Protein–Protein Interactions

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Supramolecular Protein Assemblies in the Nucleus of Human Cells**

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Abstract: *Genetically encoded supramolecular protein assemblies (SMPAs) are induced to form in living cells by combination of distinct self-assembly properties. A single fusion construct contains genes encoding the heavy chain (H) of human ferritin and the citrine fluorescent protein, the latter exposing a weak dimerization interface, as well as a nuclear localization signal. Upon expression in HeLa cells, in vivo confocal fluorescence and differential interference contrast imaging revealed extended SMPA structures exclusively in the nuclei. Assemblies were typically round and took alveolar, shell-like, or hybrid structure. Transmission electron microscopy revealed a crystalline packing. Site-specific mutagenesis of the citrine dimerization interface clarified the mechanism of SMPA formation. The constituent proteins retained their activity in iron binding and fluorescence emission, thus suggesting a general strategy for formation of synthetic cellular bodies with specific biochemical function.*

A variety of bioinspired oligo-to-multimeric protein structures, also called supramolecular protein assemblies (SMPAs),[1] such as cages, rings, tubes, and sheets/arrays have been produced in vitro.[2,3] Various strategies have been exploited to create suitable conditions for SMPA formation,[4] including genetic fusion of proteins endowed with oligomerization properties,[5,6] binding of small chemical molecules,[7] mimicry of phage assembly,[8] and metal-mediated assembly.[9] Recently, efforts have been made to create synthetic SMPAs in vivo.[10]

We choose a genetic fusion approach to express SMPAs directly in living cells. The system was built by fusing genes encoding the heavy chain (H) of the human ferritin (HuFtH),[11] with citrine,[12] a yellow variant of the green fluorescent protein (GFP) from *Aequorea victoria*. Both proteins are endowed with self-assembly properties that are expected to be combined aiming at the formation of an extended network of supramolecular interactions (Figure 1).

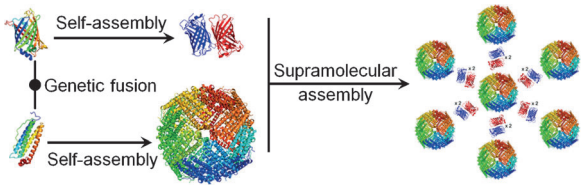

Figure 1. Design of a supramolecular assembly system. Citrine and H subunits of human ferritin were genetically fused. Upon self-assembly into the ferritin cage structure, dimerization at the citrine interface is expected to mediate contacts to form a 3D extended network of interactions.

The fluorescence of citrine provides a means for visualization of the structures by microscopy as well as a probe of correct protein folding. Additionally, we encoded a nuclear localization signal (NLS) for intracellular targeting.

Construction of the gene fusion was accomplished in steps by restriction-free PCR cloning.[13] A ferritin-specific NLS was first identified by direct alignment of the primary sequence of HuFtH with that of chicken *Gallus gallus* ferritoid, a tissue-specific nuclear transport protein[14] (Figure S1 in the Supporting Information). The N-terminal mismatching AEPRSKRPRVTLPACPAH sequence, herein called FtNLS, resembles a nuclear localization signal[15] and was initially fused to the N terminus of the HuFtH gene; the citrine gene was inserted in a second cloning. A 17 amino acid spacer (LK) was finally cloned in between the citrine and ferritin genes to permit independent folding of the two proteins (Figure S1B). The construct was finally cloned into the pcDNA3.1 mammalian constitutive expression vector (Invitrogen).

To assay the SMPA formation in vivo, human cervical cancer cells, HeLa, were transfected with the FtNLS-citrine-LK-HuFtH construct in pcDNA3.1 using Lipofectamine 2000 (Invitrogen). Protein expression was monitored after 24 h by fluorescence and differential interference contrast (DIC) imaging using an Olympus Fluoview 300 confocal scanning microscope equipped with a PlanApo 60×/N.A. 1.40 oil immersion objective (Figure 2). Intensely fluorescent spheres were observed in the nuclei of positively transfected HeLa cells (Figure 2A,D,G). Dense structures were visible at corresponding positions in the DIC images (Figure 2B,E,H); these structures will be referred to herein as citrine-HuFtH SMPAs. Preservation of the citrine fluorescence indicated that native folding was maintained during the expression of the fusion construct, and furthermore that the citrine structure suffered no significant distortions. The diameter of the citrine-HuFtH SMPAs ranged from a few hundred nanometers up to more than two micrometers. While similar overall, they varied from cell to cell by dimension, organization of mass, number, and spatial distribution (Figure 2).

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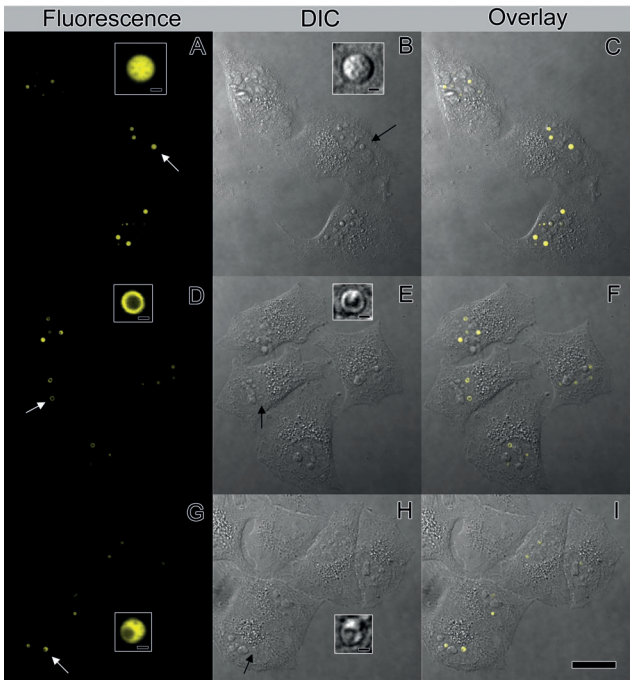

Figure 2. Confocal scanning fluorescence microscopy images of citrine-HuFtH SMPAs. Confocal fluorescence images (A, D, G), DIC images (B, E, H), and overlays (C, F, I) of citrine-HuFtH SMPAs. Several distinct organizations of citrine-HuFtH SMPAs, namely: alveolar organization (A–C), hollow shell-like organization (D–F) and hybrid organization (G–I). Insets: digital zoom of individual citrine-HuFtH SMPAs. Scale bar is 20 μm in the images and 500 nm in the insets.

Structures included: 1) an alveolar organization in which a main dense mass was interrupted by the presence of several small empty spaces (Figure 2A, B, insets); 2) a hollow shell-like organization with a single central void (Figure 2D, E, insets); 3) an hybrid organization containing both dense alveolar and hollow shell-like components (Figure 2G, H, insets).

The dimensions of the SMPAs greatly exceed the diameter of the nuclear pores. Therefore the nuclear import of FtNLS-citrine-LK-HuFtH subunits must occur before their organization into the SMPA structure. The nuclear transport must also be mediated by an active receptor-mediated mechanism addressing the FtNLS, since in its absence only cytoplasmic SMPAs are formed (Figure S2A–C).

Transmission electron microscopy (TEM) was used to obtain high-resolution structural information on the citrine-HuFtH SMPAs. HeLa cells were fixed and embedded for electron microscopy. Thin sections were imaged by using a FEI Tecnai 12 Spirit microscope. Citrine-HuFtH SMPAs were identified in the TEM images as a dark mass compared to the chromatin stain in the nucleus (Figure 3A–C). The shape of the SMPAs appeared regular and the borders well-defined, thus indicating a high degree of organization. We observed no signature of membranes surrounding the SMPAs. High-magnification images resembling those observed in the fluorescence and DIC modes were acquired for comparison (Figure 3D–F); the similarity across different imaging techniques was striking and all organizations previously observed,

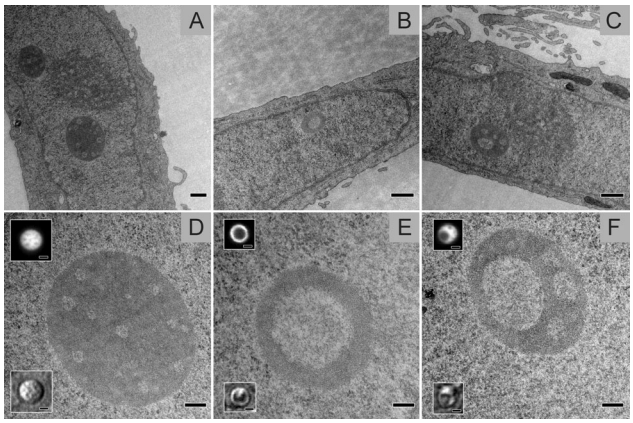

Figure 3. TEM images of the citrine-HuFtH SMPAs. TEM images of thin sections of HeLa cells embedded in epon resin showing SMPAs at low magnification in the nucleus (A–C) and at higher magnification (D–F) in comparison with the images of similar structures acquired by fluorescence and DIC microscopy (upper and lower insets, respectively). Note the presence of small structures within the voids, indicating the growth or decay of the SMPAs within a saturated protein environment. Scale bars are 500 nm in the low-magnification images (A–C), 200 nm in the high-magnification images (D–F), and 500 nm in the insets.

namely alveolar (Figure 3D, insets), hollow shell-like (Figure 3E, insets), and hybrid organization (Figure 3F, insets), were found in the TEM images as well. Diameters previously estimated from the confocal images also matched well with observations by TEM. Internal diameters were found in the range of 100–500 nm, and significantly, small SMPAs could often be seen within the void (Figure 3). This suggests growth by near-equilibrium exchange with a saturated solution of the protein components.

An ordered crystalline pattern of the citrine-HuFtH SMPAs was identified at higher magnification (Figure 4A). Application of the Fourier transform indicated a hexagonal packing of the citrine-HuFtH units, characterized by a repetitive spacing of (12.1 ± 1.4) nm (Figure 4B): this value matches the outer diameter of the quaternary structure of native ferritin (ferritin cages, consisting of 24 subunits; Figure 1D), 12 nm, determined by X-ray crystallography.[11] The added mass of citrine could be partly accommodated in the empty spaces between ferritin cages; some expansion is

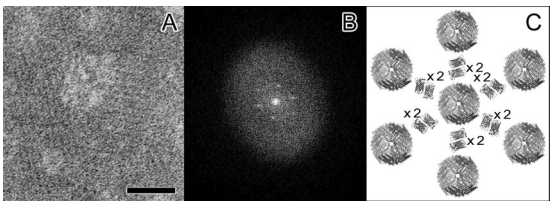

Figure 4. Ordered arrays of the citrine-HuFtH SMPAs. TEM images show a crystalline pattern (A). Scale bar is 100 nm. Graphical display of the fast Fourier transform (power spectrum) applied to the image, showing a hexagonal crystalline pattern (B). Two-dimensional representation of the organization of the ferritin cages and the contacts mediated by the dimeric interface of citrine in the hexagonal packing (C).

likely compensated by shrinkage in the embedding plastic. Hexagonal packing of native ferritin cages in ordered structures was observed in human and animal tissue sections under pathologic iron overload conditions[16] and obtained in vitro on two-dimensional arrays in material science applications.[17,18] To our knowledge this is the first report of three-dimensional crystalline packing of ferritin cages generated synthetically in vivo.

The driving force for organization of citrine-HuFtH SMPAs into a 3D hexagonal lattice should lie in the citrine dimerization, enhanced by the internal symmetry of the ferritin-cage structure. Each natively assembled ferritin cage bears 24 oriented citrine molecules at its surface, with each available for antiparallel interaction with citrine molecules belonging to neighboring ferritin cages (Figure 4C). Since hexagonal packing of spheres entails 12 nearest neighbors in 3D, two citrine molecules can form two contacts with one of the surrounding ferritin cages ("double arm" contacts, Figure 4C).

According to this model, disruption of the citrine dimerization interface should prevent the SMPA formation. A conserved patch of hydrophobic residues (Ala206, Leu221, Phe223) and a set of hydrophilic contacts contribute to the stabilization of the dimeric interface. Replacement of alanine 206 by a positively charged lysine residue has been reported to disrupt the hydrophobic patch in GFP, thereby significantly reducing its self-association constant.[19] Thus the expression of the site-specific fusion variant FtNLS-citrineA206K-LK-HuFtH was assayed in HeLa cells. Diffuse fluorescence was observed within the nuclei, with exclusion from nucleoli. No distinct fluorescent spots and no structures were visible in the DIC images (Figure 5). In the absence of the FtNLS signal,

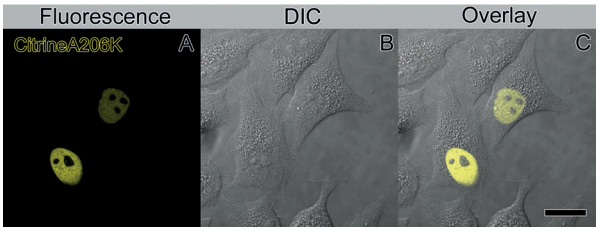

Figure 5. Confocal scanning fluorescence microscopy images of other fluorescent proteins fused to the human H ferritin. Confocal fluorescence images (A), DIC images (B), and overlay images (C) of the site-specific variant of the fusion construct FtNLS-citrineA206K-LK-FtH. Scale bar is 20 μm.

diffuse fluorescence was visible predominantly in the cytoplasm (Figure S3D–F). The dramatic difference compared to constructs involving unmodified citrine (Figure 3) underlines the primary involvement of the dimerization interface in formation of citrine-HuFtH SMPAs.

According to the assembly model based on association between fully folded proteins, the functional properties of ferritin are expected to be preserved similarly to the fluorescent properties of citrine. The main physiological role of ferritins is to bind iron, oxidize it, and incorporate it into the internal cavity.[20] Thus, the ability of the citrine-HuFtH SMPAs to accumulate iron was assayed histochemically. HeLa cells expressing the FtNLS-citrine-LK-HuFtH fusion construct were chemically fixed and permeabilized before incubation in a 100 μM Fe^{II} solution to allow iron binding and oxidation. Cells were then stained using the dye Prussian blue (potassium ferricyanide) that confers a blue/green color specifically to iron-rich cellular structures. Although fluorescence was partly quenched under the iron-loading conditions, citrine-HuFtH SMPAs could still be easily identified in the confocal fluorescence (Figure 6A) and transmitted-light DIC

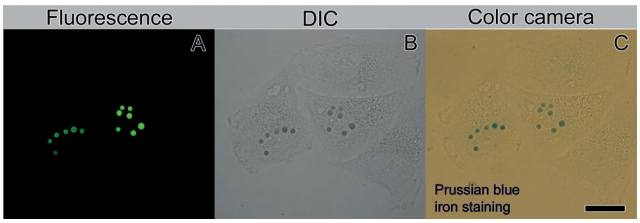

Figure 6. Histological staining for iron. Confocal fluorescence (A), DIC (B), and color camera (C) images of HeLa cells expressing citrine-HuFtH SMPAs, after chemical fixation, iron loading, and staining with Prussian blue. Scale bar is 20 μm.

(Figure 6B) images. The corresponding bright-field transmission image showed an intense and specifically localized blue/green color (Figure 6C), thereby proving unequivocally that the iron binding properties of ferritin were preserved after the formation of citrine-HuFtH SMPAs.

In conclusion, SMPAs were produced ex novo in living cells through the combined self-assembly properties of two proteins expressed as a fusion construct. Their structure, mechanism of formation, and functional properties have been elucidated. Subcellular compartmentalization of the SMPAs through the use of a genetically fused localization signal was demonstrated. The importance of polyvalent presentation of weak dimerization interfaces in generating extended three-dimensional structures has been underlined through the use of the 24-mer ferritin cage. The potential for use of fluorescent proteins as fusion partners, providing at once a dimerizing interface, a tool for in vivo imaging, and an intrinsic probe of protein folding have also been shown. Altogether the results represent a crossover between biological chemistry and cell biology for material engineering within living cells.

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