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COMMUNICATION

Insight into the structure and activity of surface-engineered lipase biofluids

Ye Zhou[a,b], Nykola C. Jones[c], Jannik Nedergaard Pedersen[d], Bianca Pérez[e], Søren Vrønning Hoffmann[c], Steen Vang Petersen[f], Jan Skov Pedersen[d], Adam Perriman[g], Peter Kristensen[h], Renjun Gao*[a], Zheng Guo*[b]

Solvent-free liquid proteins (also referred to as biofluids or protein liquids) are a new kind of hybrid nano-biomaterials[1]. They are produced via lyophilization and subsequent thermal annealing (to 60 °C) of aqueous protein-surfactant conjugates, which comprise a compact cationized globular protein core and a an electrostatically stabilized soft polymer surfactant corona. The liquid phase of the protein is stabilized via the soft polymer shell, which extends the length scale of the inter-protein attractive interactions[1,2]. As a new frontier of biomolecule-based nanoscience, protein liquids has been successfully synthesized using ferritin[2], myoglobin[3], lysozyme[4], lipase[5], where these exotic materials have exhibited some striking properties, including near-native structure, biological function, hyper-thermal stability and reversible folding[5,6].

Inspection of the crystal structures of the protein precursors for solvent-free liquid protein synthesis revealed that all the proteins show a high density of charged residues at the surface, including positively charged residues (lysine/arginine) and the modifiable carboxyl amino acid residues (aspartate/glutamate), which can react with N,N'-dimethyl-1,3-propanediamine (DMPA) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Cationization is a key step that equips the protein with a highly positively charged surface, to which a certain amount of anion polymer surfactants can be conjugated electrostatically (Figure 1A). It was reported that cationization of ferritin (cFn) gave a [cFn]:[S] stoichiometry of 1:264 (S is the abbreviation of the polymer surfactant), while conjugates prepared from native ferritin (Fn) have a [Fn]:[S] stoichiometry of only 1:96[2]. However, proteins heavily covalently modified with diamines can exhibit vital changes in their native conformation as well as a reduction in activity, especially if it is in the active site region. This can be triggered through localised changes in non-covalent bonding (e.g., loss of salt bridges), which can significantly reduce enzymatic activity if in the vicinity of the active site. For example, lipase from *Thermomyces lanuginosus* (TLL) is rich in surface carboxylates, especially in the active site region, where the lid domain is a possible modification site [7] (Supporting Information, Figure S1). TLL loses more than 90% of its initial hydrolytic activity after cationization with 100% efficiency, and conjugation of anionic polymer surfactants results in further loss in activity[5]. Apparently, the degrees of both cationization and conjugation of polymer surfactants exert crucial influences on enzyme activity[5,8].

Despite the advances in studies of solvent-free liquid protein[2,3,5,9,10], all reports to date are limited to commercially available enzymes. Critically, the same key questions remain

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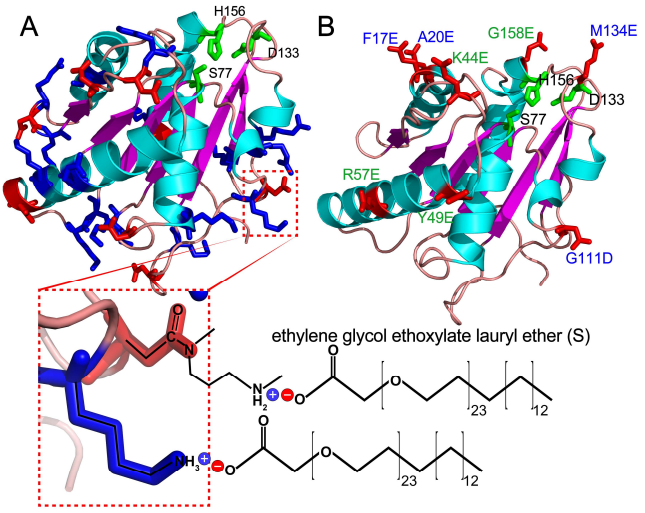

Figure 1. A) Graphic showing the carbodiimide-mediated cationization of a glutamic acid side-chain with N,N'-dimethyl-1,3-propanediamine (DMPA) to yield cBsLA, followed by electrostatic coupling with anionic polymer surfactants (S) to give [cBsLA][S]. Surface positively (Lys/Arg) and negatively (Asp/Glu) charged residues are marked in blue and red sticks, respectively. The catalytic triad (Ser77, Asp133 and His156) is marked with green sticks. (B) Ribbons diagram of the evolved 8M mutants indicating the relative positions of each mutation. The labels of mutations from 4M1 and 4M2 are colored in green and blue, respectively. The 3D structures of 4M1 and 8M were predicted with EasyModeller 4.0[26] and the figure was made using PyMol (www.pymol.org).

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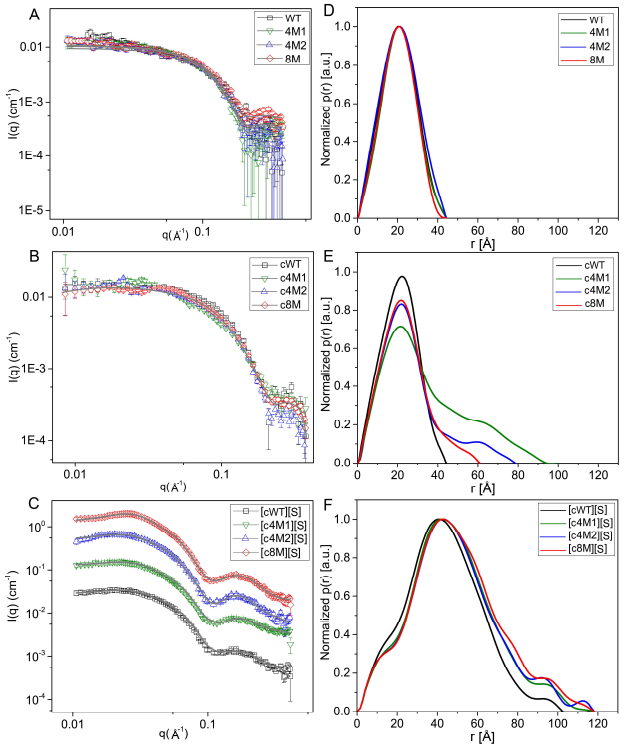

Figure 2. Small-angle X-ray scattering data (SAXS) of native (A), cationized (B), and polymer-conjugated (C) lipase variants. The fitted curves were shown in dark grey. The corresponding *p(r)* functions (a.u,) as a function of distance r (Å) obtained with the IFT procedure for native (D), cationized (E), and polymer-conjugated (F) lipase variants. The native enzymes are buffered in 50 mM sodium phosphate buffer, pH 7.2 while cationized and polymer-conjugated enzymes are all unbuffered for their high solubility in Milli-Q water.

unanswered: 1) Can an enzyme be made into a solvent-free liquid protein without sacrificing activity? 2) Can activity of the enzyme in protein liquid state be ameliorated through rational genetic engineering of the protein surface? 3) How does the number and distributions of the surfactant binding sites on the protein surface influence the property and activity of an enzyme when made into a protein liquid? 4) Is there any interplay between genetic engineering and chemical engineering of a protein surface, which might profoundly affect the structure and activity of protein liquid? To get a better understanding of the aforementioned questions, *Bacillus subtilis* lipase A (BsLA), a well-characterized enzyme with no charged residues in the active site region, was selected in the present work for biofluid synthesis. Here, a two-step strategy was applied: 1) Site-directed mutagenesis of the BsLA to introduce mutants with more chemically addressable Asp and Glu on the protein surface, and 2) Cationization of the resulting mutants to produce a highly cationic species. The resulting supercationic mutants were subsequently conjugated with anionic polymer surfactants to prepare solvent-free liquid lipases.

BsLA is one of the smallest lipases known (Mw=19.8 kDa) with a globular shape and dimensions of 35Å×36Å×42Å[11]. It shows a compact α/β-hydrolase fold but is considered to lack a "lid" covering the active site (Ser77-His156-Asp133)[11,12], leaving a large hydrophobic patch without any charged residues (Figure 1A). Previously, we reported a case of surface charge engineering of BsLA that showed additive effects in altering enzyme activity, thermostability and ionic liquid tolerance in an aqueous environment[13]. In the present study, two quadruple mutants, 4M1 (K44E, Y49E, R57E and G158E, labelled in green in Figure 1B) and 4M2 (F17E, A20E, G111D and M134E, labelled in blue in Figure 1B) were designed based on reported mutagenesis studies that were targeted towards improved thermostability and ionic liquid tolerance, respectively[14–16]. Structurally, each of the mutants, 4M1 and 4M2, contain one mutation (G158E for 4M1 and M134E for 4M2) close to the active site while the other three mutations are near the hydrophobic active site region (Figure 1B). Furthermore, the selected eight site mutations were all merged to generate a novel octuple mutant (8M).

All four of the lipase variants were cloned in expression vector pET25b (+) and protein was purified by standard protocols (Supporting Information, Figure S2). The resulting purified variants were then used as precursors for biofluid enzyme synthesis via the reported three-step method: (i) carbodiimide (EDC)-mediated cationization of solvent-accessible carboxylates to produce a fully charged polycationic protein (cBsLA), MALDI-TOF mass spectra gave cationization efficiency of 100% for all lipase variants (Supporting Information, Figure S3 and Table S1), which equated to 27 (cWT), 29 (c4M1), 31 (c4M2) and 33 (c8M) surfactant binding sites; (ii) electrostatic coupling of PEG-based anionic surfactants (carboxylated Brij-L23, we call S in the present study) and iii) thorough dialysis to remove excessive unbound anionic surfactants to yield charge-neutral aqueous constructs ([cBsLA][S]). Small-angle X-ray scattering (SAXS) spectra of the native, cationized and polymer-conjugated lipases were measured in aqueous solution (Figure 2). We found that, native enzymes showed highly similar sizes and shapes that could be fitted by the scattering calculated from the pdb structure of BsLA (Figure 2A and 2D). Upon cationization, cWT showed similar size to its native counterpart; while cationized mutants c8M1 and especially c4M1 and c4M2 had increased sizes compared to their native versions as well as cWT (Figure 2B and 2E; Supporting Information, Table S2), probably due to the very minimal crosslinking between the proteins due to excess EDC[17]. The SAXS data for the enzyme-polymer conjugates as well as the corresponding pair distance distribution function *p*(r) are shown in Figure 2C and 2F, respectively. The bump at high scattering vector modulus *q* and corresponding minima at ~0.25 $Å^{-1}$ are indicative of a core-shell structure and accordingly we fitted the data with a core-shell model with the single protein in the core surrounded by the surfactants (Supporting Information, Table S2). As mutations were introduced more surfactants were bound in the complexes, resulting in an gradual increase in radius of gyration (R_g) from 3.3 ± 0.5 nm for [cWT][S] to 3.9 ± 0.2 nm for [c8M][S] (Supporting Information, Table S2). Further analysis of the SAXS curves and modelling showed that conjugates from cWT, c4M1, c4M2 and c8M showed no free surfactant micelles and bound 39 ± 1, 48 ± 1, 55 ± 1 and 72 ± 2 S molecules per protein, respectively (Supporting Information, Table S2). To confirm the SAXS model fitting, we performed extensive lyophilization of the dialyzed aqueous conjugates followed by thermal annealing at 60 °C to form viscous solvent-free liquids[3,5]. Protein quantification of the lyophilized conjugates after re-dissolving them (with known mass) in water indicated that 38 ± 1, 55 ± 6, 59 ± 3 and 70 ± 7 S chains were attached to the surface of cWT, c4M1, c4M2 and c8M (Table S1), respectively, in an excellent agreement with the SAXS results.

All lipase variants displayed similar Circular Dichroism (CD) spectra in 50 mM sodium phosphate buffer, pH 7.2, indicating that the engineered lipases are similar to WT with no significant

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change in their secondary structures (Figure 3A). In the same buffer for the native enzymes, a significant reduction occurred in the intensity of the characteristic minima at 208 nm for all the cationized lipases except cWT (Figure 3B). Deconvolution of the CD spectra showed that surface cationization resulted in a decrease in α-helix associated with an increase of β-sheet and of unordered domains for all mutants, when compared with their native counterparts, while WT displayed negligible change in secondary structure distribution after cationization (Supporting information, Table S3). When unbuffered for conjugation of surfactants, the mutants underwent even more ellipticity loss under ambient conditions, and cWT also showed slight ellipticity loss (Figure 3C, Supporting information, Table S3). Correspondingly, all unbuffered cationized lipases displayed further reduction in α-helix, especially c4M1 and c8M which both had as low as 8% α-helix. The significant influence of cationization on the secondary structure of the mutants is similar to that on previously reported myoglobin[6], and could be associated with the absence of disulfide bridging and strong dependence of the α/β-hydrolase globular structures of BsLA on intramolecular electrostatic stabilization[18,19]. Accordingly, on the one hand, as a basic protein with the predominance of positively-charged surface residues, WT showed high resistance against removal of surface salt bridges; on the other hand, the mutants populate partially unfolded states when more cationized carboxylates and thereby more inter- and intra-domain charge repulsive interactions were introduced. Specifically, SAXS data indicated that cationization resulted in a less compact structure for 4M1 (green curve) in particular, possibly also due to the increased electrostatically repulsive interactions (Figure 2E). Interestingly, only one (D111) out of the eight introduced carboxyl amino acids is involved in salt bridges in mutant 4M2 (Supporting information, Figure S4), which still showed less change in secondary structure after cationization when compared with 4M1 (Supporting information, Table S3). The findings highlighted the long-range property of electrostatic interactions in affecting protein folding and stability, especially for highly charged proteins[20,21]. Strikingly, the polymer-conjugated lipase variants have very similar secondary structures to each other (Figure 3D, Supporting information, Table S3), indicating that conjugated polymer surfactants play an important role in recovering and maintaining the secondary structures of coated enzymes regardless of the amount of the conjugated polymer surfactants. This could be attributed to the neutralization of surface positive charges by conjugated anion polymer surfactants, and that the surrounding polymer corona might restrain the degree of mobility of the protein and reduce the local dielectric constant.

In the lyophilized solvent-free state, the role of the polymer surfactant coating in maintaining enzyme structure and activity is even more pronounced[22,23]. Genetic engineering allowed BsLA to be sterically stabilised with more polymer surfactants in the corona (Supporting information, Table 1 and 2). Differential scanning calorimetry (DSC) traces from the solvent-free liquid lipases showed reversible endothermic melting transitions at 42, 40, 40 and 40 °C for [cWT][S], [c4M1][S], [c4M2][S] and [c8M][S], respectively (Supporting Information, Figure S5), which are a little higher than the value determined for pure polymer surfactants (S = 38 °C). Although four variants in form of polymer conjugate displayed similar DSC curves, different melting behaviours were observed upon heating. Lyophilized samples from the three genetic mutants appeared as transparent and viscous liquids

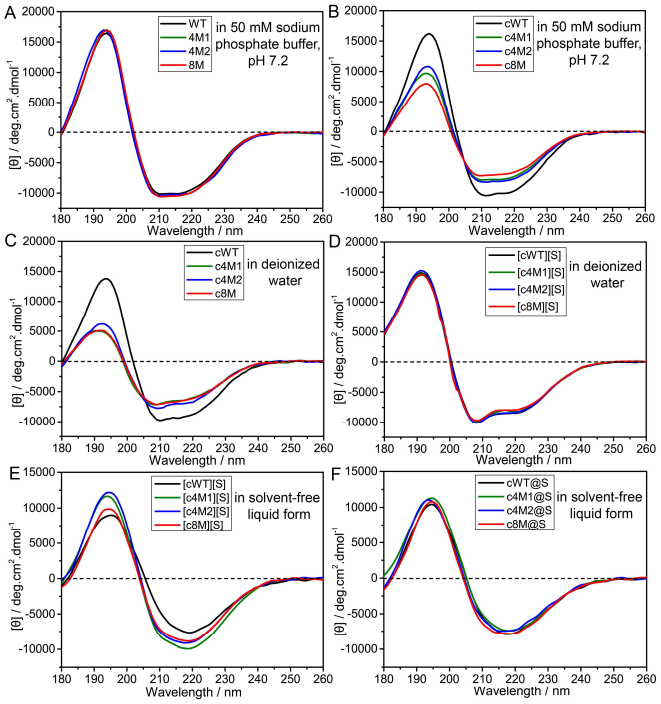

Figure 3. SRCD spectra of buffered native (A, in 50 mM sodium phosphate, pH7.2), buffered cationized (B, in 50 mM sodium phosphate, pH 7.2), unbuffered cationized (C) and unbuffered polymer-conjugated (D) lipases in aqueous solutions. (E) SRCD spectra of solvent-free liquid BsLA lipases prepared from dialyzed aqueous precursors. (F) SRCD spectra of solvent-free dispersions of all cationized lipases in excess polymers at a protein:surfactant molar ratio of 1:99.

under the microscope (Supporting information, Figure S6); however, [cWT][S] behaves like an elastic gel when heating up to 60°C, with some solid microphases, which are not observed for the [c4M1][S], [c4M2][S] and [c8M][S] solvent-free liquids. This is likely due to the lack of binding sites within the charge-free patches of cWT towards polymer surfactants, which extend the spatial range of inter-protein attractive interactions and enable a homogeneous liquid phase. This observation actually answered one of our hypothetic questions: the number and distribution of surfactant binding sites indeed affects the formation of protein liquids.

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) showed that all the resultant biofluids showed Amine I peaks at ~1,655 and 1,633 cm^{-1}, and broad amine II features at ~1,540 cm^{-1}, indicative of the presence of high levels of α-helix and β-sheet, respectively (Supporting Information, Figure S7A). This was further confirmed with synchrotron radiation circular dichroism (SRCD) measurement of the samples (Figure 3E). The deconvoluted α-helical contents of solvent-free liquid [c4M1][S], [c4M2][S] and [c8M][S] were 3-4% lower than their respective aqueous counterparts (Supporting information, Table S3 and S4). However, solvent-free liquid [cWT][S] showed the most ellipticity loss and a relatively significant reduction (~14%) in α-helical content when compared with aqueous [cWT][S], implying that a minimum number of conjugated anionic polymer surfactants and/or even distribution over protein surface is required for solvent-free protein liquid in dry state to retain its secondary structure.

In order to exclude the effect of the number of the bound polymer surfactants as a factor, we simultaneously prepared the dispersions of all cationized lipases suspended in an excess of

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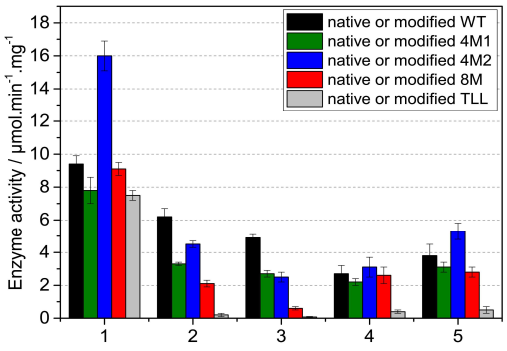

Figure 4. Hydrolytic activities of all the enzymes in native (1), cationized (2), and dialyzed polymer surfactant-conjugated (3) forms in aqueous environment. For solvent-free liquid enzymes, enzyme activities of [cProtein] [S] in stoichiometric ratio (4) and cProtein@S with fixed ratio (5) are also shown. See the details in supporting information, 2.4 Activity measurement.

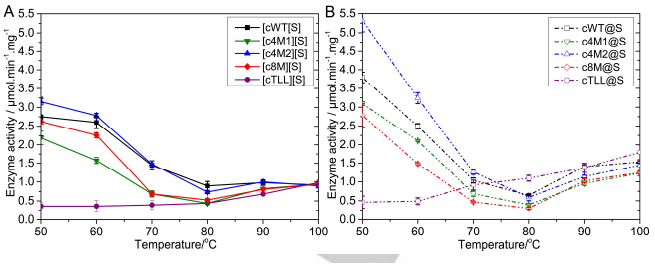

Figure 5. Temperature-dependent activity assay for solvent-free liquid BsLA lipases and TLL prepared from dialyzed aqueous precursors (A) and solvent-free dispersions of all cationized lipases in excessive polymers (B). cProtein@S was prepared by skipping dialysis. All mole ratios of cBsLAs to S is 1:99 while mole ratio of cTLL charge to S is 1:2.

unbound polymer surfactants at a uniform [protein]:[surfactant] mole ratio of 1 : 99, named as cBsLA@S (i.e. cWT@S) correspondingly in this study. The SRCD spectra for these samples are shown in Figure 3F, and the deconvoluted secondary structure distribution in Supporting information, Table S4. Here, all the samples with excess surfactant showed a reduction in the intensity of the characteristic minima at 222 nm compared with their stoichiometric counterparts. Moreover, the samples with significant levels of unbound polymer surfactants (cWT@S, c4M1@S and c4M2@S), had lower levels of α-helicity when compared with their respective dialyzed analogues, similar to that observed for solvent-free [cWT][S] in stoichiometric ratio. Conversely, c8M@S still showed a similar α-helical content to [c8M][S] at a stoichiometric ratio. The observations are well supported by FTIR spectra of the samples with more excessive polymer surfactants, displaying the collapse of amine I peak at ~1,655 cm^{-1} associated with the presence of a new peak at ~1,583 cm^{-1} (Supporting Information, Figure S7B). This suggests that excessive conjugated polymer surfactants do not behave as a diluting solvent, but in fact destabilized the cationized protein core or perturb the integrity of the corona, to different degrees. However, the difference in secondary structure might cause differences in activity and we therefore turned to test the activity of the different complexes.

A series of assays were performed to test for esterase activity in both aqueous and solvent-free liquid lipases using p-nitrophenyl butyrate (pNPB) as the substrate (Figure 4). Mutant 4M2 demonstrated the highest activity (16 $\mu molmin^{-1}mg^{-1}$), ~1.7-fold higher than WT (9.4 $\mu molmin^{-1}mg^{-1}$). Mutant 8M exhibited catalytic activity between 4M1 and 4M2 and slightly lower than WT. Mutation M134E from 4M2 was found to positively contribute to the activity, having 1.5-fold higher specific activity than that of WT[24]. Structurally, M134E is close to the active site and the local change in conformation may have induced improved binding of the substrate and facilitated the dissociation of the product[15]. After cationization, all three of the lipase mutants exhibited much lower catalytic activity (27~28% retained) than their respective precursors while cWT kept ~66% of its native esterase activity. WT was subject to the least impact of cationization and showed highest activity among cationized lipase variants, probably because its microenvironment for the active site was less altered than those of the mutants while their modifiable sites adjacent to their active sites (i.e. E134) were cationized. Electrostatic conjugation of polymer surfactants resulted in a further loss in enzyme activity, especially for c8M that lost ~70% when compared to its cationized precursor (Figure 4). Given that conjugation of polymer surfactants helps the recovery of the secondary structures of c4M1 and c8M (Figure 3D,Supporting information Table S3), the catalytic activities of the conjugates were not well correlated to the retaining of the secondary structures (Figure 4)[5]. The possible reasons include increased steric hindrance of the active sites as well as the reduction of structural mobility because of the polymer corona[5]. Significantly, stoichiometric conjugation of the c4M2 and c8M showed enhanced activity in the solvent-free state when compared with their aqueous precursors. Conversely, [cWT][S] and [c4M1][S] had higher activities in aqueous solutions. This may suggest a better catalytic conformation for [c4M2][S] and [c8M][S] after the removal of water and melting, and support the hypothesis that the polymer surfactant corona may provide sufficient mobility for catalytic conformations.

To envisage the difference of catalytic behaviours between meso- (BsLA) and thermophilic (TLL) originated lipases in biofluid form, we prepared [cTLL][S] using the standard procedure as described before[5], and performed activity assays under the identical conditions as for [cBsLA][S]s (The assay method is modified from Ref.[5], see the details in Supporting information, 2.4 Activity measurement). Significantly, all the four solvent-free liquid BsLA lipases exhibited at least 2.2 $\mu molmin^{-1}mg^{-1}$ activity, which is much higher than solvent-free liquid [cTLL][S] (0.4 $\mu molmin^{-1}mg^{-1}$). The enzyme activities for [cWT][S], [c4M1][S], [c4M2][S] and [c8M][S] were measured to be 2.7, 2.2, 3.1 and 2.6 $\mu molmin^{-1}mg^{-1}$, respectively. As mentioned above, BsLA is a lidless enzyme with its active site solvent-exposed while TLL has a lid covering the active site, which has been reported to be highly constrained in the solvent-free liquid phase[5]. To investigate the effect of excess polymer surfactants on activity, we also measured the activities of solvent-free liquid cWT@S, c4M1@S, c4M2@S and c8M@S, which displayed increased activity of 41%, 33% and 67% and 8%, respectively, compared with their respective solvent-free biofluids. c4M2@S presented the highest activity, 1.4-fold of [cWT][S]. As stated above, [cWT][S], [c4M1][S], [c4M2][S] and [c8M][S] have 39, 48, 55 and 72 S molecules per protein, respectively, whereas when all subjected to the same 99 S molecules, [c8M][S] underwent the least decrease (only ~22 molecules) in bound polymer surfactants after dialysis, which corresponded to the least change in activity (Figure 4A). This result confirmed the beneficial cooperation between free and bound anionic polymer surfactants to reduce fluid viscosity for increased substrate/product mobility so as to improve catalytic activity, regardless of the number and distribution of modifiable

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sites over the surface of protein. This may suggest that the role of excessive polymer surfactants is to contribute to an enlarged corona and as continuum through which substrate molecules can be delivered[22]. The excess surfactants increase the mobility of the enclosed protein in a way to facilitate catalysis, which is also supported by the reported finding that the polymer surfactant coating plasticizes protein structures in a way similar to hydration water[22].

To examine the responses to thermal treatment of meso-/thermophilic lipase in the form of a biofluid, we monitored the temperature dependence of the rate of pNP formation from hydrolysis of pNPB catalyzed by solvent-free liquid lipases (Figure 5A and 5B). In the case of thermophilic [cTLL][S] and cTLL@S, an increase in temperature resulted in a rate enhancement for both kinds of solvent-free liquid lipases, which is consistent with a previously reported study[5]. The researchers attributed the increase to contributions from the increase in kT (Arrhenius behaviour), as well as from temperature-induced reduction of viscosity, leading to increased substrate and product diffusion rates[5]. In contrast, all biofluids from BsLA lipases underwent distinct decrease in catalytic reaction rates with increasing temperature in spite of moderate improvement starting at 80 °C. SRCD experimental results verified that the decrease in solvent-free biofluid activity is likely resulting from thermal denaturation of protein (Supporting Information, Figure S8 and S9). In the case of cBsLA@S biofluids, the activity showed almost linear decrease for 50-70 °C, which is in good accordance with the α-helical content dropping for the cWT@S, c4M1@S, c4M2@S and c8M@S biofluids. At the extension of the heating to ~85 °C, all the solvent-free biofluids presented similar secondary structure distribution (Supporting Information, Figure S9), namely high contents of β-sheets and unordered domains, and 16-19% α-helices and 11-12% turns, indicating a dynamic equilibrium state of denaturation. Despite that, their activities were still higher than [cTLL][S] under identical assay condition. For dependence of biofluid activity and structure upon higher temperatures (over 100 °C), the present study was restricted by facility and would be expected to be further developed in the future.

In conclusion, we have demonstrated the correlations between structure and activity of a mesothermophilic lipase BsLA, when made into a protein liquid through a dual strategy that combines genetic engineering of the protein surface and cationization and subsequent anionic polymer conjugation. Our findings indicate a dependency of structure and activity on the numbers and distribution of surfactant binding sites on the surface of BsLA. We also demonstrated that improved BsLA variants can be engineered via site-mutagenesis by a rational design, either with enhanced activity in aqueous solution in native form, or with improved physical property and increased activity (i.e. 4M2) in solvent free system in the form of a protein liquid. In all, our work has answered some fundamental questions about the surface characteristics for construction of protein liquids, which is of paramount importance to recognize the limitations and potentials of solvent-free liquid protein technique. This will help identifying new strategies for developing industrially advantageous biocatalysts. Alternatively, positive supercharging by site-directed mutagenesis might be a better strategy in terms of maintaining enzyme structure and activity in biofluids, worth being further explored[25].

Experimental Section

Experimental details can be accessed in supporting information.

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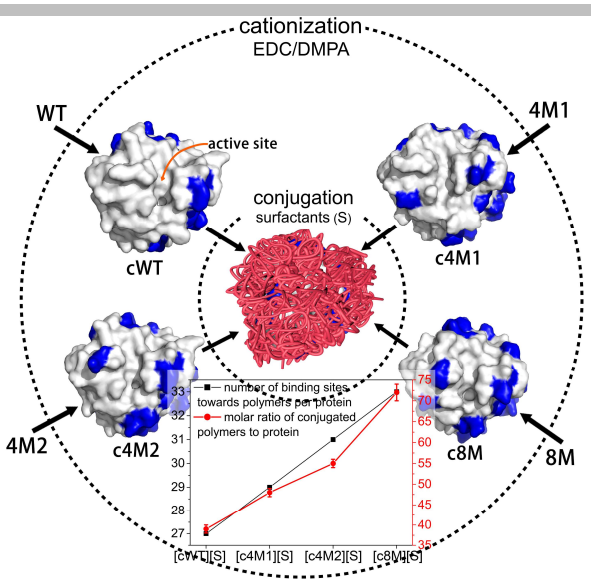
Schematic illustration showing cationization of *Bacillus subtilis lipase* A (BsLA) and its mutants using N,N-dimethyl-1,3-propanediamine (DMPA) followed by electrostatic coupling of anionic polymer-surfactant to yield a charge neutral stoichiometric conjugate [cBsLA][S]s. Blue regions on the surface of the BsLA structures show the cationic binding sites and S molecules are represented using pink tubes.



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Insight into the structure and activity of surface-engineered lipase biofluids

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