Research Article

Boost photothermal theranostics via self-assembly-induced crystallization (SAIC)

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Abstract
Owing to the intrinsic advantages of spatiotemporal selectivity, photothermal theranostics have become the advancing edge of precision medicine for cancer. Developing photothermal transduction agents (PTAs) with near-infrared (NIR) absorption, high photothermal conversion efficiency, robust photothermal stability, and good accumulation in tumors, is particularly valuable. Herein, we report a new concept, self-assembly-induced crystallization (SAIC), which can serve as a mechanism that dramatically boosts photothermal behaviors of PTA in NIR region. As a proof of concept, three heptamethine cyanine molecules with internal degrees of freedom (geometry and intramolecular interaction) are designed to fine-tune their crystallinity. Notably, Cy7-TCF-EMBI molecules with rigid and planar skeletons self-assemble into a crystalline state to maximize their packing density and improve the charge transfer, both of which contribute to nonradiative decay for energy dissipation as heat. The high packing density also renders an ideal scaffold for controlling intermolecular interactions to exhibit better photothermal stability, and endows an anisotropic three-dimensional architecture for passive tumor targeting. This “SAIC” strategy may offer a conceptually novel, practically simple but effective approach to unveil the structure–property relationship that could provide some general rules in rational design of PTAs, and paves the way for a next generation of supramolecular medicine for photothermal theranostics.

KEYWORDS
crystallization, photothermal theranostic, self-assembly, small molecule, supramolecular medicine

1 | INTRODUCTION
Photothermal theranostics, devices that make use of photothermal transduction agents (PTAs) to absorb energy from photons for fluorescence imaging, photoacoustic tomography (PAT), and image-guided photothermal therapy (PTT), have become the advancing edge of precision medicine to combat cancer, due to the high inherent specificity, minimal invasive burden, and precise spatial–temporal selectivity.[1–11] To date, most of PTAs are near-infrared (NIR) light-triggered materials including inorganic nanoparticles,[12–16] conductive polymers,[17,18] and small organic molecules.[3,19–22] Among these PTAs, small organic dyes such as indocyanine green (ICG) with the merits of NIR absorbance, great biocompatibility, nonimmunogenicity, and commercial-scale production have received increasing attention, but the therapeutic applications of the small molecule PTAs were limited by their low photothermal conversion efficiency (PCE), poor photothermal stability (PS), and lack of target specificity.[9,23,24]

According to the Jablonski diagram, small-molecule PTAs generate heat through nonradiative relaxation pathway, which is a competitive process with radiative decay.[1,6,9] The conjugated molecules with strong donor–acceptor (D–A) structure were thus employed by utilizing the inter-/intramolecular electron transfer[25,26] that narrows the bandgap to quench the fluorescence and intersystem crossing,[17,27] thereby enhancing the PCE of PTAs. Recently, Tang and colleagues reported a new excited molecular motion approach to enhance the PCE by formatting the twisted intramolecular charge...
transfer (TICT) state of PTAs in aggregation.[7] Later, pyrazine-containing units were served as bond stretching vibrators to promote intramolecular motions in the excited state for constructing PTAs with high PCE and strong PAT signals.[28] In another pioneering work of Peng and coworkers, they reported a BODIPY-derived PTA with a PCE of 88.3%, by introducing a –CF3 “barrier-free” rotor to efficiently dissipate absorbed energy as heat.[2] Li’s group further developed a double bond-based molecular motor that could twist through the conical intersection of intermolecular conversion, which possessed a high PCE of 90% in aggregates.[29] These studies gave insight into the working mechanism of intramolecular motion boosting PCE of these small molecule PTAs. However, most of those molecules are hydrophobic and usually formulated into polymeric nanoconstructs to form stable dispersion in aqueous solution.[2,7,28,29] Li’s group of 88.3%, by introducing a –CF3 “barrier-free” rotor to explain their enhanced PCE in high-viscous or solid-state environments, which prevents access to a molecular structure that enables efficient photothermal conversion. In addition, the polymeric carriers always suffer from low drug-loading ratios, batch-to-batch variation, and long-term toxicity of carriers, while increasing production costs.[30]

The intrinsic self-aggregation of small-molecule dyes has captured the attention of scientists for many years, which represents one of the most important milestones in dye chemistry and promotes the germination of supramolecular chemistry.[31–34] The strong D–A interactions and large rigid π-extended structure of NIR dyes make them prone to aggregate in physiological environment where their emission is often weakened or quenched due to the strong intermolecular dipole-dipole or π-π interactions. Such ACQ effect is a general phenomenon for traditional fluorescent dyes, which may enhance the heat generation due to the “supramolecular photothermal effects”.[3,23,35–37] However, the uncontrolled or disordered self-aggregation of the dyes in complex biological environment hindered the direct use of dyes as PTAs. The state of the art seems to be driven by the quest of designing the most versatile molecule that will intrinsically self-assemble into precisely ordered nanostructures.[38] Recently, Hu et al. demonstrated that intermolecular charge-transfer (CT) interactions of the DTTTF-TCNB cocrystal (DTC) can greatly narrow the bandgap that redshifts NIR absorption and facilitates the rapid nonradiative decay to exhibit the high-performance photothermal conversion.[39] Apparently, crystallization is an effective method to maximize the packing density of molecules, which normally can cause highly frequent intermolecular collisions to quench the fluorescence and intersystem crossing of ACQ-type PTAs. In addition, due to translation symmetry of the crystal lattice, the excitons can propagate in the crystal with a definite wave vector. The intermolecular interaction in the crystal splits the degenerate level into several components (Davydov split), which can accelerate the charge transfer in the whole network, further quench the fluorescence and intersystem crossing that could enhance PCE of PTAs. Therefore, it is conceptually proposed that realization of self-assembly-induced crystallization (SAIC) of PTAs in physiological environment can be feasible to enhance their PCE. However, the habitually irregular shape of organic dye molecules poses limitations to the way these molecules can pack into a crystal lattice. It remains highly challenging to realize controllable preparation of nanocrystals from small-molecule dyes that serve as PTAs to uncover the structure–property relationship.

SAIC can be guide through two major design considerations: intermolecular interactions and molecular geometry. First, intermolecular interactions are a complex interplay that is tremendously difficult to predict. Among them, one of the most versatile interactions that mediate the assembly of nanostructured materials is electrostatic force. By understanding the role of counter-ions, Jen’s group achieved J-type self-assembly of highly polarizable heptamethine cyanines (Cy7) in crystalline solids by rationally tuning the structure of Cy7 dyes as complementary salts.[40] However, due to their ionic nature, the complementary salt-based nanocrystals prevent their in vivo application in terms of complex interactions with multiple blood components. Hydrogen bonds occur most often between neutral molecules, which is fairly predictable in the context of supramolecular assembly owing to its directionality that can be controlled by the geometry of molecules. Recently in our group, molecular assembly strategy guided by multiple hydrogen bonds has been incorporated into the design of nonionic Cy7-based PTA (Cy7-tricyanofuran, Cy7-TCF) to boost the attractive molecular forces.[3] Second, the geometry of small molecules usually plays an important role to crystal packing, as it accounts for the minimization of the crystal free energy and the potential energy of intermolecular interactions between the molecules in the lattice. In this work, as a proof-of-concept, we explore the efficient strategy of creating the nano-sized crystalline units from NIR dyes with different geometry to boost photothermal theranostics. These NIR dyes are all based on the nonionic Cy7 structure, in which TCF group with multiple highly electronegative cyano groups represents key structural features as molecular building block to construct the self-supporting nanoaggregates.[31] Specifically, by attaching 3-ethyl-1,1,2-trimethyl-1H-benzo[e]indol-3-ium (ETBI), 1-ethyl-2-methylbenzo[c]indol-1-ium (EMBI), or 6-(diethylamino)-1,2,3,4-tetrahydroxanthyl-ium (DTX) as donors of the D–A molecular structure for fine-tuning their geometries, the new Cy7-TCF-derived PTAs are rationally designed and synthesized to exhibit strong tendency of self-assembly but different crystallization ability (Figure 1). Please note, both Cy7-TCF-ETBI and Cy7-TCF-EMBI with limited conformational freedom can form nanocrystals in water without the aid of stabilizers and excipients, thus showing an incredible phenomenon of SAIC to greatly boost the photothermal theranostics. Cy7-TCF-EMBI with higher crystallinity and closer stacking further increases the possibility of nonradiative relaxation for heat generation. In contrast, Cy7-TCF-DTX, which had stronger D–A strength but greater molecular freedom, only formed amorphous aggregates to exhibit low PCE in aqueous solutions. This SAIC strategy could be general for other ACQ-type molecules and allow for the more efficient way of generating heat through nonradiative relaxation pathway over molecular units to provide intriguing photothermal theranostic properties.
RESULTS AND DISCUSSION

Three Cy7-TCF derivatives, Cy7-TCF-ETBI, Cy7-TCF-EMBI, and Cy7-TCF-DTX, were synthesized by two consecutive condensation reactions (Figure S1, Supporting Information). Their structures were varied only at donor sites, with different geometries. Their molecular structures were confirmed by $^1$H NMR, $^{13}$C NMR, and high-resolution mass (HRMS) (Figure S2–S10). The single crystals of both Cy7-TCF-ETBI and Cy7-TCF-EMBI were obtained by slow diffusion of ethyl ether into their dichloromethane solution. Their crystal data and refinement parameters are summarized in the Supporting Information (Figure 2 and Table S1). Both Cy7-TCF-ETBI and Cy7-TCF-EMBI adopt a trans configuration of the polymethine bridge (Figure 2A and B). Structurally, the EMBI ring, the polyvinyl chain, and TCF ring in Cy7-TCF-EMBI are almost coplanar with the very-small twist of the sp$^2$ carbon skeleton, which is clearly evident from the torsion angles of C(9)-C(10)-C(11)-C(12) (–179.0°), C(9)-C(10)-C(11)-N(1) (1.2°), C(21)-C(22)-C(23)-C(24) (175.8°), and C(21)-C(22)-C(29)-O(1) (–176.2°) (Figure 2A). As comparison, Cy7-TCF-ETBI molecule adopts a slight twisted conformation, and ETBI ring lies out of the plane of the conjugated molecular backbone (Figure 2B). The average bond-length alternation (BLA) is an important parameter to characterize the electronic structure of Cy7 dyes. The conjugated polymethine chain in Cy7-TCF-EMBI is constituted of alternating long (average bond length: 1.406 Å) and short (average bond length: 1.383 Å) carbon (sp$^2$)-carbon (sp$^2$) bonds with a BLA value of 0.023 Å. This value is basically same with that of Cy7-TCF-ETBI (BLA = 0.024 Å), indicating that both Cy7-TCF-ETBI and Cy7-TCF-EMBI have the more cyanine-like resonance structure with the delocalized π electrons in their molecular frameworks. These BLA values are relatively small, which may be attributed to the favorable spatial arrangement of Cy7 dyes with the fairly symmetric environment in the crystal structure.[40] Figure 2A and B further depicts their crystal packing patterns. Cy7-TCF-EMBI crystallized in the monoclinic P21/n space group with an elemental cell containing four molecules. The Cy7-TCF-EMBI molecules packed into antiparallel dimers along the long molecule axis, with a slippage angle of 50.5° and a π−π interaction distance of 3.401 Å (Figure 2A), shorter than typical value of 3.5 Å. It is also found that there occurred multiple
C≡N—H hydrogen bonds with distances in the range of 2.5–3.0 Å between two neighboring Cy7-TCF-EMBI molecules, which could rigidify the molecular conformation to form such a construct in the solid phase (Figure 2A). The cooperation of various interactions directed Cy7-TCF-EMBI molecules to self-assemble into aggregates. As comparison, the crystal structure of Cy7-TCF-ETBI has shown unfavorable aggregation with an unexpected twisted-conformation in which the ETBI groups are on the different sides of the dimer to decrease π−π stacking. The Cy7-TCF-ETBI molecules are therefore arranged in a loose manner with a slippage angle of 65.5° and a π−π interaction distance of 4.429 Å (Figure 2B).

The increased π−π distance may suppress the nonradiative pathway to a contain extent. In addition, the crystal of Cy7-TCF-DTX is not available due to the low crystallinity. However, it is reasonable to support a looser and disordered packing in aggregates, because of the half-chain cyclohexene in DTX distorts the molecule to exhibit a more twisted conformation (Figure 2C).

These Cy7-TCF derivatives can be dissolved in most common organic solvents such as acetonitrile or DMSO. The UV-Vis-NIR absorption spectra of these Cy7-TCF-based dyes (10 μM) in acetonitrile are shown in Figure 3A. The absorption maximum (λmax) and molar extinction coefficient (ε) values are listed in Table S2. The λmax value of Cy7-TCF-ETBI (852 nm) was red-shifted compared to that of Cy7-TCF-EMBI (790 nm). The introduction of DTX led to a further bathochromic shift in the λmax value (940 nm) due to the strong D–A effect. To understand the NIR absorption spectra, we carried out time-dependent density functional theory (TD-DFT) calculations, which showed that the excitation from ground to excited state of the three Cy7-TCF-derivatives was predominantly a π−π* type transition (Figure 3B). The energy gap between the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) of Cy7-TCF-DTX is about 1.77 eV, smaller than that of Cy7-TCF-ETBI (2.02 eV), which is consistent with the bathochromic shift of Cy7-TCF-DTX in experimental spectra. Compared with the theoretical calculation that Cy7-TCF-EMBI has a smaller energy gap compared to Cy7-TCF-ETBI (1.81 eV vs. 2.02 eV), on the contrary, the experimental absorption of Cy7-TCF-EMBI has a blue shift relative to Cy7-TCF-ETBI (Figure 3A), which may be a consequence of the strong intermolecular interactions increasing molecular order. Therefore, their absorption spectra combined with TD-DFT calculations showed that Cy7-TCF-ETBI and Cy7-TCF-DTX in acetonitrile were structureless monomers, whereas Cy7-TCF-EMBI displayed some degree of aggregation, thus implying that Cy7-TCF-EMBI had higher structural rigidity to aggregate.

With the rapid injection into aqueous media, all the three Cy7-TCF derivatives showed the blue-shifted, reduced, and broaden absorbance (Figure S11), and quenched fluorescence (Figure S12), which can be attributed to the molecular interactions to form nanoscopic aggregates.[3,41,42] Dynamic light scattering (DLS) was applied to characterize their apparent aggregates in water, and revealed that the nanoaggregates from these Cy7-TCF derivatives had excellent stability without growing into larger clumps after incubating in aqueous solutions for one month (Figures 3C and S13). However, after being injected into PBS, all the Cy7-TCF derivative nanoaggregates gradually precipitated (Figure S14). PBS contains approximately 10 times more phosphate, and it has been reported that phosphate as multivalent electrolyte could more efficiently suppress the stabilizing effects of the electric double layer.[43] This may be the reason why these nanoaggregates are unstable in PBS. To further address their bioavailability as PTAs, the stability of these Cy7-TCF derivative...
nanoaggregates in cell culture medium containing serum proteins was also investigated. The DLS results indicated that these nanoaggregates were stable within 48 h (Figure S15). When these nanoaggregates were added to cell culture medium, they first contacted with albumin or other serum proteins to form the protein corona. The adsorbed proteins on the particle surface were considered to provide the increased stability of these nanoaggregates due to extra electrosteric repulsion. There was no obvious change in their UV-Vis-NIR absorption spectra, indicating that there was no significant release of cyanine dye from these nanoaggregates after incubating with serum proteins (Figure S16). Interestingly, compared with Cy7-TCF-EMBI and Cy7-TCF-DTX, the fluorescence of Cy7-TCF-ETBI increased slightly (Figure S16), which may be due to the higher binding of protein to Cy7-TCF-ETBI molecules on the surface of the nanoaggregates to decrease their internal rotation.

Transmission electron microscopy (TEM, Figure 3D–F) and atomic force microscopy (AFM, Figure 3G–I) images further exhibited these nanoaggregates were well-defined spherical particles. Impressively, at higher magnification of TEM, we observed there were some small supramolecular crystalline domains in the aggregates of Cy7-TCF-EMBI (Figure 4A and D) and Cy7-TCF-ETBI (Figure 4B and E), and these nanocrystals did not appear in the aggregates of Cy7-TCF-DTX (Figure 4C). The crystalline characteristics of the nanoaggregates were further verified by powder X-ray diffraction (XRD). Compared with their single-crystal XRD, both Cy7-TCF-EMBI (Figure 4F) and Cy7-TCF-ETBI (Figure 4G) aggregates exhibited similar XRD patterns,
FIGURE 4 HR-TEM images of (A) Cy7-TCF-EMBI, (B) Cy7-TCF-ETBI, and (C) Cy7-TCF-DTX nanoparticles, prepared by rapid injection of their acetonitrile solution into an aqueous media. SAED patterns of (D) Cy7-TCF-EMBI and (E) Cy7-TCF-ETBI, showing the characteristics of their nanocrystals. XRD patterns of (F) Cy7-TCF-EMBI, (G) Cy7-TCF-ETBI, and (H) Cy7-TCF-DTX. (I) Schematic representation of three Cy7-TCF derivatives with different geometries exhibit the different SAIC abilities in aqueous solution suggesting that Cy7-TCF-EMBI and Cy7-TCF-ETBI molecules are packed in the similar fashion as the molecules in the single crystal. For comparison, the Cy7-TCF-DTX (Figure 4H) aggregates did not show obvious crystallization peaks in XRD patterns, even with extended incubation time. These results suggested that the rapid injection process could also be able to allow the Cy7-TCF derivative molecules with proper geometries (usually planar conformation) arrange in an ordered way to form nanocrystal in aqueous solution (Figure 4I).

The photothermal conversion of three Cy7-TCF derivative nanoaggregates was evaluated under the 808 nm laser radiation (1.0 W cm⁻², Figure 5A–C). The temperature of Cy7-TCF-EMBI solution (20 μM) reached a maximum at 45°C with temperature increase (ΔT) of 25°C, which is significantly higher than that of Cy7-TCF-ETBI (ΔT = 14.9°C) and Cy7-TCF-DTX (ΔT = 10.1°C). As comparison, the water exhibited a negligible temperature change (<2°C) under same conditions. The significant temperature increase clearly demonstrated the efficient photothermal conversion efficiency (η) of Cy7-TCF-EMBI nanoaggregates, which was calculated to give a value at 63.3%, higher than that of Cy7-TCF-ETBI (η = 58.6%), and significantly higher than that of Cy7-TCF-DTX (η = 45.9%). Due to the enhanced the D–A effect, it is not surprising that the PCE of these molecularly dissolved Cy7-TCF derivatives in organic solvent increased from Cy7-TCF-EMBI (η = 26.6%), Cy7-TCF-ETBI (η = 34.9%), to Cy7-TCF-DTX (η = 39.0%). However, the ratio η[Cy7-TCF derivatives nano-aggregates]/η[Cy7-TCF derivatives monomer] gives a value of 2.38 for Cy7-TCF-EMBI, a value of 1.68 for Cy7-TCF-ETBI, and a value of 1.18 for Cy7-TCF-DTX, which decreases as D–A effect increases (Figure S17). Based on the above analysis, the maximum photothermal conversion of Cy7-TCF-EMBI could be attributed to its higher crystalline property, in which the molecular conformation was restricted, packing in dense to enhance the intermolecular interactions that cause highly frequent intermolecular collisions, and the π-electron delocalization that accelerate the charge transfer in the whole network, thereby inhibiting the photon emission process to
dissipate energy as heat. Furthermore, small-molecule PTAs often decay gradually upon irradiation by laser. Then the photothermal stability of the Cy7-TCF derivatives was investigated by reversible irradiating and cooling of the sample solutions (Figure 5D–F). All of them showed outstanding photothermal stability, remaining >97% of its $\Delta T_{\text{max}}$ after five cycles. Interestingly, the $\Delta T_{\text{max}}$ of Cy7-TCF-DTX solution even exceeded that of the initial solution (Figure 5F), which could be attributed to the local orderly arrangement of molecules in nanoaggregates during the slow heating and cooling cycle. This may further support the concept that SAIC could enhance light-to-heat conversion. However, the thermal conduction of the nanoaggregates may not allow this energy to dissipate rapidly; the thermal destruction of dyes can be observed. In addition, it is generally thought that the photodegradation of NIR dyes involves electronic transitions.

**FIGURE 5** The photothermal behaviors of three Cy7-TCF derivatives (1.0 W cm$^{-2}$, 808 nm). Temperature curves of (A) Cy7-TCF-EMBI (ICG used as control), (B) Cy7-TCF-ETBI, and (C) Cy7-TCF-DTX in water with laser on for 600 s, followed by natural cooling. Blue lines in (A)–(C) describe linear plots of time data versus $-\ln \theta$ during the cooling period. Temperature changes of (D) Cy7-TCF-EMBI (ICG used as control), (E) Cy7-TCF-ETBI, and (F) Cy7-TCF-DTX in water at the concentration of 20 $\mu$M over five laser ON/OFF cycles. The samples were irradiated with a laser at the power of 1.0 W cm$^{-2}$ for 600 s (ON) followed by natural cooling for 900 s (OFF). Absorption spectrum of (G) Cy7-TCF-EMBI, (H) Cy7-TCF-ETBI, and (I) Cy7-TCF-DTX before or after five laser ON/OFF cycles.

**FIGURE 6** In vitro photothermal theranostic. (A) Survival rate of 4T1 cells incubated with Cy7-TCF-EMBI with and without irradiation for 6 min using MTT assay. (B) Fluorescence imaging of 4T1 cells costained with EthD-1 (staining dead cells, red color) and calcein AM (staining live cells, green color). (C) Confocal fluorescence images of 4T1 cells stained with Hoechst 33342, Lyso-Tracker, and Cy7-TCF-EMBI. (D) The intensity profile of ROI lines.
excitations to triple state that generates reactive oxygen species (ROSs) to react with dyes. Both heat and light may deleteriously affect the photothermal theranostic properties. The absorbance of three Cy7-TCF derivatives was measured after irradiation, only that of Cy7-TCF-EMBI did not change significantly (Figure 5G), indicating its excellent photothermal stability. Cy7-TCF-EMBI with the face-to-face style in the nanocrystal is highly insensitive to the photothermal cycles, which could be due to their packing density that limits the diffusion of ROSs. These results demonstrated that the boosted photothermal theranostic properties of Cy7-TCF-EMBI over Cy7-TCF-ETBI and Cy7-TCF-DTX by taking advantage of SAIC to form close molecular packing in nanocrystal. Cy7-TCF-EMBI is therefore a very interesting supramolecular theranostic nano-medicine, which will be further used in the following study for both imaging and therapy of cancer in vitro and in vivo.

In vitro phototherapeutic effect of Cy7-TCF-EMBI nanocrystals on 4T1 mouse breast cancer cells was then investigated. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to show that negligible cytotoxicity was observed under dark with a concentration of up to 100 μM of Cy7-TCF-EMBI. In contrast, with laser radiation for 6 min (808 nm, 1.0 W cm^{-2}), dose-dependent cytotoxicity was detected when the Cy7-TCF-EMBI concentration high than 1.6 μM (Figure 6A). To visualize the phototherapeutic performance of Cy7-TCF-EMBI nanocrystals, LIVE/DEAD cell viability assay was performed by staining the 4T1 cells with calcein AM (green) and ethidium homodimer-1 (EthD-1, red), which further showed the photothermal effect of Cy7-TCF-EMBI nanocrystals could kill cancer cells in a spatiotemporal controlled fashion, with desirable biocompatibility and safety profile (Figure 6B). Confocal microscopy was then carried out to determine the uptake and subcellular distribution of Cy7-TCF-EMBI nanocrystals in cells. As shown in Figure 6C, Cy7-TCF-EMBI internalized into cells within a short time, as less as 5 min, and then distributed mainly in the cytoplasm, which was confirmed by the minimal colocalization of its red emission with blue emission from Hoechst.
AGGREGATE 9

**FIGURE 8** Biosafety of photothermal theranostic with Cy7-TCF-EMBI in vivo. (A) Relative body weight of mice during treatment. (B) Blood test parameters regarding blood routine and liver function of the mice 24 h after intravenous injection of saline (orange bar) and Cy7-TCF-EMBI (green bar). The blue bar represents the reference value. (C) Microscopic images of H&E-stained cross-sections of the hearts, livers, lungs, spleens and kidneys (27 days after the first treatment). Scale bar: 100 μM. (D) In vivo blood elimination kinetics of Cy7-TCF-EMBI (n = 5). (E) In vivo fluorescence images of healthy mice at different time points after intravenous injection of Cy7-TCF-EMBI. (F) The biodistribution of Cy7-TCF-EMBI in heart, liver, spleen, lung, and kidney at different time points after intravenous injection of Cy7-TCF-EMBI. (G) Relative fluorescence intensities of Cy7-TCF-EMBI in heart, liver, spleen, lung, and kidney at different time points based on the quantitative analysis of (F).

33342, the nuclei-specific probe. Additionally, cells were also costained using the lysosome-specific probe LysoTracker Green® with Cy7-TCF-EMBI nanocrystals. Colocalization percentage was quantified using Pearson’s sample correlation factors (Rr, Figure 6D). The yellow fluorescence in Figure 6C indicated that the majority of the Cy7-TCF-EMBI nanocrystals lie inside of the lysosomes (Rr = 77%), which may be due to the clathrin-mediated endocytosis. In cancer cells, the overdevelopment of lysosomes has become an important target for anti-cancer therapy, because lysosomal cell death bypasses the classical caspase-dependent apoptosis pathway, enabling the targeting of apoptosis-and drug-resistant cancers.\[44]\ However, small molecules that can specifically target lysosomes are even rare. Cy7-TCF-EMBI is a single-component small molecule that could target lysosomes via SAIC, which holds great promise as an effective agent for in vivo cancer theranostic.

Cy7-TCF-EMBI with high η generates strong photoacoustic (PA) signals, proportional to the concentration (R² = 0.9) in the agar-based phantoms (Figure 7A). This property of Cy7-TCF-EMBI is intrinsically suited for PA-guided imaging, which is highly desirable for the pre-treatment tumor location with high resolution and high contrast. After Cy7-TCF-EMBI was injected into xenograft 4T1 tumor-bearing BALB/c mice via the tail vein, time-dependent PA imaging was longitudinally recorded (Figures 7B and S18), which showed that the PA intensity at the tumor gradually increased, reaching the maximum at ~4 h postinjection. Thus, the Cy7-TCF-EMBI nanocrystals are considered to possess prominent stability in blood, and can target cancer by EPR effect or receptor-mediated endocytosis in vivo. The high contrast of PA signal further indicates that the most suitable time for phototherapy is 4 h after intravenous injection, and is expected to guide the irradiation location during PTT. Subsequently, the in vivo antitumor effect of Cy7-TCF-EMBI nanocrystals was conducted in another batch of 4T1 tumor-bearing mice. These mice were divided into four groups as follows: the saline group, mice with laser irradiation (saline + laser), mice with Cy7-TCF-EMBI administration (Cy7-TCF-EMBI), and mice treated with both Cy7-TCF-EMBI and laser irradiation (Cy7-TCF-EMBI + laser). As shown in Figure 7C and D, under laser irradiation (0.35 W cm⁻²), the temperature of tumor region in “Cy7-TCF-EMBI + laser” group increased rapidly within 10 min to reach over 55°C, which was imaged by an IR thermal mapping camera (Fluka). While the temperature increase (∆T ≈ 4°C) observed in the “saline + laser” group was negligible (Figure 7D). After laser irradiation, the tumors of mice in “Cy7-TCF-EMBI + laser” group were successfully ablated, but burned scars were formed at the tumor sites (Figure 7E). After 10–16 days, the scar healed, and no tumor recurrence was observed after prolonged time. For comparison, only laser or Cy7-TCF-EMBI alone failed to suppress tumor growth, and the average tumor volume increased by about 35-fold (Figure 7F). Furthermore, the average lifespan of mice in “saline”, “saline + laser”, and “Cy7-TCF-EMBI” groups was only 21–28 days, while the mice in
“Cy7-TCF-EMBI + laser” group survived until euthanasia (Figure 7G). These results indicated that Cy7-TCF-EMBI nanocrystals are efficient PTAs for photothermal theranostic.

To assess the biosafety of Cy7-TFC-EMBI for the potential clinical applications, the body weights of all mice were recorded to show that no changes were observed in any groups (Figure 8A). On day 7 postirradiation, a blood sample of each mouse in both “saline” and “Cy7-TFC-EMBI + laser” groups were collected and tested by blood chemistry indices. There was no significant difference between the “Cy7-TFC-EMBI + laser” and “saline” groups, and all of values were within the range of reference (Figure 8B).

Moreover, the main organs from the treated mice, including heart, liver, spleen, lung, and kidney, were harvested for the hematoxylin and eosin (H&E) staining, which indicated that no obvious damage or toxicity was observed in these main organs during PTT procedure with Cy7-TFC-EMBI. Most importantly, healthy mice were treated with the nanocrystals, and the blood decay kinetics showed that the pharmacokinetics of Cy7-TFC-EMBI followed a two-compartment mode, with the two blood circulation half-lives of 0.12 ± 0.07 and 12.95 ± 2.26 h, respectively, indicating the proper clearance of Cy7-TFC-EMBI from the body (Figure 8D). The biodistribution of Cy7-TFC-EMBI nanoaggregates was also studied in mice. With the increase of time, the fluorescence signal of the liver displayed a tendency of first increasing and then decreasing, indicating that most of the Cy7-TFC-EMBI nanoaggregates were captured by the liver and then excreted (Figure 8E-G). All these results suggested the safety and low toxicity of Cy7-TFC-EMBI for photothermal theranostic.

3 | CONCLUSION

In summary, we have put forward a new concept, “boosting photothermal theranostics with SAIC”, to enhance the photothermal conversion for PA imaging-guided PTT of cancer. The NIR nanocrystal is prepared by self-assembly of single-component small molecule (e.g., Cy7-TFC-EMB1 in this study) through multiple hydrogen bonds and π-π stacking interactions. Importantly, the planar geometry of Cy7-TFC-EMB1 is essential for the minimization of the free energy of self-assembly to pack closely in crystal, which enhances intermolecular collisions and π-electron delocalization, resulting in highly efficient photothermal conversion with outstanding stability. Furthermore, the Cy7-TFC-EMB1 molecule self-assembles into nanocrystals to endow them with prolonged blood circulation and enhanced EPR effect for passive tumor targeting, hence achieving excellent photothermal theranostic efficacy in vivo followed by few side effects. This study reveals interesting insights into the structure–property relationships to design small-molecule PTAs that can self-assemble into a crystalline state in aqueous solution, which demonstrates a new supramolecular strategy to develop the next generation of photothermal theranostic for cancer.

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CONFLICT OF INTEREST

The authors declare that is no conflict of interest.

ETHICS STATEMENT

All animal experiments were approved by the Department of Science and Technology of Shandong Province and the Laboratory Animal Center of Qingdao Hao Biological Engineering Co., Ltd.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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