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# Design and development of fluorescent nanostructures for bioimaging

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## ABSTRACT

Because fluorescence-based techniques are inherently sensitive, selective, convenient, diverse, non-destructive, potentially real time and *in situ*, they have been widely used in biological imaging. Especially those, with specific fluorescent nanostructures (FNSs) as detecting media in bioimaging, have already been intensively studied for more than a decade because of the convenient transduction of optical signal, high sensitivity and rapid response of FNSs. In this review, we summarize the major strategies to design FNSs with specific structures for biological imaging. First, recent advances are briefly introduced. Then, the specific design of FNSs and their applications are reviewed, in which their fluorescence mechanism, strategies in designing and development, preparation methods, and some representative applications in bioimaging are described. Finally, future perspectives and ongoing issues of FNSs and their applications in bioimaging are discussed. Although many FNSs have been synthesized and applied biologically, many studies still should be done before they can be widely employed as fluorescent probes in clinical tests. With further advances in design and synthesis of high quality multifunctional FNSs, the widespread application of FNSs may be expected not only in advanced bioimaging, but also in ultra-sensitive molecular diagnosis, novel light-emitting nanodevices and intracellular drug delivery.

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## Nomenclature

AAc	acrylic acid
ACQ	aggregation-caused quenching
AS-ODN	antisense oligonucleotide
ATRP	atom transfer radical polymerization
BODIPY	boron-dipyrromethene
BRET	bioluminescent resonance energy transfer
COPV	1,4-dimethoxy-2,5-di [4'- (cyano)styryl]benzene
CQD	carbon quantum dot
DMAEMA	2-dimethylamino ethyl methacrylate
DSA	9,10-distyrylanthracene
DSB	1,4-distyrylbenzene
ECM	extracellular matrix
FA	folic acid
FCNP	fluorescent capsid nanoparticle
FNS	fluorescent nanostructure
FRET	fluorescence resonance energy transfer
FQY	fluorescence quantum yield
GMA	glycidyl methacrylate
GQD	graphene quantum dot
HBC	hexa-peri-hexabenzocoronene
HCP	hyperbranched conjugated polymer
HEMA	2-hydroxyethyl methacrylate
LBL	layer-by-layer
LRP	living radical polymerization
MEF	mouse embryonic fibroblast
MMA	methyl methacrylate
MNP	magnetic nanoparticle
NC	nanocluster
NIPAM	<i>N</i> -isopropylacrylamide
NIR	near-infrared
NMRP	nitroxide mediated radical polymerization
NP	nanoparticle
NVK	<i>N</i> -vinylcarbazole
PAMAM	poly(amidoamine)
PBS	phosphate buffered solution
PCPE	phosphorescent conjugated polyelectrolyte
PDI	perylene diimide
PEBBLE	probe encapsulated by biologically localized embedding
PEGMA	poly(ethylene glycol)methacrylate
PGD	polyglycerol dendrimer

PS	polystyrene
QD	quantum dot
RAFT	reversible addition-fragmentation chain transfer
RE	rare earth
RhB	rhodamine-B
SiNP	silicon nanoparticle
SWNT	single walled carbon nanotube
TPA	triphenylamine
TPE	tetraphenylethene
TPF	two-photon fluorescence
TTA	triplet-triplet annihilation
UCNP	upconversion nanoparticle
UV	ultraviolet
VBC	4-vinylbenzyl chloride

## 1. Introduction

Biological imaging (bioimaging) has become a powerful tool in biological research today because it offers an unique approach to visualize the morphological details of cells [1]. To date, fluorescence-based techniques have been greatly encouraged in bioimaging due to their inherent superiorities, such as high sensitivity, high selectivity, convenience, diversity and non-destructive character [2]. Typically, fluorescent probes are exploited to label the target with specific chemical structures and thus to generate fluorescent signals during the fluorescence-based bioimaging. Since nanostructure-based detection platforms can provide many advantages over traditional approaches in terms of sensitivity, signal stability and multiplexing capability, a growing interest has been shown recently in the design of different fluorescent nanostructures (FNSs) as fluorescent probes in bioimaging [3–6]. Currently, the most studied FNSs in bioimaging include fluorescent proteins, organic dyes, metal complexes, semiconductor nanocrystals, and upconversion nanophosphors [7–10]. In order to obtain a better fluorescent probe, further works on FNSs with recommended chemical and optical properties have also been reported. For example, surface modification of FNSs has been done with bright fluorescence, high photostability, large Stokes shift and flexible processability in order to be further conjugated with biomolecules and/or fluorophores [6].

In this review, classification of FNSs, their fluorescence mechanisms and applications in bioimaging are summarized. According to the compositions and structures of FNSs, they are divided into three classifications, i.e., organic FNSs, inorganic FNSs and organic/inorganic hybrid FNSs (Fig. 1). Based on the classification of FNSs, their design strategies, fluorescing mechanisms, size-dependent optical properties and preparing methods are introduced and discussed. Especially, their representative applications in bioimaging at the cell- and tissue-levels are reviewed. Furthermore, potentials of FNSs and their perspectives in the field of bioimaging, based on their advantages and security, are discussed.

## 2. Organic fluorescent nanostructures

Among all of the FNSs used in bioimaging, organic FNSs are the most widely studied due to their rich chemical structures, easy chemical modification and high fluorescence quantum yield (FQY) [11,12]. Generally, these organic FNSs include carbon-based fluorescent nanostructures, fluorescent macromolecules, fluorescent polymeric nanoparticles, fluorescent supermacromolecular nanoassemblies and aggregation-induced emission fluorophores.

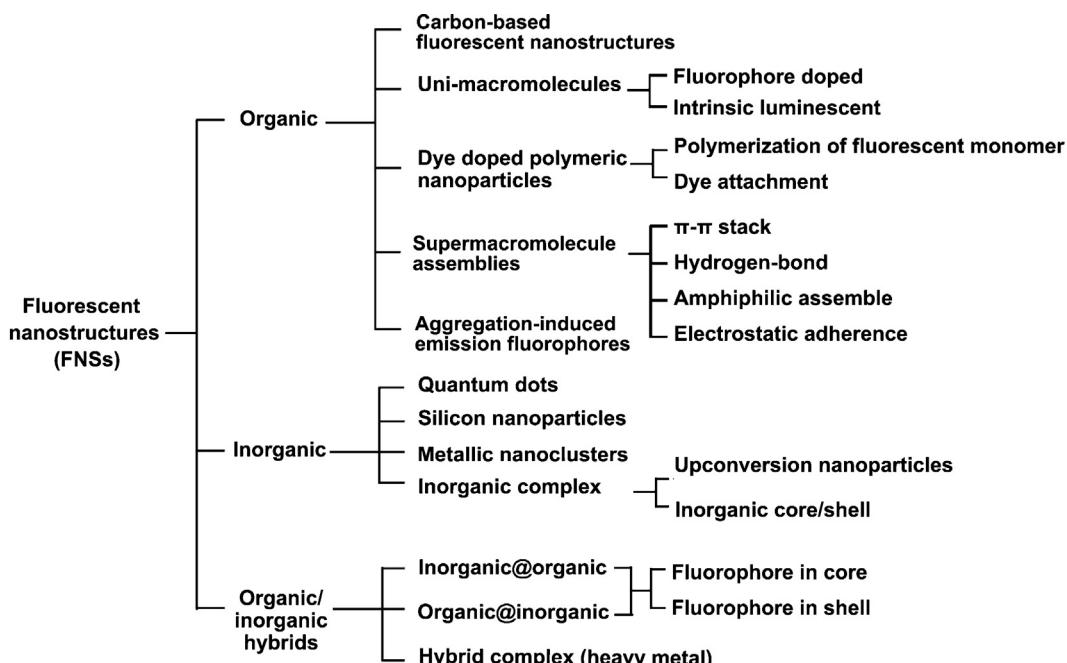
### 2.1. Carbon-based fluorescent nanostructures

Carbon-based FNSs are the basic organic fluorescent materials with only carbon elements and are currently one of the most attractive nanomaterials with different forms, such as fullerenes, single- and multiple-walled carbon nanotubes, carbon nanoparticles, nanofibers, and so forth. Such carbon-based FNSs have become important

due to their unique chemical and physical properties (*i.e.*, good thermal and electrical conductivity, high mechanical strength and optical properties), which are essential in many biomedical areas, such as bioimaging, drug delivery, tissue scaffold reinforcements, and cellular sensors [13]. However, these carbon-based nanomaterials show size-dependent toxicity. Moreover, cytotoxicity is enhanced when their surface is functionalized after an acid treatment. Therefore, further effort should be made on carbon-based FNSs [14].

Recently, zero-dimensional carbon-based FNSs, such as carbon quantum dots (CQDs) [15–18] and graphene quantum dots (GQDs) [19,20], have aroused great interest in bioimaging due to their strongly multiphoton-fluorescence and extremely large two-photon crosssections on pulsed laser excitation (800–900 nm) [21,22]. For example, Yang et al. [22] prepared nontoxic fluorescent CQDs with surface-passivation by oligomeric PEG<sub>1500N</sub>. In addition to the apparent biocompatibility, CQDs exhibited competitive (on the order of magnitude, at least) fluorescence imaging performance to that of the commercial CdSe/ZnS QDs (introduced in the following), demonstrating their potentials for both *in vitro* and *in vivo* applications. Similarly, Qian et al. [23] synthesized a series of functionalized GQDs with different small organic molecules (dialcohols, diamines and dithiols). By altering size and surface functionalization of GQDs, effective modulation of their photoluminescence could be achieved, which could render great performance of GQDs in HeLa cell bioimaging. Since many reviews have been published on the advantages of such zero-dimensional carbon-based FNSs in bioimaging [24–26], they will not be discussed extensively in this review.

Compared with the materials mentioned above, carbon nanotubes have shown unique optical properties



**Fig. 1.** Classification of fluorescent nanostrucutures (FNSs) based on their different chemical structural compositions.

in biological application [27–29]. Single walled carbon nanotubes (SWNT) are upcoming potential candidates for the fluorescence imaging agents because they generate fluorescence brightly in the 800–1600 nm wavelength range of the near-infrared (NIR) region that has greater penetration depth and lower excitation scattering [30,31] ( $FQY = 1.7 \times 10^{-4}$ – $10^{-3}$ ), which has been shown very useful in biological tissue imaging. One key advantage of SWNTs is their ability to translocate through plasma membranes, allowing their use for the delivery of therapeutically active molecules in a manner that resembles cell-penetrating peptides [28]. As labeling or imaging agents, SWNTs are bright enough to allow image deep inside living body at high frame rate without excessive excitation power [32]. For example, Robinson et al. [33] demonstrated

the application of intravenously injected SWNTs as photoluminescent agents for *in vivo* tumor imaging in the 1.0–1.4  $\mu\text{m}$  emission region with a high tumor uptake of SWNTs, which is the first time to use the intrinsic NIR photoluminescence of SWNTs for tumor imaging.

Generally, fluorescence imaging of SWNTs provide powerful tools to trace the interactions of nanotubes with cells, tissues, and organisms. And the auto-fluorescence in the NIR region is much lower than that in the ultraviolet or visible ranges. These properties make SWNTs potential imaging agents with higher resolution and greater tissue depth for NIR fluorescence microscopy and optical coherence tomography. However, aggregation of nanotubes into bundles that quenches the fluorescence through interactions with metallic tubes and substantially broadens the

**Table 1**

The most reported organic dyes conjugated to fluorescent uni-macromolecules.

Small organic fluorophores	General structural formula	Refs.
Anthracene		[44]
Coumarin		[45–48]
BODIPY		[49–51]
Fluorescein		[52–56]
Rhodamine		[57–59]
Perylene bisimides		[60–65]
Cyanine		[66–71]
Donor- $\pi$ -acceptor or acceptor- $\pi$ -acceptor fluorenyl derivatives	 $R_1 = \text{C}_6\text{H}_4\text{CO}^- \text{O}^-, \text{C}_6\text{H}_4\text{CH}_2\text{Cl}, (\text{CH}_2=\text{CH})_n \text{H}, \text{C}_6\text{H}_4\text{C}_6\text{H}_{11}, \text{C}_6\text{H}_4\text{C}_6\text{H}_{12}\text{Cl}, \text{C}_6\text{H}_4\text{C}_6\text{H}_{12}\text{Br}, \text{C}_6\text{H}_4\text{C}_6\text{H}_{12}\text{I}, \text{C}_6\text{H}_4\text{C}_6\text{H}_{12}\text{NO}_2, \text{C}_6\text{H}_4\text{C}_6\text{H}_{12}\text{SO}_3^- \text{Na}^+$ 	[72–75]

$R_1$  = Donor or Acceptor,  $R_2$  = Acceptor

absorption spectra [34]. Therefore great effort should be needed to improve the dispersion of individual SWNT for better performance of SWNT in bioimaging.

## 2.2. Fluorescent macromolecules

A fluorescent macromolecule is an individual organic macromolecule that generates fluorescence after the absorption of excitation light. Depending on the fluorescent mechanism, fluorescent macromolecules can be usually divided into two classes: (1) fluorophore-doped macromolecules and (2) intrinsic-fluorescent macromolecules.

### 2.2.1. Fluorophore doped macromolecules

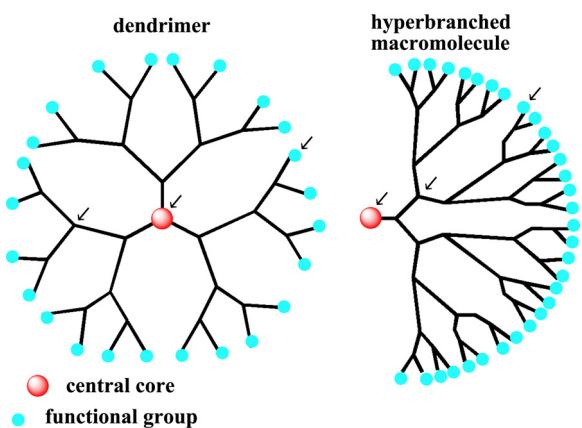
A fluorophore-doped macromolecule is a unimolecular nanostructure with organic fluorophores. And the fluorophores have been identified as small organic dye molecules or their derivatives with resonant emission [35–43], such as fluorescein, rhodamine, perylene, cyanines, boron-dipyrrromethene (BODIPY), and two-photon absorbing materials. The most often reported fluorophores are summarized in Table 1. And these macromolecules exhibited asymmetric emission spectra (400–700 nm, only a few NIR dyes could reach above 700 nm; FQY=0.5–1.0 (visible), 0.05–0.25 (NIR)) as the original fluorophores did.

The fluorophore-doped macromolecules, such as dendrimers and hyperbranched macromolecules (Fig. 2), were developed into core–shell structures *via* the following strategies [76,77]. The macromolecules could display the original fluorescence with the chromophores located in the core or the branch sites or the periphery. Meanwhile, the desired properties of probes, such as stability, biocompatibility, and specificity of labeling, would be obtained by grafting or growing the shell.

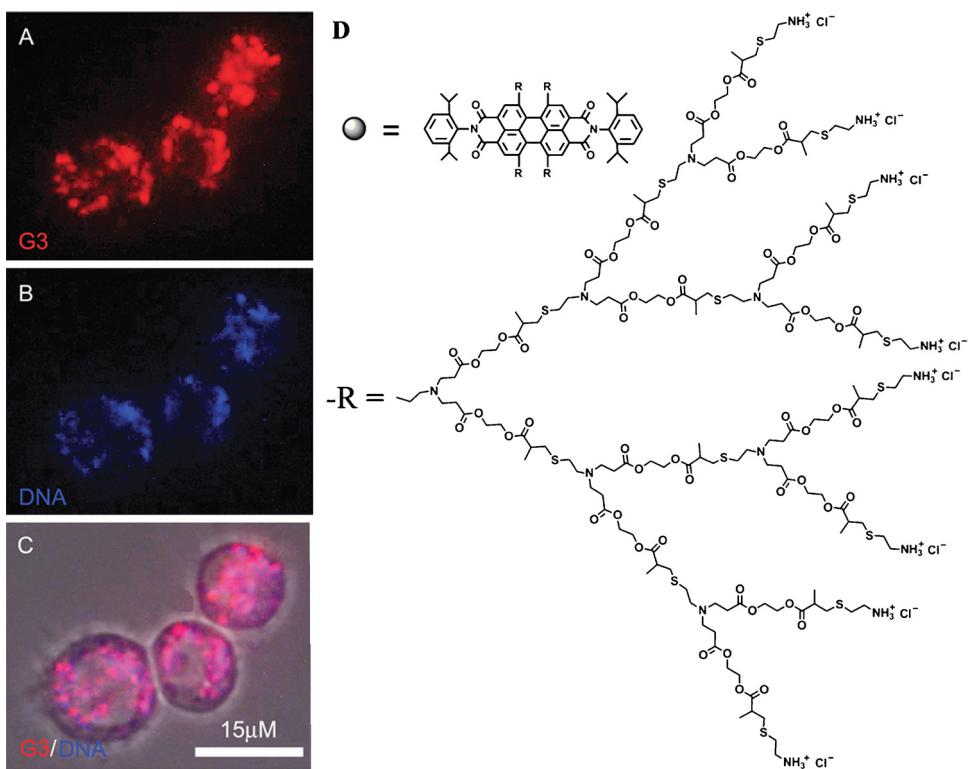
A dendrimer is a nanoscaled macromolecule with well-defined branching structure. Fluorescent dendrimer can be prepared with high regularity and controllable molecular weights, and it has a fluorophore-doped central core linked covalently to uniform repeating units (generations) and many terminal groups. Those well-defined dendrimers were reported as imaging agents in gene delivery system

[78–84], in which higher generation materials (G6 or G7) were required because low generation dendrimers could not condense siRNA into uniformly small complexes [85]. However, due to the increased toxicity of their growing generations [86], it is very important to improve the biocompatibility of dendrimers. Recently, Yin and co-workers [60] reported the preparation of G1, G2 and G3 of perylenedimide (PDI)-cored cationic dendrimer with peripheral amine groups, which could rapidly enter live cells with high gene transfection efficiency and low cytotoxicity (Fig. 3). Yang et al. [87] also synthesized monovalent probe on a ring-fused BODIPY core that is conjugated to a polyglycerol dendrimer (PGD), which has exhibited excellent brightness with an emission maximum at 705 nm.

Unlike dendrimers, fluorescent hyperbranched macromolecules suffer from difficulty in structural control, because its functional groups are located randomly, and not all the reactive sites in the repeat units have reacted. Compared with highly regular dendrimers, structural defects of fluorescent hyperbranched macromolecule offer better flexibility for its bioimaging applications, because both the branching arms and branching points could provide the reactive sites for the incorporation of functional groups. Among these fluorescent hyperbranched macromolecules, star polymers are the simplest branched material, with several linear polymer chains attached to a single branching point (core) [88–90]. The “core-first” method mostly starts from a fluorescent macro-initiator (core) and the well-defined architecture can be realized *via* living radical polymerization (LRP). Radical polymerization approach offers several advantages, especially in terms of compatibility with both aqueous and organic media as well as an excellent tolerance to many functional groups. LRP includes nitroxide mediated radical polymerization (NMRP) [91,92], reversible addition-fragmentation chain transfer (RAFT) polymerization [93–96], and atom transfer radical polymerization (ATRP) [97–99]. Among the reported LRP techniques, ATRP is most frequently used in the preparation of well-defined fluorescent star polymers. Yin and Müllen [100–105] reported several examples using this method. For example, they successfully synthesized a positively charged fluorescent core–shell dendritic star polymer [102]. They started with a macro-initiator with a central PDI chromophore and a first-generation polyphenylene dendrimer scaffold, then initiated the outer shell, which was consisted of eight-armed linear polymers with multiple amine groups (Fig. 4(a)R1). Such designed structure exhibited great water solubility and fluorescent property with positive charges, which could bind to the highly negatively charged extracellular matrix (ECM, Fig. 4(b)). Similarly, they synthesized a series of negatively charged dendritic star polymers with eight-armed linear polymers bearing multiple carboxyl groups (Fig. 4(a)R2), aiming at specifically staining the cell nucleus by binding to positively charged nuclear proteins (Fig. 4(c)) [105]. Additionally, some complicated fluorescent hyperbranched macromolecules have also been reported, in which the fluorophores could be conjugated in the branched arms [106] or cores [65] to achieve desired optical properties. Such structures offered more available strategies for site-specific covalent labeling of cells or tissues.



**Fig. 2.** Structures of fluorescent dendrimer and hyperbranched macromolecules. The chromophores could be in the central core or the branch sites or the periphery (indicated by arrows).



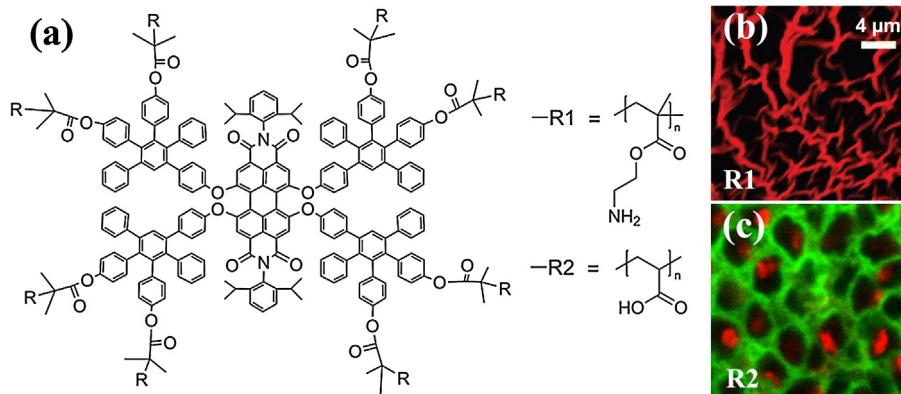
**Fig. 3.** Fluorescence images (a–c) of the G3-DNA complex inside of cells after 48 h of incubation. (a) G3 fluorescence image (red); (b) CXR reference dye labeled DNA (blue); (c) merged channels of (a) and (b); (d) structure of perylenediimide (PDI)-cored dendrimers [60]. Copyright 2013. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.) Adapted with permission from the Royal Society of Chemistry.

Generally, the optical property of fluorophore-doped macromolecule is attributed to the conjugated fluorophores, while its specific labeling capability is owing to the superficial functional groups. Moreover, the sizes of these macromolecules could be in the range of 1–20 nm [101], which are dependent on the designed structure and the chemical environment (pH, solvent, temperature, etc.). By tuning the polymer arms or the generation of macroinitiator, the size of the nanostructure could be controlled, which would further affect its performance in specific fluorescent

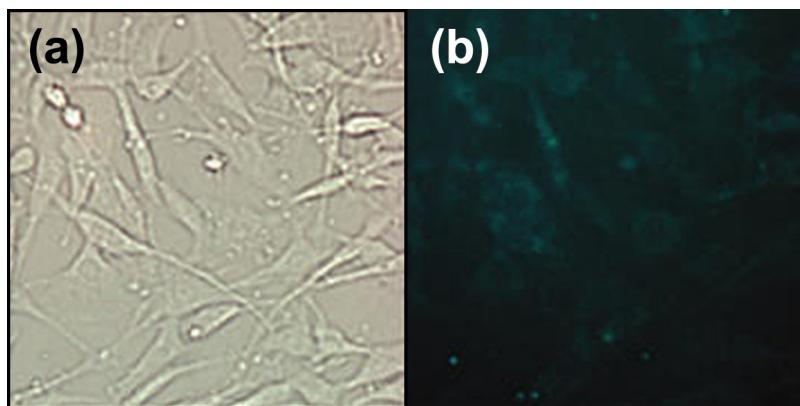
labeling in the following two ways [107,108]: (1) the fluorescence intensity of dendritic structure is determined by its generation; (2) higher dendron leads to larger size and offers more superficial functional groups to combine biological targets.

#### 2.2.2. Intrinsic-fluorescent macromolecules

An intrinsic-fluorescent macromolecule is a type of nanostructure that exhibits fluorescence (400–500 nm) without fluorophore conjugation ( $FQY = 0.025\text{--}0.98$ ).



**Fig. 4.** (a) Structure of fluorescent core–shell dendritic star polymer (R1: positively charged, R2: negatively charged); (b) fluorescent image of R1 labeled extracellular matrix; (c) fluorescent image of R2 labeled nuclear proteins [102,105]. Copyright 2008. Reproduced, respectively, with permission from the American Chemical Society and Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.



**Fig. 5.** (a) Optical and (b) fluorescence microscopic images of C6 cells transfected with the complex of fluorescent G4 PAMAM dendrimer and AS-ODN for 12 h [114]. Copyright 2011. Adapted with permission from the American Chemical Society.

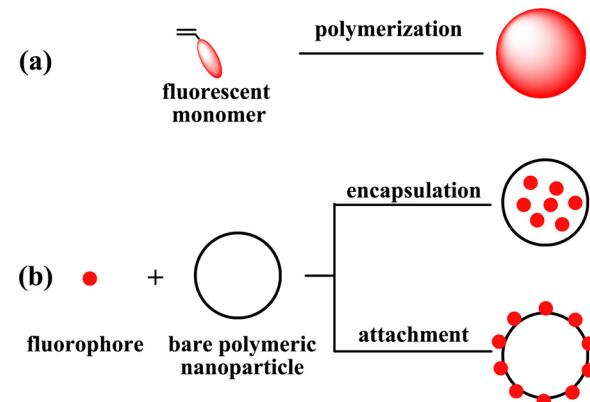
Representative examples are dendrimers or hyperbranched macromolecules with aliphatic tertiary amine, such as the hyperbranched poly(amine-ester), poly(amidoamine) (PAMAM), poly(propyletherimine) dendrimers and so on [109–113]. Since these FNSs are considered as biocompatible, nonimmunogenic and water-soluble gene vehicles, they exhibit many potential advantages in bio-applications. For example, Tsai et al. [114] successfully synthesized PAMAM dendrimers with numerous primary amines on the surface. The dendrimers with intrinsic blue fluorescence obtained were used to monitor the gene delivery and transfection toward rat C6 glioma cells directly. They also combine antisense oligonucleotides (AS-ODN) with PAMAM dendrimers electrostatically to knock down specific protein expressions. Based on fluorescence of the resulted PAMAM–(AS-ODN) complexes, the uptake process could be evaluated directly via fluorescence techniques (Fig. 5). As new fluorescent probe materials in bioimaging, intrinsic-fluorescent macromolecules attracted substantial interest to understand their luminous mechanism. However, fluorescence of these FNSs can be quenched easily and strong fluorescence has been observed only in vapor phase. Therefore, Pan and his co-workers [115–117] made great effort to retain the strong fluorescence of these FNSs. Although intrinsic-fluorescent macromolecules have been extensively studied [118,119], so far the intrinsic-fluorescent mechanism has not been studied systematically.

### 2.3. Fluorescent polymeric nanoparticles

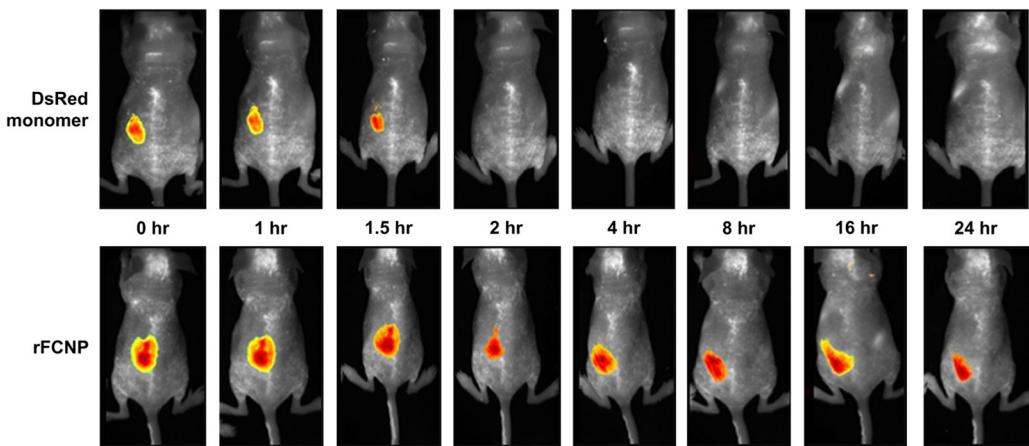
Dye-doped polymer nanoparticles (NPs) are cross-linked polymeric spheres in which fluorescent dyes were covalently attached or physically entrapped. Typically, these FNSs were synthesized through the copolymerization of fluorescent monomers with other functional monomers [120–126] or the encapsulation of fluorescent organic dye molecules inside or on the matrix (Fig. 6) [127–130]. Because of polymeric NPs supports (with sizes of ~100 nm), the composite FNSs exhibit good biocompatibility, controllable morphology and original optical property of the doped dye.

By using fluorescent monomer, the required optical properties could be introduced into the product after the polymerization. Because the chromophore was incorporated in the particle *via* chemical bond, the resulted FNPs had good stability and desired fluorescence. For instance, Marco et al. [131] synthesized a fluorescent monomer *via* binding the fluorescent dye Rhodamine-B (RhB) and 2-hydroxyethyl methacrylate (HEMA) covalently, then produced fluorescent NPs *via* the free radical emulsion copolymerization of the fluorescent monomer with methyl methacrylate (MMA) for bioimaging. Similarly, Sauer et al. [132] synthesized a fluorescent surfmer (surfactant monomer) by combining surface activity, polymerizability and fluorescent property within one molecule. By using the fluorescent surfmer, fluorescent surface-labeled polystyrene (PS) NPs were synthesized *via* miniemulsion polymerization. This approach could offer the potential to size-controlled fluorescent NPs by using specific polymerization. In addition, the steric hindrance of polymeric nanoparticle should be considered during the material design and the analysis of properties.

Due to the limited number of fluorescent monomers for polymerization, other better ways are still being explored



**Fig. 6.** Two strategies toward fluorescent polymeric nanoparticles: (a) polymerization of fluorescent monomers; (b) encapsulating fluorophores inside or attaching fluorophores onto the bare polymeric nanoparticles.

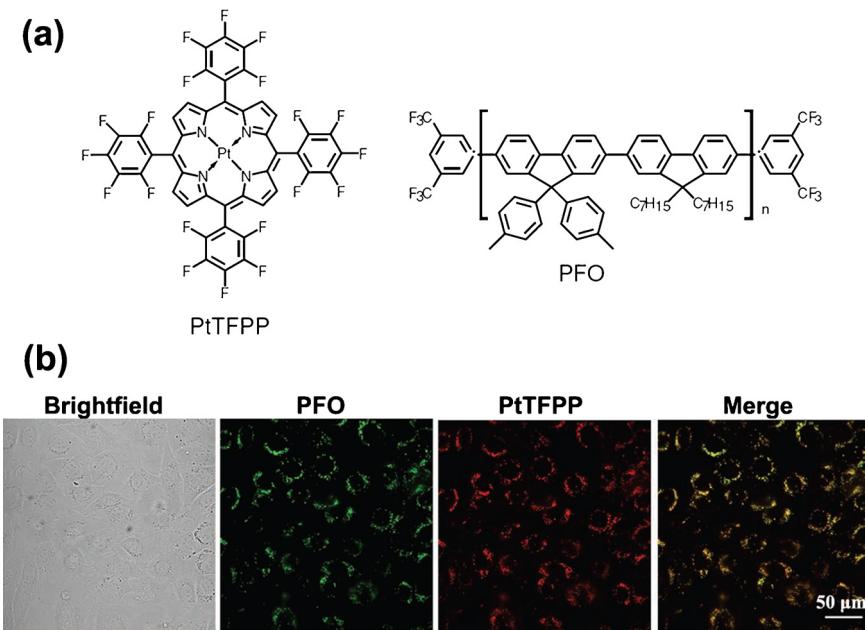


**Fig. 7.** Estimation of *in vivo* stabilities of rFCNP and DsRed that are subcutaneously injected into BALB/cSlc-*nu* mice (female 4 weeks old, JAPAN SLC, Inc.) [133]. Copyright 2012.  
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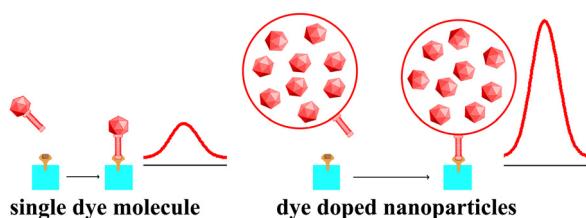
to synthesize NPs with good fluorescent properties. Wherein, the bare polymeric NPs with desired sizes were prepared first and then the combination of dyes was realized to achieve their fluorescence *via* chemical cross-links. Due to the chemical cross-links, the composite FNSs were very stable, but special functional groups were required for the integration between NPs and fluorophores. Varied sized fluorescent polymeric NPs could have different kinetic profiles, uptake and distribution rates, *etc*. Therefore, multifunctional FNSs were developed on the basis of various support polymeric materials. Yoo et al. [133] synthesized hepatitis B virus capsid particles and genetically grafted fluorescent protein (DsRed or eGFP) onto their surface, which resulted in the construction of red or green

fluorescent capsid nanoparticles (FCNPs). Because of the *in vivo* high stability inside of mice, such FCNPs seemed to have a great potential as an effective and non-cytotoxic tool for *in vivo* optical imaging, compared with the single fluorescent protein monomer (Fig. 7). Certainly, these FNSs might expose the fluorophores in external environment, causing fluorophore oxidation or photobleaching, thus their photostability could not be improved.

In the physical interaction, fluorescent molecules were usually directly involved inside the matrix NPs without any chemical interaction. The polymeric NPs acted as a protecting agent to improve the stability and compatibility of the FNSs in the physiological environment. In this way, hydrophobic fluorophores could also be considered



**Fig. 8.** (a) Chemical structures of phosphorescent PtTFPP reporter and PFO; (b) fluorescent images of MEF cells stained with MM2 (10 g/mL, 16 h) [136]. Copyright 2012.  
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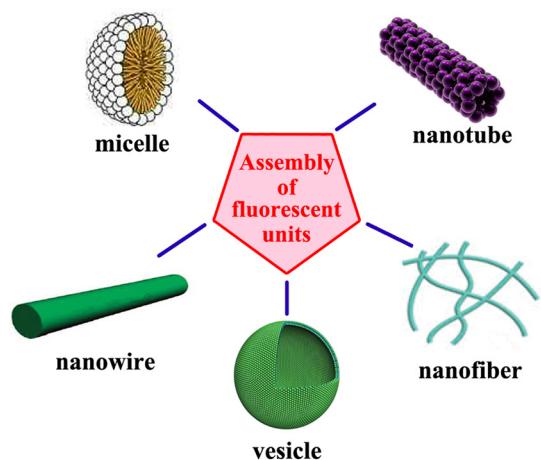
**Fig. 9.** The process of specific labeling and the comparison of fluorescence intensity between single dye molecule and dye-doped nanoparticles.

in bioimaging through encapsulation within hydrophilic polymeric NPs. Dye-doped NPs *via* physical method were probes encapsulated by biologically localized embedding (PEBBLE) and widely used as probes for bio-sensing and imaging in live cells [134,135]. For example, Kondrashina et al. [136] reported a new cell-penetrating phosphorescent nanosensor material, MM2 probe, by embedding the following two novel dyes into cationic hydrogel NPs: (1) phosphorescent reporter dye, PtTFPP (Fig. 8(e)); (2) O<sub>2</sub>-insensitive reference fluorophore (PFO, Fig. 8(e)) as one- and two-photon light harvesting antennae and fluorescence resonance energy transfer (FRET) donor for PtTFPP. The formed PEBBLEs (MM2) were applied in sensing and imaging of (intra)cellular oxygen of mouse embryonic fibroblast (MEF) (Fig. 8(a)–(d)). The procedure did not require any special functionality of doped dyes as long as they had enough affinity to the involved polymeric NPs. However, without covalent binding between the fluorophore and the matrix, dye molecules can be disassociated from the particles over time, which decreases per-particle brightness and increases the background signal. Therefore it is a challenge to improve the photostability of the fluorescent NPs by physical methods.

Generally, during inserting the fluorescent dye into matrix NPs by covalent attachment or physical entrapment to produce designed fluorescent polymeric NPs, the size of every individual FNS was determined by the size of the supporting polymeric NP, and the size-independent optical property was related to the type and the amount of incorporated fluorophores. The polymer section of the composites, either as matrix or protecting agent, contributes to the uniform distribution of the involved fluorophores, which could efficiently avoid quenching or photobleaching. Compared with fluorescent macromolecules, fluorescent polymeric NPs obtained from these procedures could produce a highly amplified optical signal (Fig. 9) due to the incorporation of several dye molecules. Although quantification of the doped fluorophores couldn't be accurately controlled in each NP, it did not exhibit any obvious negative effect on the bioimaging.

#### 2.4. Fluorescent supermacromolecular nanoassemblies

Fluorescent supermacromolecular nanoassemblies, such as micelles/vesicles [137–140], nanofibers [103,141–143], nanowires [144] and nanotubes [145], have been studied for bioimaging (Fig. 10). These fluorescent nanostructures were formed through assembly of small organic dye molecules [146] and/or fluorescent



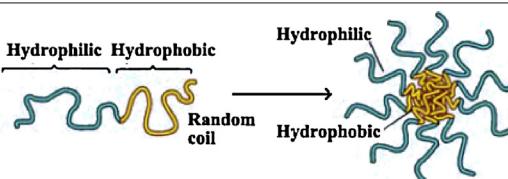
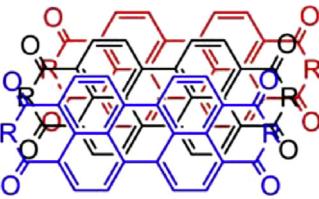
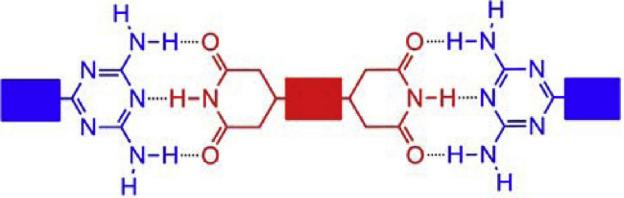
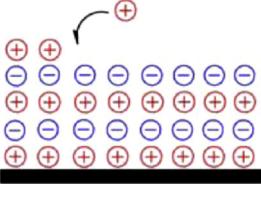
**Fig. 10.** Different supermacromolecular nano-assemblies formed by self-assembly of fluorescent units. The assemblies included micelles/vesicles [137–140], nanofibers [103,141–143], nanowires [144] and nanotubes [145].

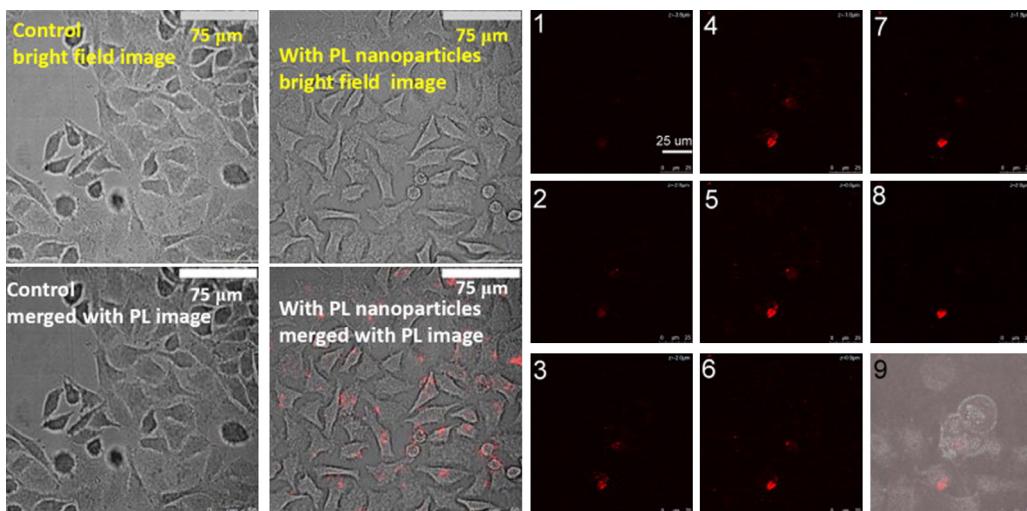
copolymers [147,148]. The reported intermolecular interactions and the self-assembly mechanism are summarized in Table 2. As reported, the assembling could occur under certain conditions, including solvent, temperature, etc., thus the assembling chance of either small organic dye molecules or fluorescent copolymers was limited. Therefore, the experimental conditions should be accurately controlled. However, the size of FNS (usually in range of 1.5–160 nm as reported) could not be easily controlled because of the complexity of assembling process.

It is well known that fluorescent units with amphiphilic structures could self-assemble into vesicle/micelle-like aggregates, which are resulted from the intermolecular interaction within hydrophobic and hydrophilic moieties, respectively. Since copolymers could be designed to have different functions in the block sections, amphiphilic polymers are generally inclined to self-assemble into uniform structures. Therefore, fluorescent amphiphilic structures are usually designed as graft [160] or block [161,162] copolymers. For instance, Chang et al. [163] synthesized a Rhodamine B (RhB)-anchored amphiphilic poly(poly(ethylene glycol)methacrylate)-*b*-poly(glycidyl methacrylate) block copolymer (PPEGMA-*b*-PGMA/RhB), in which the inter-molecular interaction of RhB resulted in the assembled fluorescent NPs. The nanoassembly PPEGMA-*b*-PGMA/RhB was then introduced into HeLa cells and the HeLa cells exhibited good fluorescence images (Fig. 11). Li et al. [160] also reported the similar results by using comb-like graft copolymer poly((N-vinylcarbazole)-co-(4-vinylbenzyl chloride))-comb-poly(((2-dimethylamino)ethylmethacrylate)-co-(acrylic acid)) (P(NVK-co-VBC)-comb-(DMAEMA-co-AAc)) to produce self-assembled hollow vesicles with multi-walls, which even exhibited good fluorescence intensity in aqueous media. Compared with liner copolymers, self-assembly of functional star polymers provides a convenient method to construct nanoaggregates with different functionalities [164]. For example, Cheng et al. [165] successfully synthesized star polymers by using a

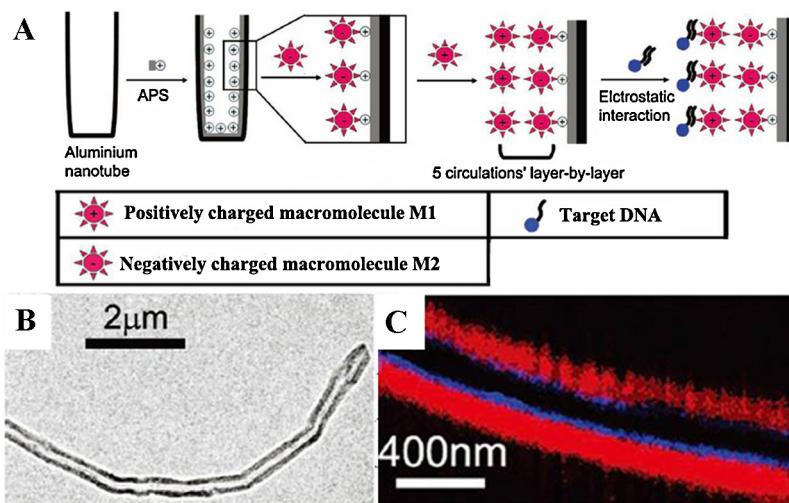
**Table 2**

Intermolecular interactions and the mechanism of self-assembly.

Intermolecular interaction	Mechanism of self-assembly	Refs.
Amphiphilic assemble		[149–154]
$\pi-\pi$ stack		[155,156]
Hydrogen bond		[157,158]
Electrostatic adsorption		[83,159]



**Fig. 11.** Left: confocal images of HeLa cells incubated without (control) and with PPEGMA-*b*-PGMA/RhB nanoparticles (50 mg/mL) for 12 h. Right: z-Stack confocal images demonstrating that the nanoparticles had been taken up into the cells (images 1–8, sampling depths are increased with 1 mm every image) [163], Copyright 2012. Reproduced with permission from Elsevier Ltd.



**Fig. 12.** (a) Fabrication of fluorescent nanotubes via LBL deposition of oppositely charged core-shell perylene derivatives (M1/M2); (b) TEM image and (c) fluorescent image of fluorescent nanotubes prepared via deposition of oppositely charged core-shell perylene derivatives [145]. Copyright 2011. Reproduced with permission from WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

novel fluorescent organo-boron crosslinker with poly(*N*-isopropylacrylamide)-*b*-polystyrene (PNIPAM-*b*-PS) as linear polymer precursors, which led to the formation of large vesicle-like fluorescent aggregates; they were subsequently tailored for applications in biomaterials and biomedical systems. Since multi-functional monomers could be introduced into the assembled copolymer, the obtained FNSs by this method also exhibited great advantages in stimuli-responsive drug delivery and release systems.

Fluorescent  $\pi$ -conjugated nanostructures could form H- or J-aggregates due to the facile  $\pi$ - $\pi$  stacking. For example, Olivier et al. [166] studied the NIR fluorescent NPs formed by self-assembly of lipidic boron-dipyrromethene dyes. The resulted supramolecular assemblies could be used as important delivery vehicles for living cells in which the entering of particle could be monitored directly with fluorescence microscopy.

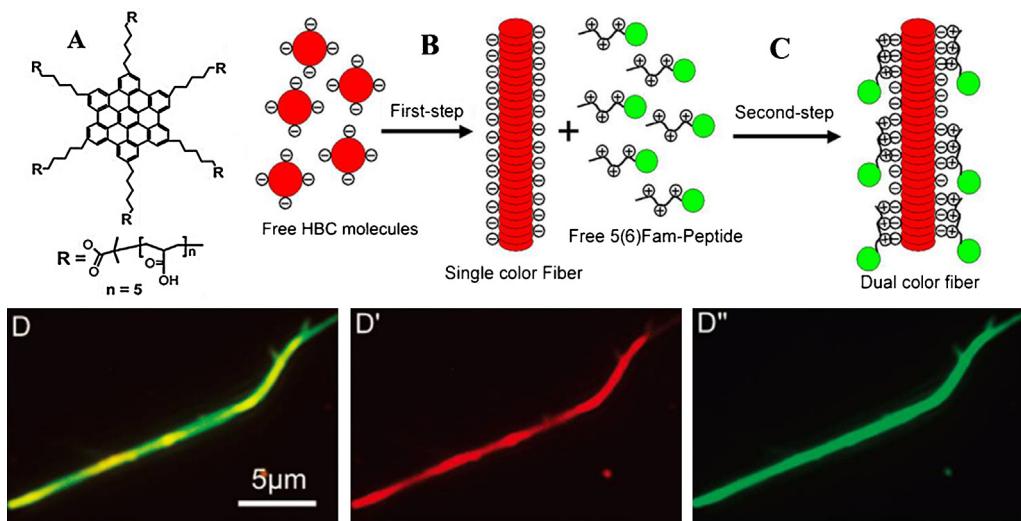
Since some  $\pi$ - $\pi$  interaction systems might show serious self-quenching of the fluorescent units, it is challenging to obtain highly fluorescent assemblies. The introduction of hydrogen bonding and electrostatic interaction has been shown as a potential solution to produce FNSs with high fluorescent intensity. For example, Gassensmith et al. encapsulated highly fluorescent NIR squaraine dyes inside macrocycles through hydrogen bonding between squaraine oxygen groups and macrocycle amide NH residues [167]. The resulting highly fluorescent, near-infrared complexes showed great promise in bioimaging [168]. Through layer-by-layer (LBL) assembly with the electrostatic interaction, Yin et al. applied aluminum nanotubes as templates and deposited oppositely charged core-shell perylene derivatives on them to generate novel fluorescent nanotubes (Fig. 12) [145]. These nanotubes can be used as biosensors to detect DNA in a dual-response manner.

Intermolecular multi-interactions induced assemblies have also been reported, which can drive the fluorescent units selectively to self-assemble in complicated

biological environments and form discrete complexes with well-defined structures. For example, Xu et al. [169] reported the formation of water-miscible organic J-aggregate nanoassemblies from the cooperation between  $\pi$ - $\pi$  stacking and hydrogen-bonding interactions of 1,4-dimethoxy-2,5-di [4'-(cyano)styryl]benzene (COPV). And the J-aggregated COPV nanoassemblies were highly emissive in aqueous media, which were also used as efficient two-photon fluorescent nanoprobe in bioimaging. Dual-color fluorescent nanofibers assembled in two strategies have been reported by Yin et al. [103]. They synthesized a novel water-soluble hexa-peri-hexabenzocoronenes (HBCs) derivative with peripheral functional groups (Fig. 13(a)), which facilitated a two-step assembly into nanofibers in water: (1) in the first step, the HBC derivative self-assembles via  $\pi$ - $\pi$  stacking into water soluble fibers with red fluorescence (Fig. 13(b)); (2) in the second step, the peripheral functional groups bind to the positively charged 5(6)-Fam-conjugated peptides with green fluorescence (Fig. 13(c)), leading to the formation of well-defined dual-color fibers (Fig. 13(d)). The advantage of hybrid design is to maintain the well-studied fluorescence of HBC chromophores, which allows the direct monitoring of the assembly process with fluorescence microscopy. This was the first report on fiber-forming HBC derivatives as a template for further functionalization of biomolecules.

## 2.5. Aggregation-induced emission fluorophores

Generally, most organic fluorescent materials mentioned in preceding perform well for bioimaging in solution state. However, fluorescence quenching or decrease in luminous efficiency occurs when their solution concentrations are increased or they are aggregated in the solid state. The phenomenon is identified as aggregation-caused quenching (ACQ). The compacted aggregation may lead to the radiationless energy conversion or the formation of detrimental species as excimers, resulting in fluorescence



**Fig. 13.** (a) Structure of HBCs; (b)  $\pi-\pi$  stacking of negatively charged HBCs into red fluorescent fibers; (c) positively charged 5(6)-Fam-conjugated peptides with green fluorescence bound to the negatively charged red fiber through electrostatic interactions, resulting in the formation of a dual-color fiber for double-fluorescence imaging; (d) fluorescence image of the formed dual-color fiber in different channels [103]. Copyright 2009. Adapted with permission from the American Chemical Society.

quenching [170]. Thus, the ACQ effect limits the bioapplication of such fluorescent materials as labeling or imaging agents.

In 2001, Tang et al. first discovered novel fluorescent materials (1-methyl-1,2,3,4,5-pentaphenylsilole) that could generate aggregation-induced emission (AIE) [171] and effectively avoided the obstacle from ACQ. These FNSs showed quite low FQY (0.03–0.3%) in solution, while high FQY (14–50%) in aggregate states. They offered a new fabrication of solid fluorescent materials with high FQY [172–174]. Up to present, a large number of new AIE luminogens have been designed and synthesized, such as silole, tetraphenylethene (TPE), triphenylamine (TPA), 9,10-distyrylanthracene (DSA), 1,4-distyrylbenzene (DSB) and their derivatives, as listed in Table 3.

In addition, these AIE fluorophores were usually further modified to improve their performance as imaging agents, *i.e.*, biocompatibility, brightness, permeability, retention effect, *etc.* For example, Qin et al. [185] had attached TPE units as terminals to 2-(2,6-bis((E)-4-(diphenylamino)styryl)-4H-pyran-4-ylidene)malononitrile (TPA-DCM) (Fig. 14), and the resulted TPE-TPA-DCM showed a novel phenomenon of AIE. Based on this result, bovine serum albumin (BSA) had been used as the polymer matrix to fabricate BSA-formulated TPE-TPA-DCM NPs with efficient far-red/near-infrared (FR/NIR) fluorescence and low cytotoxicity (Fig. 14). Their *in vitro* and *in vivo* FR/NIR bioimaging were successfully demonstrated with MCF-7 breast-cancer cells and a murine hepatoma-22 (H22)-tumor-bearing mouse model, respectively (Fig. 14).

Generally, the abnormal emissive feature of AIE fluorophores is different from “conventional” luminophores. AIE fluorophores show great potential in bio-fluorescence system since aggregation occurs in many biological processes. Because the AIE effect allows the use of highly concentrated fluorogen solution as well as their nanoaggregates

in applications of biosensor, AIE fluorogens are supposed to become practical tools in biological world very soon.

### 3. Inorganic fluorescent nanostructures

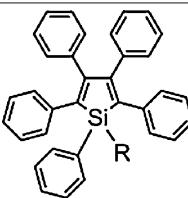
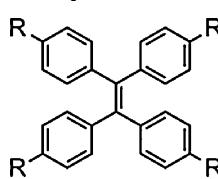
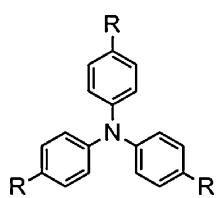
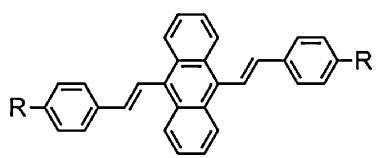
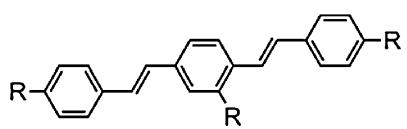
Inorganic FNSs are usually nanoclusters (NCs) or NPs with great potential advantages in bioimaging because of their interesting self-fluorescent properties. It was well known that, with very few exceptions, fluorescent lifetimes of organic dyes are too short (1–10 ns) for efficient temporal discrimination of short-lived fluorescence interference from scattered excitation light. Comparatively, inorganic FNSs have longer lifetimes (typically five to hundreds of nanoseconds) [192], which makes them as better candidates for bioimaging. According to their compositions, inorganic FNSs could be divided into various classifications, such as quantum dots, silicon nanoparticles, fluorescent metallic NPs and complexed inorganic FNSs.

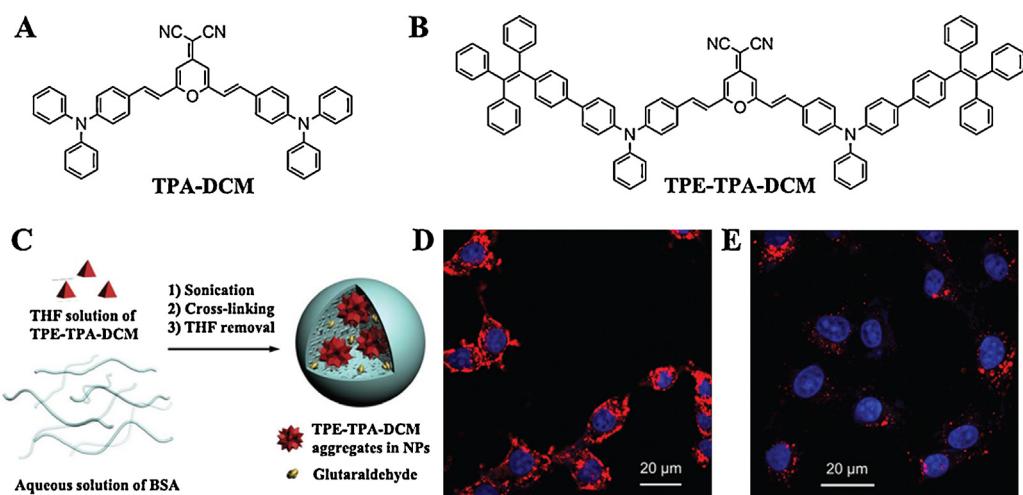
#### 3.1. Quantum dots

Quantum dots (QD), tiny semiconductor crystals, are regarded as one of the most important building blocks in modern photonics. The bright and photostable fluorescence of QD (FQY = 0.1–0.8 (visible), 0.2–0.7 (NIR)) has made it an excellent optically selective marker in bioimaging [193–195]. Compared with organic dyes, large Stock shift of QDs allows them to avoid the overlap between emission spectrum and excitation spectrum effectively, which increases the detection sensitivity of fluorescence signal. To date, the synthesis of QDs with controlled emission and absorption properties has already been a mature technique by adequately tailoring their sizes (1–10 nm) [196]. Therefore, through technically controlling the sizes and chemical compositions of QDs, their emission spectra could cover the whole visible region (Fig. 15). Furthermore, QDs provide a broad excitation spectrum and a narrow

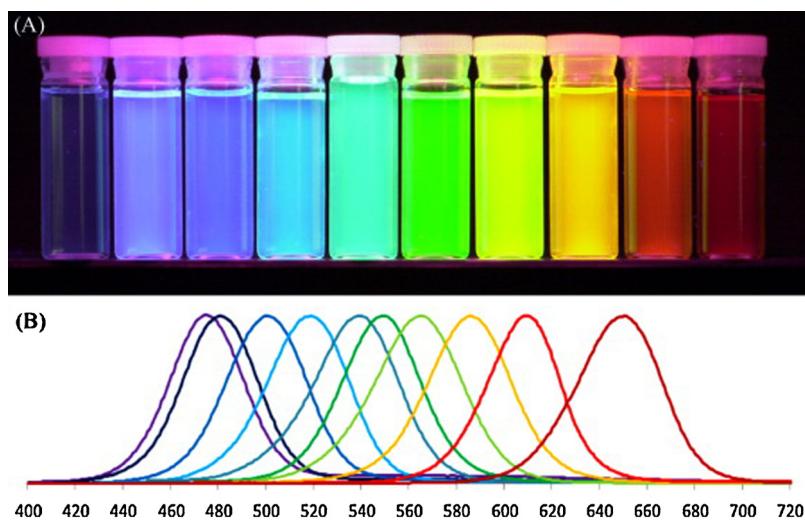
**Table 3**

The most reported fluorescent materials with aggregation-induced emission (AIE) phenomenon.

AIE fluorophores	General structural formula	Refs.
Silole		[175–179]
TPE		[180–184]
TPA		[185,186]
DSA		[187–189]
DSB		[190,191]



**Fig. 14.** Chemical structures of (a) TPA-DCM and (b) TPE-TPA-DCM. (c) Schematic illustration of the fabrication of BSA NPs loaded with TPE-TPA-DCM. (d and e) CLSM images of MCF-7 breast cancer cells after incubation with (d) fluorogen-loaded BSA NPs (with a fluorogen loading of 0.86%) and (e) bare TPE-TPA-DCM NPs for 2 h at 37 °C. [TPE-TPA-DCM] =  $0.4 \times 10^{-6}$  M [185]. Copyright 2012. Reproduced with permission from WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.



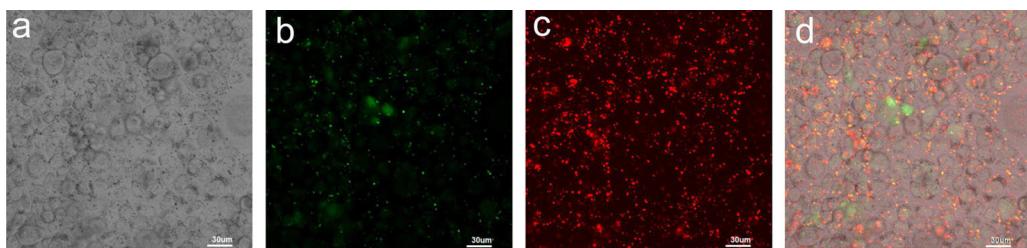
**Fig. 15.** (a) Colloidal suspension of quantum dots with different sizes viewed under long-wave ultraviolet illumination; (b) narrow emission spectra of related quantum dots with different sizes [197]. Copyright 2019. Reproduced with permission from Elsevier Ltd.

emission spectrum, so QDs with different sizes could be detected by utilizing the same excitation light source. This unique feature makes the multi-color labeling possible and greatly promotes their application in bioimaging.

Most of the highly fluorescent QDs for bioimaging contain toxic elements (Cd, Pb, Se, Te, etc.) [198–200]. Prasad and his co-workers [201] have done excellent work to summarize toxicities of different QDs in varied *in vivo* systems. Although numerous studies on low-toxic/non-toxic QDs have been reported recently, many scientists are still working on QDs with chemical stability and lower cytotoxicity [202]. For example, Peng et al. [203] reported the synthesis of CdSe QDs using CdO as Cd precursor to replace the toxic CdMe<sub>2</sub>, so QDs could be prepared in a safer and more economical way. Recently, effort has been made in developing methods to prepare non-toxic QDs. Deng et al. [204] synthesized high-quality CuInS<sub>2</sub>/ZnS (CIS/ZnS) QDs without toxic heavy metals, which avoided the intrinsic toxicity of well-developed II–VI and other semiconductor QDs (Cd, Pb, Se, Te, etc.) that was the major obstacle to their clinical applications. And they also demonstrated the versatility of the biocompatible CIS/ZnS QDs in multi-color bioimaging. Fig. 16 clearly shows their high labeling

specificities on folic acid (FA) receptor expressing Bel-7402 cells compared with that of fluorescein FITC (the green and red fluorescence signals are attributed to FITC and the encapsulated QDs, respectively). Such a toxicity study has also been done both in cellular level and in small animals. For example, Ye et al. [205] reported a pilot study in which rhesus macaques injected with phospholipid micelle-encapsulated CdSe/CdS/ZnS quantum dots did not exhibit evidence of toxicity. Blood and biochemical markers remained within normal ranges after treatment, and histology of major organs after 90 days had no abnormalities. These results indicated that acute toxicity of these QDs *in vivo* could be minimal.

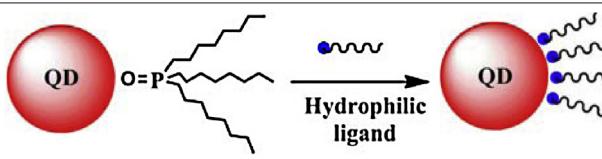
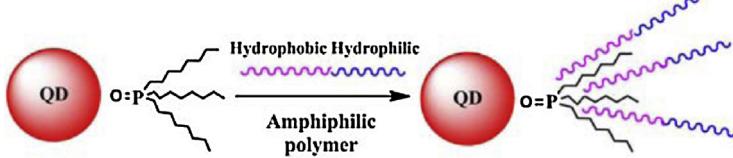
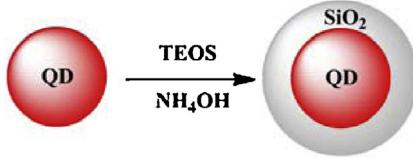
The major problems of QDs in bioimaging are their toxicity, low stability, poor solubility, poor biocompatibility and blinking effect (intermittence in emission). In order to solve these problems while maintaining their advantages in bioimaging, several strategies including surface passivation and functionalization (ligand exchange, polymer incorporated, etc.) were explored [206], as summarized in Table 4. Additionally, many reported synthetic procedure lead directly to QDs covered with polymers and small molecules, thus the process of surface passivation



**Fig. 16.** Optical microscopy of live FA receptor-positive Bel-7402 cells incubated with FITC-labeled and QDs-loaded micelles: (a) differential interference contrast (DIC), (b) fluorescein, (c) 625 nm emitting QDs-loaded micelles, (d) the merged image of the fluorescence images and DIC image.  $\lambda_{ex} = 488$  nm; scale bar: 30  $\mu$ m [204]. Copyright 2012. Reproduced with permission from the American Chemical Society.

**Table 4**

Strategies in surface passivation and functionalization of QDs.

Strategies in surface passivation and functionalization of QDs	Refs.
Hydrophilic ligand exchange	 [210–213]
Amphiphilic polymer/small molecule interaction	 [214–217]
Additional inorganic shells	 [218–221]

could be omitted [207,208]. Currently, potential applications of QDs in non-invasive bioimaging emerge as superior alternatives in bioimaging and their applications in both basic and applied biology are expanding. For example, Feugang et al. [209] reported bioluminescent resonance energy transfer-conjugated QDs (BRET-QDs) and their applications in real-time *in situ* fluorescence imaging of spermatozoa. Since spermatozoa can be incorporated with BRET-QD without affecting their motility and capacity to interact with oocytes when they are used at an appropriate ratio, the non-invasive bioimaging can be realized. The blinking effect of QDs (as well as metal, carbon, and silicon based FNSs) has limited their applicability to single molecule analysis [192], which will not be discussed in detail here in order to focus on their bioimaging applications.

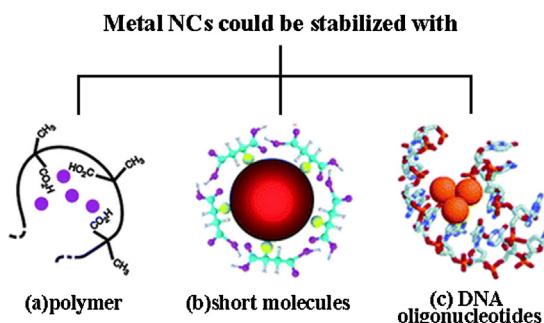
### 3.2. Silicon nanoparticles

Over the last few years silicon nanoparticles (SiNPs) have attracted many attention due to their exceptional optical properties [222–224], such as size-dependent tunable light emission, high brightness ( $FQY = 12\text{--}76\%$ ), great stability against photo-bleaching compared with organic dye molecules [225–227], and low inherent toxicity compared with the heavy elements of above-mentioned semiconductor QDs [228]. However, SiNPs usually possess poor aqueous dispersibility due to the surface-covered hydrophobic functional groups (e.g., Si–H bond) [229]. Substantial progress has been made in recent years in producing hydrophilic SiNPs [230]. For example, Zhong et al. developed a one-pot bottom-up strategy for the facile, rapid, and large-scale synthesis of SiNPs in water [231]. Significantly, the as-prepared SiNPs had excellent aqueous dispersibility, ultrahigh photo- and pH-stability, strong fluorescence, and excellent biocompatibility. And cellular experiments showed that the SiNPs were superbly suitable for long-term and real-time cellular imaging.

Currently, SiNPs have been adopted as a nontoxic alternative to toxic QDs due to their several advantages [232,233]: (1) the flexible surface chemistry, which allows them to be conjugated with biomacromolecules; (2) SiNPs exhibit NIR emission when their sizes are small, allowing deep-tissue imaging; (3) highly photostability, useful for long-term imaging; (4) tunable fluorescence with altering sizes and capping groups.

### 3.3. Fluorescent metallic nanoclusters

A fluorescent metallic nanocluster (NC), composed of a few to roughly a hundred atoms, has many attractive features, including ultrasmall size (less than 2 nm), good biocompatibility, good photostability, large Stokes shift (more than 100 nm) and high emission rate ( $FQY = 0.01\text{--}0.1\%$ , bare NCs), which make it a promising candidate for fluorescent bioimaging [234–236]. Since the 1990s, metallic NCs with self-fluorescent properties have been investigated widely [237]. However, no general mechanism has been currently known to explain the emission from any metal NC. Recent studies suggested that there might be two major sources for the fluorescence of metal NCs [238]: the metal core with its intrinsic quantization effects and the particle surface that is dependent on the chemical interaction between the metal core and the surface ligand. Therefore, the emission wavelength (typically in 400–700 nm) of metal NCs may be varied with size, coating ligand and/or even synthetic method [239]. However, the theory of size-dependent optical properties may not be suitable for all metallic NCs. In pure metallic NCs, aggregation can occur when two NCs are very close, resulting in metallic dimmers or assemblies [240]. Then their surface plasmon resonances will be coupled together, and the local electric field in the junction region will be largely enhanced [241], which is identified as surface-enhanced Raman scattering. Thus, enhanced two-photon luminescence of the



**Fig. 17.** Different strategies to functionalize metal NCs: (a) stabilization with polymer; (b) stabilization with short molecules; (c) stabilization with DNA oligonucleotides [238]. Copyright 2011. Adapted with permission from Elsevier Ltd.

metallic NCs would turn to be independent with the size or geometry.

Among the reported fluorescent metallic NCs, Au and Ag NCs gained most attention because of their monodispersity, brightness, photostability and potential optical-imaging applications [236,242]. There were many reports on the application of fluorescent Au and Ag NCs in bioimaging in the past few years [243–245]. Since Au and Ag NCs show size-dependent fluorescence, this controllable optical property offers them great advantages in bioimaging application [246,247]. In literatures, Au and Ag NCs were usually capped with different ligands during their syntheses in order to improve their water solubility and stability (Fig. 17) [238,248–251]. Further functionalization of Au and Ag NCs could be realized with different functional ligands as their capping agents, in which thiol-containing organic linkers were mostly used owing to the strong interaction between thiol and Au/Ag [252–254], and recently some zwitterionic functional ligands had also been reported [255]. Moreover, the usage of proteins as green reducing and stabilizing agents of these NCs is an additional advantageous strategy for target-specific cell imaging [242]. For example, Liu et al. [256] performed insulin-directed synthesis of fluorescent Au NCs as bioimaging agents, which had strong red fluorescence, excellent biocompatibility and preservation of natural insulin bioactivity to lower blood-glucose level. And, in C<sub>2</sub>C<sub>12</sub>, a mouse myoblast cell line, the synthesized insulin-Au NCs specifically stained the cytoplasm (Fig. 18). Similarly, Guével et al. [257] synthesized

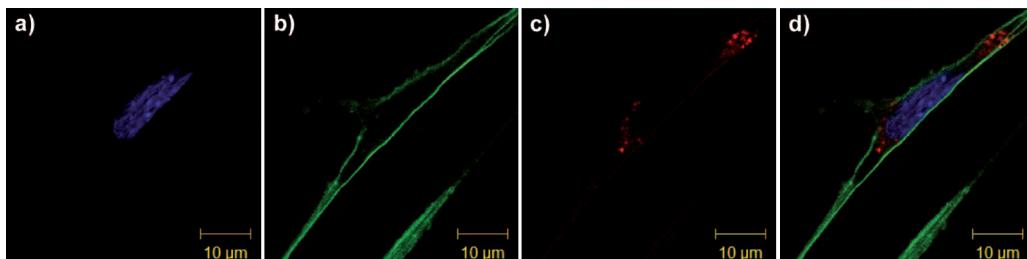
Ag NCs coated with glutathione and demonstrated their uptaking by the epithelial lung cancer, which indicated a high potential in biolabeling and bioimaging.

Apart from above-mentioned spherical morphology, extensive studies on metallic NCs with varied geometries have been reported recently, such as nanorod [258,259], nanoprisms [260,261], nanocages [262,263], and nanocubes [264,265]. Since these metallic NCs could generate visible or NIR fluorescence, their bioimaging application have also been investigated with increasing interests. For example, Wang et al. has reported a facile route to prepare radioluminescent Au nanocages without additional radiolabeling or dye conjugation [262]. The AuNCs were shown to emit luminescence with continuous wavelengths in the visible and NIR regions, enabling luminescence imaging of the whole mice *in vivo*, as well as their organs *ex vivo* for direct gamma counting. Due to their increased specific surface area, these metallic NCs showed excellent performance in many other application rather than bioimaging [266], such as photo-thermal sensors, drug delivery, and cancer diagnosis. The bioimaging performance of metallic NCs with varied geometries will not be discussed in detail here. In addition, toxicity of such metallic NCs in bioimaging have also been analyzed, which was related to the particle size, concentration, and surface modification [267]. It was believed that bare metallic NCs and their aggregates had hazardous effect to human bodies. Thus, surface modification of NCs has been paid more attention for their bioimaging during these years, which will be discussed in the following.

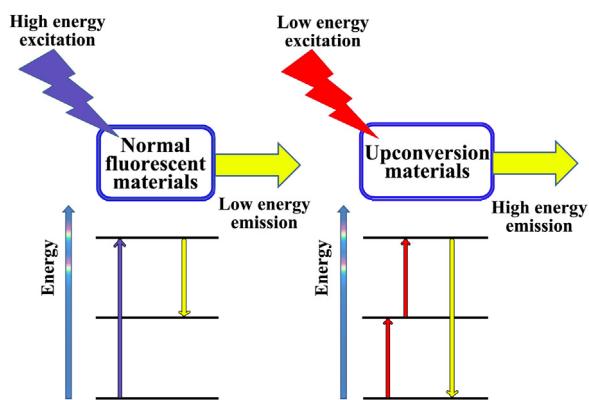
### 3.4. Complexed inorganic fluorescent nanostructures

#### 3.4.1. Upconversion nanoparticles

Traditionally, when fluorescent materials are exposed to incident light irradiation (typically ultraviolet (UV) light or X-ray) at specific wavelength, they emit low-energy photons (usually visible or NIR light) (Fig. 19). Recently, another type of FNSs with different luminous mechanism, *i.e.*, upconversion nanoparticles (UCNPs), has been studied. These FNSs could emit high-energy photons under NIR excitation [268–271]. The phenomenon of “upconversion” (UC) was defined as a nonlinear optical process in which the sequential absorption of two or more photons leads to the emission of a single photon at a shorter wavelength [272].



**Fig. 18.** Microscopic observation of the uptaking of insulin-Au NCs by differentiated C<sub>2</sub>C<sub>12</sub> myoblasts after 2 h: (a) Cell nucleus stained with 4',6-diamidino-2-phenylindole (DAPI, blue); (b) actin fiber stained with Alexa Fluor 488 phalloidin to confirm the cell boundary (green); (c) insulin-Au NCs with red luminescence in the cytoplasm; (d) fluorescence image overlay of the three images [256]. Copyright 2011. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.) Reproduced with permission from WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.



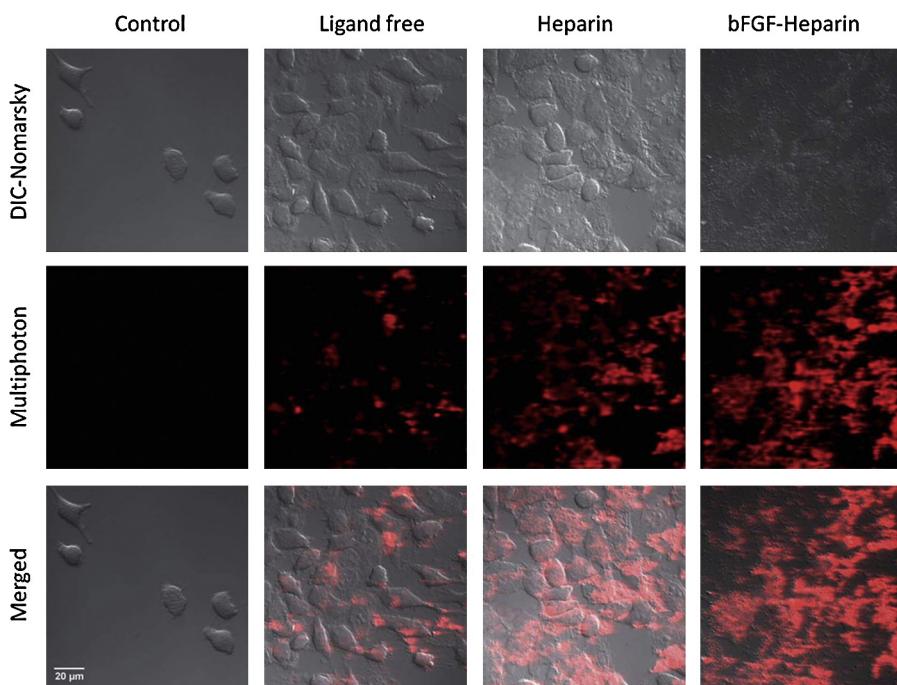
**Fig. 19.** Energy level diagram of the emission processes of normal fluorescent materials and upconversion materials.

Since the discovery of UC process in the 1960s [273], UCNPs have shown great advantages in bioimaging [274–276]. The reasons are the following: (1) UCNPs minimize the optical damage to cell tissues; (2) NIR light irradiation exhibits high tissue penetration; (3) the emission locates in visible or NIR region, so the interference of background radiation can be effectively avoided; (4) UCNPs show excellent photostability and low toxicity as bioimaging agents.

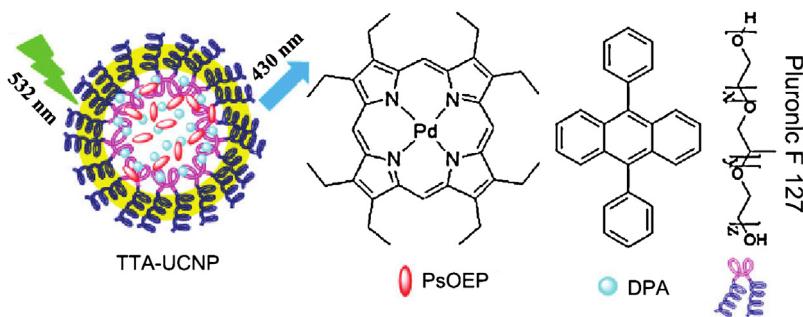
The reported UCNPs are usually a rare earth(RE)-doped inorganic host matrix. The host matrix is required to have low lattice photon energies, most of those reported as are oxide, halide, sulfide or oxysulfide [277,278]. The doping RE ions (lanthanide) play roles as a sensitizer ( $\text{Yb}^{3+}$ ) and

an activator ( $\text{Er}^{3+}$ ,  $\text{Ho}^{3+}$ ,  $\text{Tm}^{3+}$ ) [279,280]. In addition to the synthesis of new RE-doped UCNPs, many groups studied the surface modification of UCNPs to obtain better water dispersibility and biocompatibility for bioimaging. For example, Bogdan et al. reported the functionalization of ligand-free UCNPs with heparin and basic fibroblast growth factor (bFGF) [281]. These heparin-bFGF functionalized UCNPs showed specific binding capabilities to *HeLa* cell membranes, which resulted in high-contrast images of *HeLa* cells (Fig. 20). In addition, these fluorescent materials offered feasibility for bioimaging not only in the visible but also in the NIR region (first and second biological window) [276,282]. Nyk et al. [283] synthesized aqueous dispersible fluoride ( $\text{NaYF}_4$ ) nanocrystals (20–30 nm size) that were co-doped with the rare earth ions ( $\text{Tm}^{3+}$  and  $\text{Yb}^{3+}$ ), showing NIR-to-NIR ( $\lambda_{\text{em}} = 800 \text{ nm}$ ,  $\lambda_{\text{ex}} = 975 \text{ nm}$ ) upconversion for *in vitro* and *in vivo* photoluminescence imaging. This NIR-to-NIR upconversion process provides deeper light penetration into biological specimen and results in high contrast optical imaging due to the absence of an auto-fluorescence background and decreased light scattering.

However, RE-doped UCNPs have their shortcomings, such as the low absorption cross-section of  $\text{Yb}^{3+}$  and the low upconversion FQY (~0.5%) [284]. These problems inspire researchers to explore new types of UCNPs without RE ions. Liu et al. [285] successfully synthesized the water-soluble UCNPs based on triplet-triplet annihilation (TTA) by co-loading sensitizer (octaethylporphyrin Pd complex) and annihilator (9,10-diphenylanthracene) onto silica nanoparticles, whose upconversion FQY could be as high as 4.5% in aqueous solution (Fig. 21). And those



**Fig. 20.** Optical transmission images (top row), fluorescence images (middle row), and the overlay images (bottom row) of a group of *HeLa* cancer cells which were incubated at 37 °C with a phosphate buffered solution (PBS) (control) or a PBS containing 5 mg/mL of ligand-free heparin and bFGF-heparin coated UCNPs. Scale bar: 20  $\mu\text{m}$  [281]. Copyright 2012. Reproduced with permission from the Royal Society of Chemistry.



**Fig. 21.** Schematic illustration of upconversion luminescence of TTA-UCNPs, and chemical structures of PdOEP, DPA, and F127 [285]. Copyright 2012. Adapted with permission from the American Chemical Society.

TTA-based UCNPs showed low cytotoxicity and were successfully applied in the *in vivo* imaging of lymph node in living mouse with an excellent signal-to-noise ratio.

Compared with those of down-conversion FNSs, applications of UNCPs as probes in high-contrast and low-power-excited *in vivo* bioimaging (in both visible and NIR region) may expand the arsenal of currently available fluorescent bioimaging both *in vitro* and *in vivo*.

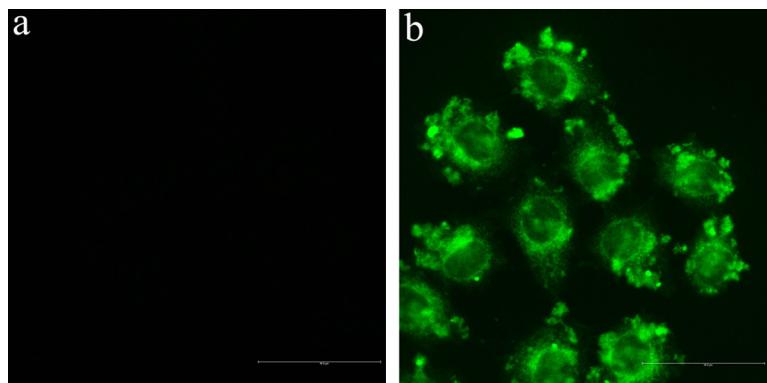
#### 3.4.2. Inorganic core/shell fluorescent nanoparticles

Even the inorganic FNSs showed great advantages in bio-application, they were often used after further surface functionalization rather than being used directly. Modified silica surfaces are preferred to achieve the desirable features of hydrophilicity, biocompatibility and low toxicity, silica surface. Zhang et al. [286] successfully synthesized silica-wrapped gold nanocluster ( $\text{Au@SiO}_2$ ) with good water solubility, exceptional biocompatibility, favorable surface properties and excellent fluorescence properties. After covalently modified with folic acid (FA),  $\text{Au@SiO}_2$  nanocomposite was exceptionally suitable for cellular imaging by effectively recognizing the expressed FA receptors on HeLa cell's surface (Fig. 22). Since similar work on both silica-coated QDs and metallic NCs have already been widely reviewed, more attention would be preferred to core/shell structural UCNPs. An increasing literature has been reported on the bioimaging of silica-coated UCNPs

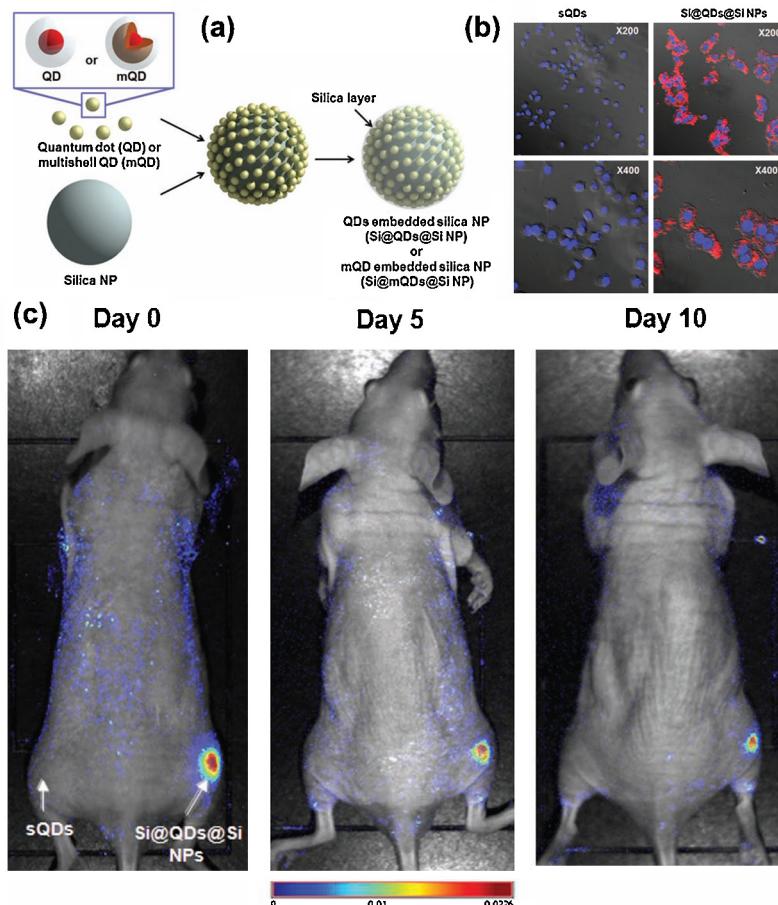
due to their strong NIR-to-visible upconversion fluorescence, attracting substantial interest in the development of various types of inorganic shell to give UCNPs better biocompatibility [287,288]. Several aspects should be taken into consideration when choosing the epitaxial shell materials, *i.e.*, the lattice mismatch with inner core, optical transparency, crystallizability and stability. For example, Chen et al. [280] developed heteroepitaxial growth of a biocompatible  $\text{CaF}_2$  shell over UCNPs ( $\alpha\text{-NaYbF}_4:\text{Tm}^{3+}$ ) to suppress surface quenching effects, resulting in core/shell ( $\alpha\text{-NaYbF}_4:\text{Tm}^{3+}$ )/ $\text{CaF}_2$  NPs that exhibit highly efficient  $\text{NIR}_{\text{in}}\text{-NIR}_{\text{out}}$  UC for high contrast and deep bioimaging.

Some studies have used silica as supporting matrix as well as coating materials [289]. For example, Jun et al. [290] synthesized highly bright QD-embedded nanoprobes ( $\text{Si@QDs@Si NPs}$ ) by embedding a large number of hydrophobic QDs onto the surface of hydrophilic silica NPs, which were then covered with a silica layer (Fig. 23(a)). These  $\text{Si@QDs@Si NPs}$  exhibited approximately 200-times stronger fluorescence than single QDs and also showed high brightness and biocompatibility for effective bioimaging of cells in mouse skin (Fig. 23(b) and (c)).

Moreover, such surface functionalization would also be realized by applying polymer network or organic biological molecules as protecting agents or scaffolds to ensure better bio-compatibility. Therefore, organic/inorganic hybrid FNSs have been rapidly developed recently.



**Fig. 22.** Confocal fluorescence images of HeLa cells treated with the following nanoparticles: (a) 11-MUA Au-NCs@ $\text{SiO}_2$  in the HeLa cells incubated at 37 °C; (b) 11-MUA Au-NCs@ $\text{SiO}_2$ -FA in the HeLa cells incubated at 37 °C. Scale bar: 50  $\mu\text{m}$  [286]. Copyright 2011. Adapted with permission from Elsevier Ltd.



**Fig. 23.** (a) Synthesis of silica-coated QD or mQD-embedded silica NPs, in which core–shell QDs (QDs) and core–multishell QDs (mQDs) were immobilized to thiol-modified silica NPs; Si@QDs@Si NPs and Si@mQDs@Si NPs were then prepared by silica–shell encapsulation; (b) fluorescence images of QD- and Si@QDs@Si-NP-uptaken cells (red color: QD; blue color: DAPI); (c) Maestro *in vivo* fluorescence image of sQD-labeled (left leg) and Si@QDs@Si-NP-labeled (right leg) cell-transplanted mouse [290]. Copyright 2012. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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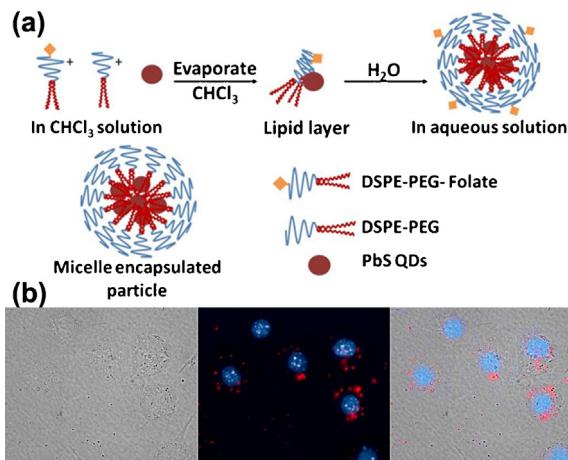
#### 4. Organic/inorganic hybrid fluorescent nanostructures

With rational design, combining multiple constituents into a single nanoobject will not only bridge the unique properties of individual sections, but also improve conventional sensing, imaging, and therapeutic efficacies. To improve the application in bioimaging, FNSs are usually designed as organic/inorganic hybrid compositions. The core and shell segments alternately contributed to the desired fluorescent properties or acted as protecting agents of matrix or scaffold, which thus offers better functionalization and biocompatibility.

##### 4.1. Inorganic@organic hybrid fluorescent nanostructures

Inorganic@organic hybrid FNSs are mostly consisted of fluorescent inorganic composition embedded inside an organic matrix. According to literatures, the organic shell could be in the following three types: (1) polymeric

network [215,291–294], which was usually induced via ATRP followed by the attachment of an ATRP initiating group to the surface. For example, Wu et al. [295] used nonlinear PEG-based nanogel network chains as a three-dimensional scaffold to protect ZnO QDs, which was followed by appropriate growth of metallic Au, and the resulted hybrid NPs performed well in lighting up B16F10 cells; (2) self-assembled micelles [296,297], in which fluorescent inorganic cores were mostly embedded physically. For example, Hu et al. [298] demonstrated the usage of PEGylated phospholipid micelles to encapsulate NIR emitting ultra-small lead sulfide (PbS) QDs to label RAW264.7 macrophage cells (Fig. 24); (3) dendrimers [299–302], which provided cavities to trap inorganic fluorophores. For example, Alcalá et al. [303,304] created a dendrimer complex by substituting thirty-two naphthalimide fluorophores on the surface of the dendrimer and incorporating eight europium cations within the branches. The resulting hybrids exhibited preferential accumulation within liver tumors and were suitable for fluorescence imaging.



**Fig. 24.** (a) Schematic illustration of the PEGylated phospholipid micelle encapsulated PbS QDs; (b) RAW264.7 macrophages labeled with micelle-encapsulated PbS QDs (red) and the cell nucleus stained with Hoechst 33342 (blue) [298]. Copyright 2012. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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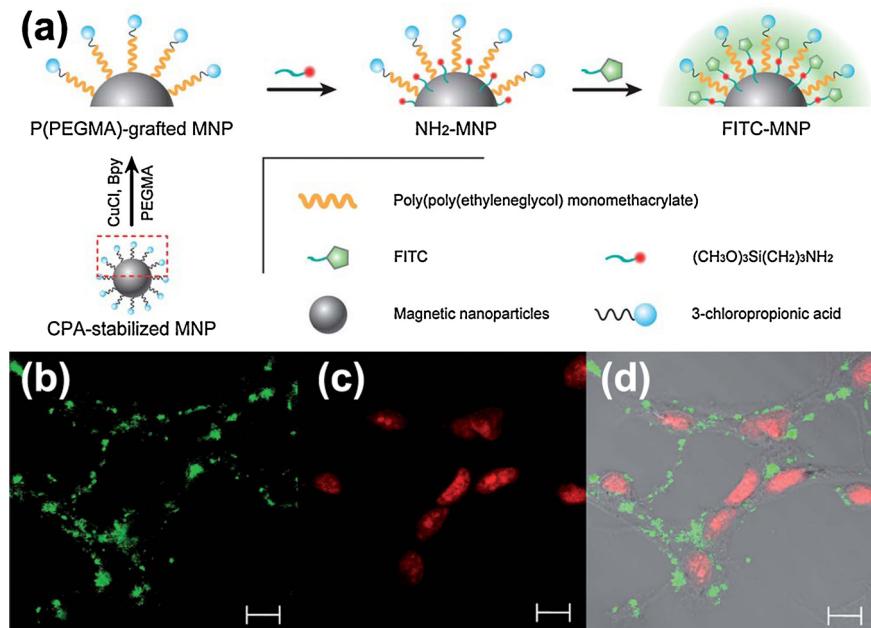
By introducing the organic shell, the sizes would be slightly increased compared with that of the original bare inorganic fluorescent NPs. The organic shell, serving as the protecting agent, effectively avoids the photobleaching of the inner fluorescent inorganic core, thus resulting in the improvement of the stability. In addition, because of the modification with polymeric cover, it is feasible to realize the functionalization of the composite FNSs, rendering

those features of hydrophilicity, biocompatibility, specificity, environmental stimuli responsive, etc.

In addition, these FNSs could be organic chromophores-decorated inorganic nanoparticles. The inorganic core served as supporting materials and the organic portion was the fluorescent source [305]. For instance, Ma et al. [306] reported the one-pot synthesis of PEGylated mesoporous silica nanoparticles which could be labeled with NIR dye Cy5.5. The produced FNSs showed great potential in bioimaging. The supporting inorganic core determined the sizes of the composites (15–200 nm) [307], which would not change much after coated with organic chromophores [308]. However, achieving high stability was a challenge when the exposed chromophores were increased. Meanwhile, these FNSs could be designed to have properties other than fluorescence by using different inorganic cores, e.g., magnetism [309–311]. For example, Lu et al. [312] synthesized water-soluble P(PEGMA)-grafted Fe<sub>3</sub>O<sub>4</sub> nanoparticles via ATRP method, and NH<sub>2</sub> groups were introduced through a facile post-ATRP modification. Fluorescent magnetic nanoparticles (MNPs) were then obtained by attaching fluorescein FITC covalently to the NH<sub>2</sub> groups. Such FITC-MNPs can be efficiently taken up by breast cancer cells (Fig. 25). These results indicate that FITC-MNPs with both fluorescence and magnetic functionalities have great potential in bioimaging.

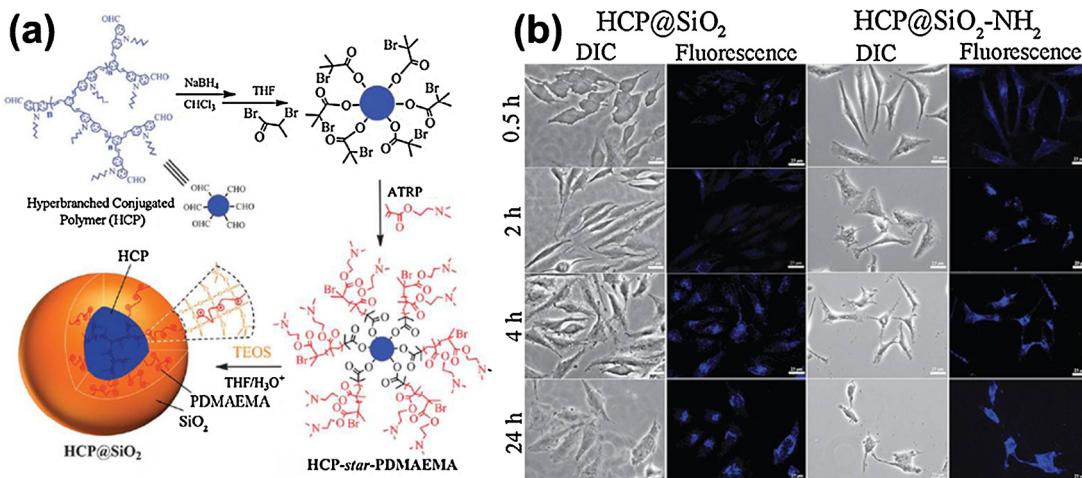
#### 4.2. Organic@inorganic hybrid fluorescent nanostructures

In organic@inorganic hybrid FNSs, fluorescent inorganic compositions were usually covalently attached onto the



**Fig. 25.** (a) Preparation route of NH<sub>2</sub>-MNPs and FITC-MNPs from P(PEGMA)-MNPs by ATRP modification. (b–d) Confocal microscopic images of MD-MBA-231 cells after 24 h incubation with FITC-MNPs at 37 °C and further staining with PI (red). The uptake of the FITC-MNPs (green) in the MD-MBA-231 cells was visualized by overlaying the images obtained by the FITC filter and the PI filter ((b) image from FITC channel; (c) image from PI channel; (d) image from combined PI channel, FITC channel and phase contrast). Figure scale bar: 10 mm [312]. Copyright 2012. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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**Fig. 26.** (a) Preparation route of core–shell HCP@SiO<sub>2</sub> nanoparticles from unimolecular HCP-star-PDMAEMA; (b) fluorescence microscope images of HeLa cells incubated with HCP@SiO<sub>2</sub> and HCP@SiO<sub>2</sub>-NH<sub>2</sub>, respectively, at different time intervals (0.5 h, 2 h, 4 h, 24 h). Compared with that of HCP@SiO<sub>2</sub>, the fluorescent imaging of HCP@SiO<sub>2</sub>-NH<sub>2</sub> is brighter after a short incubation time. Scale bar: 25 μm [320]. Copyright 2012. Adapted with permission from the Royal Society of Chemistry.

surface of organic nanoparticles. For example, Yuan et al. [313] designed a QDs-labeled PAMAM G4 derivative vector for target imaging and nucleic acid delivering. This fluorescent complex dendrimer would be useful to introduce nucleic acids or drugs into cells through a receptor-targeted mechanism because significant knockdown of expression was observed. Wang et al. [314] developed a facile strategy to synthesize Fe<sub>3</sub>O<sub>4</sub>@P(St/MAA)@Chitosan@Au NPs. The obtained multifunctional NPs had a low hemolyticity as contrast agents in magnetic resonance imaging and dark field imaging.

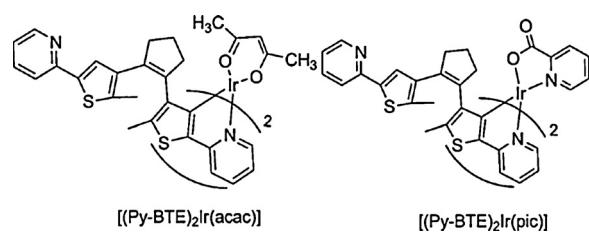
In order to improve the stability, biocompatibility and low toxicity, sol–gel derived silica was reported as an excellent host material for creating fluorescent NPs with the covalently attached organic chromophores [315–319]. In this procedure, the organic chromophores should be functionalized via terminal siliconization first, and then their sol–gel reactions with siloxanes were carried out to produce a silica shell. For example, Qiu et al. [320] used a unimolecular fluorescent hyperbranched conjugated polymer (HCP-star-PDMAEMA) as soft template and isolated them with a silica shell to fabricate highly fluorescent core–shell hybrid NPs HCP@SiO<sub>2</sub> (Fig. 26(a)). HCP@SiO<sub>2</sub> NPs with amine groups, denoted HCP@SiO<sub>2</sub>-NH<sub>2</sub>, were achieved through the surface modification. The hybrid NPs were used to stain cytoplasmic regions of the living cells. The fluorescence images (Fig. 26(b)) indicated that NPs with amino groups facilitate their cellular uptake into HeLa cells. Such silica-covered FNSs offered more opportunities for further surface modification [321], which was also a strategy for site-specific targeting in bioimaging.

#### 4.3. Heavy-metal complex

Among the above-mentioned fluorescent materials, neither down conversion nor up conversion materials could emit fluorescence without excitation light. Since phosphorescence is a fluorescence phenomenon with

characteristic decay rate which could be observed after removal of the excitation light, FNSs with phosphorescence emission can be designed based on heavy-metal complexes with a unique d<sup>6</sup>, d<sup>8</sup>, and d<sup>10</sup>-electron configuration. Unlike above-mentioned organic or inorganic fluorophores, which are singlet state emitters, heavy-metal complexes are triplet emitters [322]. Since the strong metal-induced spin-orbit coupling leads to efficient singlet–triplet state mixing, the spin-forbidden nature of the irradiative relaxation of the triplet state can be avoided, resulting in highly intense phosphorescent emission at room temperature [323]. To date, heavy-metal complexes, including Re(I)-, Ru(II)-, Os(II)-, Ir(III)- and Rh(III)-complexes with d<sup>6</sup> electronic structures, Pt(II)-complexes with d<sup>8</sup> electronic structures, and Au(I)- and Cu(I)-complexes with d<sup>10</sup> electronic structures have been reported as the most important materials with phosphorescent emission at room temperature [324].

Recent studies on heavy-metal complexes with different counter ions, solvent ligands and cyclometallated ligands demonstrated the design concept with practical applications in cell imaging [325]. For instance, Tan et al. [326] reported the synthesis of an iridium(III) complex [(Py-BTE)<sub>2</sub>Ir(acac)] (Fig. 27) with switchable phosphorescence [(Py-BTE)<sub>2</sub>Ir(pic)]. The switchable phosphorescence of



**Fig. 27.** Structures of [(Py-BTE)<sub>2</sub>Ir(acac)] and [(Py-BTE)<sub>2</sub>Ir(pic)] [326]. Copyright 2011. Adapted with permission from WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

$[(\text{Py-BTE})_2\text{Ir(pic)}]$  in solution and within living cells could be controlled by two distinguishable visible-light irradiations. As a result, this complex showed great advantages as a phosphorescence probe for monitoring living cells.

However, toxicity limited the widespread applications of heavy metal ions. In order to solve this problem, further functionalization of heavy-metal complexes has been reported recently. When conjugated with target biomolecules, such FNSs emit strong fluorescence at longer wavelength, thus the specific labeling and recognition could be realized. For instance, Shi et al. [327] synthesized phosphorescent conjugated polyelectrolytes (PCPEs) containing Ir(III) complexes and polyfluorene units, which emitted blue fluorescence in aqueous solutions. Since electrostatic interaction between the nanoparticles and heparin could improve the energy transfer between the polyfluorene units to Ir(III) complex, the red signal could also be observed with naked-eyes (Fig. 28). Therefore, the PCPEs could be used in specific labeling of live KB cell membrane with high contrast, which could be used in both confocal fluorescent cellular imaging and fluorescence lifetime imaging microscopy.

## 5. Perspective

Due to their attractive properties, including ultra-small size, high brightness, monodispersity and low

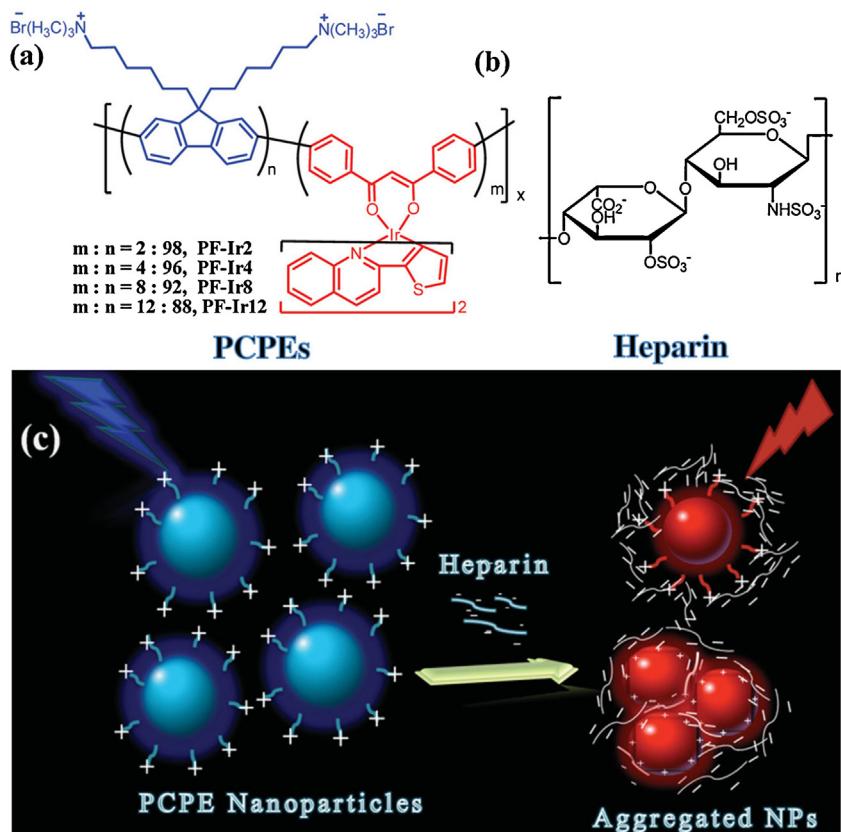
**Table 5**

Most reported bio-distribution of FNSs for *in vivo* bioimaging.

Bio-distribution	Refs.	Bio-distribution	Refs.
Liver	[279,333,334]	Lymph nodes	[335,336]
Lung	[38,337–339]	Tumor	[340–342]
Kidney	[343–345]	Brain	[55,346–348]
Spleen	[349–351]	Skin	[352]

photobleaching, FNSs represent a highly attractive platform for bioimaging [328]. Despite their great potential and promising future as novel fluorescent probes in bioimaging, further improvement of FNSs still faces many challenges.

First, progress in the design and development of better synthesis routes for different FNSs with good target specificity are needed. The required selectivity, e.g., specific labeling cell membrane, nucleus or ECM, could be realized by surface modification through multi-functionalization [35]. And the resulted structures should have special chemical or physical conjugation between FNSs and the targeted biotic components [329], e.g., macromolecules, subcellular organelles, specific cancer cells and so on. In the real *in vivo* bioimaging application, the bio-distribution of FNSs is an important point, which is dependent upon the composition or surface modification of FNSs, and the physiological environments [267,330–332] (Table 5). Up to present, liver and kidney were found to have excellent affinity with most FNSs although their real targeted bio-distribution is still a



**Fig. 28.** Structures of the synthetic (a) PCPEs and (b) heparin; (c) the possible sensing mechanism [327]. Copyright 2013. Adapted with permission from WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

great challenge. Further effort could be made in improving the functionality of complex for better stability and sensitivity in both cellular and tissue levels.

Second, FNSs should be simultaneously functionalized with other properties in addition to fluorescence. With the development and maturity of fluorescent probes in the last decades, there is a great demand on their general-purpose optimal design [353]. In addition to the application in biolabeling, FNSs in other fields, such as biological recognition, separation, orientation, delivery, bio-thermal and chemical sensors [239,354,355], are also explored. And diversification of fluorescent nanostructure design for multiple bioactivities has always been the trend of bio-science and technology development.

Third, to be fully accepted in the field of nanobiotechnology, careful investigation on the safety of FNSs in bioimaging is still needed [356]. Since bioimaging is mostly performed *in vivo*, there is an increasing concern about the toxic effects of synthetic FNSs in humans. The toxicity of FNSs is dependent on the following two factors [357,358]: (1) chemical composition of FNSs. For instance, quantum dots contain toxic cadmium (CdSe, CdS, etc.). However, potential toxicity could be avoided by coating to control the release of toxic elements [359,360]. (2) Morphology or structure of FNSs. Toxicological data from animal tests suggest that some FNSs could exert adverse effects in animals due to the retention of FNSs inside animal bodies. Therefore, it would be preferable that the sizes of FNSs are smaller than the threshold of renal metabolism [361]. In order to remove these nanostructures from cells and animals efficiently, ultrafine functionalized FNSs should be prepared in the future.

Fourth, developing fluorescent probes working for first or second biological windows (up to 1400 nm) seems to be quite important. To date, fluorescence imaging of deep tissue has been significantly hindered because of the insufficient tissue light transmission and high scattering. NIR light (700–2500 nm) can penetrate biological tissues, such as skin and blood, more efficiently than visible light [362]. Therefore, it is a challenge to prepare FNSs that could perform bioimaging in the long wavelength region (NIR region). Fluorescent nanoprobes for deep tissue *in vivo* imaging should possess following properties: (1) non-toxicity; (2) both the excitation light and fluorescent emission in the spectral range which is favorable for the penetration of light through thick tissues; (3) efficient and stable fluorescence signal. Up to present, FNSs for deep tissue imaging is gaining increasing attention, and preponderant candidates, such as carbon nanotubes, two-photon metallic NCs, and UCNPs, have been investigated for bioimaging application.

## 6. Summary

We summarize the extensive efforts on the development of FNSs, which have attractive features for their applications in bioimaging. In this review, we first introduce three classifications of bioimaging FNSs according to their chemical compositions. Their specific structural designs, synthetic strategies and the size effects on the optical properties are subsequently summarized. Great efforts

have been made to improve their fluorescence intensity, specificity in targeting, biocompatibility and non-toxicity, etc. Although many FNSs have been synthesized and applied biologically, there are still much to be done before they could be widely used as fluorescent probes in clinical tests. With further advances in the design and synthesis of multifunctional FNSs with high quality, the widespread application of FNSs could be expected not only in advanced bioimaging, but also in ultrasensitive molecular diagnosis, novel light-emitting nanodevices and intracellular drug delivery.

## Acknowledgements

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