Meso-Functionalization of Silk Fibroin by Upconversion Fluorescence and Near Infrared In Vivo Biosensing

Yang Song, Zaifu Lin, Lingqing Kong, Yao Xing, Naibo Lin,* Zhisen Zhang,* Bing-Hung Chen, and Xiang-Yang Liu*

In biomedical applications, it is very desirable to monitor the in vivo state of implanted devices, i.e., tracking the location, the state, and the interaction between the implanted devices and cell tissues. To achieve this goal, a generic strategy of soft materials meso-functionalization is presented. This is to acquire silk fibroin (SF) materials with added functions, i.e., in vivo bioimaging/sensing. The functionalization is by 3D materials assembly of functional components, lanthanide(Ln)-doped upconversion nanoparticles (UCNPs) on the mesoscopic scale to acquire upconversion fluorescent emission. To implement the meso-functionalization, the surfaces of UCNPs are modified by the hydroxyl groups (–OH) from SiO$_2$ or polyethylene glycol coating layers, which can interact with the carbonyl groups (C=O) in SF scaffolds. The functionalized silk scaffolds are further implanted subcutaneously into mice, which allows the silk scaffolds to have fluorescent in vivo bioimaging and other biomedical functions. This material functionalization strategy may lead to the rational design of biomaterials in a more generic way.

1. Introduction

Recent research reveals that the performance of soft materials is mainly determined by the mesoscopic structures, other than the atomic or nano structures.[1] It follows that the correlation between the performance and the mesoscopic structures of soft materials can be established by the following five elements:[2] (1) topology, (2) correlation length, (3) ordering/symmetry, (4) strength of linkage or interactions, and (5) hierarchy of structure. This implies that in order to modify the performance of soft materials, we need to focus on the reconstruction or modifying the mesoscopic structure of the materials. While the functionalization of typical hard materials, i.e., semiconductors, mainly concerns doping of different elements at the atomic or molecular scale, the functionalization for soft materials is to assemble functional components at mesoscopic scale. In this sense, a systematic approach of functionalization, so-called mesoscopic material assembly can be adopted. Mesoscopic material assembly is to add and bind some specific nanomaterials or molecules to the networks so as to acquire some additional functions without jeopardizing the original performance.[3]

As a typical class of soft materials, Bombyx mori silk fibroin (SF) has exhibited promising properties in biomedical applications[4] due to excellent biocompatibility,[5] minimal inflammatory reaction,[6] and controllable biodegradability.[8] To functionalize SF materials with some additional properties will provide us with vast opportunities in fabricating SF devices of next generation.

3D silk scaffolds[9] can be utilized to regenerate missing or injured tissue and serve as a kind of tunable biochemical media.[10] In this concern, the performance of scaffolds needs to be monitored.[11] Though in vitro degradation test can provide a simulation environment to elucidate the performance of scaffolds, there still are difficulties to visualize the structure of scaffolds after implantation in vivo quantitatively and precisely.[12] The conventional imaging techniques are incapable in situ observation of scaffolds in vivo.[13] Other techniques such as micro-computed tomography (micro-CT) and magnetic resonance imaging, which can solve the in situ problem to some extent, are limited by their relative low resolution.[14] In this sense, a new in vivo, noninvasive method in combination of the biological performance with bioimage functions is urgently needed. In this concern, if SF can be functionalized by particular molecules or designed into specific forms, it can offer a promising alternative for biological imaging.[13c,15] In mesoscopic material assembly, SF materials can be taken as “mother boards” and be expected to combine the advantages of specific functional components.[16] The scaffolds functionalized with one-photon or two-photon fluorescence materials provide a good choice for monitoring the evolution of the scaffolds and probing the chemical environmental change in a real-time manner.[3c,14c,17] However, the excitation wavelengths
of one-photon fluorescence materials, quantum dots (QDs) and organic dyes, can result in auto fluorescence background, low penetration depth and photodamage to tissues.[18] Moreover, the QD toxicity and the photobleaching of organic dyes are still problems in situ observations,[18b,19] and two-photon approaches are also limited with their demands for expensive high-energy femtosecond pulsed laser (10^6–10^9 W cm^-2) and focused beam of high intensity.[20]

As the exciting near infrared (NIR) light located in the second transparent biological window can deeply penetrate into biological tissues and give less biological tissue scattering in real time, advanced upconversion technique is proposed to solve the bioimaging problem. Lanthanide(Ln)-doped upconversion nanoparticles (UCNPs) for biological imaging have attracted comprehensive attention with their abilities to emit lights upon low-energy near infrared excitation.[18b,21] Compared with organic dyes and semiconductor QDs, UCNPs have been approved as promising alternatives with favorable advantages.[8a,15] In addition, a low power continuous wave laser (1–10^3 W cm^-2) has been proved efficient enough to realize upconversion process, and less photodamage to biological samples will be given comparing with UV excitation.[22] Furthermore, due to the near infrared excitation, the signal-to-noise ratio and sensitivity in quantitative analysis are remarkably enhanced owing to the absence of autofluorescence and scattering light from samples. Benefiting from the high-sensitivity and capacity of real-time monitoring, UCNPs have been adopted as a promising approach for applications in bioimaging.[8a,15]

In this work, upconversion-functionalized scaffolds were firstly proposed to realize the scaffold imaging in a real-time, noninvasive and inexpensive manner. SF will be taken as a substrate to fabricate upconversion-functionalized bioscaffolds, which are expected to gather the advantage of UCNPs and SF materials.[24] The scientific significance is to assemble UCNPs into the SF materials and to obtain soft materials with the intrinsic biosensing/imaging function using upconversion luminescence (UCL) technique. Moreover, we will also investigate the biodegradability and biocompatibility of UCL SF scaffolds. This will lead to the imaging and sensing of bioscaffold in real time and noninvasive manner in detection strategy.

2. Results and Discussion

The overall design process of UCL functionalized SF scaffolds for bioimaging is illustrated in Figure 1. Firstly, SF was extracted from B. mori cocoons via a degumming process and form 3%–5% (w/v) solution. After that, the initial UCNPs were synthesized by means of a solvent thermal process, which is capped by oleic acid (OA) on the surfaces and named UCNPs@OA.[25] In order to transfer the hydrophobic UCNPs@OA to an aqueous solution, surface modifications of SiO2 and polyethylene glycol (PEG) were utilized afterward. 3D porous upconversion-functionalized SF scaffolds were obtained after freeze-drying the mixture of as-prepared UCNPs and SF fibroin solution.

UCNPs consisting of sodium yttrium fluoride (NaYF4) co-doped with Yb3+/Er3+ were synthesized. Fluorescent spectra (HORIBA FluoroMax-4) and transmission electron microscopic (JEOL, JEM2100) images of UCNPs are shown in Figure 1Aa,b. The nanoparticles are of 41.6 ± 5.6 nm in diameter, and the X-ray diffraction (XRD) peaks could be indexed as hexagonal phase NaYF4 (JCPDS no. 16-0334) with the space group of P6_3 m̅ (Figure S1, Supporting Information). The UCL spectra of the solution was characterized with a modified fluorescence spectrometer applying laser excitation of λ = 980 nm. Upconversion emissions at 521, 540, and 654 nm can be observed, corresponding to the transitions of 2H11/2 → 4I15/2, 4G5/2 → 4I15/2, and 4F9/2 → 4I15/2, respectively (Figure 1Ac). The green fluorescent emission from the solution was observed (Inset of Figure 1Aa). Increasing the power of NIR laser, the green light intensity shows a linear relationship on a double logarithmic scale. This confirms the optical integrity of UCNPs in functionalized scaffolds (Figure S2, Supporting Information).[21e] The resulting UCNPs are covered with hydrophobic OA molecules and can be well dispersed in cyclohexane. To investigate the effect of surface groups on UCNPs, two different surface modifications are adopted to render these UCNPs water-dispersible and bioconjugatable. In Figure 1Ab, UCNPs@SiO2 particulates display silica shells around ~10 nm in thickness, and the UCNPs coated with PEG became ellipsoidal in shape with diameter of 48.3 ± 4.2 nm. Moreover, the surface modification can provide the core nanoparticles with protection against the external environment and give rise to the biocompatibility and stability of UCNPs.

The SF scaffolds functionalized by UCNPs@OA, UCNPs@SiO2, and UCNPs@PEG were fabricated with the concentration of 0.8% (w/w) (cf the details in the Experimental Section), and the control (SF scaffolds without UCNPs) were prepared by the same process. The scaffolds incorporated with UCNPs@OA are severely and irregularly shrunk, while other scaffolds display the plump cylindrical shape, implying that UCNPs@OA could not successfully interact and incorporate onto SF molecules. The porous structures of neat SF fibroin scaffolds and functionalized SF scaffolds, which can be an essential property that provides spaces for cell adhesion and nutrient exchange,[2b] were further examined by the scanning electronic microscope (Hitachi, SU-70). It follows that neat SF scaffolds have an interconnected smooth porous structure with a pore size of 100–250 µm in diameter. The porous structure of SF scaffolds with UCNPs@OA, however, shows some irregularity (Figure 1C), due to the fact that the UCNPs@OA could not incorporate onto SF molecules in the liquid mixing stage. During the freeze-drying process, the addition of UCNPs@OA leads to the liquid–liquid phase separation: a dense proteinaceous phase highly dispersed in a dilute phase. The ice crystals formed in the dense proteinaceous phase will be smaller, and the size of ice crystallites in the dilute phase will be larger. After removing the ice templates via the freeze-drying process, a multilevel porous structure can be formed (Figures 1Cb, 2a).[27] In contrast, the pore structures of UCNPs@SiO2 functionalized SF scaffolds and UCNPs@PEG functionalized SF scaffolds are similar to the neat scaffold, remaining at 100–250 µm (Figure 1Cc,d). The regular network structures were almost unchanged between neat SF scaffolds and scaffolds functionalized by surface-modified UCNPs, indicating that surface-modified UCNPs successful assembled onto the SF and the assembly process did not deteriorate the formation of...
Figure 1. A) Upconversion emission spectra of NaYF4:Yb3+/Er3+ nanoparticles coated with (1) oleic acid (UCNPs@OA), (2) silica (UCNPs@SiO2), and (3) polyethylene glycol (UCNPs@PEG), inset is the digital images of cuvettes with nanoparticles under excitation at 980 nm. B) Transmission electron microscopy (TEM) images of UCNPs@OA, UCNPs@SiO2, and UCNPs@PEG. Scale bar: 100 nm. C) Energy transfer mechanisms showing the upconversion process of Er3+ and Yb3+ doped UCNPs under 980 nm excitation. B) Illustration of the preparation process of upconversion functionalized SF scaffolds. Green emission can be observed from as-prepared scaffolds upon the excitation of NIR light. C) Scaffolds images under day light and SEM images of a) Neat SF scaffold b) UCNPs@OA functionalized SF scaffold c) UCNPs@PEG functionalized SF scaffold d) UCNPs@SiO2 functionalized SF scaffold. The scaffolds functionalized by UCNPs@OA are severely and irregularly shrunk, while other scaffolds show plump cylindrical shapes. D a) Scaffolds images under 980 nm laser. b) Fluorescence spectra of (1) Neat SF scaffold (2) UCNPs@OA functionalized SF scaffold (3) UCNPs@PEG functionalized SF scaffold (4) UCNPs@SiO2 functionalized SF scaffold. The scaffold with UCNPs@OA has the difference of emission areas and intensities of the front side and the reverse side, while the scaffolds functionalized by UCNPs@SiO2 and UCNPs@PEG, both sides of the cylinder have similar appearances.
hierarchical network (Figure 2b). Based on previous studies, the final network formation of upconversion-functionalized SF scaffolds may follow the nucleation and growth process of $\beta$-crystallites (Figure 2c).\[28\]

In order to assess the feasibility of fluorescence properties of UCL SF scaffolds for imaging applications, as can be seen in Figure 1D, the strong green emission can be observed from the tops of UCNPs functionalized SF scaffolds compared with neat silk scaffolds. This confirms the presence of UCNPs within functionalized scaffolds. The scaffolds with UCNPs@OA have the difference areas and intensities of emission between the front side and the reverse side, while the scaffolds incorporated with UCNPs@SiO$_2$ and UCNPs@PEG, both bottoms of the cylinder have similar appearances, and the fluorescence spectra also display the same results. It follows that the UCNPs@OA could not assemble into the SF uniformly due to no recognition between SF and UCNPs. In contrast, UCNPs@SiO$_2$ and UCNPs@PEG can evenly distribute, indicating the hydrogen bond interaction between the hydroxyl group ($-$OH) of UCNPs and the carbonyl group ($-$C=O) of SF molecules at the molecular level.

To further optimize the fluorescence property, different concentrations of UCNPs were incorporated into the SF materials. As the concentration of UCNPs@SiO$_2$ increases from 0% (w/w) to 0.8% (w/w), the emission intensity reaches to the highest. While the concentration exceeds 0.8%, the fluorescence intensity shows a trend to decrease (Figure S3a, Supporting Information). For the silk scaffold with UCNPs@PEG, the results are similar (Figure S3b, Supporting Information). Regarding the effect of pH on the fluorescence efficiency, pH (range: 6.0–10.0) of the mixture SF solution with 0.8% UCNPs@SiO$_2$ and UCNPs@PEG were adjusted by 1% ammonia. The maximum fluorescence intensities are both at around pH = 7.50 (Figure S3c,d, Supporting Information), which is suitable for the common pH of body fluid.\[29\]

To study the mechanism of functionalization via materials assembly at mesoscale, the structural and interaction synergy between SF protein molecules and UCNPs was examined. In this context, Fourier transform infrared spectroscopy (FTIR) was utilized to study the scaffold structure and the interaction between UCNPs and SF. The characteristic vibrational bands of SF at 1700–1450 cm$^{-1}$ for amide I (C=O stretching) and amide II (secondary NH bending) can be observed (Figure 3A).\[3c\] The stretching vibration peak of free carbonyl (C=O) group and intermolecular hydrogen-bonded carbonyl group is located at 1650 and 1628 cm$^{-1}$, respectively. Based on the fact that the same preparation process was adopted, the change in the FTIR spectra corresponds to the change of chemical environments. Compared with neat SF, the ratio of the intensity of peaks at 1650 versus 1628 cm$^{-1}$ shows a significant increase after the incorporation of UCNPs@SiO$_2$ and UCNPs@PEG, while the ratio of SF added with UCNPs@OA remains almost unchanged. This implies that the hydroxyl group ($-$OH) on these nanoparticles gives rise to the hydrogen bond interaction between the hydroxyl group ($-$OH) on the nanoparticles and the carbonyl group (C=O) of SF, and therefore there is less intermolecular hydrogen-bonded carbonyl groups between SF molecules. The C$=$N vibration absorption
Figure 3. A) FTIR spectra recorded in the region of 1700–1500 cm$^{-1}$ for 1) neat silk scaffold, 2) UCNPs@OA functionalized silk scaffold, 3) UCNPs@SiO$_2$ functionalized silk scaffold, 4) UCNPs@PEG functionalized silk scaffold. The spectra show that the addition of UCNPs@SiO$_2$ and UCNPs@PEG gives rise to the hydrogen-bond interaction between the hydroxyl group (−OH) on the nanoparticles and the carbonyl group (C=O) of SF, and therefore there is less intermolecular hydrogen-bonded carbonyl groups between SF molecules. B) FTIR spectra of SF scaffolds with different ratio of UCNPs@SiO$_2$. B a) The comparison between the secondary structure of UCNPs@SiO$_2$ functionalized SF scaffolds and their estimated values for neat SF scaffolds. It follows that β-conformations (mainly β-crystallites) are less than the estimated values. B b) The decrements of β-conformations with different UCNPs@SiO$_2$ contents. This implied that the addition of UCNPs@SiO$_2$ to SF materials will inhibit the formation of β-sheets and β-crystallites, which further disturb the formation of the mesoscopic structure. C) Schematic illustration of the formation of β-crystallite networks (nanofibril) (above) and the same process once UCNPs@SiO$_2$ was added (below). The interactions between the carbonyl group (C=O) of SF and the hydroxyl group (−OH) of UCNPs via hydrogen-bond linkages cause the blocking of β-folding for SF molecules, which leads to the β-crystallization inhibition in the structure.
band intensity show a similar shift when the surface-modified UCNPs added (Figure S4, Supporting Information). The ratio of free amino group (1232 cm\(^{-1}\)) and hydrogen-bonded amino group (1239 cm\(^{-1}\)) of the C=\(\text{N}\) vibration absorption band is significantly enhanced as the assembly of UCNPs with hydroxyl group (\(-\text{OH}\)) on the surface.

Fourier self-deconvolution was applied to quantitatively analyze the amide I region of the FTIR spectra.\(^{[29b,30]}\) The Gaussian curve fitting of the amide I region for the neat and functionalized SF scaffolds was given in Figure S5 in the Supporting Information. The total \(\beta\)-conformation and \(\beta\)-crystallites contents of neat SF scaffolds and UCNPs@OA functionalized SF scaffolds are higher than that of UCNPs@SiO\(_2\) and UCNPs@PEG functionalized SF scaffolds, while the contents of random coil show a reverse trend (Table S1, Supporting Information).

As the concentration of UCNPs@SiO\(_2\) increases in the scaffolds, in the amide I region, the intensity ratio of two peaks at 1628 versus 1650 cm\(^{-1}\) exhibits a trend of increase (Figure 3B), revealing that the breakage of the SF internal hydrogen bonds (\(-\text{C}=\text{O}\ldots\cdot\text{H-N}\ldots\cdot\)) once UCNPs@SiO\(_2\) (and/or UCNPs@PEG) were incorporated into SF materials. The secondary structure of silk displays a conversion from \(\beta\)-sheet to random-coil (Figure S6 and Table S2, Supporting Information). It follows that \(\beta\)-conformations (mainly \(\beta\)-crystallites) are less than the estimated values. In more detail, neat silk protein chains present the regular \(\beta\)-sheets; the incorporation of UCNPs with hydroxyl groups will inhibit the formation of \(\beta\)-sheets and unfold the \(\beta\)-crystallites via the hydrogen-bond interactions between silk protein molecules and UCNPs@SiO\(_2\) as illustrated in Figure 3C. Such an unfolding process may lead to the secondary structure change, which ends up with the rise in the random coils.

While FTIR quantifying the overall \(\beta\)-conformations in SF scaffolds, the structures of the blended scaffolds were further examined to determine the secondary structure changes and interactions of functional groups by XRD (Figure 3B, Figure S7, Supporting Information).\(^{[30]}\) It allows us to quantify the intramolecular \(\beta\)-sheets and intermolecular \(\beta\)-sheets. The results of intermolecular \(\beta\)-sheet (and \(\beta\)-crystallites) changes are given in Tables S1 and S2 in the Supporting Information. The decrease of \(\beta\)-crystallites can be obtained after the incorporation of the UCNPs@SiO\(_2\) into SF materials. In the assembly between hydroxyl group coated UCNPs and the SF proteins, the carboxyl group (\(-\text{C}=\text{O}\)) of SF and the hydroxyl group (\(-\text{OH}\)) of UCNPs directly interact with each other via hydrogen-bond linkages (Figure 3B). The interactions between SF molecules and UCNPs@SiO\(_2\) will cause the breakage of \(\beta\)-chain folding for SF molecules, which leads to the \(\beta\)-crystallization inhibition in the structure. This gives rise to much less \(\beta\)-crystallites in the functionalized SF materials (Figure 3C). Furthermore, the degradation rate of silk fibroin materials is correlated to the \(\beta\)-crystallinity.\(^{[31]}\) In this sense, it is feasible to regulate the degradation rate via controlling the \(\beta\)-crystallinity. After biodegradation, UCNPs are expected to infiltrate into body fluid together with the degraded silk fibroin.

In the application to tissue engineering, SF scaffolds should counter pressure during the implantation procedure; therefore, the compressive modulus is an important property. To further study the influence of the functionalization on the silk, the mechanical properties of scaffolds were examined.\(^{[32]}\) The strength–strain curves of the functionalized silk scaffolds were examined using Instron Microtester 5525× at room temperature, and the compressive modulus was calculated according to the initial slope section of the strength–strain curve. The compressive modulus of neat, UCNPs@SiO\(_2\) (concentration: 0.8\%) and UCNPs@PEG (concentration: 0.8\%) is 468.8, 408.3, and 419.6 kPa, respectively. The compressive modulus is almost same after assembling the surface-modified UCNPs (Figure 4), and the a bit lower moduli of scaffolds assembled with UCNPs@SiO\(_2\) and UCNPs@PEG may be subject to the lower crystallinity of the samples. While scaffolds assembled with UCNPs@OA show a higher compressive modulus, it may be caused by the shrink of the scaffolds after assembly process. Although these scaffolds display a higher compressive modulus, scaffolds assembled with surface-modified UCNPs are more flexible. Comparing with neat SF scaffolds, the compressive modulus of functionalized scaffolds was almost remained, which indicates that the assembly of surface-modified UCNPs process does not deteriorate the mechanical properties of scaffolds. For the UCNPs@SiO\(_2\) functionalized SF scaffolds, scaffolds with concentration above 0.8\% became more fragile and easy to deform compared to lower concentration scaffolds. The decrease of mechanical properties could be caused by the excessive UCNPs jeopardize the network formation of silk scaffolds.

Apart from the mechanical property, the biodegradability will also determine the performance of silk scaffolds in biomedical applications. In vitro degradation of SF scaffolds was further studied via incubation studies in phosphate buffer saline (PBS) with the enzyme Protease XIV, which has been widely used in the study of silk degradation.\(^{[100,33]}\) In degradation studies, all SF scaffolds degraded rapidly with a loss of more than 40\% mass after 10 d (Figure 4). Compared with neat SF scaffolds remaining ~55\% of original mass at day 10, a little bit accelerated silk degradation was observed after SF scaffolds are incorporated with UCNPs@SiO\(_2\), with ~45\% of original mass remaining at day 10. It may be caused by the unfolding of the \(\beta\)-crystallites and the transition of random coils after the assembly of UCNPs@SiO\(_2\). A similar phenomenon can also be observed in scaffolds with UCNPs@PEG. However, the SF functionalized by UCNPs@OA deformed more easily and lost significantly more mass compared with other scaffolds. It can be due to the aggregation of UCNPs@OA, after the unsuccessful incorporation into SF. This further deteriorates the porous structure of SF scaffolds.

In order to achieve the ultimate goal of bioimaging, UCL bioimaging of silk scaffolds was performed with a fluorescence microscope (Leica, DM6000B). An external continuous wave 980 nm laser was equipped with an 800 nm short-pass filter set in front of the digital camera to remove the disturb of excitation NIR light. Mouse pre-osteoblast cells (MC3T3-E1) stained with DiI were seeded in the center of the neat SF and functionalized scaffolds. MC3T3-E1 seeded on both scaffolds were stained green with DiI were seeded in the center of the neat SF and functionalized scaffolds. MC3T3-E1 stained with DiI were seeded in the center of the neat SF and functionalized scaffolds. MC3T3-E1 stained with DiI were seeded in the center of the neat SF and functionalized scaffolds. MC3T3-E1 stained with DiI were seeded in the center of the neat SF and functionalized scaffolds. MC3T3-E1 stained with DiI were seeded in the front of the digital camera to remove the disturb of excitation NIR light. Mouse pre-osteoblast cells (MC3T3-E1) stained with DiI were seeded in the center of the neat SF and functionalized scaffolds. MC3T3-E1 stained with DiI were seeded in the front of the digital camera to remove the disturb of excitation NIR light.
in Figure 5b–e, it can be observed that scaffolds still retain the intrinsic structure of SF and the network of SF can be easily captured under the green upconversion luminescence, which proves the successfully assembly of UCNPs on the SF. The clear interface between cells and scaffolds can be observed in the overlay image (Figure 5e), which provides the possibility for in vivo monitoring of the scaffold. The functionalized silk scaffolds were further implanted subcutaneously into mice (Figure 5f, g). Under the exciting light of 980 nm laser, remarkable green luminescence can be captured throughout the dorsal of mice, which verifies the possibility for upconversion-functionalized scaffolds to provide real-time in vivo imaging. And the results indicate that scaffolds with upconversion luminescent function can provide a choice for bioimaging to monitor activities under deep tissue in vivo and in vitro.

Furthermore, the strategy of mesoscopic material assembly can be applied to other functional components, such as gold clusters and quantum dots, which gives rise to an efficient route to construct functionalized SF materials. In this concern, SF materials can be functional and various forms of functionalized SF materials can be efficiently acquired via meso-functionalization, including films, sponges, gels etc., which makes it possible to apply these composite SF materials in various in vivo applications including bioimaging, biosensing, photochemistry, and soft devices (Figure 6). Apart from the in vivo bioimaging,\[14\] the upconversion-functionalized SF materials can be applied as intrinsic biosensors\[15\] to monitor the in vivo change of implanted SF materials in a noninvasive and efficient manner. Moreover, taking the advantage of intrinsic upconversion fluorescence upon near infrared, it is possible for functionalized materials to trigger in vivo photochemistry reactions.\[36\] In this regard, upconversion-functionalized SF materials can be further designed as biocompatible optical storage devices, which can realize data recording in vivo upon the excitation of near infrared. And functionalized SF materials can also be adopted in the fabrication of flexible bioelectronic devices for biomedical applications,\[37\] which may combine the advantage of biointegrated electronics and stimuli-responsive ability resulted from upconversion functionalization. This may shed light on the development of next-generation flexible bioelectronic devices.

3. Conclusion

In conclusion, a versatile approach has been utilized to successfully obtain UCL functionalized SF scaffolds for near infrared optical imaging. The approach endows the resulting composite soft materials with fluorescent properties in a moderate and controlled way. The hydrogen-bond interactions can effectively
Figure 5. a) Illustration of the in vivo bioimaging of upconversion functionalized SF materials. The UCNPs@SiO₂ functionalized scaffolds, b) bright field image, c) cell membrane (dyed in red with Dil V-22835 for visualization), d) UCL image under the excitation of 980 nm laser, e) overlay image. Scale bars are 500 μm. Functionalized scaffold was implanted into the dorsal of mice, f) bright field image, g) in vivo upconversion luminescence image under the excitation of 980 nm laser.

Figure 6. Functionalized SF materials and potential applications. Different forms of functionalized SF materials can be fabricated by mesoscopic material assembly, including films, sponges, gels, etc. and can be designed in various bioapplications.
promote the assembly process between SF molecules and surface-modified UCNPs. The obtained scaffolds not only remain the properties of neat silk scaffold, such as morphologies, mechanical properties, and cell interactions, but also provide an example of functionalization of biomaterial at mesoscale for the bioimaging and biosensing in a noninvasive and real-time manner.

4. Experimental Section

**Synthesis of UCNPs**: NaYF<sub>4</sub>:Yb,Er were synthesized via a one-pot solvothermal strategy. Firstly, 0.8 mmol YCl<sub>3</sub>:6H<sub>2</sub>O, 0.18 mmol YbCl<sub>3</sub>:6H<sub>2</sub>O, and 0.02 mmol ErCl<sub>3</sub>:6H<sub>2</sub>O were added to 6 mL OA and 15 mL octadecene. The solution was stirred under room temperature for 1 h. Then it was heated to 150 °C under argon flow and kept for 30 min until the formation of a clear and homogeneous solution, and then the heating mantle was removed and it was cooled down to the room temperature. 0.10 g NaOH and 0.148 g NH<sub>4</sub>F in methanol was added into the flask and stirred for 1 h. The solution was then heated to 110 °C to evaporate off the methanol, and the trace of remaining water was further moved by vacuum evaporation for 15 min. Next, the temperature was raised to 300 °C under argon flow and maintained at this temperature for 1 h. After cooling down to room temperature, acetone was added to precipitate the nanoparticles from the obtained solution, and then the mixture was centrifuged and washed to achieve OA coated UCNPs (UCNPs@OA). PEG coated UCNPs was carried out with as-prepared UCNPs dispersed in CHCl<sub>3</sub> (20 mg mL<sup>−1</sup>) via ligand exchange. The UCNPs solution was added dropwise into O<sub>2</sub>(Z-carboxylated) polyethylene glycol dissolved in CHCl<sub>3</sub> (20 mg mL<sup>−1</sup>) and stirred overnight. Then the mixture was centrifuged and washed three times with excess n-hexanes. Silica coating of UCNPs was carried out with as-prepared UCNPs@OA into a 50 mL flask. After 20 mL cyclohexane and 1.5 mL IGEPAL CO-520 was added into as-prepared UCNPs dispersed in cyclohexane, the mixture was stirred for 1 h. And then 0.16 mL 33% ammonium hydroxide was added into the mixture and kept for 1 h with stirring. Next, 0.08 mL tetraethyl orthosilicate was added dropwise. After stirring for 2 d, the mixture was centrifuged and washed with acetone and ethanol.

**Preparation of SF Solution**: SF was obtained from the B. mori silkworm cocoons (Guangxi Sericulture Technology Co., Ltd.). Raw silk cocoons were cut into pieces and degummed in a boiling aqueous solution containing 0.5 wt% sodium bicarbonate with frequent stirring for 30 min to extract sericin proteins. The same degumming procedure was repeated twice. Then the SF was washed five times with warm deionized water (10 min each) to remove sericin completely and dried at 40 °C in an oven overnight. Dry silk fibers were dissolved in 9.3 mol L<sup>−1</sup> LiBr solution at 60 °C for 4 h. LiBr was extracted from SF solution via a dialysis cassette for 2 d with frequent change of deionized water. Finally, the SF solution was filtered with gauze and stored in refrigerator (4 °C) for use.

**Preparation of the Upconversion Functionalized SF Scaffolds**: Regenerated SF solution (3.5% w/v) and UCNPs solution (1 mg mL<sup>−1</sup>) were mixed by gentle stirring for 5 min. Then 1-butanol solution (10% v/v) was added gradually with stirring until the predetermined volume ratio (v:V<sub>1-butanol</sub> = 2:1). Then the hybrid solution was transferred into a mold and frozen at −20 °C for 12 h. After that the freeze-drying process was adopted at −85 °C for 48 h to obtain the porous upconversion functionalized silk scaffolds. And the control silk scaffolds were prepared by the same method without the adding of UCNPs.

**Characterization of Scaffolds Mechanical Properties**: The strength–strain curves and compressive modulus of the functionalized silk scaffolds were measured using Instron Microtester 5525× with a 10 N maximum load cell at room temperature. The scaffolds were prepared to cylinder with 5 mm in height and 15 mm in diameter. The scaffolds were immersed in a bath of 0.1 mol L<sup>−1</sup> PBS for 24 h before compression modulus assay. And the measurement was carried out at 0.5 mm min<sup>−1</sup> crosshead speed. Five samples of each scaffolds group were tested to get the average modulus with standard deviation.

**Degradation of Scaffolds In Vitro**: The mass of dry cylinder scaffolds (5 mm in height and 15 mm in diameter) was weighted. 1 U mL<sup>−1</sup> Protease XIV solution in PBS was added into tube and the scaffolds were incubated in the tube at 37 °C. Every 2 d, samples were washed off the Protease XIV and dried to calculate the remaining mass. The degradation was evaluated via the comparison between the percentage of the remaining mass and the original mass. Control samples were set to baseline the degradation in the absence of Protease XIV.

**Cell Culture and Fluorescent Imaging**: Mouse pre-osteoblast cells (MC3T3-E1) were grown in Minimum Essential Medium Alpha with Eagle’s salts media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Scaffolds were prepared with a size of 5 × 5 × 1 mm, and then sterilized by 75% ethanol. MC3T3-E1 cells were seeded at the center of the scaffolds at a density of 1 × 10<sup>4</sup> per scaffolds. After culturing for 4 d, cells were stained by Dii. All in vivo implantation procedures were operated under animal care protocols approved by Xiamen University Animal Care and Use Committee. Sprague Dawley female mice (100 g) used in this study were provided by Xiamen University Laboratory Animal Center. Silk scaffolds were implanted into subcutaneous pockets of mouse.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

**Acknowledgements**

The work was supported by the 111 Project (Grant No. B16029), National Natural Science Foundation of China (Grant Nos. 21404087, 61674050, and U1405226), Fujian Provincial Department of Science and Technology (Grant Nos. 2017J06019, 2014H6022, and 2015J05109), National Science Foundation of Guangdong Province (Grant No. 2015A003100007), 1000 Talents Program, and President Foundation from Xiamen University (Grant No. 20720166088), NUS tear 1 funding (WBS: R-144-000-367-112). One of the authors, X.-Y.Liu’s primary affiliation is Department of Physics, National University of Singapore.

**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

bioimaging, hierarchical structures, silk, upconversion

Received: February 4, 2017
Revised: March 15, 2017
Published online:


