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Direct Biomolecules Binding on Nonfouling Surface via Newly Discovered Supramolecular Self-assembly of Lysozyme under Physiological Condition

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Abstract

A major challenge in the development of low cost and practical strategies for biomolecules immobilization on solid supports is that the multi-step chemical/physical activating and following deactivating procedures on nonfouling substrates often increase the cost and complexity of surface functional group types as well as deteriorate the surface integrity. Herein, we show a novel phase transition of lysozyme could be used to constitute a major step to address the above problem. It is found that when lysozyme is dissolved in a neutral buffer solution of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4) with 1–50 mM tris(2-carboxyethyl)phosphine (TCEP) added, a fast phase transition process occurs and the resulting novel fibra-like hierarchical supramolecular assemblies made by primary spherical particles aggregation would function as a “superglue” that strongly and quickly bind onto non-fouling coatings. This binding is highly selective towards lysozyme, and excludes completely tedious synthetical, chemical/physical activation/deactivation (blocking) steps. When biotin is conjugated with lysozyme, such phase transition quickly constructs a perfect biotinylated surface on nonfouling surface for avidin binding, showing great potential for the development of low-cost and practical biochips.

Keywords

Proteins; Patterning; Surfaces; Site-specific immobilization; Lysozyme

In present paper, we have solved a long-term problem in the field of biomolecules immobilization on surfaces by a newly-found supramolecular self-assembly phenomenon of lysozyme. By this approach, the conventional steps: complex surface pre-activation before biomolecules binding and deactivation after the binding on inert anti-fouling background are no longer needed.

Key to any fabrication method for biomolecules immobilization on non-fouling surfaces is to control selective surface regions being activated while inhibit reactions on the vast majority of the remained surface background that should not be activated.^[1] Looking for such an ideal method is a long term goal in research and industry realms. A core question is how to activate non-fouling surface in a facile, straightforward, inexpensive way and completely preserve/recover initial adequate “stealth” status of non-fouling surface during

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Supporting Information available.

Experimental details and Figure S1–S17.

(Supporting Information is available online from Wiley InterScience or from the author).

disturbance from multiple chemical/physical treatments on the surface.^[2] Such a consideration is coming from a fact that the acquisition of good non-fouling property, on the other hand, sacrifices surface chemical reactivity, therefore chemical activations on non-fouling surface are usually required for target biomolecules binding, which brings severe drawbacks that have great danger to suppress high throughput biomolecules binding and disturb anti-fouling property. Firstly, there is usually low number of active site available for sequential chemical reactions on non-fouling surface. Secondly, complex and multi-step reactions are usually required for reactive linker synthesis and terminal group activation, which increase the diversity of surface functional group types and deteriorate the surface integrity, since it is difficult to achieve 100% reaction yield in every chemical reaction step. For example, commonly used EDC/NHS reaction for carboxyl activation actually involves very complex multiple reaction routes^[3] and low reaction yield around 50%.^[4] Thirdly, the introduced surface functional groups are often prone to be lost due to possible side reactions.^[5] Fourthly, it is very challenging to fully deactivate all of functional groups after biomolecules binding^[2, 6] due to kinetical, thermo-dynamical and spatial limitations for surface reaction as well as heterogeneous surface chemical structures, consequently the remained unreacted functional groups would produce severe protein non-specific adsorption in further protein detection and binding experiments.^[7] Finally, complex synthesis and chemical treatments unavoidably increase fabrication cost and time. Accordingly, an inexpensive, facile, straightforward method completely avoiding complex synthesis procedures for surface modification and linker synthesis to guarantee enough binding density and keep perfectly initial adequate “stealth” status is always highly desirable and keeping challenging.^[2]

Recently, few special examples including elegantly synthesized zwitterionic polymers^[8] and poly(oligoethylene glycol methacrylate) [poly(OEGMA)] brushes^[9] are reported respectively to address this problem. In contrast to these reported methods still requiring tedious bench-top work, herein we show a new way that without any synthesis, activation and deactivation procedures, the simple use of biotin-lysozyme conjugate in neutral 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing 1–50mM tris(2-carboxyethyl)phosphine (TCEP) could provide a universal binding mode on virtually any surfaces e.g. perfect anti-fouling substrate (Scheme 1). Our strategy begins with a novel finding in research that lysozyme, a small protein molecule, could irreversibly and quickly undergo phase transition and thereby bind onto 100% tri(ethylene glycol) undecanethiol (EG3) surface in HEPES buffer with 1–50mM TCEP added (pH 7.4). Besides EG3 SAM surface, similar binding was also observed on other non-fouling coatings such as polyethylene glycol (PEG), bovine serum albumin (BSA), and dextran as well as common coatings typically used in biological studies such as polystyrene, glass and quartz. In this way, the functionality (biotin in present work) with tunable grafting density could be immobilized on 100% non-fouling or common surfaces through the bridging function of lysozyme. This binding is highly stable that could sustain during extensive ultrasonic washing, and also highly selective towards lysozyme and ineffective to other proteins.

Surface Plasmon Resonance (SPR) in Figure 1 gives the direct evidence for this binding event. We firstly inject two kinds of proteins (1mg/ml) fibrinogen and lysozyme in 10 mM HEPES buffer without TCEP added and allow both of samples to flow across 100% EG3 surface. As expected, after buffer washing, both of cases show zero binding, indicating that 100% EG3 surface is perfectly constructed in this experiment and shows classical protein-resistant property.^[10] X-ray Photoelectron Spectra (XPS) further demonstrate that the C, O atomic concentration in the obtained SAM surface is close to calculated value from theoretical EG3 SAM on surface (Figure S1). Following the first injection, the second samples, fibrinogen and lysozyme at the same concentration (1 mg/ml) in 10 mM HEPES buffer with certain amount of TCEP added (50 mM) are injected respectively and flown

across the same surfaces. The sample of fibrinogen does not result in any binding signal, and still keeps zero binding. In contrast, substantial SPR binding signal is quickly observed for lysozyme. This binding event is fast since 7 minute-flowing (injection volume is 35 μ l with flowing speed being 5 μ l/min) is enough to produce binding signal as high as 2500 RU. Quantitatively, 2500 RU (1000 RU = 1 ng/mm²) is corresponding to around 10 lysozyme molecules per surface area as 100 nm². The size of one lysozyme molecule (3 $\times$ 3 $\times$ 4.5 nm) is comparable in size to 4 nm nanoparticles.^[11] Therefore, 10 molecules per 100 nm² is higher than the saturated binding amount for lysozyme monolayer on the surface, indicating lysozyme has to adapt a compromising conformation different from its native state (vide infra). The binding density of lysozyme on the surface could be conveniently adjusted by the concentration of lysozyme and TCEP. For example, the changes of lysozyme concentration to 0.1 and 0.01 mg/ml result in a decreased binding amount as 1500 and 400 RU, corresponding to 6 and 1.6 molecules per 100 nm² respectively; a lower concentration of TCEP, e.g. 1 mM would result in a binding value as 300 RU at the lysozyme concentration being 1mg/ml (Figure S2). We also perform the separate experiments for the samples without/with TCEP added on different 100% EG3 surfaces to exclude the cross-pollution possibility from the first injected sample on the surface. The results are consistent to the measurements from continuous injection on the same surface. Both of results from continuous and separate injection experiments also have good reproducibility. Besides SPR studies, RMS (root mean-square) roughness from atomic force microscope (AFM) scanning also shows a substantial increase from initial 0.826 nm for pristine EG3 SAM to 7.761 nm for the same surface but after contacting with lysozyme HEPES TCEP buffer, supporting a notably binding amount on the surface during phase transition of lysozyme in HEPES TCEP buffer.

Based on the above SPR studies on lysozyme binding in HEPES TCEP buffer, we further design a strategy to construct biotinylated surface for avidin recognition^[12] from a solution by replacing lysozyme with biotin-lysozyme. SPR (Figure 2) has shown lysozyme-biotin could also bind smoothly onto 100% EG3 surface as it is dissolved in HEPES buffer with TCEP added. We hence infer that after biotin-lysozyme binding, the biotin moiety could be correspondingly immobilized onto the surface to form biotinylated substrate. To test this hypothesis, biotin-lysozyme in different buffers are spotted onto 100% EG3 surface and then incubated in fluorescent tag (Alexa-647)-labelled streptavidin buffer solution. After incubation, the surface is extensively washed and checked under fluorescent microscope. The first batch of samples as protein spotting from HEPES buffer at different condition are shown in Figure 3(I). Lane (A) are the spots of biotin-lysozyme in HEPES buffer containing 50mM TCEP. After incubating in fluorescent streptavidin buffer, these spots could be clearly viewed under fluorescent microscope, which fully demonstrate the binding of biotin-lysozyme on EG3 surface. Biotin conjugated with lysozyme is exposed outwards the surface so that it could serve as streptavidin recognition site. In contrast, when biotin-lysozyme at the same concentration in HEPES buffer without TCEP added is spotted on the surface (B), no spots could be observed after incubation in fluorescent streptavidin buffer, indicating that such biotin-lysozyme in HEPES without TCEP added could not be immobilized on the surface. Further control samples are provided by lysozyme without biotin moiety at the same concentration in HEPES buffer with (C) or without TCEP added (D). In both cases, the spots patterns are invisible because of the lack of biotin moiety on the surface [even lysozyme could bind onto the surface in the case of (C)], which reflect that the conjugation of biotin into lysozyme is crucial and specific for this effective streptavidin recognition on the surface.

After succeeding in protein binding from buffer solution, we further investigate the possibility to directly immobilize protein from a mixture solution, e.g. undiluted cell lysate with TCEP added. As shown in Figure 3(II), the spotting sample as biotin-lysozyme in cell

lysate containing 50mM TCEP (A) could give clear and similar binding pattern as that from the buffer solution (Figure 3I, A). As a control sample, only cell lysate containing 50 mM TCEP without biotin-lysozyme added could not produce any detectable binding pattern, indicating that fluorescence signal in Figure 3IIA is from the specific interaction between biotin conjugated with lysozyme and fluorescent streptavidin in the incubation step. This fact reflects that this lysozyme binding on EG3 surface is well compatible with cell lysate, showing the outstanding ability that direct fishing out biotin-lysozyme from a mixture solution due to the highly selective and specific phase transition of biotin-lysozyme and the well preservation on pristine non-fouling surface. As shown in Figure 3 (III), direct fishing out biotin-lysozyme from cell lysate showed ultra-high sensitivity: the detection level for biotin-lysozyme is as low as 10^{-8} mg/ml (10 pg/ml), which is corresponding to 0.001 molecules/ μm^2 . This value is even 50-fold lower than that used in single molecular study where surface density of biotin is around 0.05 molecules/ μm^2 .^[13] At low surface biotin density, the fluorescent signal from streptavidin binding is also correspondingly low. However, effective S/N ratio still could maintain because of the use of perfect 100% EG3 surface without destroying its integrity by multiple activating/deactivating reactions. As a result, high quality non-fouling surface would block noticeably the noise signal from the background and then greatly enhance S/N obtained under ultra-low concentration of analytes.

Another point that should be noted based on Figure 3 is the intact property of nonfouling surface after lysozyme binding. As shown in Figure 3I-(C), although biotin-free lysozyme in the presence of HEPES and TCEP could bind onto 100% EG3 surface (as proved by SPR in Figure 2), no fluorescence signal is observed after incubating such surface in fluorescent streptavidin buffer. This fact strongly reflects that after biotin-free lysozyme binding, the 100% EG3 surface still keeps excellent protein-repellent property to resist nonspecific adsorption of fluorescent streptavidin.

HEPES and TCEP are two kinds of classical chemicals for biotechnology and chemistry. For a long time, HEPES has been recognized as a “golden” solvent for various biomolecules and biological experiments, and TCEP is known because of its good reduction ability on disulfide bond of biomolecules with superior chemical stability in various environments. The surprising function from the combinational use of such two chemicals on lysozyme makes us focus on the characterization and analysis on this new type of supramolecular self-assembly behavior. In wealthy literatures, hierarchical supramolecular assemblies built by peptides and proteins, e.g. amyloid fibrils, have been recognized as a core in cellular homeostasis and in disease as well as a novel class of functional biological material.^[14] Self-assembly behaviours of lysozyme exhibit rich phases including crystals, dense liquid, gels and amyloid structures. Different from our present observation, the formation of these unique phases usually requires strict stimuli such as denaturants, high salt concentration, low or high pH, low temperature as well as high protein concentration, sequence mutations or strong UV irradiation. Relating to present work, the ability of aberrant small molecules such as transthyretin, catecholamines, Congo red, clioquinol and lacmoid to modulate protein phase transition has been assessed especially towards amyloid fibril formation by a variety of specific and non-specific ligand binding mechanism.^[15] In contrast to these harsh conditions, the result reported here indicates a novel phase transition occurred under quasi-physiological condition.^[16]

Time-resolved monitoring on phase transition process in the buffer by optical microscope (Figure S3, S4) reveals that the transition product is quickly discernible within 1.5 min once lysozyme is dissolved in HEPES TCEP buffer and fibra-like supramolecular assemblies are formed. With the time prolonging, these fibra-like assemblies further self-organize to form higher order pad structure consisting of interconnected fibra network. The scanning electron

microscopy (SEM) and AFM images (Figure S4F, G) with higher magnification reflect finer hierarchical structures that fibra assemblies are made of granular particles aggregation, indicating the formation of secondary fibra-like structures may be controlled by the nucleation and growth process of primary granular particles assemblies. Fiber growing kinetics are further quantitatively assessed by turbidity measurement (Figure S5), revealing the fiber is formed quickly in 15–30s and the growth reaches completion in 5–10min. Human lysozyme shows faster growth kinetics than hen egg-white lysozyme. These fibra-like assemblies are not assigned to amyloid structures, since infrared (IR) and circular dichroism (CD) spectra (Figure S6 and S7) of these lysozyme aggregates reflect random coil is predominant structure in these assemblies and typical cross β -sheet secondary structure for amyloid is not observed. Very recently, Woods et al^[15] also found by specific binding of small molecule rifamycin SV, amyloid-forming protein α 2 microglobulin would assembly to spherical particles that are distinct from amyloid fibril. The fibra-like assemblies in our work also have much bigger diameter (c.a. 1 μ m) than that from amyloid structure (tens to hundreds of nanometer). Furthermore, recent research has shown that the existence of 50 μ M TCEP would inhibit completely the amyloid formation of lysozyme through the reduction of disulfide bonds.^[17] Combining the above evidences together, this newly discovered phase transition results in a kind of novel supramolecular structure.

As already reflected by the above-mentioned fishing out experiment from cell lysate, this event is highly exclusive towards lysozyme. More control experimental results confirm that other proteins with different pI (higher or lower than lysozyme) such as fibrinogen, albumin, IgG and myoglobin do not show any phase transition behaviours (Figure S8–S11). We further find that the piperazine ring in HEPES and carboxyl groups in TCEP play a key role for this phase transition. The replacement of HEPES with other molecules for example Tris buffer while keeping other conditions unchanged would not result in the phase transition (Figure S12). In contrast, if replacing HEPES with 2-methyl piperazine which has the same piperazine ring as HEPES while keeping other conditions unchanged, similar phase transition of lysozyme is observed (Figure S13). From another side, when deliberately converting carboxyl groups in TCEP to ethylene glycol structures (Scheme S1), the transition is largely suppressed (Figure S14). TCEP is famous for its reduction on disulfide bond to form free thiol group, however, this reduction is also not mainly responsible for this phase transition since replacing TCEP with dithiothreitol (DTT), another reducing reagent having similar reduction function, would not result in obvious phase transition (Figure S2). Other recent relevant work have further shown that although TCEP is originally found as a good odor-free reducing reagent, it continuously presents interesting novel interactions with solutes such as inhibition on amyloid formation,^[17] deselenization^[18] and fracturing cysteine from proteins.^[19] Therefore, combining these references with our finding together and also referring recent finding that specific binding of small molecule on proteins can divert aggregation of an amyloid-forming protein to spherical assemblies,^[15] we conclude that specific binding of HEPES (piperazine ring) and TCEP (carboxyl group) onto lysozyme induces the conformation change of lysozyme, resulting in the observed phase transition of lysozyme. At micro-scale, such phase transition largely accelerates and diverts aggregation of lysozyme to form spherical particles distinct from amyloid fibril. These spherical particles are further self-aggregated to form fibra-like superstructures, leading the formation of novel hierarchical supramolecular organization under quasi-physiological conditions. This finding reveals a novel self-assembly pathway towards modular design of natural and biomimetic soft materials,^[20] for example, the above-mentioned binding behavior on non-fouling surface indicates that such self-assembly superstructure has a potential as a new type of biomimetic adhesive that previously is usually served by amyloid-based biological materials^[20] and catecholic amino acids.^[21]

Besides EG3-SAM substrate, the binding could be conducted successfully on a variety of chemically and physically different surfaces such as non-fouling coatings polyethylene glycol (PEG), bovine serum albumin (BSA), dextran (Figure S15) and common substrates polystyrene, glass, quartz, silica, platinum (Figure S16). Therefore the binding strength should be co-contributed from multiple physical/chemical factors. There are three basic interactions at least. Firstly, the fibra lysozyme supramolecular assemblies could be considered as a kind of colloid with hierarchical superstructures. The adhesion of such structures onto surfaces could be ascribed to osmotic pressure-driven solvent depletion force^[22] as proposed for the interaction between colloids and surfaces. Secondly, since from Figure S15 and S16, we could find that more binding amount are observed on hydrophobic substrates (e.g. polystyrene) than hydrophilic coatings (e.g. dextran), hydrophobic interaction from denaturing lysozyme aggregates onto surfaces would be another contribution. The third possible force is from fibra-induced physical entanglement that is proposed in amyloid-based adhesives^[23] and POEGMA brush-supported protein immobilization.^[9] The collective result of these activities leads to adhesion of fibra lysozyme assemblies onto versatile surfaces.

Further deep understanding on detailed mechanism for the formation of fibra-like aggregates and the adhesion on surfaces is highly possible and meaningful. For example, very recent study^[15] has demonstrated that electrospray ionization-ion mobility spectrometry-mass spectrometry (ESI-IMS-MS) technique could be used to resolve intermediates of supramolecular assembly and identify specific protein conformers during the specific binding process of small molecules. Finally, accurate molecular design could be developed to design novel stimuli-responsive phase transition system and tag. Nonetheless, in the light of modular design of natural and biomimetic soft materials, this study at present stage indeed presents a new assembly pathway of lysozyme by cooperative binding of HEPES and TCEP. The resulting hierarchical structures containing primary spherical particles and secondary fibra supramolecular assemblies from these spherical particles could function as a novel biomimetic adhesive. This study achieves a long-term goal in biomolecules microarray: directly constructing functionalized binding regions on a super-clean nonfouling background without any complex surface modification and linker synthesis. Lysozyme is available at low cost and high purity, therefore, present approach would provide an inexpensive novel class for biomolecule microarray in practical and large-scale manner. Although biotin-lysozyme is used in this work, it is strongly expected that this method should not be only limited to this example, since this proof-of-principle research reflects that in theory, any moiety which is conjugated with binding tag, lysozyme, could be immobilized onto non-fouling surface via the specific lysozyme binding. Moreover, since before present report, no one realizes the phase transition of native lysozyme is actually companied with the mixing process of three common components TCEP, HEPES and lysozyme, our results also suggest careful new considerations on this “hidden” phase transition when using these three components and even re-evaluation on existed data in such a system are needed.

Experimental

Protein immobilization

The lysozyme or biotin-lysozyme was dissolved in desired buffer for experimental use. The HEPES buffer we used was HBS-EP from Biacore containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% (v/v) surfactant P20. The additives (NaCl, EDTA, P20) were not the factors to induce the phase transition of lysozyme since the same results could be also obtained by directly using equimolar HEPES buffer (Invitrogen). Protein samples were always freshly prepared and immediately used without delay. For inducing phase transition, lysozyme HEPES solution was mixed with HEPES TCEP solution at desired concentration and the mixed solution was used immediately for characterizations.

The printing condition was settled as room temperature and 80% humidity. After that, the surface was taken out and washed extensively by HBE-EP buffer. After washing, the surface was incubated in streptavidin-Alexa 647 buffer for 2 hrs at room temperature. The surface then was extensively washed by HBS-EP buffer. The surface was always kept hydrated without drying to prevent possible denaturing on proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. SPR curves for fibrinogen (A) and lysozyme (B) flowing across 100% EG3 surface with and without the presence of 50 mM TCEP in 10 mM HEPES buffer. In both of figures, the concentration of protein samples is 1mg/ml.

Figure 2. SPR curves for biotin-lysozyme (0.01 mg/ml) flowing across 100% EG3 surface with (A) and without (B) 50 mM TCEP added in 10 mM HEPES buffer.

Figure 3.

Fluorescent image of biotin-lysozyme spotting on 100% EG3 SAM surface after Alexa 647-streptavidin recognition. (I). Protein spotting from HEPES buffer at different conditions: (A). biotin-lysozyme (0.1 mg/ml) in HEPES buffer containing 50 mM TCEP; (B). biotin-lysozyme (0.1 mg/ml) in HEPES buffer without TCEP (as a control); (C). lysozyme (0.1 mg/ml) without biotin moiety in HEPES buffer containing 50 mM TCEP (as a control); (D). lysozyme (0.1 mg/ml) without biotin moiety in HEPES buffer without TCEP (as a control). Only the spots from (A) could be detected under fluorescent microscope, indicating that by the lysozyme binding on EG3 surface, biotin attached onto lysozyme could be exposed outwards surface so that could serve as further streptavidin recognition site. (II). Protein spotting from undiluted cell lysate at different conditions: (A). biotin-lysozyme (0.1 mg/ml) in cell lysate containing 50 mM TCEP; (B). cell lysate containing 50 mM TCEP (as a control). Only the spots from (A) could be detected under fluorescent microscope, indicating that the lysozyme binding on EG3 surface has the ability that direct fishing out biotin-lysozyme from mixture solution (cell lysate), which was further demonstrated by spotting biotin-lysozyme at different concentration (mg/ml) from undiluted cell lysate containing 50 mM TCEP(III).

Scheme 1.

A schematic representation of surface functionalization by lysozyme phase-transition strategy. With the presence of HEPES and TCEP, lysozyme (purple oval) carrying a functional moiety (red dot) could be easily and quickly immobilized onto non-fouling substrate (e.g. 100% EG3) surface through its phase transition from the solution, and the functional moiety is exposed outwards the surface after this binding event finished, rendering the surface being functionalized by the functional moiety. In this paper, biotin is used as this kind of functional block. By this strategy, patterned biotinylated spots could be arrayed onto nonfouling surface. Different from conventional methods, the background in this method is always perfect non-fouling surface without activation/deactivation chemistry that would deteriorate the integrity of classical protein resistant background.