Assembly of histidine-rich protein materials controlled through divalent cations

Hèctor López-Laguna[a,b], Ugutz Unzueta[b,c,d] *, Oscar Conchillo-Solé[a], Alejandro Sánchez-Chardi[e], Mireia Pesarrodona[a,b,c,ʃ], Olivia Cano-Garrido[a,b], Eric Voltà[a,b], Laura Sánchez-García[a,b,c], Naroa Serna[a,b,c], Paolo Saccardo[a,c,f], Ramón Mangues[c,d], Antonio Villaverde[a,b,c, *] and Esther Vázquez[a,b,c, *]

[a]Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain

[b]Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain

[c] CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), C/ Monforte de Lemos 3-5, 28029 Madrid, Spain

[d]Institut d'Investigacions Biomèdiques Sant Pau, Hospital de la Santa Creu i Sant Pau, 08025 Barcelona, Spain

[e]Servei de Microscòpia, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain

[f]Plataforma de Producción de Proteínas, CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN) and Universitat Autònoma de Barcelona, Bellaterra, 08193 Cerdanyola del Vallès, Spain

[ʃ] Present address: Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology, Barcelona 08028, Spain.

[*] Corresponding authors. UU: uunzueta@santpau.cat; AV: antoni.villaverde@uab.es EV: esther.vazquez@uab.es

Abstract

Nanostructured protein materials show exciting biomedical applications, since both structure and function can be genetically programmed. In particular, self-assembling histidine-rich proteins benefit from functional plasticity that allow the generation of protein-only nanoparticles for cell targeted drug delivery. However, the rational development of constructs with improved functions is limited by a poor control of the oligomerization process. By exploring cross-interactions between histidine-tagged building blocks, we have identified a critical architectonic role of divalent cations. The obtained data instruct about how histidine-rich protein materials can be assembled, disassembled and reassembled within the nanoscale through the stoichiometric manipulation of divalent ions, in an biochemical approach to biomaterials design.

Keywords: Protein materials; functional materials; nanoparticles; genetic design; controlled oligomerization

1. Introduction

Advanced protein engineering allows designing polypeptide chains with pre-defined cross-molecular interactivity to construct peptide- and protein-based materials [1-6]. Nanostructured homomeric oligomers with self-assembling properties are potential mimetics of complex protein structures (such as viral capsids) for *in vivo* applications in regenerative medicine [4] and drug delivery [7]. Among the diverse current approaches to construct regular oligomers [4, 5, 8-12], the simple combination of cationic and histidine-tag (His-tag, H6-tag or H6-tail) regions promotes the formation of functional toroidal (cyclic) nanoparticles (NPs) potentially useable for BBB-crossing [13], tumor imaging [14] and cell-targeted drug delivery [15-17]. T22-GFP-H6, a paradigmatic representative of such engineering platform forms robust nanoparticles of around 12 nm in size. These materials are targeted to the tumoral marker CXCR4 both *in vitro* and *in vivo,* offering potential as biocompatible nanoscale vehicles for the delivery of conventional and innovative drugs [14, 18]. Derivatives of this construct that contain cytotoxic protein stretches have been exploited as potent protein-only antitumoral drugs for precision oncotherapies [15-17].

While an unbalanced electrostatic charge distribution clearly contributes to self-assembly of T22-GFP-H6 and related proteins [19], His-His and His-cationic peptide contacts have also been predicted and experimentally demonstrated [14, 20, 21], suggesting His-tags as key architectonic players in the supramolecular architecture of the material. However, the precise mechanisms by which histidine-rich regions participate in the oligomerization process remains obscure, limiting the control over nanoparticle formation. Intervening in His-mediated protein interactions would allow a more rational design of nanostructured protein-based materials through conventional protein engineering. We have explored here the role of histidine residues in the formation of protein materials and we have identified divalent metal and non-metal ions as participants in the process through unanticipated but powerful nanoarchitectonic properties.

2. Materials and methods

2.1 Protein production and purification. Genes encoding for T22-GFP-H6, GFP-H6, T22-GFP and T22-GFP-H6(Loop) proteins were designed in house and provided by Geneart (ThermoFisher) as *Escherichia coli* codon-optimized genes encoded in a pET22b plasmid. All plasmids were transformed and encoding proteins produced in *E. coli* Origami B (BL21, $OmpT^-$, Lon^-, TrxB, Gor^-; Novagen) at 20°C overnight upon induction with 0.1 mM of Isopropyl-β-D-thiogalactopyronaside (IPTG). Bacteria cells were then harvested by centrifugation (15 min, 5.000 g) and resuspended in Wash buffer (20 mM Tris, 500 mM NaCl, 10 mM Imidazole, pH=8) for T22-GFP-H6, T22-GFP-H6(Loop) and GFP-H6; (20 mM Bis-Tris Propane, pH=9.5) for T22-GFP in presence of protease inhibitors (Complete EDTA-Free, Roche) and DNAse (only for T22-GFP, 20 μg/mL, Roche). Cells were disrupted by two rounds at 1200 psi in a French press (Thermo FA-078A) and soluble fraction separated by centrifugation (45 min, 15.000 g). Traces of DNA were eliminated using centrifugal filters (Amicon® Ultra – 15 10K, Millipore) for T22-GFP. T22-GFP-H6, GFP-H6 and T22-GFP-H6(Loop) proteins were then purified by Immobilized Metal Affinity Chromatography (IMAC) using HiTrap Chelating HP 5 mL columns (GE Healthcare) in an ÄKTA pure system (GE Healthcare). Elution was achieved by a linear gradient of Elution buffer (20 mM Tris, 500 mM NaCl, 500 mM Imidazole, pH=8) and eluted proteins were finally dialyzed against sodium carbonate buffer (166 mM $NaCO_3H$, pH=8) for GFP-H6 and sodium carbonate with salt buffer (166 mM $NaCO_3H$, 333 mM NaCl, pH=8) for T22-GFP-H6, T22-GFP-H6(Loop). T22-GFP protein was purified by Ionic Exchange Chromatography (IEC) using HiTrap Q FF 1 mL column (GE Healthcare) in an ÄKTA pure system (GE Healthcare). Protein was eluted by a linear gradient of Elution buffer (20 mM Bis-Tris propane, 1 M NaCl, pH=9.5) and collected protein dialyzed against sodium carbonate with salt buffer. Protein purity was determined by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot immunodetection with anti-GFP monoclonal antibody (Santa Cruz Biotechnology) and integrity assessed by Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry. Final protein concentration was determined by Bradford assay.

2.2 Dynamic Light Scattering. Volume size distribution of all protein versions and their size changes in presence of different chemical agents were determined by Dynamic Light

Scattering (DLS) at 633nm in a Zetasizer Nano ZS (Malvern Instruments Limited). All samples were measured in triplicate.

2.3 Controlled disassembly and re-assembly. T22-GFP-H6 NPs were exposed to different free amino acids (L-Arginine, L-tyrosine, L-histidine and L-tryptophan) at two different molar ratios (1:1 and 1:3) for 1h at room temperature and samples analyzed by DLS in order to test their disassembling capacity. For all experiments in this work 1:1 and 1:3 ratio corresponds to 0.88 mM and 2.64 mM respectively. Alternatively, T22-GFP-H6 NPs were exposed to Ethylenediaminetetraacetic acid (EDTA) at 1:1 molar ratio for 1h at room temperature and samples analyzed by DLS in order to test its disassembling capacity.

On the other hand, EDTA-mediated disassembled T22-GFP-H6 protein samples were exposed to different metallic divalent cations ($NiCl_2$, $CuCl_2$ and $ZnCl_2$) and monovalent cations (CsCl and KCl) at 1:1 ratio, and to non-metallic divalent cations ($CaCl_2$) at 1:2 ratio for 15 min at R.T. Samples were then analyzed by DLS in order to test their re-assembling capacity. Alternatively, disassembled T22-GFP-H6 protein samples were exposed to increasing molar ratios of $NiCl_2$ (1:1, 1.5:1, 2:1, 2.5:1) for 15 min at room temperature and subsequently analyzed by DLS.

Reassembling was also promoted by cell extracts. Briefly, *E. coli* Origami B was grown overnight at 37°C in Lysogeny broth (LB) medium and cells harvested by centrifugation (15 min, 5.000 g). Cells were then resuspended in sodium carbonate with salt buffer, lysed by sonication (3 rounds at 10-15% amplitude for a total time of 5 min, 50 % -on and 50 % -off) and the bacteria soluble fraction (BSF) separated by centrifugation (45 min, 15.000 g). EDTA-mediated disassembled T22-GFP-H6 protein samples were then dialyzed against BSF and resulting samples analyzed by DLS.

2.4 Non-denaturing polyacrylamide gel electrophoresis. Different protein samples were charged and run into an 8% (SDS and reducing agent-free) polyacrylamide gel. Bands were then transferred to a polyvinylidene difluoride (PVDF, Bio-Rad) membrane and proteins immunodetected with an anti-GFP monoclonal antibody (Santa Cruz Biotechnology).

2.5 Ni^{2+} determination. Nickel concentration in T22-GFP-H6 NPs and EDTA-mediated disassembled T22-GFP-H6 protein samples was estimated by Inductively Coupled Plasma Mass Spectrometry (ICP-MS), model 7500ce (Agilent Technologies). For that, samples were

previously exposed to 65% suprapure nitric acid (HNO_3, Merck) in a 1:1 volume ratio and heated at 75°C for 2h in a heating block (DINKO D65). Disassembled T22-GFP-H6 samples had been previously dialyzed against sodium carbonate with salt buffer in order to remove EDTA-divalent cation complexes from the sample. All samples were analyzed in triplicate.

2.6 Molecular modelling. T22-GFP-H6 NPs were modelled as described elsewhere [20] and histidine surface accessibility calculated by Naccess2 software [22]. Those calculations were performed for His residues presented in the C-terminal tail of a previously resulted docking cluster [20] described as P1. Histidines 590 and 592 and Ni^{2+} 601, pdb code 1Q3I, presented elsewhwere [23] and structure (Crystal Structure of Na, K-ATPase N-domain from Eurasian wild boar Sus scrofa) were used as a model of interaction between His and Ni^{2+} since their structure fits with previously described results [24]. Only the atoms of the main chain were superposed over the C-terminal histidines of the P1 model by using Profit5 software [25] based on McLachlan algorithm [26], since the side chains were expected to be very mobile.

2. 7 Ultrastructural morphometry of NPs, Ni^{2+} localization and fluorescence detection. Morphometry, Ni^{2+} localization and fluorescence detection at the nanoscale were determined by Field Emission Scanning Electron Microscopy (FESEM). The analysed samples were T22-GFP-H6 NPs, EDTA-mediated disassembled T22-GFP-H6 protein subsequently dialysed in sodium carbonate with salt buffer, and sodium carbonate with salt buffer as negative control. Drops of 5 µl of each sample were deposited in carbon-coated gold grids (200 mesh) during 1 min, blotted, air dried and observed in a FESEM Zeiss Merlin (Zeiss) operating at 2 kV. Samples were then randomly checked with an *in-lens* secondary electron detector for morphology and with a Back-scattered Electron (BSE) detector for Ni^{2+} localization and fluorescence detection. Representative images were obtained at a wide range of high magnifications (from 200,000x to 500,000x).

2.8 Statistical analysis. Quantitative data are expressed as Standard Error of the Mean ($\overline{x}$ ± SEM). Comparison of Ni^{2+} concentrations was made with Friedman test and pairwise comparisons were made with Wilcoxon signed rank tests. The rest of pairwise comparisons were performed with Mann–Whitney *U*-tests. Significance was accepted at $p<0.05$ and Bonferroni correction was applied for sequential comparisons. All statistical analyses were performed with SPSS v. 18 for Windows.

3. Results

We have here investigated the involvement of His residues and cation-pi contacts in the oligomerization of the modular protein T22-GFP-H6 (Figure 1A), that self-assembles as fluorescent, 11.7 nm NPs (Figure 1 B,C,D) already at the minimal protein concentration detectable by DLS, namely 0.1mg/ml. T22-GFP-H6 and related proteins organize in form of ring-shaped oligomers (Figure 1 C) as determined by both FESEM and TEM imaging [14, 27], with morphometries that fit with *in silico* protein models ([20], Figure 1D). The presence of both the cationic peptide T22 and the H6-tail is necessary for assembly, as GFP-H6 and T22-GFP solely occur as unassembled building blocks (Figure 1 A,B). To evaluate if cation-pi interactions sustain the oligomeric form of the protein through contacts between T22 and H6 tags, we attempted a controlled disassembling of the NPs through the addition of soluble L-histidine as a competitor. At the stoichiometric molar ratio of 1:1 (corresponding to 0.88 mM) and 3:1 (corresponding to 2.64 mM), the free amino acid promoted the reduction of T22-GFP-H6 NP size to 7.5 and 6.5 nm respectively (Figure 2 A). The His-mediated disassembling was fully compatible with the H6-tag involved in cross-molecular interactions between building blocks, through the imidazole ring. To assess this hypothesis, we tried to disassemble the material with other aromatic amino acids, namely L-tryptophan and L-tyrosine, with negative results (Figure 2 A). This observation weakened the hypothesis of cation-pi interactions. In this line, free L-arginine was also unable to alter the structure of the NPs. This indicated that cationic amino acids, important to promote oligomerization, are not the unique supporters of the stability of the assembled material.

Metals remaining from the producing bacteria or residual from the Nickel II (Ni^{2+})-based chromatography might be involved in protein assembly, as in the case of amyloid fibers that interact with divalent metals [28-30]. In this regard, addition of EDTA indeed promoted efficient disassembling of the NPs (Figure 2 B). In a step further, free Ni^{2+} as well as Cu^{2+} and Zn^{2+}, but not Cs^{+} and K^{+}, recovered the original NP size when added to the solution of disassembled BBs (Figure 2 C). Note that K^{+} is unable to promote reassembly when added at the same concentration that divalent cations but also at the same ionic strength (Figure 2 C, blue bar). Interestingly, increasing molar ratios of Ni^{2+} added to EDTA-disrupted NPs promoted the occurrence of reassembled materials with increasing sizes (χ^2=15,000, p<0.0001), from 10.1 to 15.6 nm (Figure 3A). This fact confirmed an active role of Ni^{2+} in NP

formation but also in the nanoscale organization of the protein material. Furthermore, the simple use and versatility of Ni^{2+} as a structural NP modulator was fully demonstrated by the consecutive removal and addition of the cation to and from the same sample of T22-GFP-H6 (Figure 3B).

Since Ni^{2+} or other divalent cations appeared as natural molecular connectors of protein building blocks, we were interested in knowing if T22-GFP-H6 NPs might be formed already in the cytoplasm of producing *E. coli* bacterial cells. Indeed, we identified a supramolecular form of T22-GFP-H6 protein in cell extracts, by immunodetecting a slow migrating protein version in non-denaturing gels (Figure 4A). In the same context, bacterial crude cell extracts were able to increase the hydrodynamic size of disassembled T22-GFP-H6 protein upon *in vitro* incubation (Figure 4B). Finally, Ni^{2+} was analytically detected at the same stoichiometric order of magnitude (in a molar basis) for protein and ion, in samples of pure T22-GFP-H6 NPs but not in T22-GFP-H6 proteins previously treated with EDTA and further dialyzed (Figure 4C). This fact was also confirmed by direct visualization of higher backscattered electron density (BSE). The BSE imaging only showed white-light areas where NPs occurred, reporting a correlation between protein particles and the localization of high concentrations of heavy atoms (Figure 4C, bottom), compatible with the accumulation of Ni^{2+} in the nanoconstructs. In this regard, all these data indicated that an assembled version of T22-GFP-H6 does occur in the bacterial cytosol and that the soluble cell fraction contains sufficient divalent cations to ensure the *in vitro* assembling of the nanomaterial.

In this regard, we presumed that the metal detected in the oligomers could be coordinated with His residues, in the H6 overhanging tails oriented towards the geometrical centre of the structure and acting as a 'molecular glue' to sustain the architecture of the NP. This was modelled over the result of the previously done docking [20], by selecting all generated models belonging to the same cluster. In all these models, the surface accessibility of His residues of the C-terminal tail was calculated using the Nacces2 software. Data expressed as mean and the standard deviation were calculated to take into account the result for all the models. It was observed that the sum of the mean plus the standard deviation resulted in a surface accessibility greater than 50 %, for all of them. Subsequently, His 590 and 592 and Ni^{2+} 601 of structure 1Q3I3 were selected as the His-Ni^{2+} interaction model and were superimposed by the Profit5 software. Only the atoms of the main chain were used for the

superposition since the side chains, being so exposed to surface, were expected to be very mobile. This superposition generated an RMSD of 0.61 ±0.29 Å, revealing that the two His residues of the His-Ni^{2+} interaction model fit perfectly on the His residues of the particle model (Figure 5 A,B). In a further approach to experimentally validate the model we argued that His residues from the H6-tail could be unavailable for cross-molecular contacts if not oriented towards the core of the NP, where metal appears to coordinate with the proteins. In this line, we generated another protein, T22-GFP-H6(Loop) (Figure 5C), in which H6-tail was not accommodated in the C-terminus of GFP but in one of the exposed loops of GFP, that is highly permissive to peptide insertions [31, 32]. As expected, T22-GFP-H6(Loop) was unable to self-assemble as NPs but it remained in a protomeric state (Figure 5C), like T22-GFP or GFP-H6 (Figure 1B).

4. Discussion

The potential application of divalent ions in the assembling of protein-only or protein-containing materials has been largely documented [33-35]. Among others, these architectonic principles have been applied to the construction of nanoscale materials for the delivery of drugs [1, 3, 6, 10, 36], some of them in virus-inspired formats [7, 37-39]. However, the particular role of Ni^{2+} and His residues (both critical players in the purification of recombinant proteins [40]) has not been particularly addressed. Ni^{2+} had been early discussed as an important conformational arranger in biological systems (e.g., in urease) [41], as well as Zn^{2+} and Cu^{2+} that are present in carbonic anhydrase and copper B proteins [42] respectively. In fact, protein-divalent cation complexes involving Ni^{2+} and other metals have been widely described with a plethora of both catalytic and structural functions [43-48]. The configuration of electronic d orbitals in transition metals allows them to be considerable good Lewis acids for further interaction with biological molecules. Not only the d orbitals but also its geometry determines their number of possible interactions, namely an octahedral geometry for Ni^{2+} and tetrahedral for Cu^{2+} and Zn^{2+}. Those metals are able to interact with the nucleophilic unpaired electrons presented in the δ1-Nitrogen of histidine imidazole ring [49], being permanently and strongly held into the structure and thus supporting the mechanics of the His-tag based single-step protein purification. We have demonstrated here for the first time how the manipulation of Ni^{2+} and other metal and non-

metal divalent cations allows the controlled reassembling of a family of protein-only NPs (Figure 1 C,D) with high biomedical interest in protein drug delivery [50]. The hosted histidine-rich domains participate in the oligomerization process through the coordination with ions remaining from the bacterial cell factories or upon enrichment during Ni^{2+}-based chromatographic purification (Figure 4 A). Then, NPs, once purified, can be disassembled by the removal or addition of divalent ions, in a stoichiometric proportion (Figure 2 and Figure 3). In this regard, keeping these NPs at a nearly physiological pH avoids δ1-Nitrogen protonation and allow divalent cation coordination, enabling robust NP formation that ensures the *in vivo* stability of the material as previously shown [14]. The capability of that Ni^{2+}, Cu^{2+} and Zn^{2+} to coordinate more than 2 His residues simultaneously [41] makes them acting as a molecular glue at the nanoscale, fully removable by easy biochemical methods such as EDTA addition. This simple approach would allow not only fine adjustments of the NP size as demonstrated here (Figure 3), but also the easy construction of hybrid materials through the assembling of heterogeneous BBs, that might allow a functional recruitment in single nanoparticles. Apart from the emergently discovered roles of metals in amyloid formation, protein aggregation and neurodegenerative diseases [51, 52], divalent cations appear now as a powerful and simple biochemical tool to refine and manipulate, in a controlled way, architectonic properties of self-assembling nanoscale protein materials, under the umbrella of a novel and potent biochemistry-assisted nanobiotechnological platform.

5. Conclusion

The stoichiometric manipulation of divalent metals, including of Ni^{2+}, Cu^{2+} and Zn^{2+}, allows an unexpected and precise control of the assembling, disassembling and reassembling of histidine-rich recombinant proteins as functional nanoparticles. Limited by a poor control of the oligomerization process, the development of histidine-rich protein materials has not fully developed, despite the enormous potential of these entities in biomedicine, especially as intrinsically functional, biocompatible and self-delivered protein drugs.

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