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Recognition-driven assembly of self-limiting supramolecular protein nanoparticles displaying enzymatic activity†

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We report the recognition-driven assembly of self-limiting protein nanoparticles displaying enzymatic activity. Solution self-assembly of concanavalin A lectin and glycoenzyme glucose oxidase leads to the spontaneous formation of biocolloids with well-defined dimensions, narrow size distribution and remarkable stability. These biocolloids successfully recognize a glycosylated modified electrode retaining the enzyme activity.

Self-assembled colloidal nanoparticles are of paramount relevance for opening new research frontiers in nanoscience and nanotechnology[1] for a wide range of biological and chemical systems.[2] Recent progress has been made in developing nature-inspired strategies for the synthesis of self-associated hybrid particles combining organic, bio- and inorganic components.[3] The synthetic efforts have been focused on the creation of functional structures that not only combine the properties of the individual components but also exploit the interactions between constituting particles in a synergetic manner.[1c,4] Progress in this field aims to develop systems with increased complexity where specific functionalities can be introduced and displayed in a simple but efficient fashion.[5] To perform specific functions, the use of proteins, or more precisely enzymes, as building blocks is an attractive approach owing to their inherent structural and chemical properties.[6] Most precise mechanisms in nature are based on the ubiquitously self-limited self-assembly of proteins or polypeptides into diverse structures. In naturally occurring assemblies, specific multivalent molecular interactions are especially relevant in guiding the structural complexity and enhancing specific functions with respect to the individual components.[7] Mimicking of such exquisite control of protein assembly could lead to the creation of a myriad of biosupraparticles with custom-built properties, thus expanding the choice of the constituent building blocks for materials development to new horizons. Yet, it is still a challenging task to create protein-based programmable suprastructures with controlled size uniformity and composition by simple harnessing these interactions. In this work, we demonstrate that under specific conditions, the assembly between a ligand-binding protein (concanavalin A, Con A) and a ligand-presenting enzyme (glucose oxidase, GOx) proceeds through a self-limited growth process.

Colloidal bionanoparticle (BNP) formation derived from the self-assembling supramolecular structure of Con A and GOx was adopted as an initial model system because of the well-established properties of the individual components and the possibility of combining carbohydrate-recognition and electrostatic interactions (Fig. 1). Con A, the best studied of the lectins, is a tetrameric protein with four binding sites for mannose or glucose.[8] GOx from *A. niger* is a dimeric flavin containing

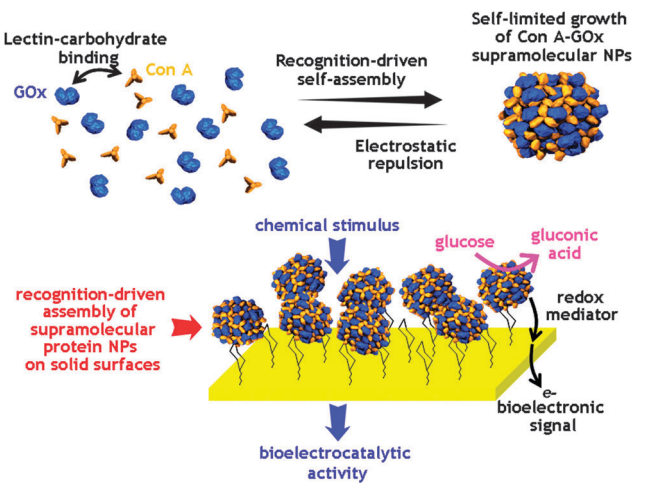

Fig. 1 Representation of the self-assembly process and the surface modification with enzyme-containing supramolecularprotein nanoparticles (NPs).

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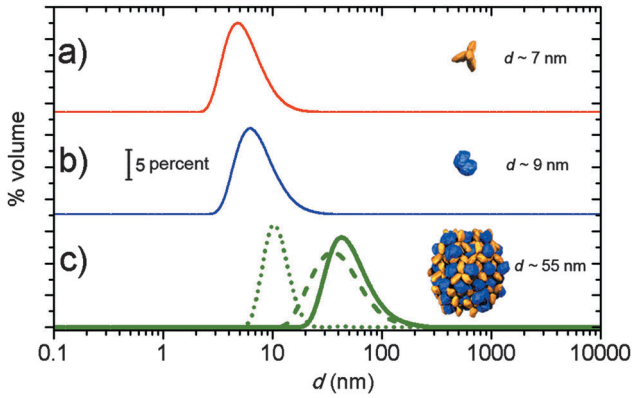

Fig. 2 Hydrodynamic diameter distribution measured by DLS of (a) Con A 1 μM (b) GOx 1 μM and (c) BNPs from Con A : GOx 1 : 2 and 1 μM total protein concentration in the absence of mannose (solid line) and in the presence of 5 mM (dashed line) and 40 mM (dotted line) of mannose.

glycoprotein with a high-mannose content that catalyzes the oxidation of β-D-glucose.[9] Con A/GOx BNPs were constructed in aqueous media by dropwise addition of Con A to a solution of GOx under smooth stirring.

Particle assembly was monitored by dynamic light scattering (DLS). Fig. 2 shows the DLS measurements for particles grown at a 1 : 2 Con A : GOx ratio and 1 μM total protein concentration. The creation of the supramolecular glycoassemblies was evidenced as a shift in the mean hydrodynamic diameter to greater values compared to the values obtained for the individual protein components (Fig. 2a and b). Interestingly, the recognition-driven assembly into multimolecular architectures proceeds to completion, *i.e.*, no unbound protein was detected. More surprising is the fact that the sugar–lectin recognition-directed self-organization process yields one single size distribution of colloidal particles. No tendency for particle association or disruption producing larger or smaller aggregates was observed within a period of 72 h.

Understanding why and how self-assembly occurs requires a detailed assessment of the forces underlying this process. Recently, Kotov and collaborators illustrated the balance between repulsive electrostatic interactions and attractive van der Waals interactions in the self-limiting growth of supraparticles made of citrate-capped CdSe nanoparticles.[10] Later, they extended their studies to the self-assembly between both positively charged CdTe stabilized with 2-(dimethylamino) ethanethiol nanoparticles and cytochrome *C* (Cyt*C*).[11] The authors proposed that the main reason for the supraparticle growth cessation is the strong electrostatic repulsion between supraparticles and between supraparticles and nanoparticles, which must counteract the intermolecular attractive interactions (*e.g.*, van der Waals forces) between nanoparticles. Although there are similarities to our observations of the assembly of Con A and GOx, it is expected that polyvalent carbohydrate–lectin binding, rather than hydrophobic or van der Waals interactions, plays the leading role in the association between Con A and GOx within the supramolecular construct. Repulsion between these biological entities can be ascribed to the fact that both proteins have charge of the same sign. The above experiments were carried out at a pH of 7.4, where Con A (pI ~ 4.5)[12] and GOx (pI ~ 4.2)[13] bear a negative net charge.

To evaluate the role of ligand recognition as the driving force for the BNP assembly, we studied protein nanoparticle growth in the presence of mannose as a competitive agent for the Con A binding sites (Fig. 2c). The mean BNP hydrodynamic diameter obtained in the presence of 5 mM mannose (dashed line) was 39 nm, while at 40 mM mannose (dotted line), it was significantly reduced to 11 nm. The presence of mannose in solution has an important impact in the protein aggregation process, highlighting the leading role of carbohydrate-recognition interactions as an attractive force for the assembly of protein nanoparticles. It is reasonable then to assume that agglomeration takes place until the electrostatic repulsion energy between proteins in the BNPs and the upcoming proteins matches the attraction energy associated with carbohydrate–lectin interactions. Nevertheless, it is unclear whether the assembly process is purely thermodynamically controlled or is also influenced by kinetic effects.

To shed light on this issue, we investigated the recognition-driven assembly between Con A and GOx under different Con A : GOx ratios and protein concentrations. The results are summarized in Table 1. It can be observed that by keeping the stoichiometry of proteins constant, variations in the total concentration of protein yield larger assemblies (Fig. S2 in the ESI†). This can be interpreted as an increase in the frequency of encounters between proteins by decreasing the average distance between biocomponents, which results in an enhanced binding rate. Proteins dynamics association seems to have a highly influencing role in determining the extension of the protein assembly. The system evolves to a new equilibrium state characterized by larger lectin–carbohydrate self-assemblies rather than by an increase in the density of particles. It is worth mentioning that in all the cases the assembled BNPs retained their integrity during the period of evaluation (at least 72 h). This suggests that the increment in binding rate has an important effect in circumventing the electrostatic repulsion between the protein constituting components, partly because electrostatic interactions cannot be maximized with globular proteins.[14] This resembles the observations of the spherical virus capsid assembly process, in which diverse size configurations are built by multiple copies of one (or few) protein subunit(s).[15] Changes in the parameter values or assembly conditions, such as concentration of viral capsid subunits,[16] can lead to a substantial variation in the binding rate, which would ultimately produce a shift in the favored assembly pathways.[16a,17]

Table 1 Characterization of BNPs by DLS

[Total protein] (μM)	Con A : GOx	*d* (nm)	PDI
1	1 : 2	55	0.19
2	1 : 2	120	0.31
1	1 : 8	12	0.38
2	1 : 8	10	0.36

d and PDI stands for hydrodynamic diameter and polydispersity index, respectively.

In the limit of high GOx concentration compared to Con A, the low density of anchoring points provided by the lectin restricted the size of the assemblies to a few molecules (Table 1). This also points out that the BNP assembly process is governed by specific carbohydrate–lectin interactions and that the influence of other operating forces, such as van der Waals, is negligible. Conversely, for the case of protein assemblies prepared with Con A : GOx x : 1 with $x \geq 1$, self-organization proceeded continuously until precipitation (Tables S1 and S2 in the ESI†). Stable discrete BNP size distributions were only achievable under certain conditions, thus highlighting the exquisite balance between protein interactions and site-directed binding constraints.

A highly desired feature of BNPs is their ability to self-organize onto appropriately functionalized surfaces as an ordered layer, *i.e.*: interfacial nanoarchitectonics.[18] Highly ordered, bio-engineered nanometer- and micrometer-sized mesostructures are of increasing technological interest exhibiting enhanced properties relevant to various application fields such as sensors, optoelectronics and biomedical devices.[6,19] Diverse strategies have been taken to produce monolayers of bio-like particles on surfaces.[3a,5] Among them, recognition directed-assembly is an attractive technique owing to its simplicity and specificity, and the fact that it is applicable to native proteins without chemical modifications.[20] The very possibility of integrating enzyme-containing supramolecular nanoparticles on surfaces is a major step as it marks a new way of interfacing bioactive elements with solid substrates.[21] To assess the binding properties of the as-synthesized BNPs, we monitored the formation of the assemblies by surface plasmon resonance (SPR) spectroscopy on gold surfaces functionalized with mannose residues. Fig. 3 depicts changes in the angle of minimum reflectance (θ_{min}) when 55 nm BNPs (Fig. S3 in the ESI†) interact with the mannosylated gold-coated sensor. The initial exposure to the BNP solution led to a rapid shift in θ_{min} followed by a plateau indicating successful recognition-driven assembly. Then, we introduced a solution of Con A, which acts as a biorecognizable building block to be assembled on the BNP layer. Subsequent incorporation of another BNP/Con A bilayer gave rise to multilayer growth upon sequential recognition-directed assembly of BNPs and Con A. The SPR angular shifts were converted into mass uptakes[22] (Table S3 in the ESI†). Compared to previous self-assemblies of Con A and glycoenzymes,[23] the incorporation of proteins into the surface assembly as BNPs instead of discrete proteins yields a 2-fold increment in protein surface density.

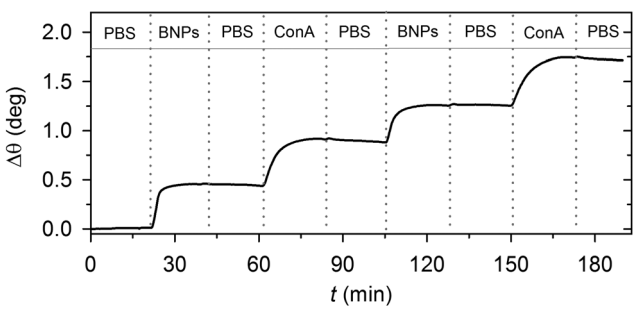

Fig. 3 Time-resolved SPR sensorgrams for the layer-by-layer assembly of BNPs and Con A in PBS buffer onto mannosylated gold sensors.

An interesting characteristic of the Con A/GOx BNPs is their ability to act as ambivalent recognition particles binding both mannose and Con A with high affinity as can be observed in the SPR trace. High affinity binding is the result of cooperative multivalent interactions.[7b] It has been demonstrated that multiple ligands located at appropriate distances can enhance the functional affinity and specificity of the protein receptor.[24] A remarkable illustration of this multivalent enhancement is exhibited by the slow dissociation rate of the mannose-BNP or BNP-Con A layers (see buffer rinse, Fig. 3). Note that during the Con A assembly on mannosylated gold sensors (Fig. S4a in the ESI†), the SPR angle shift decreases slightly before reaching the steady state, while this is imperceptible during BNP association step (Fig. S4b in the ESI†). This highlights the multivalent character of BNPs, which can be conceived as discrete particles able to interact with the surface through multiple recognition-binding sites. The strong multivalent-mediated interactions are reflected in the ability of the BNPs to hold their integrity and to remain adhered to the surface even in the presence of high concentrations of mannose in solution, a property that is less pronounced for the layer-by-layer assemblies of Con A/GOx (Fig. S5 in the ESI†). These features are expected to be effective for the development of innovative biosensing strategies (*i.e.* early stage cancer detection). Furthermore, the introduction of specific chemical functionalities to the bio-assembly, here exemplified with (although not limited to) GOx is of considerable added value for the transduction of recognition-binding events *in vivo*.

We then proceeded to characterize the response of the specific chemical stimuli-responsive element, *i.e.*, GOx, which catalyzes the oxidation of β-D-glucose in the presence of an electron acceptor to D-gluconic acid (details in the ESI†). The electron acceptor-mediated enzyme-electrochemical oxidation of glucose is transduced as an anodic current (Fig. 4a). The cyclic voltammograms of both studied BNP-assemblies reveal typical catalytic waves in the presence of the substrate of the enzyme (Fig. S6 in the ESI†). The response scaled-up linearly with increasing concentration of glucose within the range 0–20 mM (Fig. 4b). At higher concentrations the enzyme active sites became saturated with glucose, which is perceived as a plateau in the j_{cat}/[glucose] plot, as was previously reported for similar systems.[23c] We can thus infer that the supramolecularly confined enzyme within the BNPs retains its catalytic activity to a great extent.

In conclusion, we have developed a novel strategy for the construction of protein suprastructures based on bioaffinity-directed, self-limited assembly between Con A, a lectin, and GOx, a glycoenzyme widely used in multiple bioelectrochemical applications. The delicate balance between attractive and repulsive interactions led to the self-limited growth of functional and stable BNPs with defined size and composition. BNP assembly studies on gold surfaces demonstrated that BNPs display multiple recognition sites and behave as ambivalent recognition particles, while maintaining an effective glucose response. We believe that

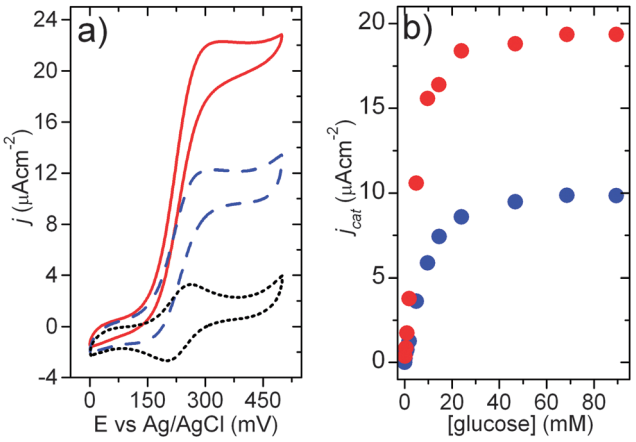

Fig. 4 (a) Cyclic voltammograms describing the bioelectrocatalysis of glucose oxidation for $(BNP)_1$ in the absence of glucose (dotted line) and $(BNP)_1$ and $(BNP)_2(Con\ A)_1$ in the presence of 100 mM glucose (dashed and solid line respectively). The experiments were performed using $[Os^{II}(bpy)_2pyCl]^+$ (100 μM) as electron mediator and v = 5 mV s^{-1}. (b) Bioelectrocatalytic current densities measured on $(BNP)_1$ (red circles) and $(BNP)_2(Con\ A)_1$ (blue circles) biointerfaces as a function of substrate concentration.

the flexibility, speed, and affordability with which this strategy provides such functional bio-supramolecular entities makes it extraordinarily attractive for technological applications in which nanoscale building blocks with multiple and adjustable recognition properties are required. For instance, there is a renewed interest in cell surface profiling aiming at characterizing various biochemical and biophysical transformations. BNP recognition properties might be of particular interest for the sensitive surveillance of surface glycans on cancer cells,[25] where increased levels of a particular glycoprotein has been associated with increased metastatic potential.[26]

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