ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

Recognition-Driven Assembly of Self-Limiting Supramolecular Protein Nanoparticles with Enzymatic Activity

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Materials

Canavalia ensiformis Concanavalin A (Con A, Jack bean), α-D-mannopyranosyl-phenyl-isothiocyanate, cysteamine hydrochloride, PBS, $CaCl_2$, $MnCl_2$, D-glucose and D-mannose were purchased from Sigma. Glucose oxidase (GOx, Apergillus niger) was obtained from Calzyme Laboratories, Inc. The $[Os(II)(bpy)_2pyCl]PF_6$ was synthesized as previously described,[1] where bpy stands for bipyridine and py for pyridine. All other reagents were analytical grade. Gold coated SPR substrates were provided by

BioNavis. All DLS, SPR and CV experiments were carried out at 20°C.

Bionanoparticles (BNPs) synthesis

Colloids made of Concanavalin A (Con A) and Glucose oxidase (GOx) were constructed in aqueous media by dropwise adding Con A to a solution of GOx under smooth stirring. The mixtures were stirred for 30 minutes at room temperature. Colloids were prepared from Con A and GOx solutions of 1 µM and 2 µM concentrations at different Con A:GOx ratios (1:8, 1:2, 1:1, 2:1 and 8:1 ratios) in PBS 20 mM pH 7.4 buffer containing KCl 100 mM, $CaCl_2$ 0.5 mM and $MnCl_2$ 0.5 mM.

Construction of biorecognition-driven architectures onto glycosylated electrodes

The construction of protein assemblies was prepared using a glass slide coated with 50 nm of titanium and 150 nm of gold by evaporation. The substrate was incubated overnight with a 5 mM ethanolic cysteamine hydrochloride solution. Afterwards, the electrode was rinsed with ethanol and dried with N_2 followed by 2 hours incubation in a 10 $\mu g.mL^{-1}$ α-D-mannopyranosyl-phenyl isothiocyanate solution in 50 mM pH 7.4 PBS buffer.[2] Then, the electrode was rinsed with PBS buffer. To prepare the BNPs biointerface we have used the colloid prepared from a Con A:GOx 1:2 and 1 µM total protein concentration. The biorecognition-driven architecture was built-up immersing the modified substrate for 1 hour in the colloidal solution. To immobilize the Con A on the BNPs-modified surface the electrode was incubated for 1 hour in a 1 µM Con A solution.[3] A 20 mM PBS pH 7.4 buffer containing $CaCl_2$ 0.5 mM and $MnCl_2$ 0.5 mM was used to rinse the electrode after BNP or Con A assembling step. The modification process was repeated to build up multilayer assemblies. All steps were carried out at room temperature.

Hydrodynamic diameter measured by dynamic light scattering

Biocolloids hydrodynamic diameters and polydispersity were determined by dynamic light scattering (DLS) using a Malvern Zetasizer ZS equipment. We used a 633 nm He-Ne laser and a backscattered detection optic arrangement, that is the detector at 7 ° of the incident light beam. All the measurements were carried out in PBS 20 mM pH 7.4 buffer containing KCl 100 mM, $CaCl_2$ 0.5 mM and $MnCl_2$ 0.5 mM. To evaluate the key role of the ligand recognition as driving force for the assembly of the BNPs, we studied the growth process of protein supraparticles in the presence of different concentration of α-D-

mannose as competitive agent (see Figure S1). The Con A-mannose dissociation constant (K_D) was found to be 1.2 mM[4]. BNPs assembly was carried out following the same procedure described before.

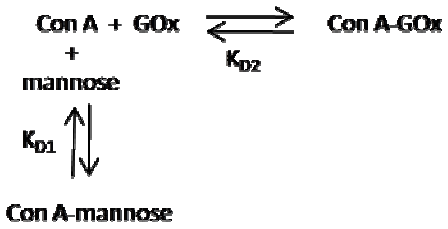

Figure S1. Competition between GOx and mannose for the Con A binding sites.

BNPs were synthesized at different Con A:GOx ratios and total protein concentrations. Stable and monodisperse BNPs were obtained under specific conditions, which illustrates the exquisite balance between attractive and repulsive forces. Figure S2 shows the size distribution of the dispersed bionanoparticles of 1 μM (Figure S2a) and 2 μM (Figure S2b and S2c) total protein concentration at different Con A:GOx ratios. Dispersed BNPs were stable for at least 72 hours at room temperature.

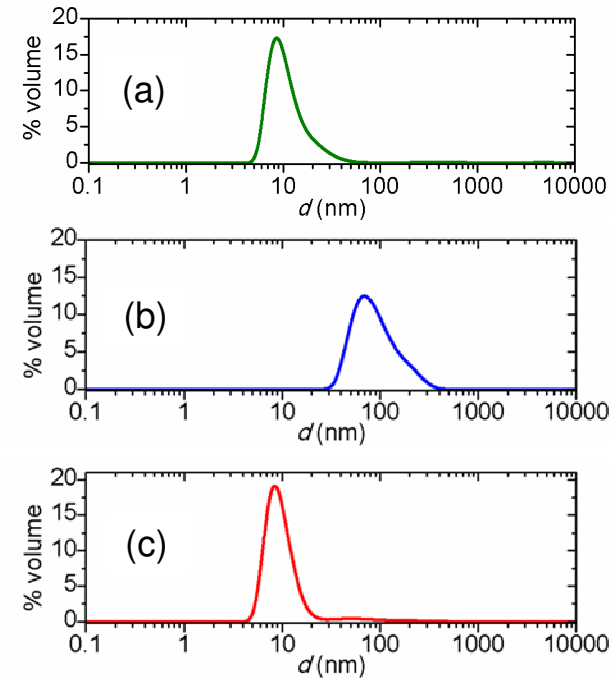

Figure S2. Hydrodynamic diameter of the following bionanoparticles: (a) Con A:GOx 1:8 and 1 μM total protein concentration, (b) Con A:GOx 1:2 and (c) Con A:GOx 1:8 both with a 2 μM total protein concentration.

Size, polydispersity and colloidal state of all the Con A:GOx mixtures prepared are summarized in tables S1 and S2.

Table S1. Size, polydispersity and colloidal state of the bionanoparticles prepared from 1 µM solution of Con A and GOx in 20 mM PBS pH 7.4 buffer containing KCl 100 mM, $CaCl_2$ 0.5 mM and $MnCl_2$ 0.5 mM.

Con A:GOx	Colloidal state	*d* (nm)	PDI
8:1	Precipitate	NA	NA
2:1	Precipitate	NA	NA
1:1	Precipitate	NA	NA
1:2	Dispersed	55	0.19
1:8	Dispersed	12	0.38

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

Table S2. Size, Polydispersity and colloidal state of the bionanoparticles prepared from 2 µM solution of Con A and GOx in 20 mM PBS pH 7.4 buffer containing KCl 100 mM, $CaCl_2$ 0.5 mM and $MnCl_2$ 0.5 mM.

Con A:GOx	Colloidal state	*d* (nm)	PDI
8:1	Precipitate	NA	NA
2:1	Precipitate	NA	NA
1:1	Precipitate	NA	NA
1:2	Dispersed	120	0.31
1:8	Dispersed	10	0.36

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

Cryo-electron scanning microscopy

A Zeiss Ultra 55 field emission electron microscope (FE-SEM) equipped with a secondary electron (SE) detectors was used for image acquisition (Zeiss SMT, Oberkochen, Germany). Acceleration voltages of about 5 keV and working distances between 3 and 10 mm were used. Cryo-SEM imaging were performed under low temperature conditions (Top = -130 ± 5 ℃). After vitrification, Con A/GOx biocolloids deposited on silicon wafers, mounted on a liquid nitrogen-cooled stage were transferred to a BAL-TECH MED 020 (BALTEC AG, Balzers, Liechtenstein) preparation device via an evacuated liquid nitrogen-cooled shuttle BAL-TECH VLC 100 (BAL-TEC AG, Balzers, Liechtenstein).

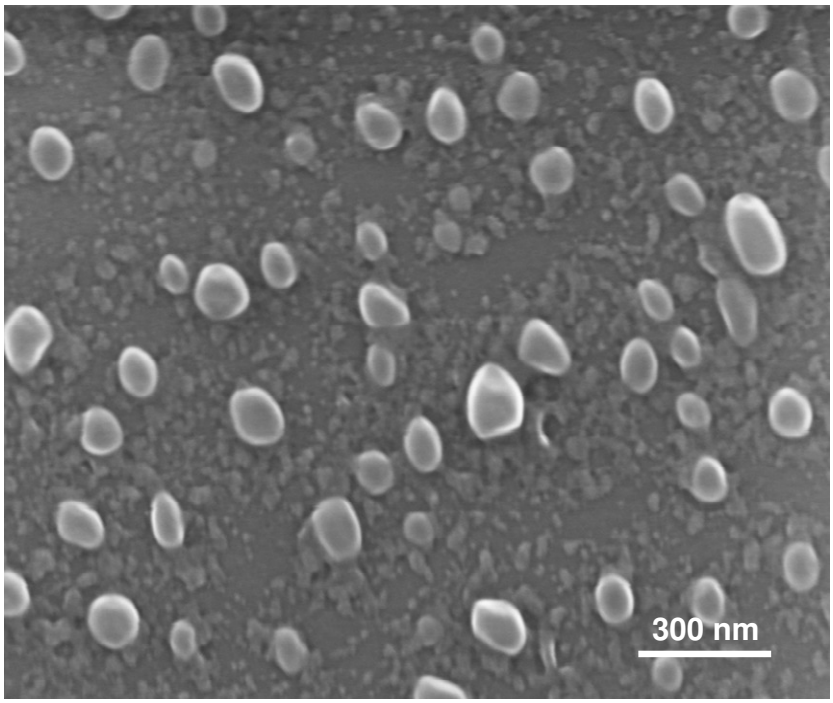

Figure S3. Cryo-SEM micrographs of Con A/GOx biocolloids on silicon prepared by mixing Con A and GOx at a 1:2 ratio and 1 μM total protein concentration.

Surface plasmon resonance spectroscopy

Molecular assemblies of Con A/GOx colloids on mannosylated gold surfaces were monitored by surface plasmon resonance (SPR, BioNavis 210A) spectroscopy using the Kretchmann configuration, a 785 nm laser and the flow rate was 10 μl/min. To estimate the biocomponents surface coverage, the SPR angle of minimum reflectivity, θ_{min}, was measured *in situ* during the recognition-driven assembly. Then, the angle of minimum reflectivity shifts, Δθ, were converted into mass surface coverage, Γ (ng/cm^2), using the following equation:[5]

$$\Gamma = \frac{\Delta\theta * k * d}{\frac{d\eta}{dC}}$$

k*d is a SPR substrate dependent parameter, in this case provided by BioNavis, with a value of 1.9x10^{-7}

cm/deg. The refractive index increment (dη/dC) of a high glucose oxidase coverage layer was obtained

from literature with a value of $1.77x10^{-10}$ cm^3/ng,[6] and the assembled Con A and BNP refractive index increment were considered equal to GOx.[7] Con A and GOx molecular mass (104 kDa and 160 kDa respectively) were used to convert the total mass surface coverage to molar surface coverage for each protein ($\Gamma_{Con\ A}$ and Γ_{GOx}). We also considered that the Con A:GOx average ratio of the immobilized BNPs on mannosylated gold surfaces was the same as BNPs in solution. The Table S3 summarizes the total mass coverage (Γ_{total}), the protein coverage for each layer (Γ_{GOx} and $\Gamma_{Con\ A}$) and the total protein coverage in the biointerfaces (Γ^T_{GOx} and $\Gamma^T_{Con\ A}$) of the $(BNPs)_2(Con\ A)_2$ assembly (Figure 3).

Table S3. Total surface coverage (Γ_{total}), and surface coverages of concanavalin A ($\Gamma_{Con\ A}$) and glucose oxidase (Γ_{GOx}) of the $(BNPs)_2(Con\ A)_2$ assembly, built-up onto the mannosylated gold sensor (Figure 3).

Assembly	Γ_{total} (ng/cm^2)	$\Gamma_{Con\ A}$ ($pmol/cm^2$)	Γ_{GOx} ($pmol/cm^2$)	$\Gamma^I_{Con\ A}$ ($pmol/cm^2$)	Γ^I_{GOx} ($pmol/cm^2$)
$(BNPs)_1$	471	1.11	2.22	1.11	2.22
$(BNPs)_1(Con\ A)_1$	941	4.52	-	5.63	-
$(BNPs)_2(Con\ A)_1$	1340	0.94	1.88	6.57	4.10
$(BNPs)_2(Con\ A)_2$	1826	4.67	-	11.24	-

Superindexes T denotes the total amount of protein assembled on the electrode surface.

Bionanoparticle affinity enhancement by multivalent biorecognition interaction

Enhancement of BNPs binding affinity towards the mannose-coated gold surface is evidenced as a decrease in the dissociation rate compared with the behavior observed for Con A. Note that during the rinsing step of Con A assemblies, the SPR angle shift slightly decrease before reaching the steady state (Figure S4a), while this is imperceptible during BNPs association and dissociation process (Figure S4b).

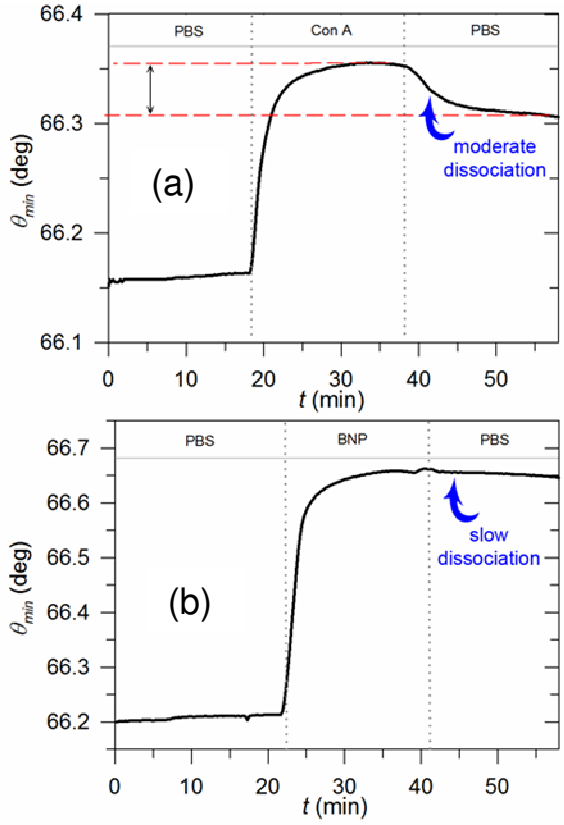

Figure S4. Time-resolved SPR sensorgrams for the assembly of (a) Con A and (b) BNPs on the mannosylated gold sensors mediated through carbohydrate-lectin biorecognition interactions.

The stability and integrity of the assemblies was studied in the presence of high concentration of mannose (ligand of strong bioaffinity for Con A). $(Con\ A)_1(GOx)_1$ and $(BNPs)_1$ assemblies were incubated in 100 mM mannose during 30 minutes and rinsed with buffer. Figure S5 shows the Reflectivity (R) as a function of the incident angle (θ) for the $(Con\ A)_1(GOx)_1$ and $(BNPs)_1$ assemblies before (blue dashed line) and after (red solid line) treatment. The $(Con\ A)_1(GOx)_1$ assembly (Figure S5a) shows a shift in the angle of minimum reflectivity after the incubation, which corresponds to a mass coverage decrease of 18 percent. In contrast, the assembled BNPs were not susceptible to such a treatment (Figure S5b).

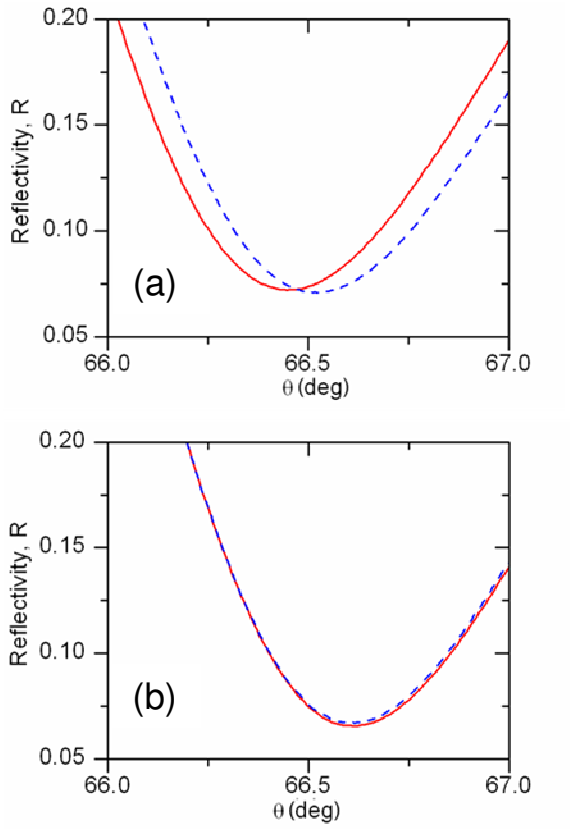

Figure S5. Angular reflectivity scans corresponding to the (a) $(Con\ A)_1(GOx)_1$-modified gold sensor and (b) BNP-modified gold sensor, prior to (blue dashed line) and after (red solid line) incubation in 100 mM mannose. In both cases the angular scans were taken in buffer solution.

Electrochemical measurements

Cyclic voltammetry experiments were performed with a TEQ-02 potentiostat using a three-electrode cell equipped with an Ag/AgCl (3M) reference electrode and platinum mesh counter electrode. All electrochemical experiments were carried out in a Teflon cell designed in a way that exposes to solution a 0.18 cm^2 surface of the electrode. 100 μM $[Os^{II}(bpy)_2pyCl]^+$ was used as redox mediator in 0.02 M pH 7.4 PBS buffer and 0.1 M KCl as supporting electrolyte. In all experiments nitrogen bubbling was used to remove dissolved oxygen from the measurement solutions for at least 30 min before using, and for 10 min between successive measurements.

Cyclic voltammetry represents an excellent tool for studying the bioelectrocatalytic properties of the supramolecular assemblies of glucose oxidase formed on the modified Au electrodes.[8] The working electrode potential is scanned in the anodic direction to generate the oxidized form of the redox mediator, which in turns triggers the catalytic process giving rise to a bioelectrochemical faradaic current.

To be more precise, the (bio)-chemical process involved in the redox-assisted GOx-mediated electrochemical oxidation of glucose consists of the following sequence of reactions:[9]

$$R \leftrightarrows O + e^-$$

$$GOx(FAD) + G \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} GOx(FADG)$$

$$GOx(FADG) \xrightarrow{k_2} GOx(FADH_2) + GL$$

$$GOx(FADH_2) + 2O \xrightarrow{k_3} GOx(FAD) + 2R + 2H^+$$

R and O are the reduced ($[Os^{II}(bpy)_2pyCl]^+$) and oxidized ($[Os^{III}(bpy)_2py]^{2+}$) forms of the mediator, respectively ($E_{1/2}$ = 0.24 V vs. Ag/AgCl). At pH 7.4, $FADH_2$ is the reduced form of the flavin prostethic group of the GOx, while FAD corresponds to the oxidized form. FADG is the enzyme-substrate complex, G is the β-D-glucose, and GL is the glucono-δ-lactone.

Cyclic voltammograms in Figure S6 depicts the bioelectrocatalytic glucose oxidation occurring at the $(BNPs)_1$ (Figure S6a) and $(BNPs)_2(Con\ A)_1$ (Figure S6b) biointerfaces at different concentrations of glucose.

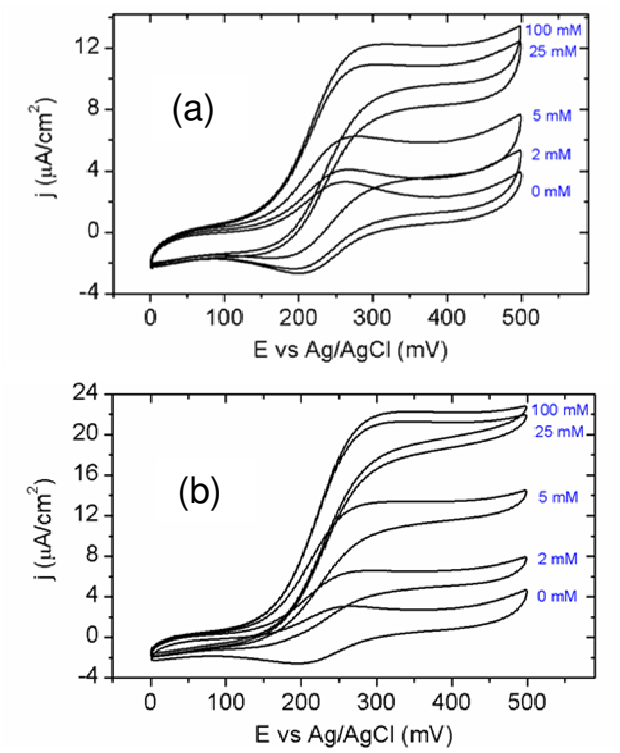

Figure S6. Cyclic voltammograms describing the bioelectrocatalysis of glucose oxidation occurring at the (a) $(BNPs)_1$-modified gold electrode and (b) $(BNPs)_2(Con\ A)_1$-modified gold electrode prepared by the bioaffinity technique. The experiments were performed using $[Os^{II}(bpy)_2pyCl]^+$ (100 μM) as mediator in different concentrations of glucose. v = 5 mV/s. Mediator: 100μM $[Os^{II}(bpy)_2pyCl]^+$.

References

[1] E. M. Kober, J. V. Caspar, B. P. Sullivan, T. J. Meyer, *Inorg. Chem.* **1988**, *27*, 4587-4598.

[2] I. Willner, S. Rubin, Y. Cohen, *J. Am. Chem. Soc.* **1993**, *115*, 4937-4938.

[3] D. Pallarola, N. Queralto, F. Battaglini, O. Azzaroni, *Phys. Chem. Chem. Phys.* **2010**, *12*, 8071-8083.

[4] P.-H. Liang, S.-K. Wang, C.-H. Wong, *J. Am. Chem. Soc.* **2007**, *129*, 11177-11184.

[5] W. Knoll, *Annu. Rev. Phys. Chem.* **1998**, *49*, 569-638.

[6] E. S. Forzani, M. Otero, M. A. Perez, M. L. Teijelo, E. J. Calvo, *Langmuir* **2002**, *18*, 4020-4029.

[7] H. Zhao, P. H. Brown, P. Schuckt, *Biophys. J.* **2011**, *100*, 2309-2317.

[8] C. Bourdillon, C. Demaille, J. Moiroux, J. M. Saveant, *Acc. Chem. Res.* **1996**, *29*, 529-535.

[9] C. Demaille, J. Moiroux, J. M. Saveant, C. Bourdillon, *Protein Architecture* **2000**, 311-335.