Aggregation-Induced Emission

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Far-Red and Near-IR AIE-Active Fluorescent Organic Nanoprobes with Enhanced Tumor-Targeting Efficacy: Shape-Specific Effects**

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Abstract: *The rational design of high-performance fluorescent materials for cancer targeting in vivo is still challenging. A unique molecular design strategy is presented that involves tailoring aggregation-induced emission (AIE)-active organic molecules to realize preferable far-red and NIR fluorescence, well-controlled morphology (from rod-like to spherical), and also tumor-targeted bioimaging. The shape-tailored organic quinoline–malononitrile (QM) nanoprobes are biocompatible and highly desirable for cell-tracking applications. Impressively, the spherical shape of QM-5 nanoaggregates exhibits excellent tumor-targeted bioimaging performance after intravenously injection into mice, but not the rod-like aggregates of QM-2.*

The development of bioimaging probes that can differentiate tumors from normal tissues are highly desirable for cancer diagnosis and therapy in vivo.[1,2] Fluorescent materials that provide dynamic and quantitative information of imaging biomolecules have become indispensable for biological analysis and clinical diagnosis.[3,4] In particular, organic nanomaterials with excellent synthetic flexibility for chemical modification are advantageous for real-time cell visualizations, diagnosis, and treatment of diseases in vivo.[5] While nanomaterial bioimaging displays a critical interdependent role of particle shape, size, and surface chemistry (such as polymers, liposomes, dendrimers, immunoconjugates, carbon nanotubes, porphysomes, and inorganic particles),[6] methods to rationally tailor small molecules to afford organic nanostructures with the desired morphology, and therefore performing ideal function in diagnosis or therapy in vivo is less understood.

Several impressive photoelectronic materials with well-defined morphologies can be obtained using tailor-made small molecules,[7] but these functional self-assembled materials remain unexplored for biomedical application, which is mainly due to several limitations: 1) inherent fluorescence quenching, which is common for most organic fluorophores during their aggregation in aqueous media; 2) lacking high-performance near-infrared (NIR) emission; and 3) the unclear relationship between tailored morphologies and targeting efficacy for in vivo diagnostics. Fortunately, since the concept of aggregation-induced emission (AIE) was originally reported by Tang et al.,[8a] AIE-active molecules exhibit highly bright fluorescence when aggregated, and weak fluorescence when separated in solution,[8] making them ideal for biosensing and imaging in vivo. However, mapping the nature of AIE-active organic molecules to finely control the morphologies and sizes of organic aggregated nanostructures is uncharted territory, especially the influence of substituents on the shape, and therefore the excellent optical properties for bioimaging in vivo.

As is well-known, far-red and NIR emission could minimize photo-damage to living cells, enable deep tissue penetration, and circumvent the spectral overlap with biosubstrate autofluorescence.[9] While great efforts have been made towards the development of high-performance AIE-active systems for long-term non-invasive bioimaging in vivo,[8b] the majority of AIE luminogens has emission wavelengths below 650 nm. Particularly, it is not clear whether AIE nanoaggregates with specific morphologies are suitable for targeted imaging in vivo. Herein we set out to construct a tailor-made far-red and NIR AIE-active system (Figure 1 A) employing the quinoline–malononitrile (QM) as AIE building block,[8f] wherein the morphology of organic nanostructures could be controlled by changing the electron donor groups and thiophene π-bridge. Different shapes of these AIE-active QM derivatives with red to NIR emission were carefully evaluated under an aggregated microenvironment, thus taking insight into the effect of specific shape on both real-time cell tracing and tumor-targeted imaging in vivo.

We performed a series of experiments to examine the photoluminescence properties of QM derivatives. As expected, all QM compounds exhibit red to NIR AIE-active

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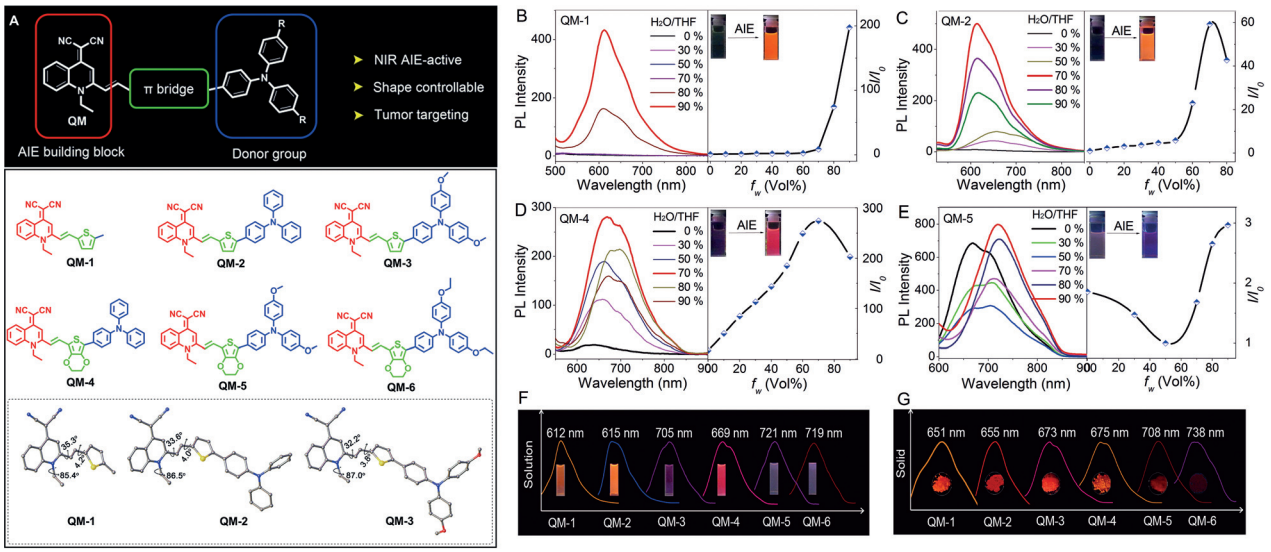

Figure 1. QM derivatives and AIE properties: A) Molecular structures and the single crystal configurations of QM-1, QM-2, and QM-3. Photoluminescence spectra and plot of the relative PL intensity of QM derivatives (10^{-5} M): B) QM-1, C) QM-2, D) QM-4, and E) QM-5 in THF/H_2O mixtures with different volume fractions of water (f_w); λ_{ex} = 480 nm. Inset: Fluorescent photoimages in pure THF solvent and THF/H_2O solution of B) QM-1 (f_w = 90%), C) QM-2 (f_w = 70%), D) QM-4 (f_w = 70%) and E) QM-5 (f_w = 90%) under 365 nm illumination. F) Normalized fluorescent spectra of QM-1 (f_w = 90%), QM-2 (f_w = 70%), QM-3 (f_w = 90%), QM-4 (f_w = 70%), QM-5 (f_w = 90%), and QM-6 (f_w = 90%) in THF/H_2O solution. G) Normalized fluorescent spectra of QM derivatives in the solid state.

characteristics along with an increasing volume fraction of water in tetrahydrofuran/water (THF/H_2O) mixtures (Figure 1; Supporting Information, Figure S1 and Table S1). Moreover, their fluorescent properties are dependent upon the aggregated microenvironment. Specifically for QM-1, a strong fluorescence was not observed until the volume fraction of water (f_w) in THF/H_2O solutions up to 80% (Figure 1 B). Its fluorescence quantum yield (Φ_F) exhibited AIE light-up enhancement by 178-fold in the mixed f_w = 90% THF/H_2O solution compared with that in pure THF solution.

When strong donor groups are introduced into the thiophene moiety elongating the π-conjugated systems of the QM molecules, their emission spectra are extended to deep red, and even to the NIR region. As shown in Figure 1 C, with a gradual addition of water into THF, QM-2 molecules containing the triphenylamine donor clustered into nanoaggregates and the emission was dramatically enhanced with an f_w increase, showing an obvious AIE effect. Successively, the emission spectra showed a little decrease when f_w > 80%, which might be attributed that QM-2 molecules aggregate and precipitate quickly at higher water fraction, leading to amorphous agglomerate formation with lower fluorescence intensity.[10] Moreover, the fluorescence quantum yield (Φ_F) of QM-2 was increased sharply by about 183-fold from pure THF solution to the mixed THF/H_2O (f_w = 70%) solution. Furthermore, when a stronger alkoxytriphenylamine donor moiety was introduced into QM-3, the emission peak located at 672 nm in pure THF was observed in its molecularly dissolved state. When water was mixed with THF (f_w > 50%), its emission spectra was bathochromically shifted to 705 nm with intensified AIE-active emission.

To further take insight into the substituent influence, a 3,4-ethylene-dioxythiophene (EDOT) unit was introduced into QM-2 to give QM-4, in which the steric hindrance of EDOT can change the initial D-π-A structure into more twist structure. In fact, the structural and conformational differences of QM derivatives are responsible for the different aggregated microenvironment, thus resulting in different AIE-active spectral features. As demonstrated by the X-ray diffraction (XRD) patterns (Supporting Information, Figure S2), QM-4 aggregates are mainly in amorphous state, which might affect its fluorescent quantum yield.[10] Indeed, QM-4 exhibited 17-fold enhancement in Φ_F value from f_w = 0 to 70% in a mixed THF/H_2O solution, and concomitantly the emission peak was red-shifted from 642 to 669 nm (Figure 1 D).

Following this line of thought, a combinational molecular strategy was employed in the design of QM-5, wherein the alkoxy-substituted triphenylamine moiety as a stronger donor would extend the NIR emission spectra, and EDOT-substituted thiophene would disrupt the initial D-π-A structure for tailoring the aggregate formation. Similar to QM-4, with an increase in water fraction, the emission spectra of QM-5 exhibited a large red shift from 668 nm in pure THF solution to 721 nm in f_w = 90% of the mixed THF/H_2O solution (Figure 1 E). To further verify our molecular design strategy, QM-6 was designed and synthesized, which also endowed similar AIE-active fluorescence properties. As a consequence, we are able to extend the long wavelength AIE-active luminescence of QM derivatives from 612 to 721 nm in THF/H_2O solution (Figure 1 F), and even from 651 to 738 nm in their solid state (Figure 1 G).

To explore the influence of substituents on the nanostructures, we employed a solution evaporation approach to fabricate QM aggregates. The progressive transformation of morphology for QM aggregates were successfully observed,

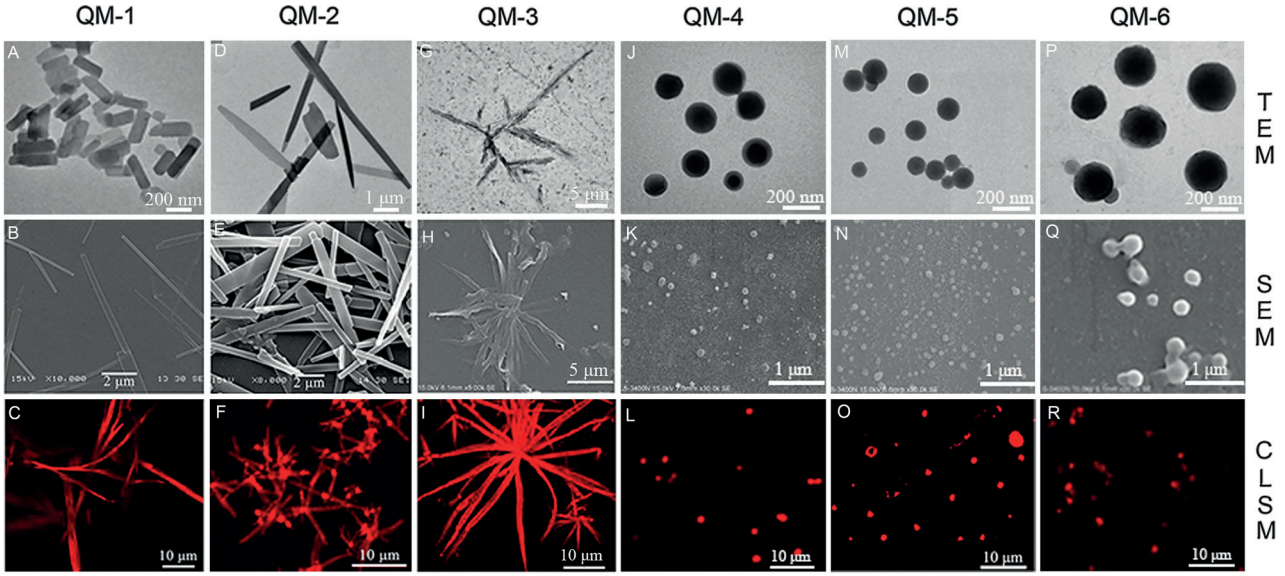

Figure 2. Multiple morphologies of micro/nanoaggregates fabricated from QM derivatives (10^{-5} M). TEM, SEM, and confocal laser scanning microscope (CLSM) images recorded for the micro/nanoaggregates of QM-1 (A–C), QM-2 (D–F), QM-3 (G–I), QM-4 (J–L), QM-5 (M–O), and QM-6 (P–R), prepared by adding different content of water into pure THF solution to afford the mixed solution of THF/H_2O then still standing for 1 h.

characterized with transmission electron microscopy (TEM), scanning electron microscopy (SEM), and confocal laser scanning microscopy (CLSM). As shown in Figure 2 A–I, the aggregates of QM-1, QM-2, and QM-3 were well-defined microrods with different sizes and diameters. For example, uniform 1D microrods of QM-2 were observed by SEM with the size of about 10 μm in length and 0.5–1 μm in diameter (Figure 2 E). The TEM and CLSM images in Figure 2 D and 2F further verified rod-like microstructures in the aggregates of QM-2. Similar morphological characteristics could also be observed in QM-1 and QM-3 aggregates, demonstrating strong AIE-active fluorescence and easily linear self-aggregation when thiophene was utilized as a π-conjugated bridge in the QM derivatives. Fortunately, single crystals of QM-1, QM-2, and QM-3 were obtained by the slow evaporation approach (Supporting Information, Table S2 and Figure S3). All QM-1, QM-2, and QM-3 display twisted conformations in their crystal structures with large torsional angles of 85.4–87.0° between the *N*-ethyl and the quinoline units (Figure 1 A). Furthermore, moderate interplanar angles of 32.2–35.3° are observed between the ethylene and quinoline units, and no obvious π–π stacking interactions can be found in the crystals, which may be ascribed to the twisted molecular structures. As a result, their aggregation states display enhanced emission.

However, for QM-4, QM-5, and QM-6 (Figure 2 J–R), instead of the microrod structures, the resulting morphology was predominated with spherical shaped nanoparticles in diameter of about 80–200 nm. This might be ascribed to the attachment of an epoxyethyl group in the EDOT π-bridge, leading to a totally different intermolecular interaction in the aggregation state. Moreover, the average diameters of QM-5 aggregates measured by laser light scattering (LLS) were about 85(±10) nm, which is exactly consistent with the data from SEM images (90 ± 10 nm). Under the same fabricating conditions, the TEM images of QM-5 had the smallest diameters and smoothest spherical morphologies with respect to QM-4 and QM-6 (Supporting Information, Figure S4).

Based on the rational molecular design, the flexibility and electron-rich properties of the epoxy ethyl groups in QM chemical structures play important roles in the morphological formation during the aggregation process when EDOT is introduced as the π-conjugated bridge. It is expected that the incorporation of both alkoxytriphenylamine group and EDOT unit in QM derivatives is a preferable design strategy to generate AIE-active spherical nanostructures, resulting in a distinct change in morphology from QM-1 to QM-6 (Figure 2).

Considering nanoprobes for bioimaging in vivo, it is necessary to assess the external influence on aggregation and photostability of AIE materials. As shown in the Supporting Information, Figures S5, S6, once QM derivatives have formed into aggregation state under the optimized f_w, external factors have little effect on the morphology of QM aggregates. The photostability of AIE-active QM materials and commercial ICG dye (approved by FDA for NIR clinical imaging agents) was also evaluated by time-course fluorescence measurement. After exposure to high density light, the half-life time of QM derivatives was about 20-fold longer than ICG dye, demonstrating that QM derivatives are more photostable materials.

Among all of the QM derivatives, QM-2 and QM-5 are typical representatives of rod-like and spherical shapes, respectively. Thus, we chose these two compounds to further explore their potential shape effects on in vitro and in vivo applications in living system. QM-2 and QM-5 exhibited low toxicity against both cancer cells and normal cells, which is highly preferable for cell imaging or tracking applications

(Supporting Information, Figures S7–S10). Flow cytometric studies were conducted to evaluate the kinetics of the cell uptake process of QM-2 and QM-5 in the HeLa cells. Although the relative uptake ratio of both compounds after 48 h incubation was close to 100% ($\geq$99.5%), the initial uptake rate of QM-5 was much faster compared to that of QM-2. Additionally, the CLSM images presented a direct observation of the spherical shape of QM-5 in cells.

The high-brightness emission for AIE-active organic nanomaterials could be used as efficient long-term cell tracers. For instance, QM-2, QM-5, and commercial ICG dye as control showed bright fluorescence after incubation with HeLa cells for 24 h despite the different retention in the cytoplasm of HeLa cells (with the potential as long-term cell tracers). For QM-2 and QM-5, even after four passages of incubation with living cells, the fluorescence of QM aggregates staining in HeLa cells was still emissive. In contrast, there was almost no fluorescence signal using ICG at two passages of incubation. Therefore, the formation of highly biocompatible and photostable AIE-active QM aggregates with different shape and size is beneficial to retain fluorescence in the cells, which is desirable for long-term cell tracing.

The promising long-term cell tracing results of AIE-active QM derivatives inspired us to further explore their feasibility as NIR bioprobes in vivo. Upon intravenous injection of QM-2 at a dose of 0.15 mg kg^{-1}, we immediately monitored the fluorescence distribution in mice at different periods of time. As shown in Figure 3A, the fluorescence was clearly observed in mice at 30 min, which is indicative of the rapid distribution of QM-2 aggregates via the blood circulation. Surprisingly, upon the paralleled intravenous injection of QM-5 (0.15 mg kg^{-1}, Figure 3B), the distinct NIR fluorescence could predominately be detected in the tumor at 30 min, rather than other organs of the mouse, and retained in tumor tissue even at 24 h after injection. The long retention of the QM-5 nanoaggreates in tumors makes this system very promising for tumor labeling and chemotherapy. However, even at 24 h after the injection, the QM-2 aggregates were still present in the whole mouse body biodistribution without producing tumor-targeted fluorescence signals in vivo. A similar phenomenon was also observed for another rod-like nanostructure QM-3 (Supporting Information, Figure S11), further confirming the non-specific in vivo imaging biodistribution of organic rod-like QM assemblies.

The ex vivo fluorescence images of the internal organs of mice sacrificed at 24 h post-injection in Figure 3D also indicated that QM-5 aggregates accumulated in the tumor and liver tissue, whereas the fluorescence of QM-2 aggregates in tumors was much weaker than other organs such as liver or lung (Figure 3C). Furthermore, the 3D fluorescence imaging of tumor-bearing mice in Figure 3E at 24 h post-injection of QM-5 (0.15 mg kg^{-1}) further confirmed the NIR fluorescence accumulation in tumors. Accordingly, the semi-quantitative analysis data of the average fluorescence intensity distribution in organs (Figure 3F) also demonstrated that the spherical shape of QM-5 aggregates exhibited much higher tumor-targeting ability than the rod-like aggregates of QM-2.

Undoubtedly, in contrast with the fast degradation in aqueous media and quick clearance from the body of small molecular imaging agent ICG dye[11] (control; Supporting Information, Figure S12), the shape-specificity of QM aggregates contributes a direct benefit for long-term retention and bioimaging in vivo. Actually, from the TEM images of cells and tissues, we clearly observed that QM aggregates almost maintained their initial aggregated morphologies in situ (Supporting Information,Figure S13). While the fluorescence of rod-like aggregates by QM-2 exhibited the whole body biodistribution in mice, the spherical QM-5 aggregates enhanced tumor-targeting capacity, which could be ascribed to the "passive" tumor-targeting by enhanced permeability and retention (EPR) effect.[12] Apparently, here the particle geometry plays an important role in the tumor-targeted bioimaging in vivo. Another potential possibility for this disparity could be the difference in shape and shape-related factors such as curvature and aspect ratio, which affect cell-particle interactions, particle transport characteristics and margination dynamics.[6k,12] Therefore, based on

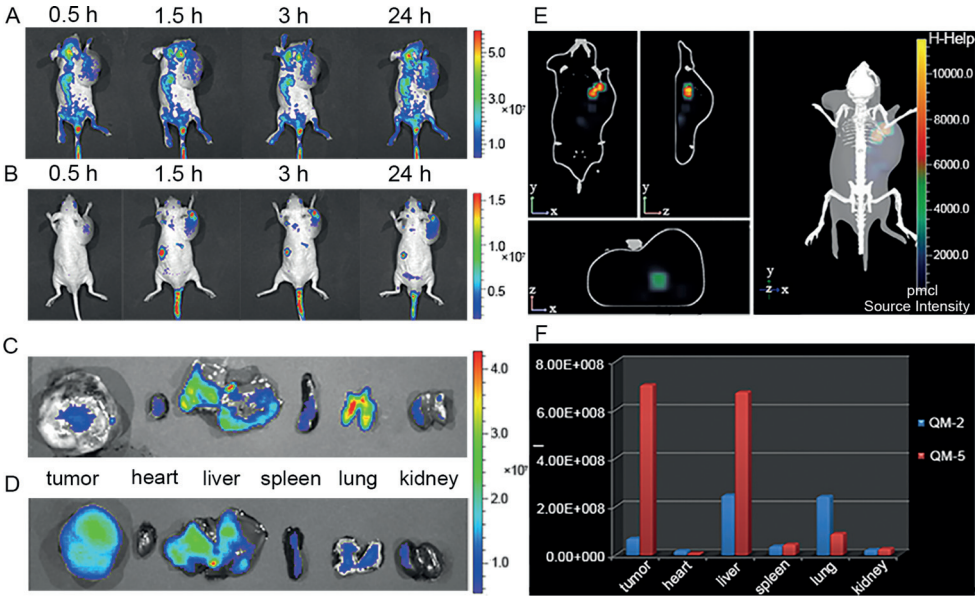

Figure 3. In vivo non-invasive imaging of tumor-bearing mice after intravenous injection of A) QM-2 (0.15 mg kg^{-1}), B) QM-5 (0.15 mg kg^{-1}) at different periods of time (0.5, 1.5, 3 and 24 h), and ex vivo fluorescence images of the internal organs of mice sacrificed at 24 h post-injection with C) QM-2 and D) QM-5. E) The 3D fluorescence imaging of tumor-bearing mice after intravenous injection of QM-5 (0.15 mg kg^{-1}) for 24 h. F) Average fluorescence intensity distribution for tumor and internal organs from mice sacrificed at 24 h post-injection with QM-2 and QM-5 (n = 3).

the TEM images of HeLa cells and tissues, the shape differences of QM aggregates formed by tailoring AIE-active organic molecules are of great value for tumor-targeted bioimaging in vivo.

In summary, far-red and NIR AIE-active fluorescent organic QM nanoprobes have been rationally designed. We specifically focused on the modulation of long emitting wavelength and the aggregated morphologies via essentially tailoring π-bridge and donor unit in molecular structures. QM derivatives from rod-like to spherical morphology were well confirmed by SEM, TEM and CLSM images. In vitro experiments have verified that these tailor-made long wavelength AIE-active organic QM nanomaterials are biocompatible and retained in the cytoplasm of living cells. The most striking feature of NIR spherical QM-5 nanoaggregates is their excellent tumor-targeting performance in mice. Conversely, the same is not true for the rod-like aggregates of QM-2, which do not display any tumor targeting properties. To the best of our knowledge, this is the first report of shape-specific tumor targeting using bare NIR AIE-active nanoprobes. Our strategy generates high-performance long wavelength AIE-active organic nanomaterials with ideal biological geometries for tumor-targeted bioimaging in vivo, providing a promising platform for in situ and in vivo tumor imaging agents.

Keywords: aggregation-induced emission · fluorescent probes · morphology effects · near infrared · tumor targeting

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