Organic dye assemblies with aggregation-induced photophysical changes and their bio-applications

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Abstract
Phototheranostics provide a safe, effective, and noninvasive way for the diagnosis and treatment of contemporary diseases, and organic dyes play a vital role. For example, chemical modification endowed dyes with powerful reactive oxygen species or heat generation ability, favoring for photodynamic therapy and photoacoustic (PA) imaging guided photothermal therapy (PTT) of serious diseases. Therefore, photophysical properties manipulation of dyes has become the focus in current dye chemistry research. The development of aggregate science has made great effort to solve this problem. In recent years, a large number of studies have focused on molecular aggregation behavior and its effect on photophysical performance. The most famous example is the discovery of aggregation-induced emission (AIE) phenomenon. Based on AIE theory, more theories for revealing the relationship between molecular aggregation behavior and photophysical properties were proposed and elucidated. The photophysical property changes caused by dye aggregation have become a unique discipline, guiding the development of molecular science and material science. With the help of molecular self-assembly, controllable aggregation of dyes can be realized, and stable nano-theranostic reagents can be obtained. Furthermore, constructing dye assemblies with various photophysical properties will greatly reduce the cost of theranostic reagents, thus, expanding biomedical applications of organic dyes. Therefore, this review focuses on the photophysical characteristic changes caused by dye aggregation and their biological applications including, fluorescence/phosphorescence/PA imaging as well as photodynamic and PTT. This review will provide guidance for the design of organic dyes, the development of controllable aggregation methods, and the construction of multifunctional phototheranostic reagents.

KEYWORDS
aggregation-induced photophysical changes, fluorescence imaging, organic dye assemblies, phosphorescence, photodynamic therapy, photothermal therapy

INTRODUCTION
With the continuous development of optical technology, optical imaging protocol for life science has become one of the indispensable methods. As a kind of widely used biological staining material, small molecular organic dyes can realize fluorescence imaging and photoacoustic (PA) imaging of both biological entities and processes. The spectral wavelength range of organic dyes can be adjusted from UV to near-infrared (NIR) and is easily degraded, which usually exhibit low long-term biological toxicity. In addition, organic dyes are not limited to offer fluorescence emission, their photophysical properties can also be tuned to a large extent by radiative transition to nonradiative transition from excited state. Thus, photodynamic effect and photothermal conversion process can be achieved. The as prepared organic photosensitizers and photothermal reagents play an important role in the optical diagnosis and treatment of major diseases such as cancer, cardiovascular, and cerebrovascular diseases. Therefore, studying the preparation methods of organic dyes and controlling the transformation of their photophysical properties are of great significance in the design and application of phototheranostic materials.

In dye chemistry, the photophysical characteristics of dyes are first regulated through chemical modification. For example, extending the conjugation structure and constructing D-A

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structure leads to the redshift of both absorption and emission band of the dye (Scheme 1A), thereby overcoming the interference of biological background and increasing the penetration depth of light.[5a] Meanwhile, these structural changes may cause the photothermal conversion efficiency increase of dyes, favoring for applications in PA imaging and photothermal therapy (PTT).[6] Owing to the intrinsic hydrophobicity of conjugated dyes, water-soluble modification of these synthetic tracers or therapeutic agents is necessary, which can significantly enhance delivery efficiency to lesions of the organisms. In the past few decades, the scientific community is devoted to modifying the organic chromophores with water-soluble polymers, peptides or dendrimers, or loading them within liposome vesicles, silica microspheres or other carriers to solve water solubility and biocompatibility issues.[7] Chemical modification and delivery strategy design endow these biomedical materials with desired functions. However, chemical modification strategy is time consuming with high cost, and delivery strategy is only suitable for very small amount of chromophores. Thus, more facile and effective methods to control the photophysical and biological properties of these materials are still highly demanding.

With the development of scientific research on dye aggregation, the construction of dye self-assembly provides new ideas for controlling photophysical properties and in vivo delivery of dyes. On the one hand, changes in the stacking and arrangement of dyes give rise to unique photophysical properties. For instance, J-aggregates discovery of pseudoisocyanine in 1937 launched the new chapter in aggregate science.[8] J-aggregates of dyes usually cause a significant red shift of both absorption and emission band, which is commonly used to construct bioimaging probes in the NIR-I and NIR-II region.[9] The establishment of Ben Zhong Tang’s aggregation-induced emission (AIE) theory also provides a new direction for the design and application of aggregated dyes.[10] At present, luminogens showing AIE characteristics (AIEgens) have played an important role in the biomedical fields including biological imaging, sensing, photodynamic, and PTT.[11] On the other hand, nano agent derived from organic dyes can pass through biological barriers such as tumor-blood vessels through the enhanced permeability and retention (EPR) effect.[12] Moreover, these nano-reagents possess controllable size and surface properties as well as enhanced stability. Recently, numerous publications focus on the construction of organic dye assembly to realize disease diagnosis and treatment.[13] Therefore, studying dye aggregation-induced photophysical property changes is of great significance for the design of multifunctional nanomedicine and its biological applications in multiple fields.

This review will focus on the photophysical property changes of organic dyes caused by the aggregation and their biomedical applications. The contents mainly include the preparation method of dye assemblies and their tunable photophysical properties. The mechanisms of aggregation-induced photophysical changes were introduced such as fluorescence change, enhanced intersystem crossing (ISC), and enhanced heat generation. Recent progress on biomedical applications of the dye assemblies with these characteristics is summarized, including fluorescence imaging, afterglow imaging, PA imaging, photodynamic therapy (PDT), and PTT. This review aims to provide guidance for the construction of organic dye assemblies and biological application strategies.

**PREPARATION OF DYE ASSEMBLY**

Nano reagents formed by dye assembly are widely used in the fields of bioimaging, disease diagnosis, and treatment. Compared with small molecular dyes, nanoparticles have better stability, bioavailability, tumor enrichment, and lower blood or kidney clearance rates.[14] The formation of special aggregation forms such as H/J-aggregation and crystals will also bring about great changes in the photophysical properties, improving the performance of dyes in biological applications. The preparation and application of organic dye assembly are summarized in Table 1. Generally, the aggregated dyes can form nanoparticles in two ways, that is, bottom-up and top-down.[15]

Owing to the operational easiness and high efficiency, bottom-up strategy is currently the most widely used method for preparing dye assemblies. During the assembly process, the molecules are driven by hydrophobicity, electric charge, π-π interaction, and hydrogen bonding to form
**TABLE 1** Preparation and application of organic dye assemblies

| Components | Assembly method | Applications                                      | Ref  |
|------------|-----------------|---------------------------------------------------|------|
| Amphiphilic BODIPY-polymer conjugate | Self-assembly | FL imaging guided drug delivery                   | 36a  |
| Amphiphilic Cy5-drug conjugate      | Self-assembly | Cancer chemo-PDT                                  | 70   |
| PMI dye and BSA                      | Co-assembly   | Deep cancer PDT                                   | 36e  |
| Cy7 and phospholipid                 | Co-assembly   | NIR-II FL imaging                                | 50   |
| Cy7 and hollow silica                | Confined self-assembly | NIR-I FL imaging                          | 46   |
| Cy7-pyrene derivative                | Nano-precipitation | PTT                                           | 75   |
| Carbazole                            | Top-down method | Afterglow/FL imaging of lymph node               | 24a  |
| Carbazole                            | Top-down method | Afterglow imaging                                | 65   |

Abbreviations: FL, fluorescence; HSA, human serum albumin; PDT, photodynamic therapy; PTT, photothermal therapy.

Regular nanostructures. Therefore, when choosing self-assembly strategy, it is generally necessary to consider the planarity of dye molecule, the hydrophilicity, and hydrophobicity of functional groups as well as charge and steric hindrance effect of the substituent. For example, perylene bisimide dyes possess a polyaromatic ring structure with good molecular planarity and rigidity, enabling the formation of strong \( \pi-\pi \) stacking, which can be assembled into various morphologies such as nanospheres, fibers, and sheets. By introducing polypeptides, polymers, cationic or anionic groups into dye molecules, amphiphilic agents are obtained to promote self-assembly of dyes and form uniform and stable nanoparticles. In addition, introducing steric hindrance groups can change the molecular configuration and stacking pattern of the dye, leading to controllable formation of H or J-aggregates with special optical properties. Commonly used bottom-up methods include self-assembly of amphiphilic molecules, co-assembly, nano-precipitation (also called solvent displacement), carrier-assisted, and confined assembly (Scheme 1B). These methods have been widely used in the preparation of nano-drugs or polymer nanoparticles. For the preparation of organic dye self-assembly, these methods are also simple and effective. Amphiphilic molecules with good water solubility can be well dispersed in water and form self-assembly spontaneously. For dyes with strong hydrophobicity, nano precipitation approach can be used to prepare nanoparticles. The dye molecules are injected from organic phase into water phase, and residual organic solvent is removed through dialysis. Co-assembly is another method to prepare nanoparticles. The intermolecular forces of dye molecules and other molecules (drugs, phospholipids, amphiphilic polymers, polyelectrolytes, etc.) are the driven force in forming stable supramolecular assemblies, and dye molecules will exhibit certain aggregation in the complex. In addition, confinement assembly is another useful method to obtain regular aggregate form of dye molecules, which is difficult to achieve under normal assembly process. For example, cyanine (Cy) dye forms J-aggregates in the pores of hollow or porous silica microspheres, leading to significantly red-shifted absorption band, which broadened the application of dyes in biomedical field.

Compared with bottom-up method, top-down method has long been a necessary approach for preparing nano-functional materials dating back to the origin of nanomaterials concept. In some cases, dye-based crystals possess enhanced fluorescence or room-temperature phosphorescence (RTP) emission performance. Most of the small molecular dye-based crystals can be easily achieved in organic solvents due to their small size and mild intermolecular interactions. However, in aqueous environment, crystal structure for these dye molecules is relatively difficult to obtain through bottom-up assembly, and the top-down method is favorable for the preparation of crystal-like nanoparticles. Since the nanoparticles prepared by top-down strategy are directly derived from the solid crystals of the dye, their molecular packing and photophysical properties can be optimally retained. Moreover, sonication makes its size miniaturized, and encapsulation with amphiphilic compounds gives the nanoparticles water solubility and stability (Scheme 1B). Therefore, top-down strategy can maintain the original solid-state fluorescence and phosphorescence emission properties of the dye to the greatest extent, which is particularly important for biological applications.

**PHOTOPHYSICAL PROPERTIES OF DYNE ASSEMBLY**

Molecular science addresses the study of structures and properties of materials at single molecular level or small interacting complexes of molecules. With the development of molecular assembly, the concept of “aggregate science” is put forward to fill the gaps between molecules and aggregates. The aggregation-property relationships established for dye assemblies are expected to make contributions for new materials and technologies development. The photophysical properties mainly include fluorescence emission, phosphorescence emission, absorption band shift, photodynamic process, and photothermal conversion of dye assemblies. These photophysical properties changes heavily rely on the dissipation pathway of molecular excitation energy. Generally, the intermolecular communications, involving mechanical hindrance effect and specific intermolecular stacking modes, significantly restrict the molecular motions or enhance the intermolecular electronic coupling in the aggregate state, leading to the variation of photophysical properties of dyes.

From the phenomenological perspective, the fluorescence emission of aggregates might be quenched or enhanced, known as aggregation-caused quenching (ACQ) and AIE, respectively. The two phenomena are often visually demonstrated by adding ACQ (such as fluorescein) and AIE (such
AGGREGATE

as hexaphenylsilole) fluorophores to a binary solvent system with different tetrahydrofuran/water ratio (Figures 1A and 1B).\cite{27} As water fraction increases, ACQ dye shows fluorescence quenching in the aggregated state. However, greatly enhanced emission was observed with AIE dye in the aggregated state. On account of the two features, strategies of avoiding ACQ or taking use of AIE effect have been investigated extensively.

The Jablonski diagram is often used to described the photophysical changes of dye (Figure 1C). The aggregation and crystallization of dyes might facilitate ISC from the singlet excited state to triplet excited state, further enhancing the triplet emission, known as phosphorescence, or the energy can be transferred to triplet oxygen to produce reactive oxygen species (ROS) such as singlet oxygen (\(^{1}O_2\)) for the photodynamic process.\cite{28} In addition, aggregation-induced strong excitonic coupling may lead to quenching of fluorescence and splitting of energy level. As a result, nonradiative decay such as internal conversion and vibration relaxation is enhanced, leading to abundant heat generation.\cite{29} Dye assembly with high nonradiative decay rate is suitable for construction of photothermal agents.

Optical spectra shift is another important issue in dye aggregation. Compared with monomer state, H-aggregate and J-aggregate mode of dye exhibit distinguished absorption band and emission feature. Generally, H-aggregation and J-aggregation refer to the face-to-face and head-to-tail aggregation of the \(\pi\)-plane (Figure 1D), respectively.\cite{30} During the translation of two parallel dipoles along the dipole direction, the energy level splits with the crossover angle \(\theta\). The aggregation of molecules is called J-aggregation when the angle \(\theta\) is less than 54.7\(^\circ\) and H-aggregation when the angle \(\theta\) is greater than 54.7\(^\circ\). Usually, H-aggregate exhibits blue-shifted absorbance band and quenched emission. Due to intense \(\pi-\pi\) stacking interactions among \(\pi\)-conjugate molecule, H-aggregation, therefore, is always considered as the major reason responsible for emission quenching of \(\pi\)-conjugate dye aggregates. In contrast, J-aggregate shows red-shifted absorbance band and retained emission peaks with narrowed full width half maxima and smaller Stoke’s shift. Therefore, the construction of H/J-aggregation is a vital methodology for tuning the photophysical characteristics of dye assembly.

AGGREGATION-INDUCED FLUORESCENCE CHANGE FOR BIOIMAGING

ACQ dye for achievable fluorescence imaging

ACQ research has a long history and has been recorded for more than 60 years since Förster discovered the concentration dependent quenching phenomenon in 1954.\cite{31} Subsequently, numerous researches on ACQ have led to a deep insight into its photophysical processes and mechanisms. Generally, most ACQ luminophores have stable disc- or rod-like \(\pi\)-conjugated planar structure, such as perylene diimide (PDI), perylene monomide (PMI), Cy, and dipyrometheneboron difluoride (BODIPY).\cite{32} Upon excitation by light in monomeric state, the stable molecular conformation blocks the excitation energy dissipated by nonradiative intramolecular motions, allowing the radiative
pathway with strong fluorescence emission. When increasing the concentration of dyes or introducing poor solvent, the strong intermolecular \(\pi-\pi\) interactions enables the stacking of planar luminophores in an ordered fashion, forming the aggregates or clusters. The \(\pi-\pi\) interactions promote that the adjacent molecules form excimers, in which the excited molecules interact with the unexcited molecules, and the excitation energy would be largely dissipated through nonradiative pathway, resulting in the observed ACQ effect with weak or even no fluorescence. At the same time, due to the excellent electron transport properties of the conjugated molecules, the formed excitions when the aggregates get excited will rapidly migrate to the defects of aggregates or undergo non-radiative energy transfer with the impurities, thus further quenching the fluorescence.

Although ACQ phenomenon is unfavorable for biological imaging, dyes with ACQ properties are still widely used in life sciences. At present, many strategies are developed to improve the dispersion of dye molecules, thus, inhibiting the aggregation. For instance, water-soluble groups are chemically attached to the dyes, and hydrophilic polymers are used for encapsulation of dye molecules.\(^{[7a,33]}\) Meanwhile, many ACQ probes with switchable fluorescence have been developed and applied to bioimaging.\(^{[34]}\) The aggregation of ACQ dyes causes fluorescence changes, which can be used to construct sensitive on-off or off-on probes for fluorescence sensing and imaging in vivo. For example, Yin et al synthesized multifunctional dithiaoacetate-modified perylenediimide (DTPDI) as a highly sensitive and selective fluorescent chemosensor for recyclable \(\text{Hg}^{2+}\) detection.\(^{[35]}\) Upon the addition of \(\text{Hg}^{2+}\) ions, the hydrophobicity of DTPDI increased significantly, resulting in rapid ACQ of the probe. With detection limit of 0.1 nM, DTPDI can detect \(\text{Hg}^{2+}\) in live cells with high sensitivity.

Another type of ACQ-based fluorescent probes are aggregated in the initial and activated after being de-aggregated under specific stimuli (such as \(\mathrm{pH}\), reductive agents, and protein), resulting in “light up” performance.\(^{[36]}\) These kinds of probes can avoid the “always on” fluorescence, improving the sensitivity and accuracy of bioimaging. Xie et al prepared self-assembly of BODIPY conjugated \(\mathrm{pH}\) responsive polymer (Figure 2A).\(^{[36d]}\) BODIPY aggregations were formed in the hydrophobic core of the nanoparticle, resulting in the fluorescence “off” state. Tumor acidic environment can trigger the dissociation of nanoparticle with fluorescence enhancement for bioimaging. Liang et al prepared the NIR probe 1 with Cy5.5 dye (Figure 2B).\(^{[36o]}\) Probe 1 was firstly reduced and subjected to a condensation reaction to self-assemble into 1-NPs with self-quenched fluorescence. Then the NIR fluorescence of 1-NPs was turned “on” by legumain-induced specific disassembling of the nanoparticles. With a fluorescence “turn on” manner, 1-NPs were successfully applied to image legumain activity both in vitro and in vivo. Yin et al designed an enzyme activatable PML-based nanoparticle (FHP) for NIR fluorescence imaging-guided PDT in vivo (Figure 2C).\(^{[36e]}\) The enzyme-triggered disassembly of FHP leads to enhanced fluorescence intensity (ca. eight-fold) and photodynamic ability, enabling in situ tumor imaging and further PDT.

Moreover, when ACQ of the dye is accompanied by other intramolecular charge or energy transfer, complete fluorescence quenching can be achieved.\(^{[37]}\) After dis-assembly, this kind of probe exhibits high contrast characteristic. Gao et al designed a series of ultra \(\mathrm{pH}\) sensitive nanoprobe based on Cy5.5 dye and tertiary amine modified polymer (Figure 2D).\(^{[37b]}\) Dye aggregate was formed in polymer micelle, and its fluorescence was completely quenched due to HOMO fluorescence resonance energy transfer process. Under acidic tumor microenvironment, the nanoprobes can be activated as a result of micelle dissociation, with a > 300-fold tumor-to-blood signal ratio. The nanoprobe achieved specific imaging of tumor tissue in multiple tumor models (Figure 2E). In other cases, off-on probes can also be constructed through the introduction of dark quencher. For instance, the famous “molecular beacon” strategy is based on such protocol. Molecular beacons are probe molecules that exhibit a characteristic stem-loop structure through which the 5’ and 3’ ends are maintained in close proximity. Fluorescence from a chromophore at one end of the probe is suppressed by a nearby dark quencher. Upon binding to the target, chromophore and dark quencher were separated to produce measurable fluorescence.\(^{[38]}\) Molecular beacons with high selectivity have been developed for the detection of mRNA, tumor markers (Figure 2F) and amyloid-\(\beta\) peptide generation.

### AIE dye for “turn-on” biosensing

AIE is another photophysical phenomenon related to the aggregation of luminophores. The concept of AIE was developed by Ben Zhong Tang in 2001.\(^{[39]}\) Different from ACQ luminophores, AIE agents have motorable molecular moieties, such as phenyl rings. The widely accepted restricted intramolecular motions theory is that these phenyl rings linked by single bonds could act as the molecular motors dissipating the excitation energy through vigorous intramolecular motions, including rotation and vibration (Figures 3A and 3B).\(^{[27,40]}\) Upon aggregate formation, intramolecular motions are restricted by the intermolecular interactions and steric hindrance effect, releasing the emission of AIE agents. Meanwhile, the highly twisted molecular conformation hampers the intramolecular \(\pi-\pi\) stacking interaction, preventing the formation of weak-emissive excimers and inducing the high fluorescence emission. Thanks to these unique features, AIEgens have shown great prospects in bio-applications such as fluorescent detection, bioimaging, and disease theranostics.\(^{[41]}\)

AIEgens have been successfully used as fluorescent “turn-on” probes and ratiometric sensors for biological applications.\(^{[42]}\) Liu et al reported HClO-activatable theranostic nanoparticles (DTF-FFP NP) for image-guided bacterial ablation in phagocytes (Figure 3C).\(^{[42]}\) Amphiphilic polymer Pluronic F127 was used to integrate a photosensitizer DTF with AIE property and an HClO-responsive NIR dye molecule FFP, resulting in DTF-FFP nanoparticles. Owing to the energy acceptor characteristic, FFP can quench the fluorescence and ROS generating ability of DTF, which endowed high biocompatibility of DTF-FFP nanoparticles within normal cells and tissues. Phagocytes can release HClO within the infection sites, triggering the response of nanoparticles (Figure 3D). When the DTF-FFP nanoparticles were delivered to the infection sites, HClO stimulation facilitated the degradation, leading to the release of red
fluorescence and ROS generating ability. Thus, the selective activation of both fluorescence and photosensitization of DTF-FPP nanoparticles exhibited potential in precise inflammation therapy. Zhao et al. reported a new fluorescent bio-probes TPA-BTTDO within polymeric matrix, which can sequentially localize in lysosome and lipid droplets (LDs) with red and cyan emissions, respectively. By monitoring the emission color change, the dynamic processes of the probes escaping from lysosome and then enriching in LDs, and finally returning to lysosome after LDs consumption were visualized. Dynamic movement and consumption of LDs was traced with a high signal-to-noise ratio. Multi-functional AIEgens integrate with bio-imaging, disease diagnosis and treatment are of vital importance. Peng’s group synthesized an AIEgen probe DQM-ALP for the imaging of alkaline phosphatase activity to facilitate early tumor detection and excision (Figure 3E). The rapid liberation of DQM-OH aggregates in the presence of alkaline phosphatase resulted in AIE effect. DQM-ALP enabled differentiation between tumor and normal tissue both ex vivo and in vivo, suggesting the probe may serve as a powerful tool to assist surgeons during tumor resection (Figure 3F).

**J-aggregation-induced red shift for NIR imaging**

The intravital diagnosis capacity of the conventional phototheranostic agents is restricted by its short absorbance wavelength that exhibit extremely limited tissue penetration. Acquiring fluorescence signals is greatly dependent on the interaction between photons and biological tissues, such
as reflection, absorbance, scattering, and autofluorescence, resulting in low tissue penetration (<3 mm) and loss of physiological and pathological information at the whole-body level (Figure 4A). Therefore, developing phototheranostic agents with suitable absorbance and fluorescence in NIR region (NIR-I region: 650–1000 nm, NIR-II region: 1000–1700 nm) for bioimaging is urgent. Benefiting from the red-shifted absorbance and enhanced emission, J-aggregates by self-assembly could be utilized for extending the spectral properties of dyes and exhibiting potential in fluorescent imaging. Meanwhile, the narrowed bands can facilitate multiplexed imaging and increased molar extinction coefficient ($\varepsilon$), thus, obtaining bright materials (Figure 4B). Based on the exciton model developed by Michael Kasha, the relationship between the molecular stacking modes and fluorescence properties can be rationalized by the dimer model, as shown in Figure 4C. Upon excitation of the dimer, the excited state energy level splits into two levels because of electronic degeneracy. When the transition dipoles are in line with the molecular axis of the dimer, the transition to lower excited level is allowed, and the maximum absorption of the dimer is red-shifted relative to the absorption of the monomer. Meanwhile, J-aggregates possess small Stokes shift with high fluorescence quantum yield.

Cy is a class of long conjugated fluorescent molecules with wide-range adjustable spectral properties. The absorption and fluorescence can be extended from the visible region to the NIR region by adjusting the length of the polythene chain. Due to large molecular polarizability, strong intermolecular attractive dispersion forces can ensure the stability of the aggregate formed by Cy. Based on that, Sletten et al reported the J-aggregates of thiazole Cy (IR-140) prepared through confined self-assembly strategy inside hollow mesoporous silica nanoparticles (Figure 4D). The IR-140 J-aggregates exhibit high stability and NIR-I absorbance, allowing the imaging with 980 nm light excitation and 1000–1700 nm acquisition (Figure 4E), providing high resolution short-wave infrared in vivo images. Zhang et al reported a J-aggregate of amphiphilic Cy FD-1080 by co-assembly with 1,2-dimyrystoyl-sn-glycero-3-phosphocholine (Figure 4F), exhibiting strong absorbance peak at 1360 nm and emission peak at 1370 nm (Figure 4G). Importantly,
the remarkable fluorescence intensity at 1500 nm leads to the superior imaging ability in vitro and in vivo in NIR-II region. The fluorescence imaging was performed to monitor the in vivo dynamic change of carotid artery in hypertensive rats after the administration of the clinically used hypotensor Isoket (Figure 4H). This work provides a novel route for the preparation of NIR-II J-aggregates, which may be extended to other NIR dyes to form J-aggregates and achieve superior bioimaging at even longer wavelength.

AGGREGATION-INDUCED RTP FOR AFTERGLOW IMAGING

RTP materials have attracted great attention for their potential applications in optical sensors, information encryption, and bioimaging. Afterglow imaging, with a certain amount of time after removal of illumination source, can minimize biological autofluorescence and background interference, which can achieve much clearer and more reliable bioimaging with high signal-to-noise ratios without real-time external excitation. Thus, high effective RTP is critical to meet the needs of bio-application.

To obtain persistent and bright RTP, not only generating sufficient but also stabilizing the generated triplet excitons is necessary. However, compared with inorganic and organometallic phosphor, most organic compounds exhibit inefficient ISC due to the weak spin orbital coupling. In addition, generated triplet excitons are easily quenched by oxygen and moisture at room temperature. Therefore, it is difficult to realize pure organic highly effective RTP luminophores. Based on a comprehensive understand-
Aggregation-induced RTP: Mechanisms and afterglow imaging applications. (A) Radiative and nonradiative decay parameters in Jablonski diagram. (B) Molecular structures of three nanoparticles and mechanism of H-aggregation enhanced ultralong RTP. (C) Design and synthesize of nanoparticles based on top-down and bottom-up methods. (D) Ultralong phosphorescence and fluorescence imaging of lymph node after the intradermal injection of OSN1-T into the forepaw of mice. (E) Schematic diagram of aggregation induced-intersystem crossing (AI-ISC) process. (F) Chemical structures of three compounds and illustrating the aggregated production of RTP excited by visible and NIR-light. (G) Image of HeLa cells at different excitation wavelengths (488 nm, 820 nm) and bright field, respectively. Scale bar: 10 μm. (A) Reproduced with permission: Copyright 2019, American Chemical Society. (B, C, and D) Reproduced with permission: Copyright 2017, Wiley-VCH Verlag GmbH & Co. (E) Reproduced with permission: Copyright 2016, The Royal Society of Chemistry. (F and G) Reproduced with permission: Copyright 2019, American Chemical Society.

 Especially, AIE can bring a new view into phosphorescence about single molecules and aggregates, which have boosted the researches on phosphorescence in recent years. Many aggregation-induced RTP systems with multiple functionality and biocompatibility have been developed and applied to biological imaging. Based on varied mechanisms, many guidances are available for aggregation-behaviour modulation of their RTP lifetimes and emission efficiency.

Aggregate-enhanced RTP lifetimes

To prolong the RTP lifetime ($\tau_p$), the phosphorescence radiative decay rate ($k_p$), non-radiative decay rate ($k_{nr}$), and quenching rate ($k_q$) should be reduced simultaneously (Figure 5A, Eq. 1). In general, phosphors in monomer state experience intramolecular motion and intermolecular collisions with oxygen, resulting in no phosphorescence. However, aggregation can restrict the structures via various
intermolecular interactions, which can suppress intermolecular motion, and thus reduce $k_{ew}$. In addition, aggregation can reduce the quenching of triplet excitons caused by oxygen, heat, and moisture.

As an ordered aggregation form, crystallization can effectively promote RTP emissions. Tang groups proposed the concept of “crystallization-induced phosphorescence,” which can stabilize the triplet exciton and prevent it from being quenched by oxygen and moisture, thus inducing persistent RTP. In this study, the crystal with denser aggregation mode exhibits obviously longer RTP lifetime. Moreover, these crystals show the presence and disappearance of triplet emissions with the transformation between crystalline and amorphous states. The change of aggregation forms has a great influence in RTP properties, which is also confirmed in dopant-based and polymerization systems. Zhao’s group reported a single-molecule RTP phosphorescence in PVA polymer matrix, while RTP phenomenon can be completely suppressed with the formation of aggregates. In this system, PVA provides a confined environment to stabilize the triplet state, and naphthalene compounds exhibit an anti-aggregation effect. Theoretical calculations suggest that the aggregation depresses spin-orbit coupling between the excited singlet and triplet states and enhances the nonradiative quenching process.

Nowadays, some crystallization-based RTP materials have been developed for biological applications. Pu et al designed and synthesized a series of organic nanoparticles with ultralong RTP for application in afterglow imaging in vivo (Figure 5B). Through a top-down nanoparticle preparation strategy, the phosphors molecules formed H-aggregates in nanoparticle, which can immensely stabilize the triplet excited states and prolong the lifetime (Figure 5C). Compared with solution state, the molecules in nanoparticle aggregate state show such ultralong lifetime, which has been permitted imaging of lymph nodes in living mice with weakened biological background autofluorescence (Figure 5D). This study provides a universal design principle to prolong the lifetime of RTP molecules to the level that can be effective for bioimaging.

Overall, aggregation can influence $k_p$, $k_{ew}$, and $k_{nr}$, thus modulating the RTP lifetime. Similarly, the aggregation strategies used to reduce $k_{ew}$ and $k_{nr}$ for prolonging RTP lifetime are also all applicable for enhancing the RTP efficiency, which will be discussed in the next section.

Aggregate-enhanced RTP efficiency

Phosphorescence emission is originated from the triplet exciton radiative process, in which ISC process plays a significant role. ISC is the transition of excitons from singlet state to triplet state, in which electrons of excited molecule spin in reverse and change the multiplicity of the molecule. In general, ISC processes are forbidden, and spin-orbit coupling can mitigate this resistance. Due to the weak spin-orbital coupling, traditional luminophore shows low triplet exciton yield. Jang et al proposed a new mechanism called aggregation-induced ISC (AI-ISC) to understand the effect of aggregation on increasing the ISC efficiency. Compared to monomer, more excitonic couplings cause excited-state energy splitting and overlapping of singlet and triplet in aggregate (Figure 5E). The energy splitting and overlapping significantly produce many ISC channels with very small $\Delta E_{ST}$ in aggregates, which is available for ISC processes. The AI-ISC effect is conductive to highly efficiency phosphorescence emission (Eqs. 2 and 3).

As discussed above, crystallized aggregates can better confine nonradiative relaxation for effectively bright RTP emission. However, crystal is too large to be utilized within living body. Nano-crystallization is also a way of recrystallization to prepare dye nanocrystals. This method is based on compounds that can crystallize in a good/bad solvent medium such as THF/ water. The asprepared nanocrystal can provide appropriate size and good water dispersity for biomedical applications, which show effective cellular uptake and bright phosphorescence emission. Liu and Chi et al reported a nanocrystal strategy to realize high brightness of red-emissive RTP molecules for biomedical applications. Introducing a soft alkoxy spacer based on C-Br results in different crystallized aggregation due to more easily formed Br-H bond, which also facilitates intermolecular electron coupling and intermolecular heavy halogen effect. This design strategy results in more than 200% enhancement in the phosphorescence quantum efficiency of C-C4-Br (11%) as compared to C-Br (5%).

In addition, crystallization also have facilitated more channels for ISC than their monomers, leading to an enhancement in the RTP efficiency. Huang’s group focuses on a quantitative investigation about the relationship of aggregation and RTP properties based on aggregation engineering by varying the position and number of CN substituents of cyanophenyl carbazoles. It is found that thermally activated reversed phase transformation H-aggregation plays a key role in the splitting energy ($\Delta \varepsilon$) and the RTP emission. The H-aggregation of DCNPPhCz molecules results in more facile ISC channels, and thus exhibiting high RTP quantum yield (8.6%). Through top-down method, the nano-aggregates are constructed, which has been applied for afterglow bioimaging. Similarly, difluoroboron $\beta$-diketonate compounds were assembled to form water-soluble nanoparticles (Figure 5F). Through nanoprecipitation method in solvent/antisolvent upon sonication, self-assembled organic nanoparticles were prepared, showing efficient RTP in aqueous media. It is revealed that the efficient RTP is originated from aggregated dimers in their excited states. The multiple intermolecular interactions and intermolecular charge transfer in the aggregation state play a significant role in promoting the production of ISC channels and suppressing the nonradiative decays to boost the RTP in water. Particularly, the nanoparticles show a bright RTP irradiated by visible- and NIR-light, which is suitable for bright imaging of HeLa cells (Figure 5G).

Phosphors need to maintain sufficiently emissive in biological environment, while they usually display RTP in crystal aggregate but only weak emission in amorphous aggregate. Moreover, plenty of reported organic RTP materials exhibit short-wavelength emissions, which also limits the bio-applications. Thus, through molecular design, RTP materials with enhanced crystallization ability and red-shifted emission band can be achieved. On the other hand, water solubility, particle size and biocompatibility issues of organic RTP materials should be addressed to better suit the biological applications.
Aggregate enhanced ISC for PDT

PDT takes use of ROS (especially singlet oxygen) produced by photosensitive reagents to kill cancer cells and treat tumors. PDT possesses the advantages of non-invasiveness, high selectivity, and little side effects. Organic photosensitizers such as methylene blue, Rose Bengal, and porphyrin derivatives have shown great potential in clinical PDT. However, traditional fluorophore has low efficiency of ISC, thus resulting in a low yield of triplet excitons and $^1$O$_2$. The $^1$O$_2$ quantum yield of organic dye can be effectively enhanced by introducing heavy atoms such as iodine and metal to the chromophore, resulting in higher synthesis cost and potential cytotoxicity.

According to the AI-ISC theory, dye aggregation can promote the generation of triplet excitons. Apart from phosphorescence radiation, the generated triplet excitons can also decay to the ground state by transferring energy to molecular oxygen ($^3$O$_2$), and thus produce singlet $^1$O$_2$, which is termed as aggregation enhanced photodynamic effect. Yoon et al prepared a boronic acid-functionalized phthalocyanine, which can self-assemble into uniform nanoparticle in water (Figure 6A). The self-assembly PcN4-BA exhibited enhanced ROS generation efficiency due to AI-ISC process. PcN4-BA displays excellent photodynamic antimicrobial activity against both common and antibiotic-resistant bacterial strains (Figures 6B and 6C). In another case, a traditional imaging agent Cy5 dye was turned into photosensitizer through aggregation. Yin et al prepared an amphiphilic Cy5 dye-drug conjugate ICy5-CPT-RGD (Figure 6D). ICy5-CPT-RGD can self-assemble into nanodrug (PTN) with 10-fold enhancement of $^1$O$_2$ quantum yield (Figures 6E and 6F). Upon light irradiation and weak acidic environment, PTN disassembled into ultrasmall nanoparticles with enhanced fluorescence. Based on these features, PTN achieved in situ tumor imaging and potent tumor inhibition by deep chemopDT (Figures 6G and 6H). When it comes to the ACQ dyes, radiative decay and ISC process are competitive. Proper
aggregation can suppress the fluorescence emission and increase the probability of ISC, achieving aggregation enhanced photodynamic effect. Switch of photosensitizer and fluorescent agent can be realized through controlling of dye aggregation/deaggregation process. However, severe aggregation will lead to photodynamic ability decrease of the dye and quenching of the singlet oxygen generated. Therefore, construction of amphiphilic photosensitizers, self-assembly manipulation, and aggregation degree control of the dyes in the water environment are essential parameters for enhancing the photodynamic performance.

AIE dyes with aggregation-enhanced photodynamic properties have also been reported. Some AIE dyes cannot generate ROS in the solution state but process high ROS generation efficiency in the aggregated state.[70] It is assumed that active intramolecular motion accelerates the nonradiative decay and result in inefficient ISC and destabilization of the triplet state, leading to poor ROS generation ability in solution. However, when the dye underwent aggregation process, effectively suppressed nonradiative decay, significantly improved ISC efficiency, and triplet excitons stability was achieved, resulting in high ROS generation efficiency.[10b] Tang et al report a strategy of generating strong intramolecular charge transfer in electron-rich anion-$\pi^-$ AIE-active AIEgens (Figure 6a).[71] Upon aggregation, nonradiative IC of AIEgens was suppressed while radiative decay ($k_r$) and $k_{isc}$ were promoted to boost free radical generation through both type I and type II photochemical mechanisms (Figure 6j). These AIEgens showed high-efficiency cancer cell killing in vitro under normoxia/hypoxia and imaging-guided PDT in vivo.

**Aggregate enhanced heat generation for PTT**

PTT can induce the ablation of tumors through elevating the temperature of tumor tissues and attracted much attention due to its high selectivity, low invasiveness, high effectiveness, and overcoming of cancer multidrug resistance.[72] Aggregation of dye (especially H-aggregation) also boosts heat generation through enhancing internal conversion and vibration relaxation process.[47b] H-type aggregates are easily obtained due to the strong intermolecular $\pi-\pi$ interaction. In general, the transition from H-type aggregates to the upper excited-state levels is allowed. Thus, the maximum absorbance peak is blue-shifted relative to the monomer absorption. In contrast, transition to the lower level is forbidden, resulting in suppressed radiative decay or even quenched emission. The enhanced nonradiative pathway facilitates heat generation and enables PA imaging guided PTT.

Wang et al reported the self-assembly of squaraine (SQ) dyes embedded in phospholipid bilayers of liposomes (SQCL) with variable mixing ratios of SQ and phospholipids (Figure 7A).[73] H-aggregation of SQ was formed, leading to increased photothermal conversion efficiency. Furthermore, the PA signal was enhanced with higher SQ content. Both fluorescence and PA signal of SQCL can be detected under 1:100 (w/w) of SQ and liposomes (Figure 7B). SQCL was applied as a dual-modal imaging probe in vivo for solid tumor. Li et al reported a self-assembly of Cy into H-aggregates as a new supramolecular strategy to fabricate small-molecule-based photothermal nanomaterials.[74] The conjugating of the pyrene and TPE moieties on Cy backbone provide significant steric hindrance, leading to the H-type dimers among Cy backbones, which exhibit sharp blue-shifted absorbance peak and completely quenched fluorescence (Figure 7C). Meanwhile, two H-aggregates show excellent photothermal ability with the photothermal conversion efficiency of about 9.5% for Cy7-TPE and 22.3% for Cy7-pyrene, respectively. Not only fluorescent dye but also photosensitizer can be converted into photothermal agent when aggregated.[29] Yan et al constructed supramolecular self-assembly of dipeptide conjugated porphyrin with photothermal conversion efficiency of 54.2%.[75] The tight molecular packing of porphyrin units facilitated intermolecular charge transfer or energy transfer, which completely quenched fluorescence and inhibited ISC and resulted in heat generation, realizing a PA-guided PTT for cancer (Figures 7D and 7E).

Construction of dye J-aggregation for PTT was also well-documented. For example, Zheng et al reported the formation of highly ordered porphyrin J-aggregates and disordered aggregates in lipid. Porphyrin-lipid J-aggregates are photostable with a photothermal efficiency of 54 ± 6%, and disordered aggregates are photodynamically active.[176] In other cases, the J-aggregation of dye possessed all features of red shifted fluorescence emission, photodynamic ability, and photothermal conversion.[148] The phenomenon was attributed to the balance of radiative decay, ISC, and heat generation-related photophysical processes. In addition, the dye aggregations possess enhanced stability compared with that of their monomer state.[46a,77] The resistant of photo bleaching of dye assembly leads to more effective photothermal conversion under long time and multiple cycles laser irradiation.

**CONCLUSION AND PROSPECT**

Dye aggregation is a tricky problem that needs to be solved in the biomedical applications. Instead of inhibiting dye aggregation, this review focuses on how to control dye aggregation through self-assembly, thus obtaining desired photophysical properties to achieve diversified biological applications. This review discusses and summarizes: (1) the method of constructing nanomaterials with different aggregations and crystal states through self-assembly of dyes, (2) the mechanism by which dye aggregate changes its photophysical properties through affecting molecular motion, energy level, or de-excitation mode, (3) application of dye aggregates in the fields of fluorescence detection, phosphorescence imaging, PDT, and PA imaging guided PTT.

Despite the great progress achieved, several issues are yet to be addressed. For instance, how to maintain the photophysical properties of dye aggregates and achieve self-assemblies with proper size, and high stability is of vital importance. In this case, nano-crystallization is a facile method to prepare dye nanoparticles through recrystallization. In an aqueous environment, the effect of ultrasound maximizes the nucleation rate of crystals and minimizes the growth rate of crystals, leading to the formation of very small crystals retaining the properties of dye crystal.[62,78] In the long run, facile and effective self-assembly methods suitable for aqueous environment should be developed to better serve the above purposes. Moreover, the tissue penetration depth and background
interference are also critical to be addressed. Chemiluminescence (CL), the result of chemical reactions that produces light through chemical excitation, has become a new tool for in vivo bioimaging and therapy.\textsuperscript{79} Unlike fluorescence imaging techniques, CL imaging offers ultra-high sensitivity without the background noise of biological tissues because of the elimination of excitation light, allowing for deep tissue imaging with extremely high signal-to-noise ratio, which pave the way for CL to guide cancer diagnosis and surgery.

On the other hand, most of these dye aggregates are used in cancer-related applications. At present, diseases such as cardiovascular disease and Alzheimer’s disease are serious threats to human health.\textsuperscript{80} The combination of nanotechnology and photo-theranostics brings new opportunities for early diagnosis, targeted intervention, and treatment of these diseases. Dye aggregation can provide fluorescence, phosphorescence, photodynamic, and photothermal functionalities for disease diagnosis and treatment. On the other hand, the use of dye aggregates to specifically bind to the signal molecules and proteins at the disease site can diagnose and interfere with the progress of disease for further treatment.

Thus, aggregation of dye bridges the gap between the molecular science and photophysics, which facilitated the development of dye assembly-based probes for biomedical applications. Studies in organic dye aggregate will ultimately help unveil certain life operating mechanism and guarantee life quality and health.

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