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Biomimetic Fabrication of Genetically-Engineered Collagen Peptide-Assembled Freestanding Films Reinforced by Quantum Dot Joints

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Abstract

Genetically-engineered collagen peptides were assembled into freestanding films when QDs are co-assembled as joints between collagen domains. These peptide based films show excellent mechanical properties with Young's modulus of ~20 GPa, much larger than most of multi-composite polymer films and previously reported freestanding nanoparticle-assembled sheets, and it is even close to the bone tissue in nature. These films show little permanent deformation under small indentation while the mechanical hysteresis becomes remarkable when the load approaches near and beyond the rupture point, which is also characteristic to the bone tissue.

Over the past decade, biomimetic fabrication of nanomaterials revolutionized a variety of technological applications by unveiling novel mechanical properties compared with their traditional bulk counterparts. Recent efforts have been focused on the assembly of nanometric building blocks into complex macro-scale superstructures that exhibit excellent functionality, customizability and mechanical stability.¹ For example, the nacre in abalone consists of interleaved laminate composites of 95% calcium carbonate (one of the most fragile minerals) and proteins (which is soft), however the integration of these materials in the specific assembly geometry makes the resulting materials very stiff and strong.² By mimicking this type of structure, alternated layer-by-layer assembly of soft aluminum and titanium stacking pressed at 1300°F creates an extraordinary strong film that can be applied to bullet-proof vest.³ Another important application of nanoparticle assemblies is freestanding films.⁴ If these films possess excellent macroscopic mechanical features while reserving nanoscale effects of nanoparticles(NPs), the resulting flexible and robust two dimensional (2D) structures could be promising materials for the next generation of electronics, which may take the place of current rigid and brittle devices. This type of films could also be useful platforms for the investigation of substrate-free ultrafast transport phenomena.⁵ For the fabrication of such films, the molecular self-assembly approach should be investigated more as an alternative fabrication methodology due to the robust incorporation process of nanomaterials into the self-assembled films. Previously, NPs capped with small ligands were assembled into freestanding films via random van der Waals interactions; however, these films are usually fragile and their Young's moduli are less than 10 GPa.⁴

Living organisms are efficient, inexpensive and environmentally benign material generators which produce composite materials exhibiting multiple functions and extraordinary physical

[†]Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here].
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properties at room temperature.⁶ Therefore, biomimetic assembly is an emerging technique to design novel approaches for the nanocomposite processing. An intriguing example of such strong and elastic biomaterials in nature is bone tissue consisting of collagens and hydroxyapatite particles. The bone tissue is ten to twenty times stiffer than collagen molecules while its fracture strain is much larger than the mineral crystals.⁷ This excellent combination of mechanical properties is due to the building block of the collagens that can dispatch stress to the inorganic nanocrystals through complicated molecular deformation and intermolecular shear.^{7–8} If biological molecules are assembled into freestanding films with synthetic inorganic particles as joints by mimicking the bone tissue structure, the resulting films may have superior mechanical property close to the practically applicable quality. On the basis of this concept, DNAs were examined to assemble into monolayered superlattice with gold NPs; however, these DNA based films do not have excellent mechanical property since the 2D arrangement of the NPs is mediated by the nonspecific electrostatic DNA interactions rather than the strong Watson-Crick base-pairing force.^{4f} This work demonstrates that it is possible to program the 2D biomolecular assembly for the fabrication of nanoparticle films, but further improvement of their mechanical property is necessary. Another interesting biomolecular building block to generate freestanding nanoparticle films is the collagen-like triple helix peptide. While wild-type collagen lacks specific recognition for synthetic NPs, the genetically engineered peptide designed to have both collagen's assembling ability and the biorecognition capability is a promising candidate to fulfill the bone-mimicking assembly. Previously, we have shown that 3D superlattice structure can be generated by co-assembling streptavidin-functionalized NPs and the collagen-mimetic peptides whose N-terminus was biotinylated.^{1b} If these peptides and functionalized NPs are casted on a porous substrate, 2D freestanding films are expected to form on the top of pores via molecular recognition, and the resulting films could have excellent mechanical property as compared with other films due to the protein-protein interactions.

Here, we demonstrated this protocol to produce freestanding films from the collagen-like biotinylated triple helix peptides and the streptavidin-functionalized quantum dots (QDs). While casting only the collagen peptides on a porous substrate do not produce any freestanding films, co-assembling the QDs and the collagen-mimetic peptides via the biotin-streptavidin connections enables forming strong and elastic freestanding films across the pores. The resulting peptide-based freestanding films show very interesting mechanical features, despite they are different from bone tissues whose properties we initially tried to exactly mimic. The Young's modulus of the peptide-QD freestanding films is ~20 GPa, much larger than most of polymer films and previously reported freestanding NP-sheets (< 10 GPa), and it is even close to the bone tissue in nature.^{5d} However, it is interesting that the fracture toughness of the peptide-freestanding film is much smaller than the one for bone tissues. This observation indicates that the small degree of disorder of peptide alignment in the peptide-QD film seems to reduce the toughness while the stiffness of peptide-QD film is still comparable to the one for bone tissues. In addition, these films show little permanent deformation under small indentation while the mechanical hysteresis becomes remarkable when the load approaches near and beyond the rupture point, which is also characteristic to the bone tissue.⁹

To examine our hypotheses in the hybrid peptide-nanoparticles assembly mediated by biomolecular recognition of peptide, we firstly produced the collagen-mimetic peptide, a triple helical molecule containing a segment of the α_1 chain of type I collagen with biotin acceptor peptides (BAP) fused at both N- and C-terminus. Next, the resulting peptide (B877B) was co-expressed with biotin ligase in *E. coli* BL21 (DE3) to complete the biotinylation. Then, the biotinylated B877B peptides were assembled with streptavidin-labeled QDs through the streptavidin-biotin connections (Scheme 1). After drying one drop of an aqueous solution containing biotinylated B877B peptides and streptavidin-labeled QDs

on the holey copper substrate whose porous size is 2 μm in diameter, the NPs spontaneously cross-linked at the air/water interface during the drying process at ambient conditions. Figures 1-a and b show atomic force microscopy (AFM) images of an intact and a crumpled freestanding film, respectively. The profile analysis suggests that the freestanding film on the hole is flat and smooth with recession below the substrate surface to 22 nm (Figure 1-a, inset). Transmission electron microscopy (TEM) images in Figures 1c – f show that the distribution of QDs are not close-packed in peptide films, and the non-uniform interparticle distance observed in these TEM images indicates that the peptide assembly is not completely parallel to the substrate, and we hypothesize that the collagen peptides are assembled in random directions in the film (Scheme 1), which is a common phenomenon for films assembled from particles at interfaces.^{5a} It should be noted that in our control experiment with using collagen peptides alone, no freestanding films were formed on the holes without cross-linking with streptavidin-QDs, which indicates that the structural reinforcement with biomolecular linkage between collagen domains and QDs is critical for the generation of the freestanding films. We examined another control experiment by drying the solution containing the biotinylated B877B peptides and carboxyl acid-capped QDs, which are water-soluble but unable to bind the biotin motif at the ends of peptide. Again, freestanding films were failed to be generated (Figure 2-a), and QDs were accumulated near the holey wall of the grid after drying (Figure 2-b and c). These observations suggest that the strongly cross-linked peptide assembly through biomolecular recognition can resist the surface tension created in the drying process to generate the smooth freestanding films. Since the structure of the NPs assembly is determined kinetically when they are dried at the liquid–air interface¹⁰, we hypothesize that the morphology of the peptide-QD films should be influenced by the drying speed. To verify this hypothesis, the collagen peptide-QD assembly was dried on the holey grid in different humidity. As shown in Figures 2-d and 2-e, when the sample was dried slowly in 70–90% relative humidity at 4°C^{1b}, the morphology of the peptide film was quite different from the one observed at ambient conditions as shown in Figure 1-c. The slow drying process forms islands of peptide-QD assemblies in the film. While the mechanism to grow highly aggregated areas in the films in higher humidity is not quite understood yet^{1b}, it is clear by this control experiment that the drying speed is one of the important factors to create smooth peptide films.

To quantify the mechanical property of the collagen peptide-QD films, nano-indenting tests were carried out at the center of the film with an AFM probe. Figure 3-a shows a typical trace of the force-displacement curve for the peptide–QD films. As the AFM probe approaching to the film, the attractive force starts pulling down the cantilever at ~170 nm away from the surface and it extends up to ~350 nm in the retracting trace where the AFM cantilever suddenly breaks free from the membrane surface. The attractive force can be attributed to the interaction between B877B peptide and the silicon probe.¹¹ Figure 3-b shows a rescaled extending plot (highlighted in the rectangular area of Figure 1-a), which reveal the nonlinear elastic behavior of the film. By fitting the experimental data to the Equation (1), Young's modulus can be derived.¹²

$$F = \sigma_0^{2D}(\pi r) \left(\frac{\delta}{r} \right) + E h r q^3 \left(\frac{\delta}{r} \right)^3 \quad (1)$$

where F is applied force, δ is the deflection at the center point, and σ_0^{2D} is the pretension; E is Young's modulus; r and h are the radius and thickness of the membrane which can be measured at 1 μm and 50 nm, respectively (Figure S1 and S2). $q \approx 1/(1.0491 - 0.1462\nu - 0.15827\nu^2)$ is a dimensionless constant related to Poisson's ratio (ν) and ν is assumed as 1/3, which is usually used for nanoparticle monolayer sheets.⁴ Histograms in Figure 3-c and d represent the distributions of the derived value of σ_0^{2D} and E on the basis of 70 force

curves (Figure S3). The average value of E is at 20 GPa, which is remarkably enhanced as compared with the assemblies of wild type collagens (~ 1 GPa),^{7, 13} and this stiffness is even closer to the elastic modulus of bone tissue (15–24 GPa).¹⁴ The exact enhancement mechanism of the stiffness is not clear, but it is hypothesized that this property is due to the assembled structure of QDs cross-linked by the triple helix peptides since the common phenomenon is also observed in bone tissues and other nanocomposite materials.⁷

A rupture point by the large scale indentation (Figure 3-e and f) is used to determine the fracture strength of the peptide-QD film. The fracture strength of the film is defined as F_{rup}/A where F_{rup} is the maximum force at the rupture point and A is the area where the force is applied.^{4c} Based on Qin's method^{4c}, the vertical deflection of the freestanding film is neglected and A is determined by the contour of AFM probe (Figure S4). By this calculation, the fracture strength of the peptide-QD film is determined as 81 MPa, much stronger than the film previously generated with metal nanocrystals alone (< 30 MPa).^{4c}

Energy dissipation is calculated by integrating enclosed areas between loading and unloading force–displacement curves.^{9, 15} Similar to the bone tissue, the peptide-QD freestanding films show little permanent deformation under small indentation (Figure 3-a). However, it is remarkable that the mechanical hysteresis appears when the load extends near and beyond the rupture point (Figure 3-e and f).⁹ In Figure 3-e, the energy dissipation is calculated at 14 fJ. Taking A as $4.2 \times 10^3 \text{ nm}^2$ (Figure S4), the fracture toughness of the freestanding film is estimated at 3.3 J m^{-2} , much smaller than that of bone tissue ($\sim 10^3 \text{ J m}^{-2}$).^{15b} The lower toughness of the peptide-QD films as compared to the bone tissue could be attributed to more disordered arrangement of collagen-like peptides in the peptide-QD film. It really makes sense since high toughness of bone tissues is due to the highly-ordered collagen alignment enabling a series of molecular-scale deformations such as twist, stretch and molecular uncoiling to relax and dissipate the external force, and the lack of toughness in this collagen-like peptide assembled films could be attributed to the disorder of the peptide alignment in the film, and the reduced number of sacrificial bonds that absorb mechanical impacts.^{9, 15} It should be noted that values of fracture strength and toughness are underestimated since the real force applying area (the effective contact area) could be much smaller due to the vertical movement of the film under point indentation.¹² In addition, TEM image of the fractured film in Figure 1-f also supports that the fracture strain is effectively relaxed through the inelastic deformation of B877B peptides in the drying process since the arrangement of QDs is not localized at the edge of the films after rupturing.

In summary, we demonstrated that genetically-engineered collagen peptides can be assembled into freestanding films when QDs are co-assembled as joints between collagen domains. These peptide-QD films show excellent mechanical properties with Young's modulus of ~ 20 GPa, much larger than most of multi-composite polymer films and previously reported freestanding nanoparticle-assembled sheets, and it is even close to the bone tissue in nature.^{5d} However, the fracture toughness of the peptide-freestanding film is much smaller than the one for bone tissues. We hypothesize that the alignment of peptide in this peptide-QD film is more disordered than the collagen alignment in bone tissues, which is the major reason to reduce the toughness while it maintains high stiffness. From this result, it is indicated that bone tissues have such superior mechanical properties as compared to synthetic counterparts including this peptide-QD film and the structure needs to be mimicked precisely to generate freestanding films with the similar mechanical performance as bone tissues. But of course, among synthetic freestanding films incorporating NPs, this peptide-QD film stands out in both stiffness and toughness. This outcome gives important knowledge that for some biomimetic systems coarse mimicking of the structure could mimic one mechanical property as far as QDs are assembled with the peptides via biomolecular recognition but precise mimicking of the structure such as the distribution of QDs is critical

to produce materials with the exact set of targeted mechanical properties. Since the collagen-like peptide can be assembled to films with a variety of nanomaterials such as metal NPs, QDs, and carbon nanotubes as joint building blocks as they are functionalized by streptavidin or other binding ligands genetically or chemically^{1, 16}, these peptide-assembled freestanding films could potentially be applied to substrate-free electronics, ultrafast filtration and tissue engineering.^{4–5}

Experimental

Streptavidin-labeled QDs (QDot 525) were purchased from Invitrogen. Carboxyl acid-coated QDs (QSH-530-04) were purchased from Ocean NanoTech. The synthesis and biotinylation of B877B peptide followed the method shown in Supplementary Information and our previous work¹⁷. Holey copper grids (2 μm diameter hole, 2 μm separation) were purchased from Ted Pella. For the preparation of the freestanding peptide films, typically, equal amount of aqueous solutions of QDs (50 nM) and biotinylated B877B peptide (50 nM) were mixed and immediately casted onto the holey copper grid. After drying overnight at room temperature, the resulting film was analyzed by TEM (JEM 2100 TEM (JEOL)) at an acceleration voltage of 200 kV. AFM analyses were performed on a stand-alone MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA). Ultrasharp silicon tips (MikroMasch) with a typical probe tip radius of 10 nm were used in the tests with the resonant frequency of 325 kHz. All of the images were taken in the tapping mode, and treated with the WSxM software to enhance the imaging contrast. For the force plots, prior the measurements the silicon tip was blunted by scanning a mica surface for several minutes to prevent piercing the peptide film during the force measurement. The spring constant (22.55 nN/nm) of the cantilever was determined by the thermal noise method.¹⁸ The sensitivity measurements were performed on mica. A typical tip speed of 500 nm s⁻¹ was used in all force measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Images of the collagen peptide-QD freestanding films on holey substrates. (a) AFM image and height profiles (between arrows) of the intact peptide-QD film. (b) AFM image of a broken part of the peptide-QD film. (c) TEM image of the intact peptide-QD film. (d) Magnified TEM image of the rectangular area highlighted in (c). (e) TEM image of the broken peptide-QD film. (f) Magnified TEM image of the rectangular area highlighted in (e).

Figure 2.

TEM images for control experiments. (a–c) Casting the solution containing B877B peptides and carboxyl QDs instead of streptavidin-QDs on holey Cu grids. The magnified TEM image of a square area in (b) was shown in (c). (d–e) Casting the solution containing B877B peptides and streptavidin-QDs on holey Cu grids by drying slowly in 70–90% humidity at 4°C.

Figure 3.

(a) A typical force (F)-displacement (δ) curve of the freestanding peptide-QD film. (b) The rescaled loading curve (highlighted in the rectangular area of Figure 1(a) and a fitting with Eq. 1 (blue line). The curve approaches cubic behavior at high loads (inset). (c–d) Histogram of film pretensions and Young's modulus of the freestanding peptide-QD film, respectively. Dashed lines in (c) and (d) represent Gaussian fits. (e) A typical F - δ curve of the freestanding peptide-QD film on large scale of nanoindentation. (f) Rescaled loading curve highlighted in the rectangular area of Figure 3(e). The rupture point is marked by an arrow. $F_{\text{rup}} = 340$ nN, and $\delta_{\text{rup}} = 52$ nm.

Scheme 1.
Schematic representation of the formation of peptide-QD freestanding films.