activity to achieve high efficiency and specificity. Adjustment of conditions during the reaction is only one possibility, whereas timely activation and termination of enzymatic functions further tailor the outcomes. Existing methods for enzyme activation include temperature, pH, ultrasound, and magnetic and electric fields, but each approach struggles with limited applicability to different enzyme classes and faces challenges with reversibility. In our research, we employed hydrophobic ion pairing to modulate lysozyme activity through complexation with hydrophobic counterions, sodium docusate, and sodium laurate. The resulting complex agglomerates were stabilized with nonionic surfactants and characterized by their colloidal properties, including size and zeta potential. A fluorescence-based enzymatic assay was used to monitor the activity of the nanosystems under varying dissociation conditions. Lysozyme nanosystems with sodium docusate were completely inactivated, while those with sodium laurate retained only a partial activity. Increasing the ionic strength reversed the inactivation and restored enzymatic function. We developed a method for reversible, conditional modulation of enzymatic activity as a function of the ionic concentration. This work demonstrates a tunable strategy for regulating enzyme activity in cases where conventional control mechanisms are not suitable.



KEYWORDS: *hydrophobic ion pairing, lysozyme nanosystems, conditional enzyme reactivation, enzyme−surfactant complex, colloidal enzyme formulation, nanoparticle formulation*

Enzymes play a pivotal role in biotechnology, molecular biology, and a wide range of industrial applications, where their activity is essential for both biological and chemical processes. Achieving optimal reaction efficiency and specificity often requires that the enzymes are active only when the enzymatic reaction is needed and not prior, e.g., during storing or preprocessing, preventing undesired byproduct formation and improving overall process efficiency and control.[1] This need for conditional reactivation is evident in various applications, such as molecular diagnostics or biocatalytic cascade reactions, in the industry. For instance, in polymerase chain reaction, the premature activity of DNA polymerase is typically prevented by antibodies or aptamers that denature at elevated temperatures resulting in enzyme activation, thus improving the specificity and sensitivity of reactions.[2] A range of stimuli-responsive methods have been developed to achieve conditional inactivation and reactivation of enzymes, including not only temperature shifts[3−5] but also pH changes,[6] application of magnetic field,[7] light,[8] or ultrasound.[1,9] Each method presents limitations, such as restricted applicability to certain enzyme classes or challenges with reversibility, highlighting the need for new, broadly applicable concepts.

Existing research indicates that surfactant interactions can inhibit enzymatic activity.[10−12] Building on this, hydrophobic ion pairing (HIP),[13,14] a well-established method primarily used to enhance the incorporation of hydrophilic molecules into hydrophobic carriers, such as nanoparticles, potentially could offer a promising strategy for conditional control over enzymatic activity.[15,16] HIP increases the hydrophobicity of charged molecules through complexation with surfactants bearing polar head groups and hydrophobic tails, for instance, a molecule containing only positive charges on the surface can be complexed with anionic surfactants,[13] while the decrease in enzymatic activity in aqueous solutions, e.g., due to partial unfolding in the presence of hydrophobic counterions is well-known,[17] unpairing the complexed enzyme from HIP counterions under defined conditions would allow for an alternative pathway toward conditional reactivation of enzymes.

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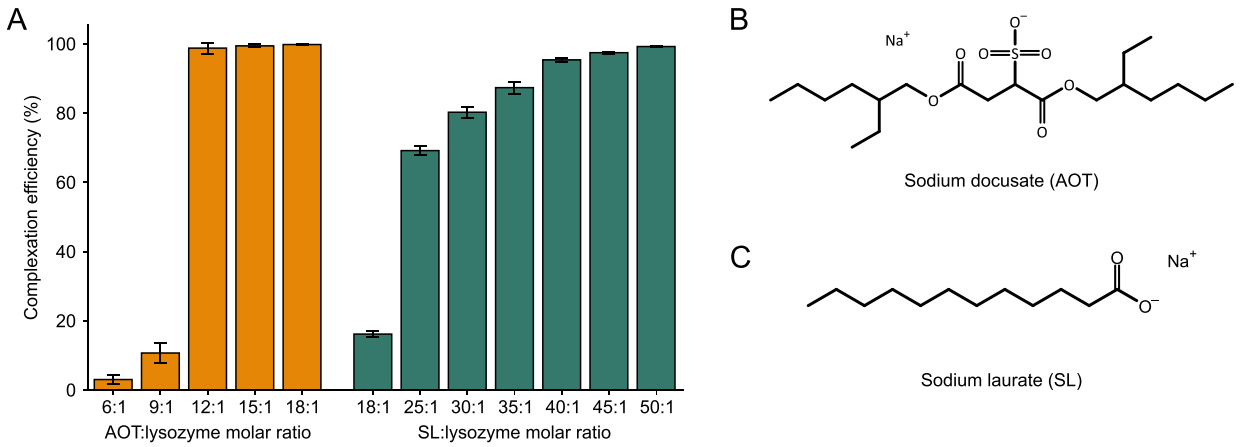

Figure 1. A) Complexation efficiency at different counterion/lysozyme molar ratios; (B) chemical structure of AOT; and (C) chemical structure of SL.

Indeed, HIP is reversible and governed by changes either of pH or of ionic strength.[14] In the case of the latter, ions present in the surrounding medium compete with the original hydrophobic counterion, thereby facilitating its concentration-dependent dissociation from the complex.[14] Although prior studies have examined changes in enzymatic performance before complex formation and after dissociation, as a function of the surfactant-to-enzyme ratio or of the counterion type,[18−20] the systematic use of ionic strength to modulate activity in a controlled manner remains underexplored.

In this study, we address this by investigating the reversible regulation of lysozyme activity through a HIP with anionic surfactants as a model system. We adjusted the enzymes' surface charge by lowering the pH below the isoelectric point, enabling efficient complexation and formation of HIP complex agglomerates with limited solubility. The agglomerated complexes were further stabilized with nonionic surfactants and characterized in terms of their colloidal properties, including hydrodynamic diameter and zeta potential measurements. Using a fluorescence-based enzymatic assay, we monitored changes in the activity of the lysozyme within the nanosystems under varying dissociation conditions. By systematically varying the ionic strength of the surrounding medium, we demonstrate a method for reversible, conditional modulation of enzymatic activity as a function of the ionic concentration. This proof-of-concept, if successful with other enzymes, could open many possibilities in applications where enzyme activity needs to be actively regulated and where traditional mechanisms, such as temperature-controlled denaturation and dissociation of antibodies, are not feasible, e.g., due to the thermolability of some enzymes.

2. RESULTS AND DISCUSSION

2.1. Preparation of HIP Complexes

Complex molecules, such as enzymes, bear both positive and negative charges on their surface. Lysozyme contains 129 amino acids and is characterized by a high isoelectric point (pI ≈11).[21] In this study, the lysozyme at pH 3.9 was paired with two anionic surfactants, sodium docusate (AOT) and sodium laurate (SL), to decrease its solubility in water and allow for its precipitation and inactivation. At this pH, the overload of protons neutralized the negative charges on the surface of the molecule, thus leading to a net-positive charge, which can be neutralized by hydrophobic ion pairing (HIP) with anionic surfactants.[14]

The search for the most suitable surfactant-to-enzyme ratio aimed at identifying how many surfactant molecules per enzyme are needed to render the enzyme fully insoluble in water. To determine the success of this process, we used UV−vis absorption spectroscopy of the supernatants after HIP and centrifugation. To this end, we first determined the extinction coefficient of the lysozyme to be 31,622 $M^{-1\bullet}$ cm^{-1} at 280 nm. In a control experiment, we verified that the free, unmodified lysozyme could not be pelleted by centrifugation and fully remained in the supernatant, thus ensuring that centrifugation is a suitable method to evaluate the (in)solubility of lysozyme−HIP complexes. Absorption spectra of both anionic surfactants were determined prior to the experiment and did not contribute to the absorption signal at 280 nm. When pairing with AOT, the complexation efficiency was low for molar ratios of 6:1 and 9:1 and increased rapidly for higher ratios, reaching full precipitation at an 18:1 ratio of AOT to lysozyme. Interestingly, for SL, much higher molar ratios were necessary to fully precipitate the lysozyme (50:1) (see Figure 1A). All subsequent experiments were prepared by freshly mixing equal volumes of counterion and enzyme solutions to reach 18:1 AOT-to-lysozyme ratios and 50:1 SL-to-lysozyme ratio.

We attribute this difference in the final molar ratios to two factors. First, the anionic surfactants, when used at pH 3.9, exhibit different degrees of ionization, AOT, a with pK_a value of −0.75, remains fully deprotonated, whereas SL, with a much higher pK_a of 4.95, is only partially deprotonated.[14] Therefore, ion pairing of SL with positively charged residues on the surface of lysozyme is expected to be less efficient than that for AOT under these conditions. Second, the difference likely reflects a higher affinity of AOT to lysozyme compared to SL. Interestingly, AOT precipitates the enzyme fully, even before the theoretical number of positive charge bearing amino acids (1_x His, 6_x Lys, and 11_x Arg) is matched by the number of provided AOT molecules per enzyme. It remains unclear where the extra SL molecules locate once the 18 basic amino acids are fully paired.

AOT has a sulfonate group and two branched alkyl chains (Figure 1B), which are responsible for its hydrophobicity, while SL is a linear fatty acid salt with a single hydrophobic chain (Figure 1C). Each of the surfactants contains one negatively charged group, which allows it to interact with the

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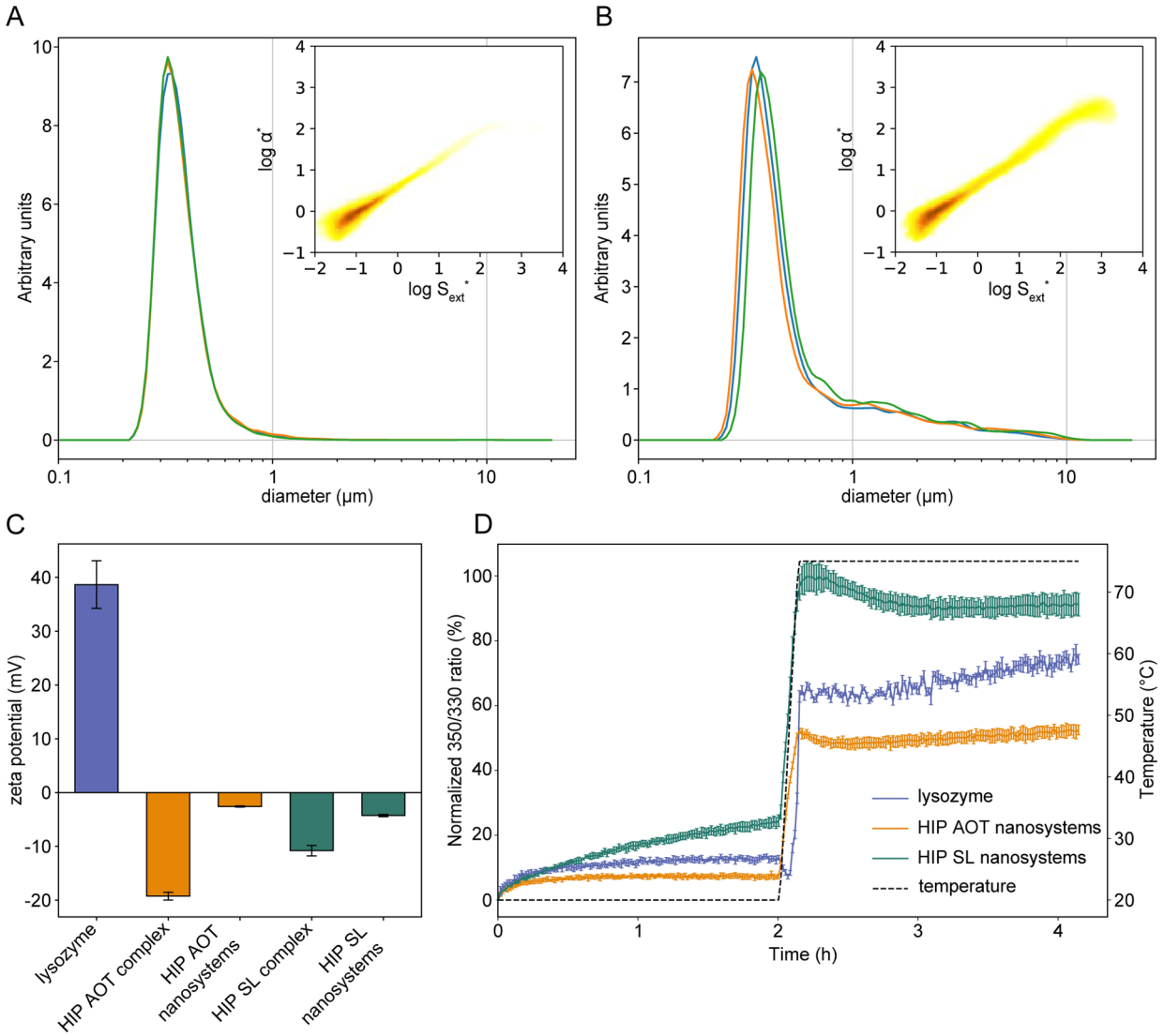

Figure 2. Colloidal properties: (A,B) SPES data: PSD and raw data for (A) triplicates of HIP AOT nanosystems (Peak: 330 ± 10 nm; fwhm: 150 ± 10 nm); (B) triplicates of HIP SL nanosystems (Peak: 360 ± 20 nm; fwhm: 170 ± 10 nm). In the raw data diagrams, color intensity (yellow to brown) increases when more particles are present; arbitrary unit: relative number of particles per ml per bin; (C) zeta potential of: lysozyme, HIP complexes without nonionic surfactant stabilization, and HIP nanosystems stabilized with nonionic surfactants; (D) nanoDSF fluorescence ratio at 350/330 nm normalized to their individual minimum and maximum values.

positive residues on the surface of the lysozyme.[14] According to Ristroph and Prud'homme, there are two main mechanisms in which the counterions act during complexation: (1) they mask the natural charge of the protein decreasing its readiness to interact with polar solvents and (2) their hydrophobic residues coat the surface of the molecule, thus excluding water from its surrounding.[14] Our experiments showed that at pH 3.9, AOT saturates the positive residues of the lysozyme more efficiently (by around a factor of 3 regarding the surfactant/lysozyme molar ratio needed for efficient complexation), thus indicating that it has a higher binding affinity toward the enzyme in comparison to SL. This stronger interaction likely results in a HIP complex more densely coated with anionic surfactants. We expected the complexes with AOT to exhibit a more negative zeta potential than those with SL, reflecting the more extensive charge shielding and enhanced formation of a hydrophobic surface layer.

The difference between the optimal ratios may also arise from the distinct molecular structures of the surfactants, which translate to their different hydrophobic−lipophilic balance (HLB). AOT is considered hydrophobic,[22] whereas SL is located at the strongly hydrophilic end of the HLB scale in Griffin's classification.[23] Consistently, AOT has a larger hydrophobic surface area when compared to SL and thus could be able to shield lysozyme's charged regions more effectively. In contrast, SL provides less hydrophobic coverage per molecule, requiring more counterion molecules to achieve a comparable effect. This interpretation aligns with studies suggesting that, at the same molar ratios, surfactants with branched hydrophobic tails are more effective in enhancing lipophilicity.[24]

2.2. Colloidal Properties

All the following experiments were performed at an AOT/lysozyme ratio of 18:1 and an SL/lysozyme ratio of 50:1. Freshly prepared HIP complexes agglomerated and rapidly precipitated out of solution, necessitating stabilization prior to reliable colloidal characterization. To enhance reproducibility in subsequent analyses, various nonionic surfactants were

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evaluated for their ability to provide steric stabilization of the hydrophobic complexes. The objective was to achieve a uniformly turbid suspension with minimal immediate precipitation, as assessed by a visual inspection over time. Among the surfactants tested, Tween 20 (T20) at a ratio of 100 mol per mol of lysozyme proved the most effective for stabilizing HIP AOT complexes (this formulation stabilized with T20 is further referred to as HIP AOT nanosystems), while Poloxamer 407 (P407) at 5 mol per mol of lysozyme provided optimal stabilization for HIP SL complexes (this formulation stabilized with P407 is further referred to as HIP SL nanosystems). The colloidal properties of the resulting HIP AOT and SL nanosystems were subsequently characterized.

2.2.1. Size and Polydispersity. We employed the single-particle excitation and scattering (SPES) method to characterize the size distribution of HIP nanosystems. SPES offers significant advantages for heterogeneous systems, as it analyzes individual particles, yielding accurate measurements even in the presence of aggregates or broad size distributions.[25] The particle size distributions (PSD) of the diameters obtained by SPES are presented in Figure 2 A (HIP AOT) and B (HIP SL). Both HIP AOT and HIP SL nanosystems exhibit peaks in a similar size range, presenting comparable particle diameters above 300 nm. Individual lysozyme molecules are only 3−4 nm in diameter,[26] indicating that the larger diameters detected for HIP nanosystems result from aggregation of enzyme−counterion pairs. This aggregation likely arises due to increased hydrophobicity caused by complexation, promoting the formation of larger complexes in aqueous solutions.[14] Similar clustering behavior has been reported previously, with HIP complex sizes reaching up to 170 nm and varying in response to the molar ratios of counterion to lysozyme.[10]

Notably, the full width at half-maximum (fwhm) of the distribution for HIP AOT nanosystems is slightly narrower than that of the HIP SL nanosystems, reflecting lower polydispersity in the former. In addition, the nanosystems with AOT show less agglomeration, as shown by both the PSD and the raw data (Figure 2A). The corresponding 2D histogram displays a highly focused region with only a narrow streak, indicating a high concentration of particles within a narrow size range. Conversely, the HIP SL nanosystems exhibit a pronounced shoulder toward larger diameters, which is also reflected in the raw data as a prolonged streak toward higher log of reduced polarizability (log α^*) and log of reduced extinction cross section (log S_{ext}^*) values (Figure 2B), proving increased heterogeneity and a greater tendency to form agglomerates. The HIP complexes with SL had a much higher molar ratio of surfactant to lysozyme (50:1) compared to AOT (18:1), thus the higher tendency for agglomeration observed in the case of HIP SL nanosystems is likely due to the high hydrophobicity of a resulting mixture.

To prevent further uncontrolled aggregation and improve liquid handling and characterization of the complexes, nonionic surfactants were added. Establishing colloidal stability was a necessary prerequisite, as only sufficiently stabilized nanosystems can be reliably used in applications without the danger of aggregation compromising the accuracy.[27] This stabilization provided steric hindrance, enabled reliable size analysis, and improved colloidal stability, facilitating subsequent handling during enzymatic activity and dissociation tests.

2.2.2. Zeta Potential. To evaluate the interactions between lysozyme and surfactants, zeta potential measurements were performed (Figure 2C). The free lysozyme (pH 3.9 after reconstitution in water) exhibited a positive zeta potential, while complexation with anionic surfactants resulted in negatively charged molecules. Interactions between positively charged residues on the surface of lysozyme and negatively charged counterions resulted in a negative overall charge of the nanosystems. The zeta potential of HIP complexes with AOT was slightly more negative than that of HIP SL, confirming that charge shielding and hydrophobic surface layer formation are counterion-dependent, and the larger net negative value for AOT could be a result of a higher binding affinity of the counterion to the lysozyme. Our findings are consistent with those of Mu et al., who also investigated zeta potential changes upon HIP with the lysozyme and obtained a slightly negative zeta potential for the 18:1 AOT-to-lysozyme ratio.[10] The addition of nonionic surfactants shifted the zeta potential toward neutral values for both HIP nanosystems. This effect may be attributed to the chemical composition of T20 and P407, both of which contain units of polyethylene glycol (PEG).

As described by Rabanel et al.,[28] absolute zeta potential values decrease with increasing distance between the nanoparticle surface and the slipping plane. Previous studies have shown that PEG forms an adsorbed layer on nanoparticle surfaces, thus moving the slipping plane outward and thereby reducing the measured zeta potential.[29,30] Given the structural similarity of the surfactants used, it can be postulated that the interactions between them and the HIP complexes are also based on the mechanism described. This observation is in alignment with the research of Redhead et. al, who documented this phenomenon for P407.[31]

The decrease in the absolute zeta potential values of HIP nanosystems (i.e., the zeta potentials become less negative) indicates interactions between the surfactants and the complexes, which play an important role in the colloidal stabilization of these protein−surfactant nanosystems.

2.3. Influence of HIP on Lysozyme Structure

To further investigate the differences between the HIP AOT and HIP SL nanosystems, we employed nanodifferential scanning fluorimetry (nanoDSF) and monitored the intrinsic fluorescence ratio (350/330 nm) (Figure 2D). This ratio reflects changes in the local environments of tryptophans and tyrosines. In the lysozyme, mostly tryptophan residues, in particular, Trp 62 and 108, located in its active center are responsible for the emission.[32] Changes in this ratio typically occur when these residues become more solvent-exposed, suggesting conformational rearrangements or the unfolding of the protein. In a recent study involving spectroscopic measurements, Mu et al. have explored changes in the maximum emission wavelength and showed a blue shift of the lysozyme with increasing AOT concentration, followed by a slight redshift for the higher surfactant concentrations where the free lysozyme and surfactant micelles are present again.[10] We tracked the fluorescence ratios to validate if there are changes overtime once the enzyme is coupled with the surfactants.

At constant temperature, the lysozyme complexed with AOT closely resembles the behavior of the native enzyme, and both fluorescence ratios are stable. This suggests that the structure of the enzyme remained intact after HIP. In contrast, the HIP SL nanosystems showed a gradual shift in the fluorescence ratio over time, indicating ongoing structural rearrangements. Our observations point toward reduced stabilization of the

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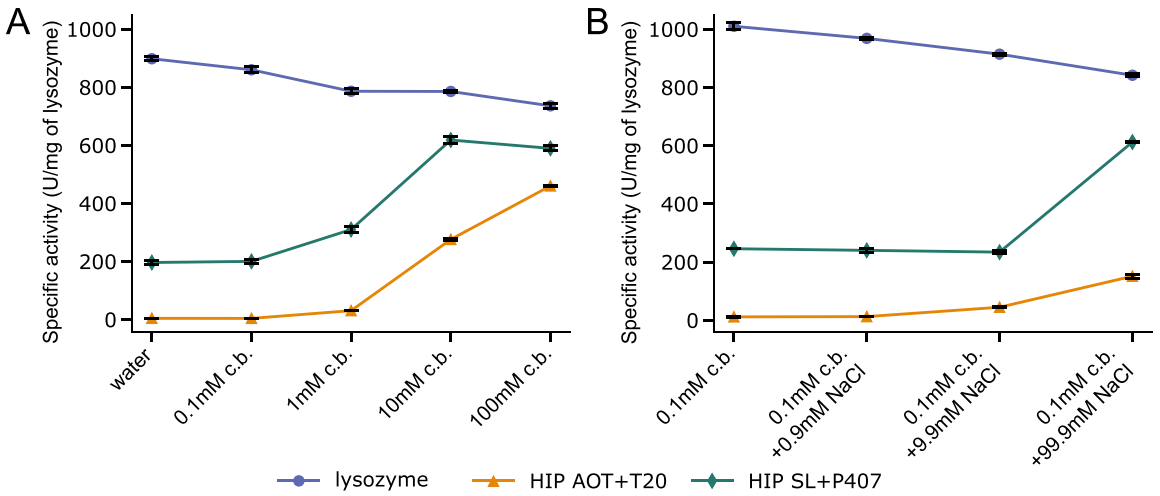

Figure 3. Specific activities of the free lysozyme, HIP AOT nanosystems, and HIP SL nanosystems upon incubation in (A) water and citrate buffers (c.b.) ranging from 0.1 to 100 mM; (B) 0.1 mM (c.b.) without or with varying concentrations of NaCl. The lines were added for better readability; all experiments were performed in triplicates.

protein structure by SL as compared to AOT, possibly due to the differences in how these surfactants interact with the protein surface.

To gain insight into the influence of HIP on the conformation of the lysozyme, we applied a rapid temperature increase. Increasing the temperature from 20 to 75 °C within less than 20 min provided information about the nanosystems' thermal responsiveness. Upon heating, both HIP AOT and HIP SL nanosystems showed an increase in the 350/330 nm fluorescence ratio, which implies that the lysozyme remained at least partially folded after complexation and that final unfolding primarily occurred in response to temperature. Overall, these results demonstrate that while both counterions allow retention of structural flexibility, pairing of the lysozyme with AOT results in nanosystems that more closely resemble the native lysozyme and do not undergo immediate conformational changes, whereas complexation with SL influences the protein's stability over time even at ambient temperatures.

2.4. Influence of HIP on Lysozyme Activity

2.4.1. Inactivation of Lysozyme via HIP. Pairing of enzymes with hydrophobic ions has been shown to result in their decreased activity or complete inactivation in aqueous solutions.[12] After preparation of HIP AOT and HIP SL nanosystems, we tested their activity in water and compared it to that of the native lysozyme (see Figure 3A, "water"). Pairing of the lysozyme with AOT resulted in a complete inactivation of the enzyme, whereas the HIP SL nanosystems retained only partial activity compared to the native enzyme.

Thus, it can be concluded that coupling of the lysozyme with counterions via HIP not only increases its hydrophobicity[13] but also decreases its activity in a low salt aqueous environment. This may be attributed to an altered charge at lysozyme's active center, leading to a limited substrate recognition.[10] Moreover, the aggregation of individual enzyme-surfactant particles into larger HIP complexes described above likely hinders substrate access to lysozyme's active center. Hydrophobic surfaces of counterions are known to create a mass transfer barrier that slows diffusion of water to the complexes,[14] we believe that this mechanism also likely impedes diffusion of the substrate toward the active center. Furthermore, such a diffusion barrier could lead to partial exclusion of water from the proximity of the active center, which in turn may decrease the efficiency of hydrolysis.[33]

2.4.2. Reactivation of the Lysozyme by Increasing Ion Concentration. We postulated that due to the reversibility of HIP, the interactions leading to the decreased activity could be reversed when an appropriate competing compound is present. To test this, we incubated the HIP nanosystems in citrate buffer with varying ionic strengths and monitored the lysozyme activity in each reaction. The activity profiles for all samples were linear over 18 h (see Figures S1 and S2). The free lysozyme was tested in parallel and was most active in water (900 U/mg of lysozyme), while its activity decreased slightly with an increasing citrate buffer concentration, reaching the lowest value in 100 mM buffer (736 U/mg of lysozyme) (Figure 3A). In contrast, HIP nanosystems showed the opposite trend. Notably, an additional control experiment (see Figure S3) showed that the presence of nonionic surfactants slightly decreases the specific activity of lysozyme in all tested solutions, whereby the effect of T20 is more pronounced than that of P407.

The lysozyme within HIP AOT nanosystems was inactive both in water and in the lowest concentrated citrate buffer, but its activity was restored up to 460 U/mg of lysozyme in 100 mM citrate buffer. This demonstrates that the reversibility of HIP can be harnessed to conditionally inactivate and reactivate the lysozyme, while using AOT as an anionic surfactant. Full inactivation was achieved for HIP AOT nanosystems, whereas the enzyme in HIP SL nanosystems retained some activity in water and in the 0.1 mM citrate buffer. Although complete inactivation could not be obtained for HIP SL, the activity of lysozyme paired with SL decreased in water as compared with the native enzyme and increased upon exposure to more concentrated citrate buffers.

Experiments preceding the activity tests, such as the search for an optimal counterion to lysozyme ratio, have shown that a substantially larger number of SL molecules was required to achieve full complexation of the enzyme as compared to AOT. Strong protonation of SL at the working pH, resulting from its higher pK_a, reduces the fraction of anionic groups available for ion pairing with lysozyme and thus necessitates higher SL concentrations to achieve optimal complexation efficiency. The resulting excess of SL in the final solution promoted increased

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aggregation within the HIP SL nanosystems, as confirmed by SPES measurements.

The literature on HIP formation indicates that counterions with higher hydrophobicity and lower pK_a facilitate ion pairing and tend to form stronger, less water−soluble complexes.[34] Our data follow this trend, with AOT (more hydrophobic, lower pK_a) leading to more efficient complexation. The higher hydrophobicity of AOT as compared to SL most likely results in HIP AOT nanosystems being more hydrophobic, translating to their higher stability in aqueous solutions, while HIP SL nanosystems retain greater capacity for structural rearrangements. In agreement with this hypothesis, nanoDSF measurements showed that HIP AOT nanosystems at a constant temperature resembled the behavior of free lysozyme, while HIP SL nanosystems indicated ongoing structural rearrangements reflecting lower colloidal stability of the latter. The incomplete inactivation of HIP SL nanosystems further supports our hypothesis that AOT exhibits a higher binding affinity toward the enzyme as compared to SL. Despite these differences, both nanosystem types were influenced by the surrounding medium in a similar manner, and a mechanism in which lysozyme activity is suppressed at low ionic concentration and subsequently increased in higher-concentrated citrate buffers could be shown.

These findings are particularly interesting when compared with state-of-the-art knowledge. Previous studies postulated that the lysozyme in the form of a HIP complex may lose its activity due to risks of denaturation or irreversible aggregation in the complexed state.[10,20,21] Li et al. investigated interactions between the lysozyme and an anionic surfactant and showed, using a bacteriolytic activity assay based on turbidity measurements, that inactivation in the complexed state is possible and depends on the dose of the counterion.[12] Our study quantitatively confirms a decrease in lysozyme activity in its HIP complexed form in aqueous media using a fluorescence assay based on 4-MU that directly reports product formation. This method avoids potential optical interference from turbid HIP complexes and directly quantifies soluble product formation, thus allowing monitoring of the activity directly in the environment that led to the dissociation of the complexes. Activity measurements in water revealed that intact HIP nanosystems substantially reduce lysozyme activity in the aqueous environment and that the extent of the inactivation is counterion-dependent. Furthermore, monitoring 4-MU formation over time allowed us to notice that the rate of product formation is constant over 18 h, suggesting that a certain level of dissociation is established within moments after the HIP nanosystems contact the reaction mixture, and further dissociation over time is negligible.

To further ensure that the observed reactivation was driven by changes in ionic competition and to exclude any effects coming from the buffer itself, we performed a test with 0.1 mM citrate buffer and varied the ionic strength only by increasing the concentration of NaCl (Figure 3B). The results confirmed that the increase in enzymatic activity upon HIP dissociation was indeed caused by changes in ionic competition rather than buffer-related influence. Similarly, Hassan et al. noted that an increased NaCl concentration in lysozyme-sodium dodecyl sulfate samples led to dissociation of the complexes and enhanced lysozyme activity.[35]

Multiple other studies have explored the relationship between the dissociation of HIP complexes and the presence of ions.[10,19,36] In cases where a higher ionic concentration is the reason for the dissociation, the mechanism is based on ionic competition by salts: ions from the surrounding medium access the HIP complex and outcompete the anionic surfactants, causing complex dissociation.[14] It has been shown previously that the lysozyme, which undergoes complete inactivation in the form of a HIP complex, can be turned active via competition between the lysozyme paired with an anionic surfactant and an organic compound.[12] Our findings suggest that the concept of enzyme reactivation is not limited to a single competing substance but can be extended to various ions, with ionic competition serving as the main force driving the dissociation. Moreover, the fluorescent assay employing 4-MUC in the presence of all HIP nanosystem components establishes a foundation for future studies of hydrophobic ion pairs. It enables quantitative analysis of enzyme kinetics and may help to elucidate further mechanisms governing changes in lysozyme activity upon HIP complex dissociation.

It is well established that pairing of hydrophobic ions with hydrophilic molecules is a reversible process.[14] Here, we repurpose this reversibility to conditionally inactivate and reactivate the lysozyme, thus presenting a novel application for the HIP method. The lack of enzymatic activity in HIP nanosystems can be treated as an advantage and used in situations where the enzyme must remain inactive, whereas regulation of the ionic concentration of the surrounding medium can be employed to reactivate the enzyme once its function is desired. Already existing solutions for regulation of enzymatic activity in nanocarriers include triggers such as light,[37] magnetic field,[38] redox signals,[39] or near-infrared irradiation.[40−42] Our nanosystems could potentially be used in areas where undetected changes in ionic strength play a role or are the starting signal for a reaction. Possible applications include storage of stabilized and inactive enzymes within nanosystems, as long as the surrounding environment exhibits a low ionic concentration and reactivation of enzymes once the ionic strength increases. Subsequently, the enzymatic activity could lead to visible reaction effects such as changes in turbidity or fluorescent signal, resulting in detection of changes within the system. The model nanosystems developed with the lysozyme could be of interest in the food industry or clean water surveillance. Adapting the system to other enzymes would allow the area of applications to be extended to other fields. Potential directions for future studies could include reactions in molecular diagnostics, which could be started by the addition of ions or patient sample, e.g., for thermosensitive reactivable enzymes, as well as other industrial reactions, where ionic strength changes could easily be obtained.

3. CONCLUSIONS

The present study investigates changes in the lysozyme activity upon pairing with two different anionic surfactants. HIP with AOT allows for complete inactivation of the enzyme, whereas coupling with SL reduces its activity in water. Thus, the extent of the activity decrease is counterion-dependent. The reversibility of HIP, induced by introducing competing ions, from sources such as citrate buffers with increasing ionic competition or simple addition of salt, enables the reactivation of the lysozyme. Varying ionic competition allows modulation of the dissociation of HIP nanosystems and consequently the level of enzymatic activity. HIP is primarily used to increase the hydrophobicity of molecules prior to their encapsulation within lipid-based nanocarriers, yet its effects on the activity of

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enzymes are often overlooked. Our research not only provides a better understanding of these effects but also proposes a new method for reversible modulation of enzymatic activity. In future work, this concept should be validated with additional enzymes to assess its broader applicability, for example, in contexts such as molecular diagnostics or biocatalytic reactions in the industry, where controlled enzyme regulation is desirable.

4. MATERIALS AND METHODS

The lysozyme (from chicken egg white, 14,400 g/mol) was purchased from AppliChem as lyophilized powder and prepared fresh for each experiment by reconstitution in water to the concentration of 1 mg/mL. The pH of the lysozyme solution was 3.9 after reconstitution in water, and no further pH adjustment was performed in order to avoid additional ions that could interfere with HIP between lysozyme and the anionic counterions.[14] AOT, SL, T20, Tween 80, Span 80, P407, 4-Methylumbelliferyl β-D-*N*,*N*′,*N*″-Triacetylchitotriosid (4-MUC), 4-Methyl-umbelliferon (4-MU), and citric acid were purchased from Merck (Darmstadt, Germany). Trisodium citrate dihydrate was purchased from Carl Roth (Karlsruhe, Germany). Dimethyl sulfoxide (DMSO) was purchased from Thermo Fisher Scientific. Double deionized water was used in all of the experiments.

4.1. Hydrophobic Ion Pairing

The most efficient conditions for HIP were defined as those in which the entire lysozyme is complexed and rendered insoluble in water. To identify them, various molar ratios of the counterion to enzyme were tested. For this purpose, 350 μL of lysozyme solution (1 mg/mL equivalent to 69.4 μM) was mixed with 350 μL of the counterion solution.

AOT aqueous solutions were prepared within the concentration range of 0.42−1.25 mM with exact concentrations adjusted to achieve AOT/lysozyme molar ratios (*X*:1), where *X* spanned the range of 6−18. Similarly, SL aqueous solutions were prepared in the concentration range 1.25−3.47 mM, corresponding to SL/lysozyme molar ratios (*Y*:1), with a *Y* of 18−50. To prevent micelle formation, surfactant concentrations were maintained below their commonly reported critical micelle concentrations in water at 25 °C, which are 2.5−4.1 mM for AOT and 24 mM for SL.[21,43] All samples were prepared in triplicate, vortexed for 20 s, and allowed to equilibrate for 2 min.

4.2. Complexation Efficiency

Following equilibration, the efficiency of HIP was assessed by centrifuging all samples at 17,000*g* for 5 min. As a control, the free lysozyme at the corresponding concentration was also subjected to centrifugation. The concentration of the free lysozyme remaining in the supernatant was determined spectrophotometrically by measuring the absorbance at 280 nm using a NanoDrop Lite (Thermo Scientific). The extinction coefficient for the lysozyme at 280 nm was determined experimentally according to Stephenson.[44] If the pairing was successful, the resulting complexes could be pelleted by centrifugation, while the free, unbound lysozyme remained measurable in the supernatant. Complexation efficiency (%) was calculated as follows with *C* referring to the lysozyme concentration

$$\text{Complexation efficiency (\%)} = \frac{(C_{\text{initial lysozyme}} - C_{\text{lysozyme in the supernatant}})}{C_{\text{initial lysozyme}}} \times 100$$

4.3. Stabilization of HIP Complexes

The following nonionic surfactants were employed for the stabilization of HIP complexes: T20, Tween 80, Span 80, and P407. Initial selection was based on an optical evaluation of sample turbidity and precipitate formation. The most promising candidates were then used to prepare solution variants with different nonionic surfactant-to-HIP complex ratios. Single Particle Extinction and Scattering measurements were employed to determine optimal stabilization conditions.

4.4. Preparation of HIP Nanosystems

Once the optimal molar ratios of counterion to lysozyme were established for maximal complexation efficiency, the nanosystems were prepared by combining 6 mL of lysozyme solution (1 mg/mL) with 6 mL of aqueous counterion solution (0.56 mg/mL AOT or 0.77 mg/mL SL) under magnetic stirring at room temperature. The mixture was stirred at 600 rpm by using a 1.5 cm magnetic stir bar in a 5 cm diameter beaker for 2 min. Subsequently, nonionic surfactants were added (525 μL of 9.75% T20 to HIP AOT and 525 μL of 5% P407 to HIP SL), and mixing was continued for an additional 30 s.

4.5. Characterization of HIP Nanosystems

The diameter of the nanosystems was determined with Single Particle Extinction and Scattering using a Classizer ONE (EOS Instruments, Italy). This method allows one to determine diameter, refractive index, and nanosystem concentration by measuring the extinction and scattering of particle-like nanosystems in solution. Particles are measured individually, in a diluted state, when they pass through a laser beam, thus avoiding ensemble averaging and allowing analysis of polydisperse samples.[25] Double-deionized water used for dilutions was prefiltered via a 0.22 μm PES membrane filter. Dilutions of particles (21- to 81-fold) were measured on SPES in triplicate under magnetic stirring at 600 rpm.

To investigate the changes in lysozymes' surface charge following complexation and stabilization, the zeta potential in Nuclease-Free water (Thermo Fisher Scientific) was determined using a Zetasizer instrument (Malvern Panalytical, UK).

To monitor the stability of HIP nanosystems over time, we used nanodifferential scanning fluorimetry (nano-DSF) with a Prometheus NT.48 system (NanoTemper Technologies, Germany). The method uses the intrinsic fluorescence of aromatic amino acids to determine protein unfolding as well as it monitors shifts in backscattering that indicate aggregation.[45] The sample replicates (n = 5) were excited at 280 nm, and the fluorescence ratio 350/330 nm was recorded. The temperature of the measurement was set to 20 °C for the first 2 h, and, subsequently, changed to 75 °C for the next 2 h.

4.6. Lysozyme Activity Testing

Lysozyme activity was measured using a fluorescence-based assay with 4-MUC as the substrate.[46] Briefly, 4-MUC and 4-MU were reconstituted in DMSO to a final concentration of 6.4 mM. On the day of the experiment, both stock solutions were diluted in water or in citrate buffer (pH 5,0) in varying salt concentrations. In each reaction, 90 μL of freshly prepared lysozyme (0.5 mg/mL) or HIP nanosystems was incubated with 10 μL of 4-MUC (final concentration in the reaction 40 μM) at 34 °C. The enzymatic hydrolysis of 4-MUC released 4-MU, which was quantified by monitoring fluorescence changes over 18 h, at excitation/emission wavelengths of 355/450 nm using a microplate reader (Tecan Infinite M Nano+, Switzerland). The product concentration was calculated using standard curves (prepared separately for each of the solutions) (see Tables S1 and S2) with known concentrations of 4-MU. Lysozyme specific activity was defined as 1 pmol of product released per hour under assay conditions/mg of the enzyme. All measurements were performed in triplicate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnanoscienceau.5c00160.

Lysozyme activity kinetics and standard curves for 4-MU concentration (PDF)

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Notes

The authors declare no competing financial interest.

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