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3D Directed Self-Assembly of Peptide Nanowires into Micron-Sized Crystalline Cubes with Nanoparticle Joints**

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In the last decade, nanoscale materials have been created with superior physical properties. The next grand challenge is to assemble nano-building blocks into complex and larger-scale superstructures so that these nanomaterials with unique properties can be integrated as macroscale components in solar cells, microelectronics, metamaterials, catalysis, and sensors.^[1] Recent trends in the complexity of device design demand the fabrication of three-dimensional (3D) superstructures from multi-nanomaterial components in precise configurations.^[2] Biomimetic assembly is an emerging methodology in such pursuits since living organisms are efficient, inexpensive, and environmentally benign material factories allowing low temperature fabrication.^[3-7] While DNA bionanotechnology has recently been used to precisely assemble 3D shapes,^[8-16] the methodology to develop highly ordered macroscopic materials from these nanostructures remains limited^[17] and for practical applications the production scale, size, and the yield of the assembled materials need to be amplified.^[18, 19] Peptides are another of nature's building blocks with high specificity, versatility, and robustness for directed assembly that can be exploited to design novel 3D

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superstructures.^[20] Here we applied the directed self-assembly of peptides and ligand-functionalized Au nanoparticle (NP) joints for the development of the micron-scale 3D cube-shaped hybrid superlattices, creating a physical framework for the proposed biomimetic assembly strategy. In this approach, we took advantage of the naturally robust assembling nature of collagen triple helix peptides and used them as nanowire building blocks for the 3D peptide-NP superlattice generation. Assembling streptavidin-functionalized Au NPs and recombinant peptides modeling a segment of the $\alpha 1$ chain of type I collagen that is specifically modified to be biotinylated at the N-terminus *in vivo*, we created micron-sized cubes with peptide nanowires as frames and Au NPs as joints. These collagen-mimicking peptides can self-associate laterally,^[21, 22] and this intrinsic peptide-peptide interaction allows us to create unit cells of various scales as well as predicts the ability to create an extensive latticework of cubic microcrystals. The NPs can join the peptide nanowires through the streptavidin-biotin interaction to create cubic unit cells with extremely high yield and the resulting peptide-NP superlattices are ordered in the long range with the isotropic crystalline orientation. This simple and rapid fabrication protocol produces high yields of 3D materials in tailored shapes, dependent on the geometry of the peptide-NP unit cells, promising ease and flexibility in manufacturing functional devices. To our knowledge this is the first example of 3D NP superlattice assembly in micron-sizes from peptide nanowires.

The collagen-peptide is derived from the F877 peptide that was previously used as a nanowire template.^[23, 24] This new BAP877 peptide is modified by the insertion of a 15 amino acid residue of Biotin Acceptor Peptide (BAP) at the N-terminus (Fig. 1-a). Other design features of this collagen-peptide include a triple helix domain consisting of 63 residues from the $\alpha 1$ chain of type I collagen with additional repeating Gly-Pro-Pro sequences for increased stability, a bacteriophage T4 fibrin foldon domain at the C-terminus serving as a nucleation site to facilitate the correct folding, and a Cys-knot sequence (Gly-Pro-Cys-Cys) to cross-link three polypeptide chains of the triple helix through a set of disulfide bonds (Fig. 1-a).^[23, 24] The newly inserted BAP site is designed to be biotinylated *in vivo* to generate the complementary binding motif for streptavidin-coated Au NPs. To accomplish this modification, BAP877 peptide was expressed as a fusion protein in *E. coli* where endogenous bacterial biotin ligase BirA, biotinylates the specific lysine residue within the BAP sequence.^[25, 26] As seen in Fig. 1-b, the purified biotinylated triple helix peptide was monodisperse in size, 4×40 nm, (see supplementary information for size distribution) and adapted to rigid conformation with no sign of bending.

In the proposed 3D peptide-NP superlattice assembly scheme shown in Fig. 1-c, the biotin-streptavidin interaction joins the ends of triple helix peptides to the Au NPs to form a unit cell for the cube microcrystal. The design of this triple helix peptide mimics the staggered lateral association of natural collagen to scale these unit cells for the assembly of larger cubic crystals. As Au NPs with streptavidin on the surface bind the biotinylated triple helix peptides, we anticipate the size ratio between the triple helix peptide and the Au NP is important to define the shape of the unit cell of the peptide nanowires (Fig. 1-c). When 10 nm diameter Au NPs decorated with six streptavidin molecules were incubated with the biotinylated triple helix peptide in solution for 1 hr, the peptides were assembled in cubic structure as seen in Fig. 2-a. The yield of $1 - 2 \mu\text{m}$ cubes was extremely high with respect to uniformity in shape and dimension of the crystals as observed in this TEM image. Repeating this assembly with a concomitant 10-fold reduction of both the peptide and Au NP concentrations reduced the size of the cubes on the order of $100 - 200$ nm (see supplementary information). When the TEM grid of the sample was tilted, the edges of the cubes become visible, indicating that these cubic assemblies are three-dimensional (inset of Fig. 2-a). A circular dichroism (CD) spectrum of these cubic superlattices indicates that the genetically-engineered peptides maintain the triple helical conformation with a small positive

peak at ~225nm and a deep negative peak at ~ 197nm (see supplementary information).^[24] Fig. 2-b shows the small-angle X-ray scattering (SAXS) pattern of the cubes measured at 30° C with diffraction peaks located at $q = 0.06, 0.11$ and $0.18 \text{ \AA}^{-1}$ respectively. The q_x/q_1 ratios of these peaks are $1 : 2^{1/2} : 5^{1/2}$, matching the characteristic diffraction pattern of body-centered cubic (b.c.c) structure (see supplementary information). On the basis of this SAXS spectrum, the interparticle distance of Au NPs in the diagonal direction of the b.c.c. unit cell is 7 nm. A characteristic sharp increase in the scattering at small q in the SAXS profile indicates the long range order of Au NPs in the cubes.²⁷ To further confirm the arrangement of Au NPs in the cubic peptide-NP superlattices, we also imaged these cubes by polarized light microscopy. The contrast for the cubic crystals was diminished with cross-polarized light (see supplementary information), supporting that the arrangement of Au NPs in the cube is isometric in the long range. In Fig. 3-a, high resolution TEM (HR-TEM) of the peptide cube reveals the array of Au NPs with their lattice fringes and the crystalline faces of Au NPs are oriented in the isotropic direction. In this image, the periodic alignment of Au NPs and the peptide frame are visible when the peptides were stained by ammonium molybdate to increase the contrast of the peptide lattice in the cube. In Fig. 3-b, an electron diffraction of this cube matches the diffraction pattern of the single crystalline Au indicating that the crystalline orientation of Au NPs in the peptide frame is aligned in an isotropic direction as observed in the HR-TEM image in Fig. 3-a. It should be noted that the same streptavidin-functionalized Au NPs mixed with non-biotinylated triple helix peptides did not produce any peptide-NP aggregations (data not shown), demonstrating the crucial role of the biotin-streptavidin interaction for the assembly of the cube-shaped microcrystals. Previously, mesocrystals of calcium carbonate were observed to undergo the oriented assembly to form single crystals by dipolar interactions with polymer adsorption,^[5] and the NP alignment in an isotropic crystallographic direction in this peptide-Au NP cube could also be originated from the dipole interaction of peptides and NPs.^[27]

Since collagen fibrils are known to be disassembled into triple helix peptides in an acidic environment,^[21] it follows that cubic peptide-Au NP superlattice crystals should also be dismantled at lower pH. When the solution containing peptide-NP cubes was incubated overnight at a pH slightly lower than neutral, these peptide frames were slowly disassembled into smaller domains of the micro-cube, whose sizes are in the range of 20 nm – 80 nm, as shown in Fig. 3-c. When pH of the solution with the cubes was decreased to 5.5, the micron-scale cubes were further disassembled into individual peptides and then these peptides followed their inherent reassembling process. After reassembly into the wire structures, these peptide wires were aligned side-by-side to form peptide sheets, resembling natural collagen assembly (see supplementary information).

To further test our hypothesis that the shape of the peptide-Au NP assembly can be changed by altering the geometry of unit cell, larger 30 nm diameter Au NPs were assembled with the same triple helix peptides assuming that the size ratio between the diameter of Au NPs and the length of peptide nanowires can change the shape of unit cell. When the size of Au NPs was comparable to the length of the peptide nanowires, these Au NPs and peptides were assembled into micron-sized hexagons (Fig. 3-d). This result indicates that the size ratio indeed plays an important role in the assembly of the structure. It is plausible that the larger size of Au NPs changes the interaction and the structure of peptide-NP unit cells and the difference in the peptide-NP angle is responsible for the resulting shape change as these unit cells are assembled in a micro-scale.

In summary, this report introduces a novel peptide-directed nanomaterial assembly technology for the construction of macroscale multi-component materials that still retain superior nanoscale domains and properties (i.e., technology to bridge between nano- and macro-assemblies). By applying this methodology, nanomaterials can be assembled with

peptides in the ordered dipole orientation and long periodicity resulting in 3D peptide-inorganic superlattices in defined 3D shape. The unique features of peptides' molecular recognition and large-scale 3D self-assembling nature enable to create such multi-component 3D materials in precise designs and high yields, difficult to obtain by other templates such as DNAs and polymers. The type of 3D peptide superlattice assembly with hybrid NP building blocks we have described here shows potential to impact the future fabrication of complex 3D functional device building blocks which demands precise arrangement and periodicity of NPs in a long range. This programmable recognition-based assembly technology provides the flexibility to modify the NP arrangement and the final crystalline structure by altering the size ratio between triple helix peptides and NPs and/or the number of ligands on NPs, as observed in the DNA-Au NP assembly.^[10] It is also expected that more systematic investigation of the robust large-scale assembly nature of peptides on 3D microcrystal structures will provide further insight into the design and fabrication of novel 3D crystals in practical sizes.

Experimental Section

Expression and Purification of Recombinant Protein

The recombinant collagen-like fragment BAP 877 peptide was generated using the original F877 bacteria expression construct^[24] with the insertion of a 15 amino acid sequence of Biotin Acceptor Peptide (BAP)^[25, 26, 28] at the N-terminus (Figure S-1). The insertion of the 15 amino acids of Biotin Acceptor Peptide (BAP) is achieved by PCR using the PrimeSTAR HS DNA Polymerase (Takara Bio Inc) with the pET32a-877 (foldon) plasmid as the template and the following forward and reverse primers described in supplementary information. The fusion protein was sub-cloned into a pCR4 blunt TOPO (Invitrogen, Carlsbad, CA) plasmid and the sequence was verified by DNA sequencing (ABI PRISM 3100-Avant Genetic Analyzer, Applied and Biosystems, Carlsbad, CA). The constructed plasmid was transformed into chemically competent *E. coli* BL21 (DE3). The protein in the supernatant was purified using nickel affinity chromatography, and the final protein was purified by RP-HPLC (Beckman Coulter) with C18 column (Vydac) with a 20-70% acetonitrile –water with 0.1 % TFA. To confirm in vivo biotinylation of fusion proteins Western blots were performed.

The self-assembly of streptavidin–gold conjugate with biotinylated BAP877 peptide

Gold nanoparticles with bound streptavidin were purchased from Nanocs (New York, NY). The diameter of the gold nanoparticles is 10 nm. The density of streptavidin is 4 to 6 per Au NP. The conjugated nanoparticle solution was diluted with 0.1mM HEPES buffer (pH 7) in 1:10 ratio. After the diluted streptavidin-gold conjugate was allowed to equilibrate for 30 min at room temperature, biotin-conjugated BAP877 peptide (10 mg/ml) was incubated in the molar ratio of 1:2 for 1 hr.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Structure of engineered triple helix peptide with biotin at the N-terminus. (b) TEM image of biotinylated triple helix peptides. Scale bar = 50 nm. (c) Proposed 3D superlattice structure of the biotinylated triple helix peptides and streptavidin-functionalized Au NPs assembled by the streptavidin-biotin interaction and lateral peptide-peptide association through the collagen motif.

Figure 2.

(a) TEM image of the cubes assembled from the biotinylated triple helix peptides and streptavidin-functionalized Au NPs. Scale bar = 20 μm . Inset: A tilted TEM image of the cubes in (a). Scale bar = 1 μm . (b) SAXS pattern of these cubes. Scale bar (inset) = 2 μm .

Figure 3.

(a) High resolution TEM image of the cube assembled from biotinylated triple helix peptides and Au NPs. Scale bar = 30 nm. (b) An electron diffraction of the cube. (c) TEM image of the cubes after the peptide frames were disassembled by lowering the pH. Scale bar = 100 nm. (d) TEM image of the micron-sized hexagons assembled from larger Au NPs (diameter = 30 nm) and the same triple helix peptide nanowires (length = 40 nm). Scale bar = 1 μ m.