CJC Chinese Journal of Chemistry 中国化学 - An International Journal

For submission: https://mc.manuscriptcentral.com/cjoc
For published articles: https://onlinelibrary.wiley.com/journal/16147065

Critical Review

Cite this paper: *Chin. J. Chem.* **2023**, *41*, 2035—2046. **DOI: 10.1002/cjoc.202300043**

Fluorescent Silk Obtained by Feeding Silkworms with Fluorescent Materials†

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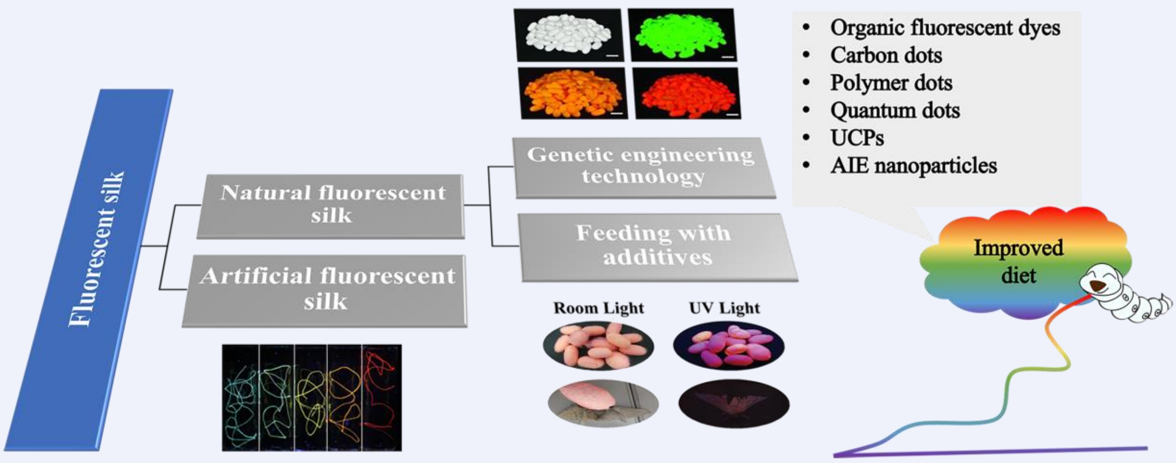
Keywords

Fluorescent silk | Fluorescent materials | Feeding silkworms | Carbon dots | Nanoparticles | Nanotechnology | Multi-color emission | Bioimaging

Comprehensive Summary



Fluorescent silk has potential application in many fields such as bioimaging, tissue engineering scaffolds, luminescent marks, and dazzling fabrics. Among the methods to endow natural silk with fluorescent properties, feeding silkworms with fluorescent additives is facile, low-cost and environment friendly, which has the prospect of large-scale production. In this paper, we reviewed the research progress for this aim in the past ten years, and summarized the unified characteristics for the substances that can enter the silk gland by digestive tract of silkworms. The advantages and disadvantages of various fluorescent materials for this application are compared in detail. And the future research directions are suggested to overcome the shortcomings of the present research.

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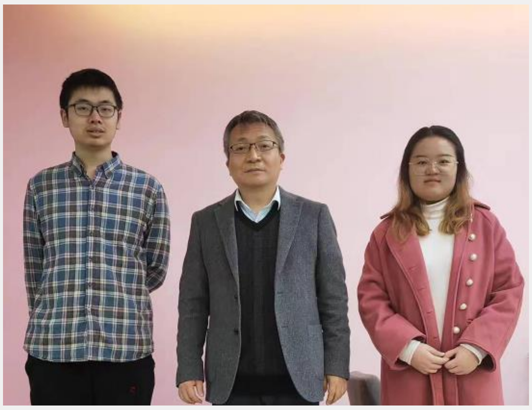
† Dedicated to the Special Issue of Carbon Dots Based Functional Materials.



Left to Right: Zhao-Nan Sun, Huan-Ming Xiong, Zhao-Fan Wu

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1. Introduction

Natural silk is the most widely studied and applied biological protein fiber, and it is also the most productive natural protein fiber in the world. Due to the advantages of soft texture, excellent mechanical properties, good biocompatibility and good degradability, natural *Bombyx mori* silk is known as the queen of fiber.[1] China is the earliest country engaged in mulberry planting, sericulture, filature and silk weaving in the world, possessing a history of more than 5700 years. As an important trade goods on the Silk Road, sericulture has brought great economic benefits to people since ancient times.[2] Today, China still produces more than 78 percent of the world's raw silk, generating an annual income of \$30 billion. With the development of modern civilization and science and technology, people are not satisfied with using silk as only textile raw materials. After the various treatment of physical, chemical and biological techniques, as shown in Figure 1 a, silk has been gradually applied in the field of drug delivery, tissue engineering, bioimaging, *etc*.[3-8] Modified silk mainly includes two categories: artificial silk obtained by chemical modification of natural silk, and functional natural silk directly produced by living silkworms.[9] The post-modification method usually involves various chemical reagents (Figure 1 b), with problems such as poor modification effect, complex process, environmental pollution.[10] It is also easy to damage the internal structure of silk and reduce the biosecurity of silk.[11-14] In contrast, natural modified silk can significantly improve the safety of silk, which has attracted special attention of researchers.[4,15]

Silkworm (*Bombyx mori*), an invertebrate insect, is widely used as a model organism in life science because of numerous advantages like requiring less sophisticated infrastructure for rearing, strong reproductive ability, short life cycle, moderate body size and easy manipulation of organs.[16] As displayed in Figure 1 b, there are two main methods to obtain natural modified silk from silkworms. One is at the genetic level, through genetic engineering technology to transform silkworm genes.[17] This method is cumbersome, requires huge human and economic costs, and risks disturbing the silkworm gene pool.[18-20] The second method is to optimize the performance and function of silkworms by adding functional materials to the food of silkworm (mulberry leaves or artificial diet) and taking advantage of the absorption or digestion of functional substances by the living silkworms.[21-22] Different from the long and difficult process of genetic modification for only one modification purpose, this method can design different additives according to requisite silk function and achieve a variety of modification effects.[9] The method also has the advantages of simple operation, short experiment period, low cost, environmental protection, and so on,[23] indicating a great potential for large-scale production.

The silk spinning progress of silkworms is that fibroin and sericin (two proteins of silk) secreted in the silk gland, after a series of physiological activities, assemble together and pass through the spinning tube and spinning mouth, and solidify rapidly under the stretching and swinging of the silkworm head to form silk.[24] The essence of feeding method with adding substances is taking silkworm organism itself as a natural material processing lab, and using silkworm's own absorption of exogenous substances, and organically combining exogenous substances with silk protein to form composite silk with excellent performance or special functions. At first, various artificial dyes were added to obtain different colors of natural silk.[25-26] Later, various substances were employed to improve other aspects of silk.

In 2014, Wang *et al.* developed a simple process for obtaining high strength silk fibers from silkworms by feeding carbon nanotubes, and the stress, strain, conductivity and thermal stability of silk fibers have been enhanced visibly.[27] After that, different nanoparticles were fed to silkworms, including graphene,[28] metal nanoparticles,[29-30] silver nanowires,[31] TiO_2 nanoparticles,[32] ion precursors[33] and carbon nanotubes[27] to obtain silk with enhanced mechanical and electrical properties, thermal stability, antibacterial properties and UV resistance.[15,23,34-35] In addition to the mechanical properties of silk fibers, some materials with excellent fluorescence performance have been fed to silkworms to obtain intrinsic fluorescent silk with different photoluminescence colors (Figure 1 c), including organic dye molecules,[36-37] carbon dots,[38-40] polymer dots,[41] quantum dots,[38] AIE nanoparticles,[42] rare earth up-conversion nanoparticles,[43] and biocompatible luminescent nanoparticles encapsulated by complex.[44] In the future, fluorescent silk will not only be applied to silk fabrics, but also have great application potential in biosensing and bioimaging, drug delivery, and tissue scaffolds.[45-46]

During the history of feeding silkworms with additives, there have been many reports about feeding silkworms with fluorescent materials, and some of them have made considerable achievements. However, there is no comprehensive review by far.

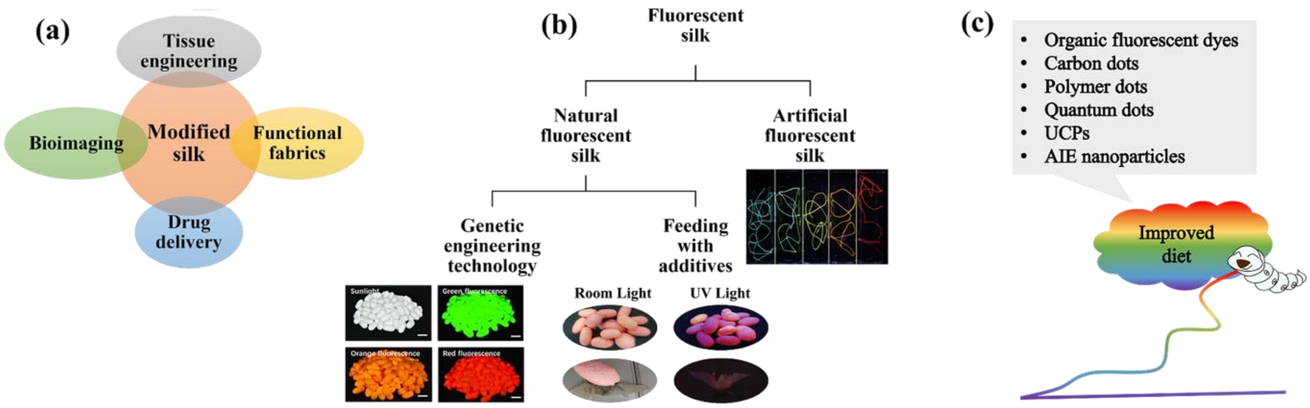

Figure 1 (a) The application fields of the modified silk in literature. (b) The kinds and the modified strategies of fluorescent silk. Reproduced with permission.[10,20,40] Copyright 2013, 2021, 2022 Wiley Online Library. (c) The representative fluorescent materials added in silkworm diet for producing fluorescent silk.

For the future exploration, it is urgent to review and discuss fluorescent materials for silkworm rearing specifically. In this review, we summarize the species, synthesis, and luminescence characteristics of these fluorescent materials, as well as applications of natural modified silk. We also summarize the common properties of substances that can cross the midgut barrier and enter the silk gland of silkworms, which benefits the development of full-color fluorescent silk. Table 1 shows the specifics of various fluorescent materials and the applications of modified silk. Afterwards, the corresponding research works are described in detail according to the types of materials.

2. Fluorescent Materials

2.1. Organic fluorescent dyes

In the 19th century, people found that some substances absorb light energy to emit fluorescence, belonging to a photoluminescence phenomenon.[47] Since then, people have used fluorescent materials for lighting,[48] bioimaging,[49] anti-counterfeiting encryption,[50] solar energy storage[51] and conversion, *etc*. More and more fluorescent materials were developed, including semiconductor quantum dots,[52] carbon dots,[53] complexes,[54] *etc.* Among them, organic fluorescent small molecular dyes are the traditional class,[47,55] which includes the famous rhodamine,[56] coumarin,[57] fluorescein,[58] cyanine,[59] BODIPY,[60] *etc.* Rhodamine derivatives have been widely applied in fluorescence sensing and imaging owing to their low cost, mature preparation process and outstanding fluorescence quantum yield.[61] Hence in 2011, natural multicolor fluorescent silk was firstly obtained by adding rhodamine derivatives to the silkworm feed.[36]

Tansil *et al*. mixed Rhodamine 101, Rhodamine 110, Rhodamine 116, Rhodamine B, sulforhodamine 101, acridine orange, and fluorescein sodium (50 g) with normal feed (100 g), respectively, and used them on the third day of the fifth instar of silkworms. As shown in Figure 2 a, among the dyes, Rhodamine 101, Rhodamine 110 and Rhodamine B are successfully taken in by the silkworms which produce purple, green, and orange yellow fluorescence cocoons finally, with the corresponding PL emission

Table 1 Synthesis and properties of fluorescent materials used to feed silkworms, and applications of the modified silk

Materials	Raw materials	Synthesis	Purification	Size/nm	PL color	PL peak/nm	QY/%	Zeta potential/mV	Application	Ref.
Rh101, Rh110, RhB	Commercial		No further purification		Purple, green, orange				Multicolor-fluorescent silkworm cocoon	[36]
Rh101, Rh110, RhB	Commercial		No further purification		Purple, green, orange				Luminescent silk as tissue engineering scaffold	[37]
GQD	Commercial		No further purification	~3	Blue	440			Fluorescent silk and imaging	[38]
CND	Citric acid and ethylenediamine	Hydrothermal		1—5	Blue	450	42.7		scaffold for cell culture and imaging	[39]
R-CDs	Mulberry-Leaves	Solvothermal	Extraction	~3	Red	676	72.6		NIR-fluorescent silk, *in vivo* imaging	[40]
CQD	Commercial		No further purification	~10	Green	530			Fluorescent silk fiber cell scaffolds and fluorescent fabrics	[38]
COOH-Pdots, NH2-Pdots	MEH-PPV, PS-PEG-COOH, MMANH2	Co-precipitation		65, 200	Red	600		-20	Scaffold for cell culture and imaging	[41]
HPS nanoparticles	HPS			~116	Blue	410—550		-30	Natural super-strong and uniform fluorescent silk	[42]
UCP	Commercial	Er^{3+}, Yb^{3+}, oleic acid, hydrothermal	No further purification	~200	Green	540			Natural degumming resistant upconversion fluorescent silk and imaging	[43]
LNP	Alq3, PMMA-*co*-MAA	Stirring at room temperature		~100	Blue green	515			Fluorescence imaging and functional fabrics	[44]

peaks at 602, 518 and 578 nm, respectively. Since the molecular structures of these rhodamine derivatives are very similar, the relationships between their log *P* (a parameter of hydrophobicity) and the amounts of dyes incorporated into the silk fiber after feeding were further analyzed. The results in Figure 2 a show that the amounts of dyes in silk are positively correlated with the degrees of hydrophobicity when the log *P* is below 0.5, but negatively correlated when the log *P* is in the range of 0.5—2.0. Obviously, the higher hydrophilicity and the negative log *P* of fluorescein sodium and rhodamine 101 make them metabolize rapidly, and thus both of them failed to accumulate in the silkworms. In addition, when log *P* is below 2, the content of each dye in sericin is higher than that of sericin, leading to the low practical application values. However, when log *P* is greater than 2, not only the dye contents in the total silk increase sharply, but also the dye in silk fibroin exceeds that in sericin. The above results indicate that only using dyes with higher hydrophobicity as additives, can obtain the desired silk by feeding silkworms.

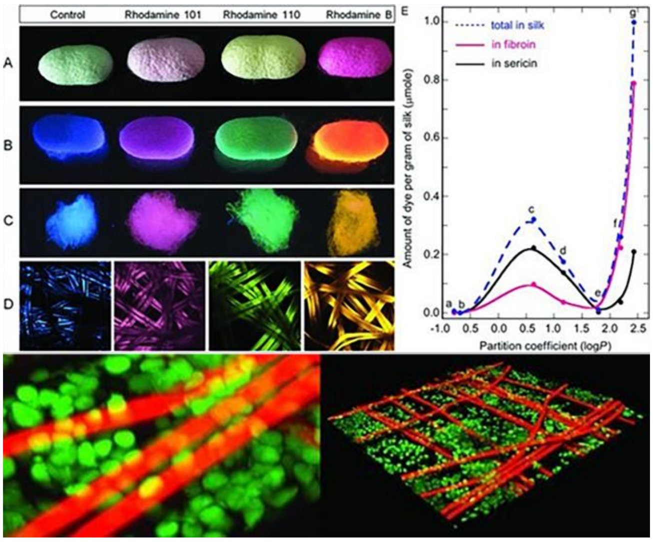

Figure 2 (a) Intrinsically multicolored fluorescent silk produced by silkworms that have consumed various fluorescent dyes and a control silk with normal feed. The relationship between various fluorescent dyes in sericin, fibroin, silk and their lipo-hydro partition coefficient (log P). The curves plotted here are to show the correlation between hydrophobicity and uptake amount and are not representative of a model or equation. Sample a: Fluorescein sodium, Sample b: sulforhodamine 101, Sample c: rhodamine 116, Sample d: rhodamine 110, Sample e: acridine orange, Sample f: rhodamine 101, and Sample g: rhodamine B. (b) 3D confocal image of human colon fibroblast cells stained with fluorescein diacetate after culturing for 10 d on a silk fibroin scaffold made of intrinsically luminescent silk containing rhodamine B. Reproduced with permission.[36] Copyright 2011 Wiley Online Library.

As displayed in Figure 2 b, a silk scaffold is constructed on a glass cover slip by degummed RhB-modified silk, and human colon fibroblasts (CCD-112CoN) grow normally after 10 d of inoculation on it. When cells are stained with fluorescein with different emission wavelengths, the intrinsic fluorescence of the scaffold allows the interface between scaffold and cells to be clearly visualized and to monitor scaffold degradation easily. It proves that the modified silk had good biocompatibility and broad application prospects in the visualization of tissue engineering scaffolds. Based on the above study, Tansil *et al.* took silkworms as a model for screening xenobiotic, focusing on the distribution of fluorescent dyes in silkworm's silk gland, metabolism in silkworm body, and biological toxicity to silkworms.[37] According to the photos of silk glands under UV light in Figure 3 a, during the 2-day feeding period, RhB is found to be mainly in the silk gland wall and then diffuses into the silk gland cavity, while acridine orange always stays in the silk gland wall. RhB could be metabolized in time by the withdrawal of feeding, while acridine orange was strongly retained. As depicted in Figure 3 b, in RhB group, cocoon breaking and reproduction of moth are normal, while silkworms in rhodamine B hexyl ester group die after 3 d. Autopsy of dead silkworms shows that rhodamine B hexyl ester is mainly packed in the wall of silkworms, and it is the high lipophilicity that leads to strong retention in the lipid of cell membrane, resulting in the higher toxicity. So that, the hydrophobic particles with hydrophilic groups at the front and back, acidic groups and positive surface charge are most likely to penetrate the midgut barrier and biofilm of silkworms to reach the silk gland.

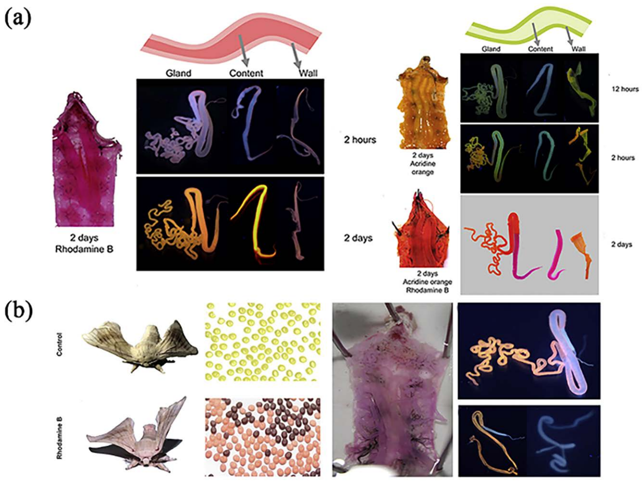

Figure 3 (a) Molecular structure-dependent uptake led to spatial and temporal distribution of fluorescent molecules in silkworms, with dominant uptake of Rhodamine B into the lumen and acridine orange in the epithelium of silk gland. (b) Passage of uptaken Rhodamine B and normal feed from silkworms to moths and eggs and the dissection situation (the distribution of the dye in gland wall and gland cavity of the silkworm) of the dead silkworm that consumed Rhodamine B hexyl ester, a highly lipophilic xenobiotic. Reproduced with permission.[37] Copyright 2011 ScienceDirect.

A series of research of Tansil *et al.* has demonstrated the feasibility of obtaining natural intrinsic fluorescent silk by adding fluorescent dye molecules to the feed of silkworms, and the application potential in fluorescent tissue engineering scaffolds. Most importantly, the characteristics of various fluorescent xenobiotic that entered silk gland cavity through silk gland epithelial cells were studied and quantified, and the rules of non-toxic and harmless exogenous substances that can be successfully taken up by silkworms were summarized.

2.2. Carbon dots

Since the discovery of fluorescent carbon nanoparticles with diameters between 1—10 nm from carbon nanotubes in 2004, carbon dots, as a new type of nanomaterials of the carbon family, have rapidly attracted intensive attention.[62-63] Research about carbon dots has risen nearly exponentially in recent years.[64] As described in Figure 4 c, due to the excellent photoluminescence performance, good biocompatibility, and low cytotoxicity, carbon dots have been widely applied in the field of biosensing, medical phototherapy, photoelectric lighting, energy storage, *etc.*[65-68] CDs are composed of a core centered on the carbon with sp^2/sp^3 hybrid and a shell modified by a variety of organic functional groups. In general, CDs are further divided into graphene quantum dots (GQD), carbon quantum dots (CQD), carbon nanodots (CNDs), and carbonized polymer dots (CPD) based on the microstructure of the carbon core.[68-70] The photoluminescence mechanism of CDs has not been completely determined. There are three main recognized PL mechanisms of carbon core, surface state and molecular state luminescence and two influence factors of the internal conjugation and external environmental effect.[71-73] As shown in Figure 4 a and b, the synthesis methods of CDs usually include top-down method and bottom-up method. The “top-down” method refers to the decomposition of large-volume carbon sources into nanosized CDs by means of light, electricity, heat, and chemistry, such as arc discharge, electrolysis, acid etching, laser ablation, combustion, and so forth.[63,68] The “bottom-up” method generates CDs through dehydration, condensation, carbonization, and other steps from the carbon sources, including hydrothermal/solvothermal method, microwave synthesis method, and solvent-free method, and this method is the main current to prepare novel carbon dots.[74] CDs have irreplaceable advantages in the fluorescent materials family. Compared with organic fluorescent molecules, CDs have more simple synthesis steps, stronger photostability and chemical stability.[75-76] Compared with metal quantum dots/metal oxide nanoparticles, CDs are easier to degrade, more environmentally friendly, and have better biocompatibility which is based on the biomass sources.[77-78] These characteristics endow CDs with wide application potential in environmental monitoring and biological techniques.

With the excellent PL properties and biocompatibility, carbon dots can be used as ideal addition agent for silkworms to obtain intrinsic multi-colorful fluorescent silk. On one hand, carbon sources for the synthesis of carbon dots are very rich, ranging from organic molecules to flowers, trees, fruits, vegetables, grains, animal hair, eggs, milk, and so on.[77,79-82] These raw materials for the synthesis of CDs with high fluorescence quantum yield are cheap and nontoxic, which benefit the large-scale preparation for feeding silkworms.[83-86] On the other hand, the properties of CDs are easily adjustable. The solvents,[87-90] reactant ratios,[91-93] reaction temperature,[94-95] precursors,[69,96-97] and time,[98-100] can be finely adjusted to regulate the fluorescent emission, quantum yield, size, chemical reactivity of CDs. Flexible tuning methods and various tunable items make it possible to precisely and rationally design CDs according to the required functionality. In general, the large-scale production, rich properties and good biosafety ensure the application potential of CDs in this field.

In 2019, Cheng *et al.* used commercialized graphene quantum dots (GQDs) to spray on fresh mulberry leaves for feeding silkworms and obtained highly stable natural blue fluorescent silk with excellent mechanical properties and good biocompatibility, demonstrating the feasibility of carbon dots used to feed silkworm.[38] GQDs are a type of carbon dots, with a diameter of about 3 nm, and a clear lattice on individual nanoparticles under high-resolution TEM, emitting blue fluorescence at 440 nm under a UV lamp. Silkworm (*Bombyx mori*) larvae were raised normally to the third day of the fifth instar under appropriate temperature and humidity. Then as shown in Figure 5 a, GQDs are sprayed on mulberry leaves to make improved diet and fed until spinning. The SEM images of the two groups of cocoon surfaces have similar microstructure. The outside and inside of modified silkworm cocoons show bright blue fluorescence under 346 nm light irradiation of stereo microscope. Fluorescence image (under the confocal laser scanning microscope) of the anterior, posterior silk gland of silkworm after dissection show that the blue fluorescence of the experimental group was significantly stronger than the control, proving that GQDs had indeed been transferred to the silk gland through digestive tract. Confocal images and fluorescence spectra of the degummed silk after long immersion show that the optimal fluorescence intensity of GQDs decreased by 20% after 30 d of immersion, explaining good fluorescence stability. The survival rate of cells cultured on modified silk exceeded 85% after 72 h. In addition, the quasistatic mechanical properties of the naturally modified silk obtained by adding GQDs to the food of silkworms were also greatly improved.

In the same year, Fan *et al.* mixed carbon nanodots (CNDs) into artificial silkworm diet to obtain natural modified silk with super mechanical properties and intrinsic fluorescence.[39] Carbon nanodots (CNDs) were prepared by a modified hydrothermal method using citric acid and ethylenediamine as precursors, with particle sizes of 1—5 nm, excitation-independent fluorescence emission at 450 nm, absolute fluorescence quantum yield of 42.7%, exhibiting excellent water dispersibility. Instead of spraying the additive on mulberry leaves, this research mixed the feed with CNDs in water suspension evenly and microwaved it into chips with different mass fractions of CNDs. As described in Figure 5 b, silkworms are fed the improved diet with gradually increasing CNDs content on the second day of the fifth instar. The silkworms of all groups grew and produced silk normally, with similar body weight at the fifth instar and similar cocoon weight, and the increased fluorescence intensity at 450 nm coincided with the CNDs itself with the CNDs adding concentration. The fluorescence images in Figure 5 b of the silk gland under 405 nm laser excitation display that the whole silk gland of the control group is light yellow-green fluorescence. With the increase of CND content, the medial and posterior silk glands of the experimental group changed

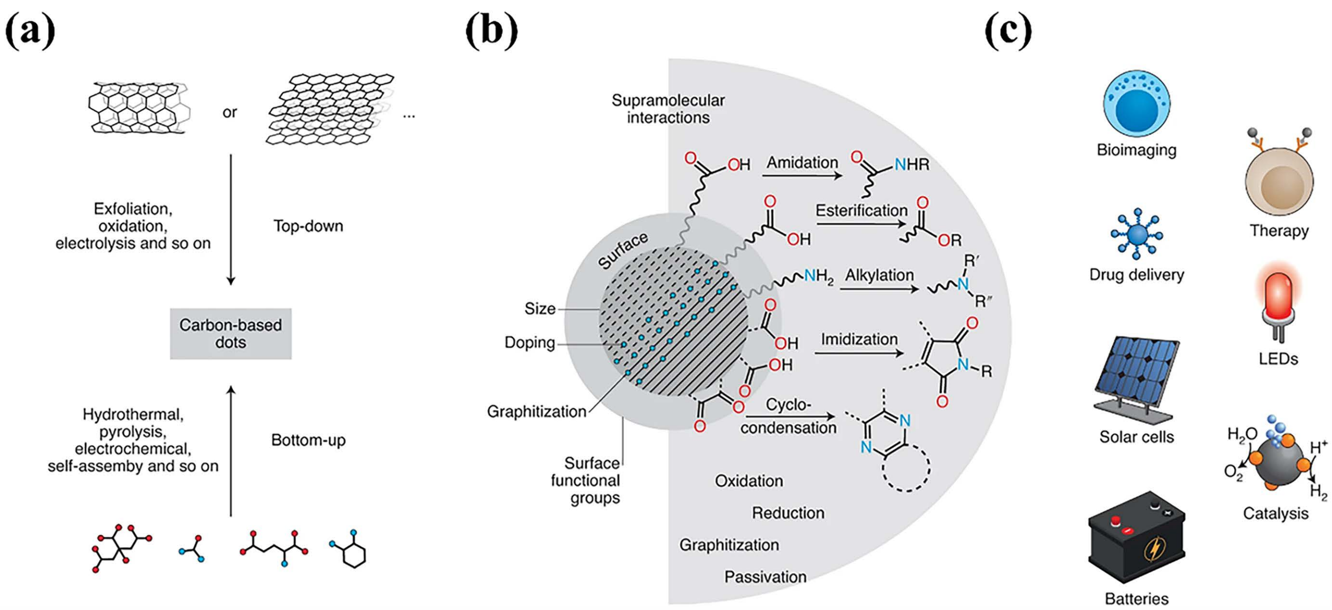

Figure 4 Overview of synthetic approaches (a, b) and applications (c) of CDs. Reproduced with permission.[67] Copyright 2022 Nature Portfolio.

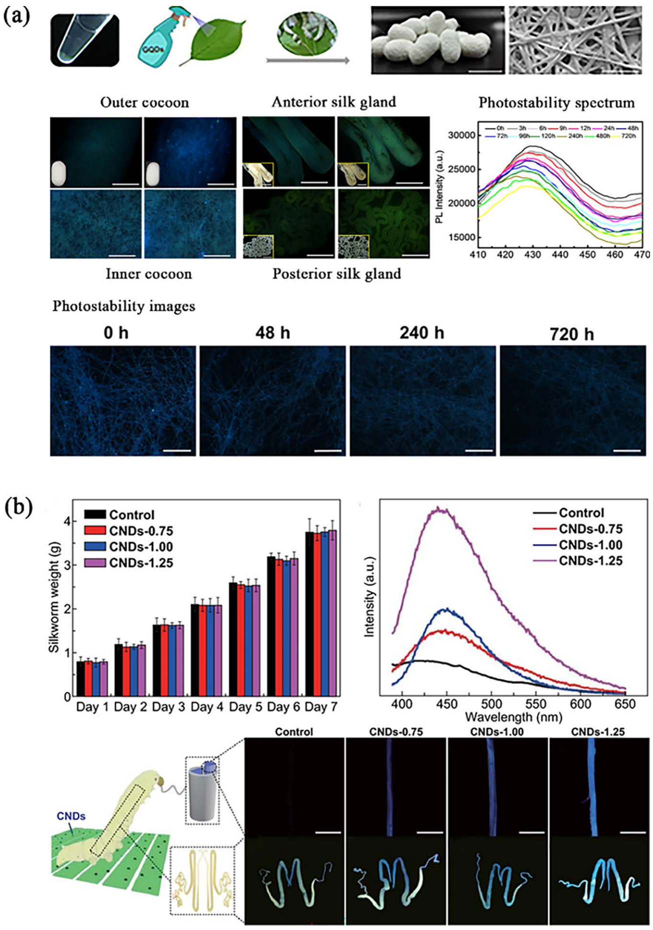

Figure 5 (a) Schematics of feeding silkworms with mulberry leaves sprayed with GQDs, and the macroscopic and microscopic structures of the as-obtained silkworm cocoons. Scale bars: 3 cm for photographs; 200 µm for SEM. Fluorescence images of the inner and outer cocoons, middle and posterior silk glands of the silkworms in experimental group, as well as the photostability images and spectral test of the degummed silk. Reproduced with permission.[38] Copyright 2019 Springer. (b) The silkworm weight record and PL spectra of degummed silk under 370 nm excitation. Schematic representation of silkworms fed the CNDs modified diet and the corresponding silk glands and fibers. Confocal laser scanning microscopy images (the scale bars represent 50 µm) of silk glands and fibers of silkworms fed modified diets with different concentrations of CNDs. Reproduced with permission.[39] Copyright 2019 Springer.

from light blue to dark blue, and the respective PL spectra in the article are also consistent with the above results. These results indicate that a small quantity of CNDs can endow silk with strong blue fluorescence, and CNDs can successfully accumulate in the silk gland of silkworms and exist in silk fibroin protein. Moreover, on the cell scaffolds constructed by the obtained fibers, the fluorescence of the modified fibers improves the visualization of cells, allowing the determination of scaffold and cell position based on the fluorescence color, as well as monitoring scaffold degradation.

In order to expand the further biomedical application of fluorescent silk, it is necessary to develop long-wavelength PL CDs to meet the bioimaging requirement. In 2022, our group synthesized a new type of red fluorescent carbon dots (R-CDs) from mulberry leaves and used them to feed silkworms, obtaining bright natural red fluorescent silk which is visible to the naked eyes.[40] The R-CDs were prepared by a solvothermal method, as displayed in Figure 5 a. The ethanol extract of fresh mulberry leaves was heated at 150 °C for 4 h, and the product was extracted by a dichloromethane and water mixture with a volume ratio of 1 : 1. R-CDs have uniform particle of about 3 nm, broad absorption covering UV-visible region, a narrow fluorescence emission peak at 676 nm with excitation independency, high absolute fluorescence quantum yield of 72.6 % in ethanol, and the deep red fluorescence can be clearly seen in sunlight. From the second day of the fifth instar of silkworms, R-CDs as additive was sprayed on the mulberry leaves until the cocoon formation. The silkworms in the experimental group grew healthy and produced bright natural red fluorescent cocoons. As performed in Figure 5 b, significant red fluorescence can be observed from living silkworms of the experimental group under a fluorescent microscope. After dissection, the digestive tract, anterior and middle silk glands of the silkworms in the experimental group showed red fluorescence under UV-light. Moreover, the ethanol extracts of cocoon coat, shell and all the ethanol extract of organs, had the same absorption and the fluorescence features, as well as similar TEM images, confirming that R-CDs were successfully ingested by silkworms and one of the metabolic pathways was from the digestive tract to the silk gland and finally to the cocoon.

For the first time, the deep red fluorescent cocoon can be observed under normal UV-light, which is different from the previous silk luminescence observed by a laser irradiation. This may be attributed to high fluorescence quantum yield of R-CDs, high uptake rate of R-CDs in silkworm body, and high transfer rate of R-CDs from the midgut to the silk gland. In addition, the distribution of R-CDs in living silkworms was studied in detail, and it was confirmed that carbon dots were successfully absorbed into the body of silkworms as proven by the spectral analyses, morphology

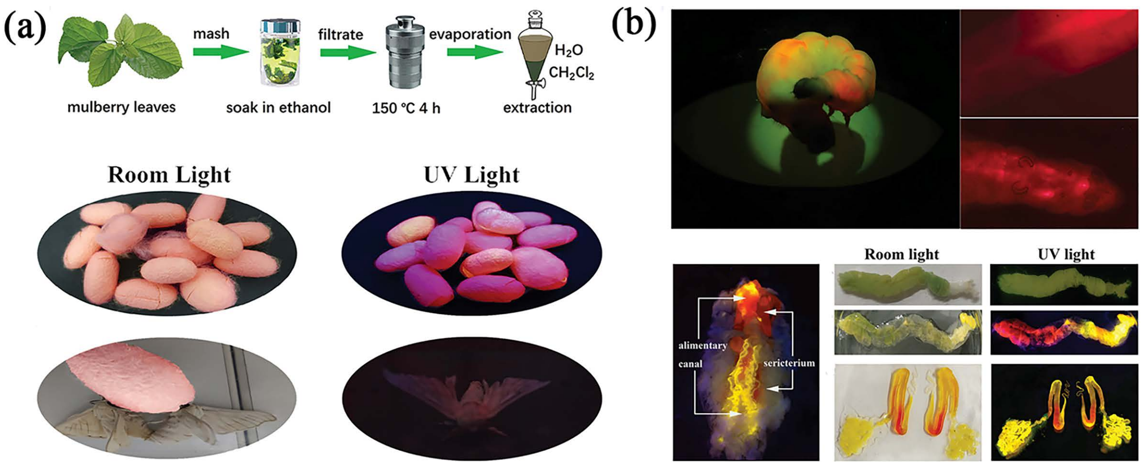

Figure 6 (a) Preparation process of R-CDs and the photos of the obtained cocoons under room and ordinary UV light. (b) Fluorescence images of living silkworms fed with R-CDs as well as the photos of the digestive tract and silk gland of silkworms in experimental and control group under room and UV light respectively. Reproduced with permission.[40] Copyright 2022 Wiley Online Library.

and fluorescence image, which strongly verified the feasibility and great application potential of CDs as fluorescent materials for feeding silkworms. Furthermore, the corresponding fluorescent silk cannot be produced by feeding another synthesized aqueous CDs to silkworms, which indicates that only hydrophobic substances can be accumulated in the body of silkworms.

In comparison with the above silk obtained by feeding silkworms with metal quantum dots, RhB molecule,[38] graphene and TiO_2 nanoparticles,[39] it is clear that CDs derived natural silk has the better fluorescence properties and good biocompability. However, the studies of CDs for silkworm feeding are still very few at present, in which only blue and red fluorescent silks are obtained, along with the shortcomings of high metabolic rate and low accumulation rate in silkworm body. It is necessary to develop natural multicolor fluorescent silkworm cocoons. Besides, to improve the PL quantum yield of CDs and balance the conversion rate of CDs into silkworms and biological toxicity are also the future striving directions.

2.3. Semiconductor quantum dots

The commercial CdSe/ZnS core-shell quantum dots (CQDs) were also used to feed silkworms, and the natural obtained green fluorescent silk was visible under laser (Figure 7 a).[38] CQDs used in this research are actually semiconductor quantum dots (QDs), rather than carbon quantum dots. It is well known that these Cd-based QDs, such as CdS, CdSe and CdTe, are toxic to life, so they are often coated by less toxic shells like ZnS before biological experiments.[101-104] The CQDs have a uniform particle size of about 10 nm and the green fluorescence emission peak at 530 nm. In Figure 7 b, the green fluorescence of CQDs is also observed in the middle and posterior silk glands of silkworms under the confocal laser microscopy, verifying the successful absorption of CQD into silkworm body. Similar with the degummed silk containing GQD, the CQDs derived silk was also tested for fluorescence stability, and the CLSM images in Figure 7 c indicate that the properties of CQDs group are better than that of the RhB group. And, the cell staining images on the silk fiber scaffolds and the strong fluorescence emission on the fabric sewn with modified silk showed broad applications in biomedicine and tissue engineering, fluorescent fabrics and anti-counterfeiting marks.

2.4. Polymer dots

Polymer dots (PDs) are new type of nanoparticles composed of π-conjugated organic polymers, with the advantages of high absorption coefficient, high quantum yield, good photostability,

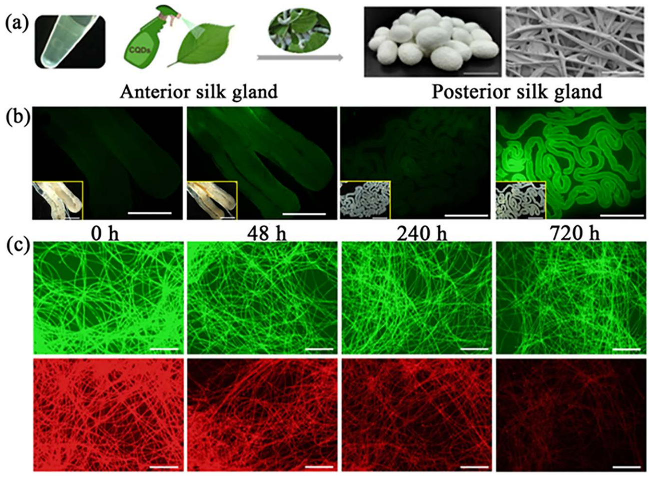

Figure 7 (a) Schematics of feeding silkworms with mulberry leaves sprayed with CdSe/ZnS QDs, and the macroscopic and microscopic structures of the as-obtained silkworm cocoons. Scale bars: 3 cm for photographs; 200 μm for SEM. (b) The CLSM images of the middle and posterior silk glands of the silkworm in experimental group. (c) The CLSM images of the degummed silk of CQDs and RhB group at different times. Reproduced with permission.[38] Copyright 2019 Springer.

and dense emission centers, usually synthesized by nanoprecipitation, minimal emulsion or less common self-assembly (electrostatic or hydrophobic).[41,105-107] Due to the tunable optical properties of PDs and their easy coupling with biomolecules, PDs have been applied in many complex biological environments, such as cell imaging, tumor imaging, and cancer phototherapy.[108-109] As shown in Figure 8, Li *et al.* incorporate PDs into the silkworm diet to obtain naturally modified silk with red fluorescence and optimized mechanical properties.[41] This study directly used the synthetic route of PDs in the previous report.[106] COOH-PDs and NH_2-PDs have the mean hydrodynamic diameters of 65 and 200 nm, respectively, with the polymer dispersion index (PDI) of less than 0.2, and the zeta potential of about −20 mV, and red fluorescence emission peak at 600 nm. The fluorescence intensity of the obtained cocoon showed that the NH_2-PDs group had a dose-dependency, but the COOH-PDs group was more suitable for low-dose adding. The two PDs existed in both sericin and fibroin, exhibiting the typical fluorescence. Moreover, a large number of H1299 cells survived healthy 24 h after seeded on the modified

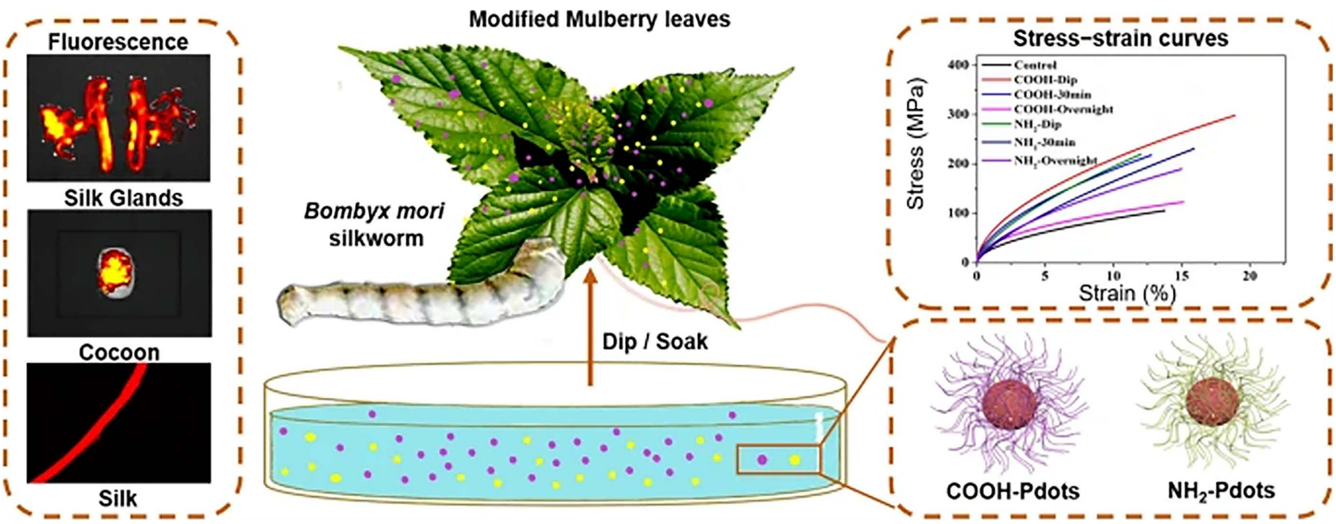

Figure 8 The approaches of artificial diets used to feed silkworms and the fluorescence images of the silkworm gland, cocoon and silk. Reproduced with permission.[41] Copyright 2022 Springer.

silk fibers, demonstrating the practical application prospects of PDs-modified silk as a cell scaffold. And, PDs were excreted from the silk glands after normal diet of 6 h, avoiding the biological toxicity caused by excessive accumulation.

2.5. Aggregated Induced Emission (AIE) nanoparticles

In the reports that use additive method to obtain intrinsic fluorescent silk, the content of the additive in the diet of silkworm is always high.[39,43] Although the high dose didn't affect the health of silkworm, it is easy to cause agglomeration and fluorescence quenching of additive in produced silk, leading to the weakness and unevenness of fluorescence.[43] Based on the above viewpoints, as depicted in Figure 9 a, Zhan *et al.* used HPS nanoparticles to feed silkworm to obtain modified silk with uniform green-blue fluorescence.[42] HPS is an important AIE molecule, whose luminescence induced by aggregation makes it an ideal material for feeding.[110-113] The HPS was prepared by high-gravity reactive method, with the particle size of 116 nm, the uniform Zeta potential of –30 mV, the fluorescence emission at 500 nm in the water, and the weight percent of 0.012% in the modified diet of silkworms. The enlarged SEM images of the degummed silk showed that there were wrinkles and particles on the surface of the modified group, and the normal group was smooth and flat. As shown in Figure 9 b, the blue fluorescence of the modified silk can be observed under a confocal laser scanning microscope (CLSM), the uniformity degree of which is higher and the content of additives used is lower, in the existing reports. The speculation was that the inhibited intramolecular rotation of HPS under the physical constraints in silk, resulted in fluorescence enhancement. As well, the HPS structure in the twisted state also weakened the intramolecular π-π interactions and effectively prevented fluorescence quenching. In addition, the mechanical properties of the modified silk displayed a great improvement in this respect.

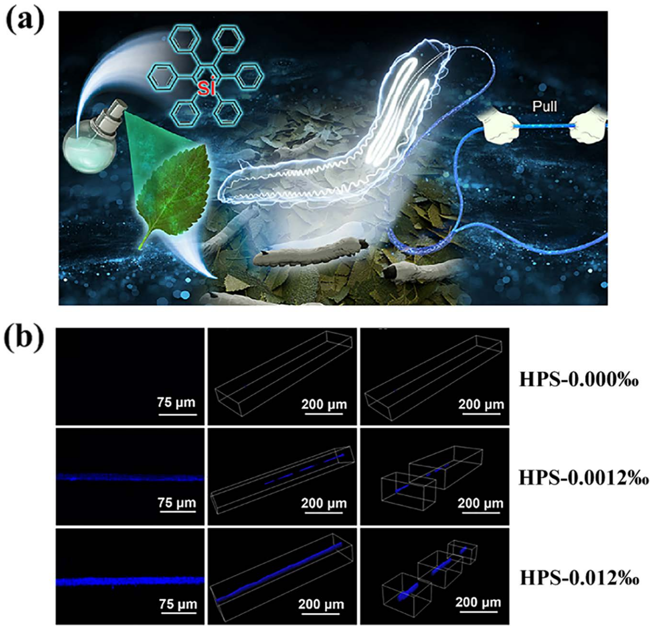

Figure 9 (a) Schematic representation of feeding the silkworm with trace AIE nanoparticles to obtain silk with high strength and uniform blue fluorescence. (b) CLSM images and corresponding 3D reconstructed images of degummed silks containing different concentration of HPS nanoparticles at 405 nm excitation. Reproduced with permission.[42] Copyright 2020 Elsevier.

2.6. Rare-earth upconversion nanoparticles

Upconversion fluorescent powders are an important class of fluorescent materials, being famous for the feature of anti-stokes shift phenomenon, and can perform short-wave emission under long-wave excitation.[114-115] Due to the compensation to high spontaneous background fluorescence, high radiation injury, and low tissue penetration depth brought by short excitation wavelength, upconversion nanoparticles have been widely used in biomedical fields such as biosensing and imaging, as well as tumor diagnosis and therapy.[116-118] In 2018, Zheng *et al.* used the upconversion fluorescent powder (UCP) as an additive to silkworm diets and obtained the successfully modified upconversion fluorescent silk.[43] The synthetic path of UCP is very common, similar to the work of Wu *et al.*,[119] which is "a hydrothermal method with lanthanide (Yb and Er) doping and oleic acid assisting". The synthesized UCP has a particle size of about 200 nm and emits green fluorescence at 540 nm under the excitation of 980 nm (Figure 10 a). From the second day of the fifth instar, the silkworms of the experimental groups fed with UCP of different concentrations could spin and make cocoons normally. Demonstrated in Figure 10 b—h, after degumming, the modified silks display uneven bright green fluorescence under 980 nm laser, and the fluorescence intensity of silk has an additive dose-dependency within a certain concentration range. In addition, high UCP content can cause morphological defects of degummed silk, decreased crystallinity of fiber and poor mechanical properties, which may be derived from the large size and easiness to aggregation of UCP particles.

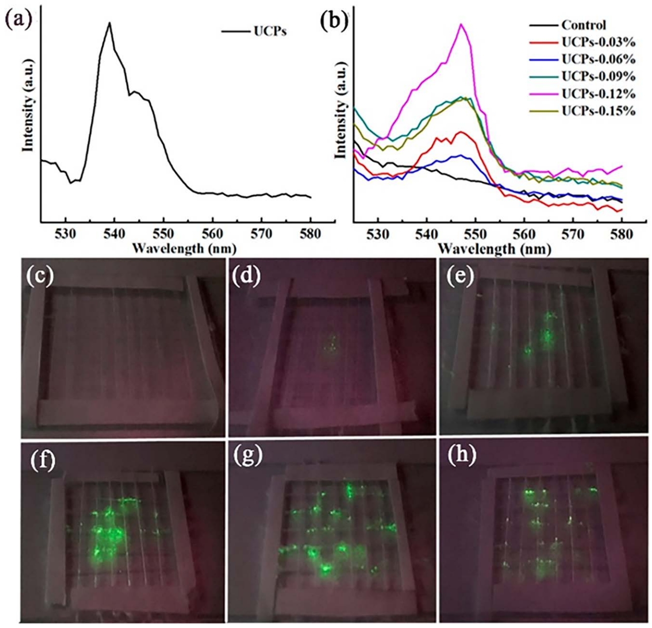

Figure 10 PL spectra of (a) UCPs and (b) degummed modified silks. Fluorescent pictures of degummed silks: (c) control, (d) UCPs-0.03%, (e) UCPs-0.06%, (f) UCPs-0.09%, (g) UCPs-0.12%, and (h) UCPs-0.15% under a 980 nm miniaturized solid-state laser. Reproduced with permission.[43] Copyright 2018 ACS Publications.

2.7. Other fluorescent nanoparticles

In 2018, Yan *et al.* successfully obtained green modified fluorescent silk by feeding silkworms with biocompatible luminescent nanoparticles (LNPs).[44] Moreover, as displayed in Figure 11, the silk is woven into silk fabric with stable fluorescence, which greatly proves the possibility of producing functional textiles by feeding silkworms with additives. An established method was chosen to encapsulate the traditional luminescent complex tris (8-hydroxyquinoline) aluminum (Alq_3) in a nanoparticle poly (methyl methacrylate co-methacrylate) (PMMA-co-MAA) with water dispersibility and good biocompatibility. The obtained LNP has a particle size of about 100 nm and a blue-green fluorescence emission at 515 nm. After LNP was sprayed on mulberry leaves and fed to silkworms, blue-green fluorescence was observed in silkworm body and cocoons. The silk fibers were twisted into yarns and then hand-woven into plain patterns, showing a blue-green fluorescence emission which could be seen under UV light. The modified silk was further processed and applied to woven into simple cloth.

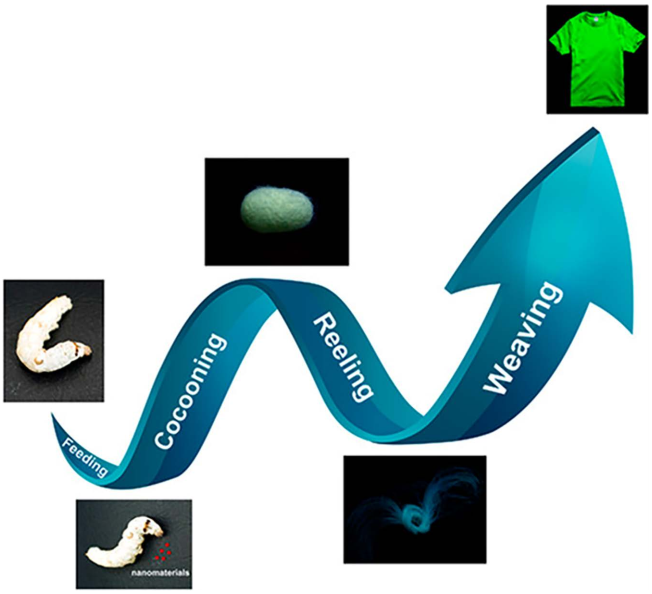

Figure 11 The preparation process of fluorescence textiles by feeding additive to silkworms. Reproduced with permission.[44] Copyright 2018 ACS Publications.

3. Conclusions and Perspectives

For a long time, the field of fluorescent materials serving as additive in silkworm feeding has been explored, but only recently it has attracted much attention. This paper reviews all the reports in the past decade to our knowledge, which modify silk by adding fluorescent materials to silkworms. It is found that the diet additive method can avoid the high cost in genetic engineering modification and the complex process in post-modification of silk, and obtain considerable modification results. However, there are still many gaps in this field: for example, except the direct use of commercial fluorescent dyes, most of the reports have obtained blue-green fluorescent silk, with a few red fluorescence, but no modified silk has been found in yellow, orange, and purple bands. Secondly, for the reported fluorescent materials, their properties such as particle size, excitation dependent situation, quantum yield, photostability, cytotoxicity, lipid-water partition coefficient (log *P*), *etc.*, have not been analyzed and summarized in detail. Thirdly, the feeding time of silkworms, the preparation method of the modified diet (directly spraying on mulberry leaves or mixed with artificial feed) and the dosage of fluorescent additive were not systematically optimized, though these factors would also affect the final performance of the modified silk. Fourthly, the distribution ratio of fluorescent materials in silk fibroin and sericin, and the absorption, transport, distribution and metabolism in silkworm were unclear by far. Fifthly, most of the related reports only present fluorescent silk, but do not show specific applications in bioimaging, tissue engineering scaffolds, photothermal therapy, luminescent fabrics and other deep aspects, which means the production yields of those modified silk are actually very low.

From a viewpoint of material research, to successfully obtain modified intrinsic fluorescent silk, the following requirements are necessary for additives. First of all, excellent biocompatibility, high quantum yield and long term photostability are basic requirements. In addition, small particle size, strong hydrophobicity with suitable hydrophilic groups on the surface, and negative charge on the surface are allowed to penetrate the midgut barrier and the silk gland epithelial cells by digestive tract, and successfully reach the silk gland of silkworm. Furthermore, the materials used had better possess a certain ability of anti-aggregation induced quenching effects to ensure the intensity and uniformity in silk luminescence.

The fluorescent additives discussed in this article include organic dye molecules, carbon dots (CDs), polymer dots (PDs), semiconductor quantum dots (QDs), upconversion nanoparticles (UCPs), AIE nanoparticles, *etc.* Each of them has its own superiorities and defects. Organic dye molecules are traditional fluorescent materials. Although the process is mature, they are usually highly biotoxic and easy to cause environmental pollution when used in a large scale. In the family of nanoparticles, although QDs and UCPs have strong fluorescence stability and narrow emission peak width, their heavy metal elements contents are toxic to life. Moreover, the complex preparation process and the large particle size are not beneficial for them to the uniform distribution in silk glands and modified silk. In contrast, metal-free carbon-based nanomaterials have better advantages in biocompatibility, so CDs, like graphene quantum dots (GQDs), carbon nanodots (CNDs), carbonized polymer dots (CPDs) and CDs with AIE properties, are ideal fluorescent additives for feeding silkworms. However, at present, this application field has not been fully developed and the number of reports is very few, but it has shown great potential and bright prospects.

What needs to be done in the future is to design more fluorescent CDs according to the existing rules in silkworm feeding experiments, and to conduct research on synthetic raw materials, optical properties, feeding scheme, silkworm body distribution, biological application and other aspects. The most urgent task is to expand the fluorescence band of the modified silk and conclude more precise rules of additives properties. It is suggested that the luminophore and mechanisms of organic dyes and AIE materials should be referenced in the development of novel CDs to prepare ideal ones with various excellent properties. Finally, we also look forward to emerging more reports in which other fluorescent materials are used for feeding silkworms to obtain modified fluorescent filaments.

Acknowledgement

This work was financially supported by the National Natural Science Foundation of China (21975048, 21771039), and the Shanghai Science and Technology Committee (19DZ2270100).

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Manuscript received: January 28, 2023
Manuscript revised: March 9, 2023
Manuscript accepted: March 21, 2023
Accepted manuscript online: March 23, 2023
Version of record online: May 16, 2023

