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Assembly of designed protein scaffolds into monolayers for nanoparticle patterning

Sara H. Mejias,^{ab‡} Pierre Couleaud,^{ab‡} Santiago Casado,^a Daniel Granados,^a Miguel Angel Garcia,^{ac} Jose M. Abad^{ad*} and Aitziber L. Cortajarena.^{ab*}

^a *IMDEA-Nanociencia, Universidad Autónoma de Madrid ,Cantoblanco, 28049 Madrid, Spain; Tel: 34 912998857; E-mail: aitziber.lopezcortajarena@imdea.org*

^b *Centro Nacional de Biotecnología (CNB-CSIC) - IMDEA Nanociencia Associated Unit, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain.*

^c *Instituto de Cerámica y Vidrio (ICV-CSIC), Cantoblanco, 28049 Madrid, Spain.*

^d *Departamento de Química Analítica y Análisis Instrumental, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain. E-mail: josemaria.abad@uam.es*

[‡] *These authors contributed equally to this work.*

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^a *IMDEA-Nanociencia, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain; Tel: 34 912998857; E-mail: aitziber.lopezcortajarena@imdea.org*

^b *Centro Nacional de Biotecnología (CNB-CSIC) - IMDEA Nanociencia Associated Unit, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain.*

^c *Instituto de Cerámica y Vidrio (ICV-CSIC), Cantoblanco, 28049 Madrid, Spain.*

^d *Departamento de Química Analítica y Análisis Instrumental, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain. E-mail: josemaria.abad@uam.es*

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Abstract

The controlled assembly of building blocks to achieve new nanostructured materials with defined properties at different length scales through rational design is the basis and future of bottom-up nanofabrication. This work describes the assembly of the idealized protein building block, the consensus tetratricopeptide repeat (CTPR), into monolayers by oriented immobilization of the blocks. The selectivity of thiol-gold interaction for an oriented immobilization has been verified by comparing a non-thiolated protein building block. The physical properties of the CTPR protein thin biomolecular films including topography, thickness, and viscoelasticity, are characterized. Finally, the ability of these scaffolds to act as templates for inorganic nanostructures has been demonstrated by the formation of well-packed gold nanoparticles (GNPs) monolayer patterned by the CTPR monolayer.

Keywords

repeat protein; protein design; self-assembly; monolayer; gold nanoparticles; biomolecular templating; building blocks, nanoparticle 2D arrays, nanopatterning.

1. Introduction

The use of building blocks and self-assembly is acknowledged as a versatile strategy for the fabrication of multifunctional nanostructures. Inorganic building blocks and nanoparticles have already shown great potential in the fabrication of new materials and multifunctional structures.[1-4] Additionally, the synthesis of hybrid organic-inorganic multifunctional nanostructures has also been developed.[5, 6] At the contrary, the use of purely organic building blocks, such as biomolecules, has not yet been fully exploited in nanotechnology. Self-assembly of biological molecules into defined functional structures has tremendous potential in nanopatterning and in the design of novel materials and functional devices.[7-15] Moreover, templating inorganic nanostructures, such as gold nanoparticles, using biomolecules is a challenging way to generate ordered inorganic materials[16, 17] for example to produce memory devices, non-linear optical and sensing devices, and nanowires.[18, 19] These strategies permit to have a high control at the nanometer scale, that is the major limitation of the conventional lithographic top-down processes used actually.

Molecular self-assembly is a process by which complex three-dimensional structures with specified functions are constructed from simple molecular building blocks. These methodologies enable the precise control over the structure but also over the function that can be encoded in the biomolecules. In order to take advantage of the great potential of using biomolecules to generate functional templates, it is key to have a molecular understanding of the building blocks and a control over interactions that drive their assembly. Additionally, green, easy and innovative strategies to have a precise control at the atomic scale for the development of scaffold and patterned structures are still to be found.

In that sense the use of proteins is of great interest as a new strategy for bottom-up approaches in nanotechnology.[20] For example, compared to other approaches used for patterning nanoparticles, such as nanolithography, any bottom up approach is more cost effective and easier to implement. In this sense, interfacial bottom up 2D assembly of nanoparticles has become a novel and widely used strategy, however it presents some limitations, including the difficulty of assembling nanoparticles with different coatings and mostly the low adhesion of the film when transferred to the substrate. [21] Nanoparticle templating by proteins combines the cost efficiency of bottom-up approaches with the capability of covalent bioconjugation with sub-nanometric resolution. The complexity and sophistication of protein-based structures in nature hints to the great potential of designed protein-based templating. In particular, the use of designed repeat proteins as the building

blocks for fabrication and patterning provides several advantages.[22] Repeat proteins present a modular structure defined by local repeated interactions and are composed of tandem arrays of the same small structural motif.[23, 24] Their simple architecture makes easier to understand the basic rules that relate sequence to structure for these repeated modules and make them ideal molecular building blocks.[25, 26]

Consensus tetratricopeptide repeat (CTPR)[23] is an idealized 34 amino acid helix-turn-helix repeat module that can be combined in tandem to form CTPR proteins with different numbers of repeats from 2 to 20 so called CTPR2 to CTPR20 proteins.[27] CTPR proteins display superhelical structures, in which eight repeats comprise one full turn of the superhelix (**Fig. 1A**).[27, 28] Additionally, the stability and ligand binding properties of CTPRs can be modulated in a predictable manner, which opens the opportunity to tune the building block properties for specific applications.[29-32] We have recently used these modules to show the specific functionalization of patterned polymeric surfaces.[33, 34] Moreover, it has been shown that under certain conditions CTPR proteins self-assemble into ordered structures including ordered protein films comprised,[35] and linear nanofibers in solution [36, 37], mimicking the packing observed in the crystal forms of CTPRs.[27, 28, 38] These results illustrate the potential of these protein modules as self-assembling building blocks.

One key component for the assembly is the side-to-side lateral interactions between CTPR superhelices.[35, 39] **Fig. 1A** schematically shows the potential alignment of CTPR superhelices. In this work, we focus on the self-assembly of the CTPR20-Cys protein composed of 20 identical CTPR repeats with a single C-terminal cysteine. CTPR20 is chosen since it is the longest CTPR that can be produced recombinantly with high yield. The longer the CTPR protein the higher the aspect ratio,[26] thus CTPR20 is expected to have higher propensity to be oriented on the surface and form more stable side-to-side interactions. In addition, longer CTPRs show higher stability[40] which is important for future applications. CTPR20-Cys is immobilized on a gold surface through gold-sulfur bond and, driven by the lateral inter-molecular interactions, can generate ordered and oriented protein self-assembled monolayers (SAMs) (**Fig. 1B**). To characterize the protein's specific adsorption on gold surface and its self-packing, we monitored the immobilization by different techniques including quartz crystal microbalance (QCM), surface plasmon resonance (SPR) and atomic force microscopy (AFM).

Finally, to demonstrate the potential of the repeat protein scaffolds to act as templates for nanoscale patterning, gold nanoparticles (GNPs) were grafted onto immobilized CTPR20-Cys through the formation of a covalent amide bond between the terminal amine of the

protein and the carboxylate groups of the GNPs (**Fig. 1C**). The resulting functionalized surface with a monolayer of GNPs was characterized by scanning electron microscopy (SEM) providing a final evidence of the oriented monolayer formation of CTPR20-Cys protein and a potential application in the controlled patterning of GNPs (**Fig. 1D**). The use of GNPs in an ordered monolayer organization is of great interest in field of plasmonics [41] and organic solar cells progress[42] or for the development of localized surface plasmon resonance-based sensor.[43]

2. Results and discussion

The main goal of this work is to demonstrate that repeat proteins can be used as scaffolds for the patterning of inorganic nanostructures, such as gold nanoparticles.

The first step of the study is the adsorption of CTPR20-Cys onto gold surface. Thus, we first compared the immobilization on gold surface of two proteins: CTPR20-Cys for oriented thiol mediated immobilization and CTPR20 without Cys as a non-oriented deposition control. The process was monitored by quartz crystal microbalance (QCM), which is a powerful tool to study interfacial process at surfaces. After stabilization of the temperature, frequency and resistance, purified proteins solutions prepared as previously reported [13, 44] were flowed through the cell. **Fig. 2A and 2B** present the resulting frequency and resistance changes as a function of time after protein injection. As it can be seen in the **Fig. 2A**, upon flowing CTPR20-Cys protein, a rapid decrease in the resonant frequency was observed during the first 2 minutes as a result of the protein binding to the surface by chemisorption of cysteine on gold. Afterward, the frequency decreased more slowly until a steady state was reached. Simultaneously with the frequency decrease, an increase in motional resistance was also observed (**Fig. 2B**). It indicates that the binding of the protein does not result in a rigid load but viscoelastic causing an energy loss by dissipation from the QCM sensor.

The ratio between the motional resistance change and the frequency shift can be used to evaluate the relative influence of viscoelastic and mass loading effects (see below). The shape of the frequency-time profile can be employed to study the kinetics of adsorption by fitting to a first-order kinetics equation (**Fig. 2A** and Materials and methods section). The values of $\Delta F_{\max}$ and k obtained from the data fit were -73.2 Hz and 0.57 min^{-1} , respectively. From the $\Delta F_{\max}$ at the equilibrium, assuming that the frequency decrease is only due to the change in mass arising from the adsorption of the protein, the mass of CTPR20-Cys immobilized on the gold electrode surface was estimated to be $1285 \text{ ng}\cdot\text{cm}^{-2}$ using **Equation 1**. This value corresponds to a surface coverage of about $15.2 \times 10^{-12} \text{ mol}\cdot\text{cm}^{-2}$ considering a molecular mass of 85242 Da for the CTPR20-Cys ($9.0 \times 10^{12} \text{ CTPR20-Cys molecules}\cdot\text{cm}^{-2}$). This value is in agreement with the number of molecules for a compact monolayer of oriented CTPR20-Cys proteins, considering a projected area of about 11 nm^2 per molecule based on the dimensions from the crystal structure.[27] Additional support to this fact is provided by the comparison with the extensively studied monolayers of n-alkanethiolates onto gold surface [45, 46] which result in a maximum coverage of $\sim 4.2 \times 10^{14} \text{ molecules}\cdot\text{cm}^{-2}$ for molecules that cover 0.217 nm^2 .[47] For an equally packed CTPR20-Cys molecule (11 nm^2) the

theoretical maximum coverage would be reached with 50 times less molecules 8.4×10^{12} molecules·cm⁻², which is in the same range of the experimental value of 9.0×10^{12} molecules·cm⁻².

These results show that the protein units assemble in a compact manner on the surface by thiol chemisorption of cysteine residues onto the gold surface. As it is the case for more simple thiol derivatives, CTPR20-Cys proteins are not randomly deposited since the amount of protein per surface area would be otherwise smaller. To serve as comparison, QCM adsorption measurements of CTPR20 without cysteine were also carried out (Electronic supplementary material). As shown in **Fig. S1** flowing CTPR20 gives a final surface coverage of 6.5×10^{12} mol·cm⁻², significantly lower than for CTPR20-Cys. This result can be associated to the fact that direct adsorption of CTPR20 proceeds through unspecific multipoint attachment of the protein to the surface contrary to the oriented thiol-derivative protein that is tightly adsorbed on the gold surface by an unique single-point interaction.[48, 49] This fact was also put in evidence by studying the thermodynamics of adsorption. The calculated equilibrium proteins coverages from ΔF_{max} for different protein concentrations are fitted to a Langmuir adsorption isotherm (Materials and methods section, **Eq. 4**) to calculate the saturation protein coverage ($\Gamma_s = 15.2 \times 10^{12}$ mol·cm⁻²) (**Fig. 2C**). In contrast, for CTPR20 without cysteine the Γ_s was 7.3×10^{12} mol·cm⁻² (**Fig. S2**). The coverage at saturation for CTPR20 is half of the one obtained for CTPR20-Cys, presumably due to a random immobilization of CTPR20 in different orientations.

In order to confirm the validity of conversion the change in frequency to molecular mass from QCM data using Sauerbrey's equation, surface plasmon resonance (SPR) experiments were conducted, as detailed in the Materials & Methods section,[50, 51] to quantify the amount of protein bound. **Fig. 3A** shows a SPR experiment carried out employing a CTPR20-Cys protein concentration of 2.5 μ M to saturate the surface. The mass measured by SPR from the resonance angle shift upon protein binding to the gold surface was approximately 1000 ng·cm⁻². This mass is slightly lower than the value obtained from QCM (1285 ng·cm⁻²), probably due to the uncertainty of the refractive index of the adsorbed protein and to the effect of the hydration water in the QCM measurements. The SPR mass can be considered comparable to the QCM mass confirming that the frequency changes observed in the QCM experiments are mainly ascribed to mass changes as consequence of the protein adsorption since viscoelastic effects should induce a bigger difference between Δm_{QCM} and Δm_{SPR} , as previously reported.[52]

We used AFM to image the CTPR20-Cys proteins immobilized onto gold surfaces. Single CTPR20-Cys molecules were imaged first at a low protein concentration (10 nM) to characterize the features of the individual monomers (**Fig. 4A**). A single size distribution peak was obtained for individual CTPR20-Cys molecules with an average height of approximately 8 nm, indicating that each bright dot on the image corresponds to a protein monomer (**Fig. 4A**). The width measurements are not reliable due to the tip convolution.[53] The end to end distance of the CTPR20 superhelix calculated from the crystal structure is approximately 19 nm (**Fig. 1A**).[27] There are several reasons that will account for the smaller size measured by AFM: (1) CTPR20-Cys adsorption onto gold surface through its thiol group might be tilted with an angle relative to an orthogonal orientation to the surface; (2) vertical elastic tip compression of the elongated superhelical structure, as it has been described for other repeat proteins;[54] (3) AFM characterizes single protein molecules in solution, which might have some flexibility[55] compared to the dimensions determined from the rigid crystal structure of the protein.[56]

Following, CTPR20-Cys monolayer assembly on gold surface was studied. CTPR20-Cys at 2.5 μ M concentration, which guarantees full coverage of the surface according to the equilibrium immobilization studies by QCM (**Fig. 2C**), was deposited onto annealed gold surfaces. The AFM image shows a densely packed array of protein particles that completely covers the gold surface (**Fig. 4B**). Under the solution deposition conditions and in the range of protein concentration used (10 nM-2.5 μ M), CTPR20-Cys protein is stable, folded, and fully monomeric [27, 40], thus is not expected the deposition of multimeric particles. The AFM images at saturation provide insights about the tight packing of the protein molecules on the monolayer and clearly show a full coverage of the surface (**Fig. 4B**), corroborating a higher occupancy than expected from simple random distribution of individual CTPR20 monomers. The overall roughness analysis of the surface resulted in a RMS roughness of 0.86 nm with an average height of 3.42 nm, smaller than the one observed for individual CTPR20-Cys proteins (RMS roughness of 1.88 nm and average size of 6.36 nm), in agreement with a compaction of the molecules. The CTPR20-Cys covered surface shown in figure 4B was incubated an excess of dithiothreitol (DTT) that will displace the previously adsorbed CTPR20-Cys, in order to study the specificity of the interaction between CTPR20-Cys and the gold surface. After the incubation and wash of the surface the AFM image (**Fig. 4C**) shows some dots corresponding

to individual protein molecules, while most of the protein has been detached from the surface. This result confirms the specificity of the thiol-gold immobilization of the CTPR20-Cys.

In order to determine the thickness of the monolayer imaged by AFM, profilometry experiments were performed on a CTPR20-Cys layer deposited on one half of a gold surface using 2.5 μM protein concentration. Three protein layers were measured to give a thickness value of $17.93 \pm 1.05\text{nm}$ (Fig.3B), consistent with the formation of a single CTPR20-Cys protein monolayer.

In addition, the ratio between the motional resistance (ΔR) and the frequency (ΔF) provides information about the viscoelastic properties of the deposited protein layer.[57] In **Fig. 5A**, the ΔR during the course of CTPR20-Cys protein assembly is plotted against ΔF . Two separate regions are observed with different $\Delta R/\Delta F$ slopes indicating that surface coverage has a strong influence on the viscoelastic properties of the protein film. The first region (ΔF from 0 to -20) shows a linear relationship between $\Delta R/\Delta F$ presenting a steeper slope. It corresponds to the beginning of the adsorption process associated to lower protein densities and therefore less lateral interactions between protein molecules. The deposit tends to be more flexible and more dissipative at this stage. When higher protein densities are reached (ΔF from -20 to -60), protein molecules can establish lateral interactions with neighbouring molecules, yielding finally to a more rigid and less dissipative film. The change in slope between two regions indicates the transition from individual proteins to a compact state. The intersection of the two straight segments from the linear fit occurs at 22.2 Hz and at a $\Delta R/\Delta F$ ratio $0.130\text{ Ohm}\cdot\text{Hz}^{-1}$.

To confirm that the observed difference in $\Delta R/\Delta F$ between high and low protein density on the surface could be attributed to the protein compact state, experiments using different protein concentrations and recording ΔF and ΔR simultaneously were performed (**Fig. 5B**). It is observed that at low protein concentrations the $\Delta R/\Delta F$ ratio increases as the protein concentration increases, associated to a higher viscoelasticity of the protein film, reaching a maximum of $0.124\text{ Ohm}\cdot\text{Hz}^{-1}$ at $0.075\text{ }\mu\text{M}$ of protein. It is noteworthy that this $\Delta R/\Delta F$ value is comparable to that obtained from the intersection in **Fig. 5A**. This result shows that at low concentrations there are not enough neighbouring CTPR20-Cys molecules immobilized for them to assemble, as previously suggested. When the protein concentration is increased, a subsequent $\Delta R/\Delta F$ decrease is obtained until reaching a stable value of $0.06\text{ Ohm}\cdot\text{Hz}^{-1}$ at

surface-saturating protein concentrations. The surface becomes more densely packed with CTPR20-Cys molecules, promoting lateral interactions and leading to an increase in the rigidity of the film. These results clearly suggest that the mode of attachment through the C-terminal Cys residue leads to a spatial arrangement, with parallel alignment between protein molecules, favouring the establishment of lateral protein interactions. In the case of CTPR20 protein without cysteine this analysis shows a different behaviour with a linear increase in the viscoelasticity of the deposit as the protein is adsorbed (Electronic supplementary material Figure S3).

The final step of this study is the functionalization of the protein monolayer with gold nanoparticles (GNPs) in order to monitor the templating capabilities of the CTPR monolayer. In addition, the attachment of GNPs will allow us to achieve a higher resolution characterization of the surface using SEM. GNPs stabilized with thioctic acid (TA-GNPs) displaying free carboxylic acid functions, required for the immobilization step, were used. As the CTPR20-Cys was selectively immobilized onto the gold surface through its single C-terminal cysteine, the N-terminal moiety of the protein was selected as the anchoring point. The anchoring reaction is achieved through the formation of an amide bond between amine of the N-terminal of the CTPR20-Cys (previously immobilized on gold surface) and the carboxylic acid functions of TA-GNPs, which were activated *in situ* by EDC/NHS. After the reaction of the TA-GNPs with the gold surface coated with CTPR20-Cys, scanning electron microscope (SEM) was used to characterize the functional surfaces obtained. The AFM results showed the evidences of a monolayer of compacted CTPR-20-Cys (**Fig. 4**) onto gold surface and a homogenous coverage of protein over surfaces in the order of μm^2 . Each CTPR20-Cys molecule should be able to interact only with one GNP through an amide bond formation, and the area of the protein section is about 11 nm^2 , in the same range of the GNPs diameter. Therefore it is expected to observe a homogenous coverage of a single layer of GNPs over the CTPR20 surface.

Fig. 6A shows a significant GNPs surface coverage yield (clearer areas) in a highly packed distribution. The entire surface was not fully covered by GNPs but CTPR20-Cys can be observed on almost the whole surface (as black/grey background). An important observation is that when the functionalization with GNPs occurred, it formed preferentially a monolayer of GNPs with a high level of compaction, leading to islands of close-packed GNPs monolayers (**Fig. 6B**). At a higher magnification, the monolayer organization of the GNPs

functionalized onto CTPR20 immobilized onto gold surface can be clearly confirmed (**Fig. 6B**). The controls of GNPs onto gold surface and GNPs onto CTPR20 without coupling agents gave very different results in SEM, with a majority of isolated GNPs aggregates and/or randomly dispersed (Electronic supplementary material). The comparison of both SEM (**Fig. 6B**) and AFM (**Fig. 4B**) at the same scale confirms the monolayer organization of the GNPs and that the same kind of organization is observed for the protein onto gold surface, presenting in both cases a high compaction. This was not observed when the GNPs were deposited on gold surfaces without prior CTPR20-Cys protein layer formation (**Fig. S5** and **S6**), which corroborates that the CTPR20-Cys monolayer is templating the GNPs.

3. Conclusions

In conclusion, we have demonstrated that we can immobilize oriented long designed CTPR proteins on gold surface through thiol-gold specific interaction and that the protein modules present intrinsic lateral assembly properties. By controlled immobilization we generated a tightly packed protein SAM and characterized the packing by QCM, SPR, and AFM. We monitored the assembly transition by the change on the viscoelastic properties of the immobilized materials. It is clear that the oriented immobilization of the long protein arrays allows the arrangement of the modules and the formation of a rigid protein film on the surface. Regarding the mechanism of interaction between the proteins and the forces that drive the assembly, we can speculate based on the previously observed lateral packing of superhelices in solid films[35] and in crystal forms.[27] Long CTPR proteins are structurally rigid with anisotropic shape (**Fig. 1**), that allows for preferential alignment along one direction and lateral packing when the proteins are immobilized directionally oriented on a surface. The crystal packing provides hints that CTPR protein superhelices have a tendency to interact through salt bridges between positively and negatively charged residues on the protein surface.[58] Similar interactions can mediate side-to-side packing within monolayer. The functionalization with GNPs additionally confirms that most of the protein molecules are oriented as expected (N-terminal free) and in a monolayer disposition. This work is an example where the molecular understanding of the biomolecular blocks and the control of the immobilization and assembly process on surfaces can lead to the generation of materials with defined macroscopic properties. For example, by changing the protein packing density, the rigidity of the protein film can be tuned at will. Additionally, these ordered and structured biocompatible surfaces can be functionalized by introducing unique groups with atomic

resolution at defined positions in the protein. The results of this work show the potential of these designed scaffolds as platforms for patterning GNPs. The assembly of GNPs into closely packed thin films has great interest because of their potential applications in electronics, nanophotonics, data storage, and plasmonics.[59] The templating strategy described here is versatile and can be easily applied to a variety of nanoparticles and other molecules for the generation of closely packed arrays. The application of these biomolecular templates can result in novel types of hybrid materials within the bottom-up approaches.

4. Materials and methods

4.1. Protein design and purification

CTPR20 protein with a single cysteine at the C-terminal was expressed as His-tagged fusion and purified using standard affinity chromatography methods as previously described. [13, 27, 44] The protein was dialyzed into PBS buffer (150 mM NaCl, 50 mM phosphate buffer pH 7.4) and stored frozen at -20°C. The protein concentration was determined by *UV* absorption using a Cary 50 *UV*-visible spectrophotometer (Varian) at 280 nm using the extinction coefficient calculated from the amino acid composition.[60]

4.2. Protein reduction for gold surface immobilization experiments

Prior to any immobilization experiment on gold, purified CTPR20-Cys at a protein concentration of about 23 μ M was freshly reduced with 1 mM dithiothreitol (DTT) during 20 minutes to ensure full reduction of the sample. DTT was removed by buffer exchange over a NAP-5 column (GE Healthcare Life Science) in PBS-2 buffer with lower salt and buffer concentration (10 mM NaCl, 10 mM phosphate buffer pH 7.4) or water. Protein fractions without DTT are collected at 4°C and their concentration measured by *UV* absorption spectroscopy.

4.3. Quartz Crystal Microbalance (QCM)

QCM is an extremely sensitive mass sensor capable of providing real-time monitoring of immobilization events in the nanogram range and determination of kinetic rate constant. QCM measures frequency changes in the resonant frequency of the quartz crystal according

to Sauerbrey's equation (**Eq 1**), where a decrease in mass corresponds to an increase in frequency and vice versa. Moreover, QCM with motional resistance monitoring also allows obtaining information related to the structure, conformation, and interactions of the molecules on the surface through the acoustic dissipation. In situ mass changes were measured with a SRS QCM200 Quartz Crystal Microbalance from SRS Instruments (Sunnyvale, CA., USA) with AT-cut quartz crystals (5 MHz) of 25 mm diameter with gold electrodes deposited over a Chromium adhesion layer. An asymmetric electrode format was used with the side having the larger gold area facing the solution. The electroactive working area (front side) was 1.370 cm² and the piezoelectric area (backside) was 0.317 cm².

Prior to use, the quartz crystals were cleaned by exposure to "piranha" solution (3:1 concentrated H₂SO₄/30% H₂O₂) followed by exhaustive rinsing with distilled water and a final rinse with ethanol/water (2:1). *Caution: piranha solution reacts violently with most organic materials and must be handled with extreme care.* The quartz crystal resonator was set in a probe made of Teflon and immersed in water-jacketed beaker thermostated at 25°C. The crystals sensitivity was 17.68 10⁻⁹ g·Hz⁻¹·cm⁻². [61-63]

Under constant flow protein samples in PBS-2 buffer at concentrations from 5 nM to 2.5 μM are injected in the system and both the changes in motional frequency and in resistance were recorded until saturation is reached.

Sauerbrey's equation describes the correlation between frequency changes in the resonant frequency of the quartz crystal and the immobilize mass for QCM experiments:

$$\Delta m = -C_f \Delta F \quad \text{Equation 1}$$

where Δm is the mass change (ng·cm⁻²), C_f (17.7 ng Hz⁻¹·cm⁻²) the proportionality constant for the 5.0 MHz crystals used in this study, and ΔF the frequency change (in hertz). Although this equation rigorously applies to the system in air and mass additions forming an evenly distributed rigid layer on the active sensor area, [64] it is widely accepted to estimate the adsorbed mass of protein in liquid environments. [65-67] Sauerbrey equation can therefore be reliably used to measure the mass of thin, evenly distributed, rigid and non-porous overlayers even in the liquid phase. [64] In this way, it has been reported that the linearity between adsorbed mass and frequency response may persist under certain conditions. [68]

Assuming that the immobilization process is kinetically controlled the frequency curves were fit to a first-order kinetics equation:

$$\Delta F = -\Delta F_{\max}(1 - e^{-kt}) \quad \text{Equation 2}$$

where ΔF is the frequency change (in hertz), $\Delta F_{\max}$ the frequency change between the initial and the steady-state frequencies, and k the first-order rate constant (min^{-1}).

The frequency-time data for CTPR20 can be well fitted to a first order double-exponential decay with two adsorption processes, one fast decay rate and a subsequent slower adsorption:

$$\Delta F = \Delta F_{\text{fast}} \exp(-k_{\text{fast}} t) + \Delta F_{\text{slow}} \exp(-k_{\text{slow}} t) + \Delta F_{\max} \quad \text{Equation 3}$$

From the $\Delta F_{\max}$ at the equilibrium and using Sauerbrey's equation (**Equation 1**) the total mass of protein can be estimated assuming that the frequency decrease is only due to the change in mass arising from the adsorption of the protein immobilized on the gold electrode surface.

From the frequency change at the equilibrium ($\Delta F_{\max}$), the total amount of protein immobilized per surface area can be calculated at the different protein injections. The equilibrium protein coverage values were represented versus protein concentrations and fitted to a Langmuir adsorption isotherm:

$$\quad \text{Equation 4}$$

$$\Gamma_e = \frac{\Gamma_s K_a C_s}{1 + K_a C_s}$$

where K_a is the thermodynamic binding constant, C_s is the bulk protein concentration, and Γ_e and Γ_s are the equilibrium and saturation protein coverage.

4.4. Surface plasmon resonance (SPR) measurements

A gold film of approximately 30 nm was first deposited on sodalime glass substrate (1 mm thick) by electron beam evaporation in a Molecular Beam Epitaxy system with a base pressure of $P_b = 1 \times 10^{-10}$ Torr. The SPR spectrum of the gold surface was measured to calculate the exact thickness of the film. Then, a CTPR20-Cys protein solution at 2.5 μM concentration was deposited on the gold surface. After incubation for 30 minutes at room temperature the SPR spectrum of the protein film in solution was acquired.

SPR spectra were acquired using the Kretschmann-Raether configuration, [69, 70] with a home-made device described elsewhere.[50] SPR was excited with a 632.8 nm laser. The angular response of the photodiode was corrected as described before.[51] For each sample 4 scans were recorded from different spatial locations. The spectra shown here correspond to the average (with the line thickness being the standard deviation). Any possible drift in the motor position was corrected by fixing the position of the critical angle to 39.5 deg; this value depends only on the glass and the surrounded air, and it is independent of the gold and protein films. To calculate the total mass of protein immobilized on the gold surface, a shift of 0.1 deg on the resonance angle is related to a mass of $1 \text{ ng}\cdot\text{mm}^{-2}$, as described before for proteins.[71] SPR curve simulation was carried out using Winspall freeware by RES-TEC (<http://www.res-tec.de/applications.html>) including the correction of refraction for triangular prisms.

4.5. Atomic force microscopy (AFM)

For AFM measurements CTPR20-Cys protein was immobilized on gold surfaces. The gold substrates were commercial gold-coated borosilicate glass substrates (Gold ArrandeeTM) annealed using a Bunsen burner prior to use. First, we monitored gold surfaces under different annealing conditions without protein to achieve sub-nanometric roughness and ensure that the surface roughness is small enough to clearly image the nanometric-scale protein molecules. CTPR20-Cys freshly reduced protein as described above was incubated on the gold surface during 1 hour. After incubation the surface is thoroughly washed with water.

The protein coated surface is imaged in solution using a Nanotec Cervantes system in jumping mode.[72] Olympus standard silicon nitride probes of $0.05 \text{ N}\cdot\text{m}^{-1}$ and 18 kHz (OMCL-RC800PSA) were employed. Images were processed using the WSxM software (Nanotech Electronica, Madrid, Spain <http://www.nanotec.es/>).[73]

4.6. Profilometry measurements

CTPR20-Cys protein was immobilized on gold surfaces using the same protocol used for the generation of the AFM samples. Three different layers were obtained by incubation of $2.5 \text{ }\mu\text{M}$ protein. The layer thickness was measured using a “Dektak XT” mechanical profilometer with a $2.5\mu\text{m}$ radio stylus and 1mg force (the minimal force).

4.7. Gold nanoparticle (GNP) synthesis

Gold nanoparticles were synthesized following the well-established Turkevich protocol[74] with slight adjustments as described previously.[75] Briefly, 100 mL of an aqueous solution of HAuCl_4 (Sigma-Aldrich) at 1 mM was refluxed at 140°C and reduced for 15 min with 10 mL of an aqueous solution of sodium citrate (55 mM). The reaction led rapidly to a red-wine solution of citrate based GNPs. The sample is then cooled to room temperature and filter through $0.2\ \mu\text{m}$ pore-size filter to eliminate large aggregates. The final concentration of GNPs (9 nM) was determined by *UV/VIS* spectrometry using an extinction coefficient at 520 nm, $\epsilon = 2.7 \times 10^8\ \text{M}^{-1} \cdot \text{cm}^{-1}$. The as-modified Turkevich protocol for the synthesis of GNPs leads to a well-dispersed colloidal suspension of citrate-stabilized GNPs with an average diameter of $12.4 \pm 1.2\ \text{nm}$ (measured by TEM, **Fig. S6**). A second step of ligand exchange with thioctic acid was carried out to introduce a more stable surfactant with carboxylic groups. To 10 mL of GNPs at 9 nM was added 1.15 mL of thioctic acid at 10 mM in ethanol to react overnight at room temperature. Then, sample was centrifuged 40 min at $10000 \times g$ and supernatant discarded. The red-wine pellet was redispersed in 7 mL milliQ water, resulting in a light purple suspension. Then, drops of NaOH 10 mM were added until the solution turned to red-wine color, indicating a good colloidal dispersion of thioctic acid stabilized GNPs (TA-GNPs) due to negative charges of the carboxylic acids.

4.8. GNPs conjugation with CTPR20-Cys immobilized onto gold surface

The conjugation between TA-GNPs and CTPR20-Cys, previously immobilized onto gold surface by the C-terminal Cys residue, was carried out through the reaction between carboxylic acid functions of the GNPs ligands and the free exposed terminal amine of the protein. First, 9 μL of ethyl(dimethylaminopropyl) carbodiimide (EDC, 1 mM) and 6 μL of N-hydroxysuccinimide (NHS, 1 mM) were added to 1 mL of TA-GNPs at 30 nM for 10 min to perform the activation of the carboxylic groups. Then, 200 μL of activated TA-GNPs were incubated onto the gold surface covered by CTPR20-Cys during 3 days at room temperature. Controls of the same experiment without adding EDC/NHS coupling agents and the immobilization of TA-GNPs onto protein-free gold surface were also carried out under the same experimental conditions.

After the incubation time, the liquid phase was removed from the gold surface and the surface was washed several times with MilliQ water and dried under nitrogen flux.

4.9. Scanning Electron Microscopy (SEM)

SEM measurements of the surfaces with gold nanoparticles were carried out at low acceleration voltages with an ultra high resolution Scanning Electron Microscope, Auriga Cross Beam FIB-SEM from Carl Zeiss equipped with a Gemini electron beam column and in-lens detector technology, at IMDEA Nanociencia. The samples were deposited on gold substrates and imaged using a 2-3 kV beam acceleration voltages.

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Figures

Fig. 1. **A.** Representation of the crystal structure of CTPR20-Cys protein. **B.** Schematic representation of CTPR20-Cys adsorption onto gold surface through thiol-gold interactions. **C.** Overview of the functionalization process of gold-adsorbed CTPR20-Cys with gold nanoparticles (GNPs) through amide bond formation in the presence of EDC and NHS. **D.** Schematic representation of the GNPs monolayer patterned by the previously adsorbed monolayer of CTPR20-Cys onto gold surface.

Fig. 2. CTPR20-Cys adsorption on gold surface monitored by quartz crystal microbalance. **A.** Adsorption kinetics of CTPR20-Cys onto quartz crystals with gold electrodes at 2.5 μM protein concentration. The frequency changes of the quartz crystal resonator are recorded upon protein injection. The curve was fit to a first-order kinetics equation (solid line), obtaining -73.2 Hz and 0.57 min^{-1} values for $-\Delta F_{\text{max}}$ and k , respectively. **B.** Simultaneous resistance increase recorded by QCM upon CTPR20-Cys protein injection. **C.** Equilibrium surface coverage by QCM. Equilibrium surface coverage of CTPR20-Cys adsorbed on gold surface represented versus the protein concentration and fitted using a Langmuir adsorption isotherm. The calculated value for Γ_s was $15.2 \times 10^{-12} \text{ mol} \cdot \text{cm}^{-2}$ for CTPR20-Cys.

Fig. 3. A. Surface protein coverage by surface plasmon resonance. SPR spectra of bared gold surface (solid line) and CTPR20-Cys deposited on the gold surface (dashed line). The protein spectrum fits with a gold film thickness of 32 ± 2 nm, a protein coverage of approximately $1000 \text{ ng}\cdot\text{cm}^{-2}$. **B.** Height profile of the CTPR20-Cys monolayer deposited on the gold surface acquired by a stylus profilometer. The thickness of the protein layer was $17.93 \pm 1.05 \text{ nm}$.

Fig. 4. AFM image of CTPR20-Cys protein immobilized on gold surfaces. **A.** AFM image of individual CTPR20-Cys molecules deposited on gold surface at 10 nM protein concentration after 1 hour incubation at room temperature. Each bright spot corresponds to a single protein. The right panel shows the height profile of two single protein molecules. Note that the nanometer-scale widths obtained by AFM are not reliable due to the size and shape of the contacting probe. **B.** AFM image of a large area of completely saturated CTPR20-Cys monolayer deposited on gold surface at 2.5 μM protein concentration. The inset shows a zoom-in image in which individual protein units can be observed. The panel below shows the global roughness analysis of the image that showed an RMS roughness of 0.86 nm and an average size of 3.42 nm. **C.** AFM image of the same region as image B after the addition of DTT to the sample in order to remove the CTPR20-Cys. The panel below shows the height profile along the line to show a single protein molecule.

Fig. 5. Viscoelastic properties of deposited protein material measured by QCM. **A.** The change in resistance during the course of protein immobilization and assembly kinetics *vs.* the change in frequency for CTPR20-Cys at 2.5 μ M protein concentration. For CTPR20-Cys two regions are observed. The fit to linear equations with two different slopes are shown. **B.** The change in resistance divided by the change in frequency ($\Delta R/\Delta F$) for different protein concentrations of CTPR20-Cys measured after steady state was reached.

Fig. 6. **A.** SEM image of a large area (30 μ m $\times$ 30 μ m) of gold surface covered by CTPR20 and functionalized with GNPs. **B.** Representative SEM images of GNPs immobilized onto CTPR20 covering gold surface. Two areas in which is clear the close packed GNPs monolayer are displayed. SEM images are in the same scale as the AFM images of the protein monolayer displayed in Fig. 4.