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Subcellular Behaviour Evaluation of Nanopharmaceuticals with Aggregation-Induced Emission Molecules

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Abstract

Nanopharmaceuticals possess a myriad of advantages for disease treatment, not only in delivering therapeutic agents, but also in deciphering their innate intracellular or subcellular behaviours, providing detailed diagnostic and prognostic information, quantifying treatment efficacy and designing better therapeutics. To evaluate the subcellular behaviour of nanopharmaceuticals, colourful fluorescence is the most potential technique, because it is capable of painting the subcellular detail in three dimensions with high resolution. Furthermore, the fluorescence is switchable, and thus the subcellular details can be lightened specifically without the undesirable background. However, most nanopharmaceuticals lack a fluorescent report group, and its introduction requires extra steps. Moreover, the introduced fluorescent groups can suffer from concentration quenching or aggregation-caused quenching (ACQ) when they are embedded in nanopharmaceuticals at a high concentration. The unique aggregation-induced emission (AIE) effect provides a straightforward solution. The aromatic cores of AIE molecules are always hydrophobic and do not undergo the ACQ effect even at high concentrations. Hence, AIE molecules can be directly introduced as building blocks to provide the driving force for the self-assembly of nanopharmaceuticals and can allow us to develop label-free, ACQ-free and luminescent nanopharmaceuticals that can simultaneously implement drug delivery and subcellular behaviour evaluation. This review presents different types of AIE molecules-based nanopharmaceuticals and their biological properties and applications for imaging subcellular behaviours, including the drug releasing process, metabolism of nanopharmaceuticals, subcellular distributions of drug and carriers, and therapeutic effect. With detailed acquaintance of these subcellular behaviours, we anticipate that the research we discuss in this review can inspire other scientists to develop next generation nanopharmaceuticals that can be guided by fluorescence imaging and thus can realize concisely controllable drug delivery.

1. Introduction

Nanopharmaceuticals are pharmaceuticals produced using nanotechnology or manufactured in the form of nanoformulations.^[1–3] Hitherto, various nanomaterials have been employed to develop nanopharmaceuticals with therapeutic agents, such as nanoparticles,^[4–6] micelles^[7–9] liposomes,^[10–12] polymers^[13–15] and dendrimers^[16, 17]. When precisely designed and engineered, nanopharmaceuticals show advanced functionalities compared to conventional pharmaceuticals.^[18, 19] The nanoformulations enable enhancements that can improve the solubility and stability of drugs,^[20, 21] prevent them from being degraded^[22, 23] or escape from reticuloendothelial system (RES) and prolong blood circulation time.^[24–26] Moreover, by virtue of the unique enhanced permeability and retention (EPR) effect of nanoscaled materials,^[27–29] nanopharmaceuticals always exhibit specific tumor targeting ability, which greatly mitigates the side effect of conventional pharmaceuticals on normal tissues.

To develop sophisticated and controllable nanopharmaceuticals, it is a requisite to have detailed information about the pharmacokinetic of the nanoformulations. To date, most studies have macroscopically focused on *in vivo* pharmacokinetic evaluations.^[30–32] However, micro-level evaluations are just capable of providing general information about the transportation and distribution of nanopharmaceuticals in the whole organism, but lack descriptive details. The cell is the most fundamental element in an organism. After being through a long journey in blood vessels, cells are the final stations of nanopharmaceuticals. Therefore, the subcellular pharmacokinetic process of these nanopharmaceuticals is the key determinant that influences their binding to the intracellular target and thus affects the therapeutic effect. The disposition of nanopharmaceuticals in the targeted cells involves many complicated processes, including drug releasing dynamics, cell absorptions, intracellular distributions, metabolism and efflux.

Deciphering these subcellular behaviours of nanopharmaceuticals can provide detailed information to guide drug design, drug efficacy evaluation, drug targeting and cytotoxicity assessment, and could further allow us to understand the drug delivery process better and control over the drug release more precisely, thus enhancing the effectiveness of pharmaceuticals. However, most nanopharmaceuticals are not easy to be tracked, because they usually lack traceable groups. To solve this problem, researchers introduce various contrast agents into nanoparticles, and obtain information emitted from the contrast agents by corresponding imaging techniques, such as magnetic resonance imaging (MRI),^[33, 34] positron emission-computed tomography (PET),^[35, 36] computed tomography (CT)^[37] and optical imaging^[38, 39]. Among these techniques, the most commonly used method is fluorescence-based optical imaging, especially in subcellular level research. Compared to other “always ON” imaging techniques, the fluorescence is tunable, which can be switched between “OFF” and “ON”.^[40, 41] The distinct wavelengths of fluorescence exhibit different colours, and thus the subcellular organelles can be vividly painted in three dimensions. With introduction of fluorescent tags into nanopharmaceuticals, the dynamic pharmacokinetics in living cells can be visualized by observing the variations and distributions of different fluorescence. However, fluorescent tags always suffer from a notorious aggregation-caused quenching (ACQ) effect,^[42–44] which tends to occur in conventional fluorescent dyes, and

even in quantum dots.^[45] The ACQ effect greatly limits signal readouts of the fluorescent tags and abates the sensitivity of the pharmacokinetics details. Thus, nanopharmaceuticals should be fabricated with self-luminance and ACQ-free features that are capable of indicating their subcellular behaviours with high sensitivity.

Unlike ACQ dominated dyes, Tang's group inaugurated new fluorescent molecules that emit weak fluorescence when they are dissolved, but exhibit high luminance in the aggregation state. They termed this phenomenon as "aggregation-induced emission (AIE)".^[42, 46] The AIE molecules generally contain hydrophobic aromatic components that experience intramolecular rotation or/and vibration when they are dispersed. The intramolecular rotation or/and vibration can consequently dissipate the exciton energy they receive, causing non-radiative decay of the excited-state energy and leading the fluorogens to a non-emissive situation. In the aggregated state, the intramolecular rotation and/or vibration will be physically restricted and will release the exciton energy in the form of fluorescence.^[47, 48] The molecular conformation of AIE molecules is propeller-like, and therefore they will not suffer " π - π stacking" induced ACQ, even in the aggregated state. Furthermore, the hydrophobic chromophores of AIE molecules are prone to stay in an aggregated state in water, and the propeller-like structures tend to assemble into the ground shape. The self-assembly feature enables AIE molecules to gain access to guide the hydrophilic molecules into the aggregated form in physiological circumstances. By virtue of the unique photophysical and self-assembly characteristics of AIE molecules, they are extensively employed in the fields of cell imaging,^[49–51] molecules detection^[52–54] and drug delivery.^[55–57]

In this review, we highlight recent research efforts on the integration of AIE molecules into nanopharmaceuticals, and fluorescent visualization of their subcellular behaviours by virtue of the unique AIE features (Figure 1). We mainly discuss different types of nanopharmaceuticals, such as AIE nanoparticles, AIE micelles, AIE polymeric nanopharmaceuticals and other AIE-based nano-drugs, and their subcellular behaviours unveiled by AIE-based fluorescence. We hope our discussions can inspire scientists to develop advanced and controllable nanoscaled drug formulations, based on their being better acquainted with the subcellular details.

2. AIE nanoparticles

Nanoparticles are microscopic materials with size in the nanoscale. To date, many nanoparticles have been used for developing sophisticated nanopharmaceuticals with various superiorities, such as mitigated side effects and targeted therapies. Unveiling the subcellular behaviours of these nanopharmaceuticals allows us to develop more controllable and efficient therapeutic regimen. However, most nanoparticles-based pharmaceuticals lack traceable groups. To decipher the subcellular behaviours of these nanopharmaceuticals, the most commonly used method is fluorescent tagging, because the colours of different emissions are capable of painting a picture of the three dimensional cell structures. However, fluorescent tagging methods still suffer some drawbacks. For instance, first, the fluorescent tagging method needs extra chemical steps to modify the nanoparticle; second, some surface modifications of fluorescent dyes may alter the physiochemical properties of nanoparticles,

such as the surface charge, hydrophilic or hydrophobic properties, and can lead to the labelled nanoparticles exhibiting different cell uptake pathways from the naked ones; finally, the fluorescent molecules may be hydrolyzed or released during their transportation, so that the subcellular location marked by fluorescence may not represent the real positions of the nanopharmaceuticals. Therefore, to aid evaluation of the real subcellular behaviours, nanopharmaceuticals with self-luminescence need to be developed.

AIE molecules are the best candidates to fabricate self-luminant and label-free nanoparticles (AIE nanoparticles), because they do not suffer from the ACQ effect in the aggregated state. Hence, an AIE nanoparticle-based self-indicating drug delivery system (SIDDS) was developed by our group^[55] As shown in Figure 2, a typical AIE molecule, tetraphenylethylene (TPE), was assembled into self-luminescent nanocarriers with blue fluorescence, and it carried the red luminescent anticancer drug doxorubicin (DOX) by electrostatic force, forming new nanopharmaceuticals (TD NPs). Because the TPE nanocarriers and DOX both showed different colours under confocal laser scanning microscope (CLSM) observation, the TD NPs exhibited a purple colour (merging the “blue” and “red” colours). By observing the co-localization, dissociation and distribution dynamics of these different colours, we were able to spatiotemporally visualize the drug releasing dynamics at the subcellular level, including the drug releasing site, and the real distributions of carriers and drugs. TD NPs (purple colour) were first uptaken into cells and translocated into lysosome, then DOX (red colour) was detached from the TPE NPs (blue colour). After TD NPs detachment, TPE and DOX escaped from lysosome, TPE located and lightened the cytoplasm, and DOX translocated to the nucleus and executed its anticancer function.

While constructing SIDDS, we found that a fluorescence resonance energy transfer (FRET) phenomenon occurred between the carrier (TPE) and drug (DOX). We speculated that the drug dynamic kinetics may be more concisely unveiled if we integrate the dynamic energy transfer into nanopharmaceuticals. Thus, we conjugated TPE and DOX together with a hydrazone bond, and assembled the conjugates into pH-responsive nanopharmaceuticals (THyD NPs).^[58] In THyD NPs, TPE transferred emissive energy to DOX through FRET, leading to its fluorescence emission being quenched; and the fluorescence of DOX was also quenched by means of ACQ, thus resulting in a “double quenched” effect. Due to the energy transfer relay between TPE and DOX, THyD NPs stayed in the fluorescence quenching state before they released the drugs. When THyD NPs encountered a low pH environment and caused the energy transfer relay to be invalid, the fluorescence of TPE and DOX were both woken up. TPE and DOX then experienced a fluorogenic process accompanied with drug dynamic release, and then DOX and TPE were transferred to their subcellular locations (Figure 3, Panel I). The drug releasing process realized a “from darkness to brightness” process, and by recording the fluorogenic process with real-time CLSM, the spatiotemporal drug releasing kinetics could be visualized in a non-invasive manner (Figure 3, Panel II). THyD NPs were first transferred to lysosomes after being uptaken, and then they released the drugs in lysosomes. The released drug invaded into the nucleus, and the carriers were distributed in the cytoplasm.

Based on the design and experimental results of our AIE-based nanoparticles, we combined TPE and DOX with different chemical interactions and composed three types of AIE-based

DOX formulations.^[58] The differences of their intracellular behaviours were visualized by fluorescence variations, and are summarized in Table 1. THyD NPs, the hydrazone bond composed of nanopharmaceuticals experienced a sustained drug release process in lysosomes and released DOX slowly and persistently; however, for TAmD NPs, the amide bond composed nanopharmaceuticals cannot release the drug effectively. For TD NPs, the electrostatic interaction composed nanopharmaceuticals released drugs in lysosomes with a faster speed, whereas free DOX was most likely to enter cells through diffusion and directly invade the nucleus from the cytoplasm without being postponed in lysosomes. AIE carriers were finally distributed in the cytoplasm when they had finished the drug carrying role, and the drugs would enter their therapeutic effects loci.

Liu and Tang's group developed AIE-based theranostic nanoparticles, which enable co-delivering *cis*-platin and DOX, and could be utilized for the visualization of subcellular drug behaviours.^[59] The prodrug was composed of a targeted cRGD (cyclic arginine-glycine-aspartic acid) moiety, a TPE derivative with AIE characteristics, a fluorescent anticancer drug (DOX), and chemotherapeutic Pt (IV) as the linker (Figure 4, Panel I). Between TPE and DOX, a FRET effect occurred and dominated the fluorescence variations of the nanopharmaceuticals. The cRGD ligand could specifically promote the accumulation of the nanopharmaceuticals to the $\alpha_v\beta_3$ integrin (overexpressed cancer cells) through receptor-mediated endocytosis. After being translocated into cells, Pt (IV) would be transformed into its active form, Pt (II), by intracellular reduction. With breakage of the Pt (IV) linker, DOX was detached as well. Before drug release, the FRET effect induced the fluorescence quenching of TPE, while the red fluorescence of DOX acted as an intracellular indicator of the nanopharmaceuticals. FRET became invalid accompanied with drug release; the fluorescence of TPE was then recovered, which can be used for tracking the drug releasing behaviours (Figure 4, Panel II). In 1 h of incubation, the cells clearly showed DOX fluorescence, indicating the cellular uptake of the nanopharmaceuticals. In the TPE channel, very weak blue fluorescence was detected, indicating that most nanopharmaceuticals stayed intact. With prolongation of the incubation time to 2 h, the fluorescence of TPE became bright and was distributed in cytoplasm; DOX also stayed in the cytoplasm and was co-localized with TPE, while the fluorescence recovery of TPE indicated that Pt (IV) began to be transformed into its active form, Pt (II). After further 4 h incubation, TPE became brighter, while DOX began to invade the nucleus and became co-localized with the nucleus indicating dyes (DRAQ5). In this design, the spatiotemporal drug releasing behaviours were clearly visualized by real-time tracking of the transitions of the different colours in cRGD-TPE-Pt-DOX. The nanopharmaceuticals were first taken up by cells via receptor-mediated endocytosis and transported into the cytoplasm, then Pt (IV) was activated by the reduction force in cytoplasm, DOX invaded into the nucleus and executed its anticancer functions, and finally TPE was distributed in the cytoplasm.

AIE molecules, exhibiting excellent ACQ-free imaging features, were highly motivated to develop label-free, self-luminescent nanopharmaceuticals, which not only can fluorescently indicate their real distributions at the subcellular level, but can also be used to visualize the dynamic drug releasing process in a real-time manner.

3. AIE micelles

Micelles are commonly assembled by amphiphilic lipids, which have excellent drug delivery abilities, and show great potential to be used in clinical trials. Nevertheless, the traditional micelles are typically “one trick ponies”, and their sole role is to deliver therapeutic agents. After finishing the drug delivery mission, the subcellular location of the micelles can be a mystery; thus, their destinations, whether they are digested or dispatched, are unknown. Therefore, it is desirable to develop “visible” micelles to unveil these invisible “underground” activities.

To achieve this, we covalently conjugated an AIE molecule (TPE) to amino-methoxypolyethylene glycol (mPEG₂₀₀₀-NH₂), and synthesized AIE-based micellar building blocks: TPE-mPEG (Figure 5, Panel I). Then, the hydrophobic TPE molecules drove the TPE-mPEG assembling into visible AIE micelles.^[60] The AIE micelles were then employed to carry anticancer drug (DOX) and applied to unveil their subcellular behaviours. In our AIE micelles, the fluorescence intensities of TPE and DOX were both decreased, because the energy transfer relay occurred between TPE and DOX. The fluorescence of TPE and DOX would then be increased when drugs were released from the micelles, breaking the energy transfer relationship. The unique AIE effect endows the micelle with ACQ-free and high-quality imaging abilities. We incubated DOX-loaded AIE micelles with a MCF-7 breast cancer cell line for different times to evaluate the spatiotemporal subcellular behaviours. The imaging results are shown in Figure 5 (Panel II), where it can be seen that after 0.5 h incubation, AIE micelles and DOX showed less fluorescence, and both were distributed in cytoplasm. With prolongation of the incubation time to 1 h, the fluorescence of AIE micelles and DOX both increased, indicating that DOX was starting to be released from the micelles. By tracking their fluorescence disposition, we found that AIE micelles mostly located in cytoplasm when they released the drugs, and did not enter into the nucleus to interrupt the anticancer activities of DOX, and DOX then entered into the nucleus after being released.

Polyethyleneimine (PEI) serves as the gold standard for gene delivery. However, the gold standard is also lacking traceable groups that can indicate the subcellular locations during its gene transfecting process. Otherwise, PEI suffers from undesirable cytotoxicity, which greatly hinders its biological applications. To overcome these drawbacks, we introduced an AIE molecule (TPE) and palmitic acid (PA) to the PEI backbone, and formed an amphiphilic TPEI molecule (Figure 6, Panel I). The TPEI molecules can self-assemble into a luminescent micelle-like gene delivery system (TPEI NPs).^[61] The TPEI NPs exhibited bright blue fluorescence and ACQ-free characteristics, which showed it was not only suitable for long-term cell imaging, but also could play a role with gene transfection systems. By observing the blue fluorescence of TPEI NPs, its co-localization with LysoTracker and pDNA expressed GFP, (Figure 6, Panel II), we concluded that the TPEI NPs transported and released the pDNA in lysosomes, and encoded the pDNA efficiently. Otherwise, the micelle-liked TPEI NPs stayed in cytoplasm after they finished the gene transfection.

To illustrate the pH-responsive intracellular behaviours of the nanopharmaceuticals, Wang's group constructed AIE polymeric micelles with excellent pH responsibility.^[57] As shown in Figure 7 (Panel I), they synthesized an amphiphilic polymer by conjugating the AIE molecule (TPE) and dextran through a pH-sensitive hydrazone bond. AIE molecule conducted both functionalities of the ACQ-free report group and a hydrophobic part that can guide the polymer aggregation to nanoformulations. The drug-loaded AIE polymeric micelles showed sensitive pH responsiveness of drug releasing, and improved therapeutic efficacy. By observing the fluorescence of the carriers and drugs with fluorescence microscopy (Figure 7, Panel II), it was inferred that the subcellular behaviours of the AIE polymeric micelles obeyed a spatiotemporal drug releasing pattern, and the cells were turned up gradually with time elapse. A blue fluorescence of TPE was clearly observed and it was distributed in the cytoplasm, suggesting that the nanoparticles were internalized by cells and concentrated in endosome. The drugs were distributed into the nucleus to execute their anticancer functions eventually.

With introduction of AIE molecules to a hydrophilic lipid, the building units of micelles would convert to amphiphilic, so that the micelle structures can be easily self-assembled and become steady in physiological circumstances. More important, AIE molecules endow self-luminant functionality to the common micelles, which is capable of indicating the post-destinations of micelles when they finish their drug delivery mission. The ACQ-free AIE molecules are able to lighten the subcellular translocation pathway of the micelles, even in a real-time manner, and thus can accurately provide valuable information for the pre-design and post-evaluation of the nanopharmaceuticals.

4. AIE polymeric nanopharmaceuticals

Polymeric nanopharmaceuticals are pharmaceuticals consisting of a polymer or copolymer in the forms of nanoformulations and complexed with therapeutic agents. The building blocks of polymer or copolymers contain hydrophilic and hydrophobic parts that can aid self-assembly into nano-structures under physiological circumstance. The complexed therapeutic agents are released when the external stimuli break the nano-structures or molecular structures of the polymer/copolymer. However, the changes (metabolisms) of the nano-structures or molecular structures are always speculated by the chemical architectures of the polymers or copolymers, because they tend to be invisible. To visualize the intracellular changes of the polymers, AIE molecules are smartly introduced into the building blocks of the polymers or copolymers, and act as both hydrophobic parts and fluorescent reporters to track metabolisms and the drug releasing process of the polymeric nanopharmaceuticals. The direct introduction of AIE molecules can save the extra steps usually required to modify the nanopharmaceuticals, and also provide the hydrophobic driving force for the self-assembly.

Wu's group synthesized an AIE amphiphilic block copolymer, PEG-b-P(S-co-PPSEMA), in which the AIE molecules (PPSEMA) acted as both hydrophobic building blocks and fluorescent reporters.^[62] This smart design directly employed the fluorescent group as the hydrophobic part of the polymers, which achieved a straightforward solution to decrease the extra chemical modification steps to introduce the fluorescent reporter, and realized a label-

free nanopharmaceuticals construction method. The fabricated polymeric micelles were employed to deliver the anticancer drug doxorubicin (DOX). Interestingly, FRET occurred between PPSEMA and DOX (Figure 8, Panel I). The intracellular uptake of empty and DOX-loaded micelles was studied in HT-29 cells using CLSM. Here, drug encapsulation was evaluated by observing the FRET occurrence, and the drug release with the subsequent FRET decrement was fluorescently visualized. By virtue of the unique fluorescent features of their AIE polymeric micelles, the group also unveiled the bio-distribution and release kinetics of the antitumour drug *in vitro* (Figure 8, Panel II).

Tang's group developed AIE-based polymeric nanoparticles to deliver siRNA for pancreatic cancer treatment.^[63] As shown in Figure 9 (Panel I), they employed two FDA-proven surfactant polymers, namely, Pluronic F127 and DSPE-PEG, to encapsulate the deep-red emissive AIE dye (PE-TPA-DCM), and fabricated the AIE polymeric nanoparticles by a nano-precipitation method. The prepared polymeric nanoparticles were then complexed with siRNA for the gene silencing treatment of pancreatic cancer. By virtue of the excellent imaging features of AIE dyes, they observed the dynamic fluorescence variations and the fluorescence co-localizations between nanocarriers and siRNA, and demonstrated detailed information about the subcellular distributions and high transfection efficiency (Figure 9, Panel II)

By taking advantage of AIE molecules, Liu's group developed a photoactivatable AIE polymer for light-controlled gene delivery, which could escape from endo/lysosomes and unpack DNA by force of the AIE-generated ROS.^[56] As shown in Figure 10 (Panel I), the polymer contained an AIE molecules (TPECM) conjugated with oligoethylenimine via ROS-responsive linker (aminoacrylate, AA), which was then further grafted to the PEG chain. The polymer can self-assemble into nanoparticles with bright red fluorescence because of the AIE behaviours of TPECM. Furthermore, the TPECM enables generating ROS under light irradiation. The polymeric nanoparticles then carried the DNA through electrostatic interaction and could be applied for cell evaluation. Upon light irradiation, the generated ROS can facilitate the escape of the DNA from endo/lysosomes by disrupting the membranes, and the released DNA will then execute their therapeutic effect. In their design, AIE molecules exhibited not only excellent fluorescence for bio-imaging, but also acted as the trigger for DNA release. By observing the fluorescence co-localization, dissociation and distribution of the AIE molecules and other auxiliary fluorescence, this smart design was capable of unveiling the dynamic polymer breaks and DNA releasing process, as well as the carriers and DNA distribution (Figure 10, Panel II). Furthermore, the polymer structure metabolism process was also unveiled, such as the reversion of the high molecular weight complex back to their low molecular weight counterparts, and the DNA unpacking.

In AIE-based polymeric nanopharmaceuticals, AIE molecules are not just the fluorescence reporter, but also the building blocks and provide the driving force for the polymers to assemble into nanoformulations. Moreover, scientists have smartly reported the metabolisms of nanopharmaceuticals by virtue of the unique fluorescence behaviours of AIE molecules. The AIE nanopharmaceuticals emit a tunable fluorescent signal when they disassemble or release the therapeutic agents, and even the post-metabolism can be visualized when the polymers have finished the drug delivery. The visualization of nanocarrier's metabolisms

can definitely pave the way for study of the toxicology of nanomaterials, which is very controversial for using nanomaterials for biological implementation.^[64, 65]

5. Other AIE nanopharmaceuticals

AIE molecules were also employed to indicate the therapeutic effect of the nanopharmaceuticals. Liu and Tang's group developed a targeted theranostic platinum (IV) prodrug with an embedded AIE light-up apoptosis sensor for non-invasive evaluation of its therapeutic responses *in situ*.^[66] The prodrug was composed of a chemotherapeutic agent, platinum (IV), an apoptosis sensor (TPS-DEVD) based on an AIE molecule (tetraphenylsilole, TPS), and a cyclic (RGD) peptide as the targeting ligand (Figure 11, Panel I). The prodrug could preferentially recognize the overexpressed $\alpha_v\beta_3$ integrin on tumor cells, and be translocated into cell lumen. The Pt (IV) was then released by intracellular reduction and transformed into the active Pt (II) form. The released Pt (II) could induce cell apoptosis and activate caspase-3 to cleave the DEVD peptide and thus make the apoptosis sensor (TPS-DEVD) become aggregated and trigger the fluorescence "turn on" of TPS. The AIE-based prodrug was employed to treat the cancer cells, as shown in Figure 11 (Panel II), where a fluorogenic process in cells was vividly performed with time elapse. In 1 h incubation, the cells showed no obvious fluorescence, indicating that the prodrug did not make the cell apoptosis. Then, they further prolonged the incubation time to 2 h, and the cells began to stain with green fluorescence, indicating that the cells became apoptosis, and the prodrug had started to become active. The cells finally showed extensive apoptosis-related fluorescence when the incubation time was increased to 4 h; the time-dependent fluorogenic process demonstrated that the prodrug enabled visualization of the therapeutic effect of the administrated drugs directly in the form of fluorescence.

6. Perspective and conclusions

This review highlights recent study into the development of AIE-based self-luminescent nanopharmaceuticals. Unlike the conventional fluorescent dye tagged nanopharmaceuticals, AIE molecules are introduced as internal building blocks that drive the hydrophilic parts of the pharmaceuticals to assemble into nanoformulations. As building blocks, the introduction of AIE molecules avoids the extra modification steps to make the nanopharmaceuticals become luminescent. AIE molecules also act as report groups of the nanopharmaceuticals, which then never suffer from high concentration induced ACQ-effects, and thus offer higher imaging efficacy for their subcellular behaviour tracking. Thus, AIE nanopharmaceuticals allow fluorescent visualization of real pharmacokinetics at the subcellular level, including the distribution, metabolism, drug releasing kinetics and therapeutic efficacy.

AIE-based nanopharmaceuticals are self-indicating and exhibit various excellent characteristics, where i) the unique fluorescence imaging features can directly indicate their own subcellular behaviours; ii) the fluorescence exhibited by AIE molecules also indicates the aggregation state of the AIE nanopharmaceuticals, because AIE molecules will only show fluorescence when their intramolecular rotation or/and vibration are restricted in structures/aggregates of nanopharmaceuticals; and thus iii) the metabolisms of AIE nanopharmaceuticals can be performed by observing the fluorescence variations.

Furthermore, by introducing specific energy transfer between the therapeutic agents and AIE building blocks, AIE nanopharmaceuticals can unveil the dynamic drug kinetics by observing the energy transfer triggered fluorescence variations in a real-time manner.

To date, research into AIE molecules has been highly motivated for the fabrication of label-free, ACQ-free, and self-luminant nanopharmaceuticals. Nevertheless, AIE-based nanopharmaceuticals still have some imperfections that need to be noted. The basic working mechanisms of AIE molecules are the restriction of intramolecular rotation or/and vibration, and therefore, where the AIE fluorescence comes from should be clearly understood, because either the AIE molecules may get stuck in biomacromolecules or they may stay in an aggregated state under physiological circumstances, which would restrict the intramolecular rotation or/and vibration and trigger the AIE molecules becoming luminant. Furthermore, the AIE nanopharmaceuticals must be precisely designed and engineered to obtain high signal readouts, because the AIE fluorescence outcomes are directly correlated with the restriction intensities of the intramolecular rotation or/and vibration.

Future development of AIE nanopharmaceuticals should put much effort into a drug efflux study, which intimately relates to drug resistance^[67–70] and decreases the therapeutic efficacy of the administrated agents. Visualization of drug efflux will provide detailed information about the drug resistant process, and thus pave the way to the development of more sophisticated nanopharmaceuticals for drug resistant diseases treatments. Near-infrared AIE fluorogens also need to be urgently developed for unveiling the *in vivo* behaviours of nanopharmaceuticals, because subcellular pharmacokinetics could finally exhibit the influence on the whole organism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Subcellular behaviours evaluated by AIE-based nanopharmaceuticals

Figure 2.

Panel I: Schematic showing the formation of TD NPs. The drug releasing process, drug releasing subcellular location and real subcellular distributions of TPE NPs and DOX were fluorescently visualized by the colour transitions of SIDDs. Panel II: CLSM illustrated the spatiotemporal distributions of TD NPs in MCF-7s cells. TD NPs were incubated with cells for 2 h and 4 h. The blue colour denotes TPE NPs, red denotes DOX, purple colour stands for TD NPs and green denotes lysosomes stained by LysoTracker. Adapted with permission from Ref. 55. Copyright 2014 Wiley-VCH.

Figure 3.

Panel I: Intracellular trajectory of THyD NPs in living cells. The THyD NPs fabricated with two fluorophore groups (TPE and DOX) and conjugated with a pH-responsive hydrazone bond. In THyD NPs, the energy transfer relay effect, mediated by FRET and ACQ, inactivates the fluorescence of TPE and DOX and forms “silent” nanopharmaceuticals. By observing the dual-colour responsiveness of TPE and DOX, we could observe the following: THyD NPs were first translocated into lysosomes, the drugs were released, and then TPE stayed in the lysosomes or diffused to the cytoplasm, whereas DOX entered the nucleus and performed its antitumor duty. Panel II: Real-time visualization of the spatiotemporal drug release by filming the fluorogenic process of the THyD nanopharmaceuticals. The drug releasing kinetics exhibited a “from darkness to brightness” pattern. The scale bars are 20 μm. Adapted with permission from Ref. 58. Copyright 2015 American Chemical Society.

Figure 4.

Panel I: Schematic of the targeted theranostic dual-acting prodrug for real-time drug tracking and activation monitoring. Panel II: Confocal images of MDA-MB-231 cells after incubation with cRGD-TPE-Pt-DOX for 1 h (A), 2 h (B) and 2 h followed by incubation in fresh medium for another 4 h (C). Blue: TPE fluorescence; masked green: DOX fluorescence; red: cell nuclei stained by DRAQ5. All images share the same scale bar (20 μm). Reproduced with permission from Ref. 59. Copyright 2014 Royal Society of Chemistry.

Figure 5.

Panel I: Schematic of a DOX-loaded self-assembly micelle (TPED) with AIE characteristics as a novel multifunctional theranostic platform for intracellular imaging and cancer treatment. Panel II: Spatial distributions of TPEM and TPED in MCF-7 cells. CLSM images of the distribution of self-indicating TPEM and TPED. MCF-7 breast cancer cells were incubated with TPEM and TPED for 0.5 and 4 h. Scale bars are 20 μm . Reproduced with permission from Ref. 60. Copyright 2014 American Chemical Society.

Figure 6.

Panel I: Synthetic route of TPEI and schematic of the self-assembly of micelle-like TPEI nanoparticles (TPEI NPs). Panel II: (A) CLSM images of 293T cells treated with micelle-like TPEI NPs. Lysosomes, stained by LysoTracker® Deep Red, and exhibiting red emission. (Scale bar = 20 μm); (B) CLSM images of 293T cells treated with 10-1 and 20-1 TPEI-NPs/pDNA complexes for 24 h. (scale bar = 10 μm). Reproduced with permission from Ref. 61. Copyright 2015 Royal Society of Chemistry.

Figure 7.

Panel I: Illustration of a drug-loaded micelle with AIE properties as a novel theranostic platform for intracellular imaging and cancer treatment. Panel II: Fluorescence microscopy images of HeLa cells incubated with drug-loaded micelles for different times. From left to right: bright field, TPE (blue), DOX (red) and merging of the three images. (a) 1 h and (b) 3 h. Reproduced with permission from Ref. 57. Copyright 2015 Royal Society of Chemistry.

Figure 8.

Panel I: Synthesis of PEG-b-P(S-co-PPSEMA). Panel II: Confocal images of HT-29 cells incubated with empty micelles (A–C), DOX-loaded micelles (D–F) and free DOX (G–I). The intracellular images were obtained using three different microscopic settings: donor channel (A, D, G); FRET channel (B, E, H) and acceptor channel (C, F, I). Scale bar are 20 μm . Adapted with permission from Ref. 62. Copyright 2013 Wiley-VCH.

Figure 9.

Panel I: Schematic of the construction of nanoparticle formulations based on PEGylated phospholipid and triblock copolymer Pluronic F127. Panel II: Fluorescent imaging of MiaPaCa-2 cells treated with AIE dye doped nanoparticle formulations: (A) Blank control, (B) Phos-10% and (C) F127-10%. The FAM labelled siRNAs and AIE dye were rendered in green and red, respectively. Cell nuclei are stained with DAPI and rendered in blue. Reproduced with permission from Ref. 63. Copyright 2015 Springer-Verlag Berlin Heidelberg.

Figure 10.

Panel I: A) Chemical structure of the ROS-responsive polymer P(TPECM-AA-OEI)-g-mPEG. B) ROS-sensitive nanoparticles (S-NPs) self-assembled from P(TPECM-AA-OEI)-g-mPEG in aqueous media and their complexation with DNA to form S-NPs/DNA. C) The itinerary of S-NPs/DNA to the transgene expression. S-NPs/DNA was endocytosed by the cells and entrapped in endo/lysosomes. Upon light irradiation, the generated ROS can concurrently destroy the endo/lysosomal membrane to facilitate the vector escape and break the S-NPs to favor DNA unpacking, leading to DNA release for nuclear entry and transcription. Panel II: A) CLSM images of HeLa cells stained with S-NPs/DNA (A1) and LysoTracker green (A2); overlay of the images A1 and A2 (A3); intensity profiles of region of interest (A4: red line in image A3; solid line = LysoTracker, dashed line = S-NP/DNA). B) CLSM images of HeLa cells incubated with S-NPs/YOYO-1-DNA complexes in the dark (B1), with light irradiation for 2 min (B2), 5 min (B3) and 5 min in the presence of VC (B4). Green: YOYO-1 fluorescence; Red: S-NPs fluorescence. Yellow: co-localization of red and

green pixels. C) Changes in co-localization ratios between the fluorescence of YOYO-1 and S-NPs after different treatment. D, E) CLSM images of HeLa cells after incubation with D) S-NPs/YOYO-1-DNA pretreated with chloroquine (CQ), E) inS-NPs/YOYO-1-DNA in the dark (D1, E1) or after 5 min light irradiation (D2, E2). Scale bar for all images is 10 mm. All images share the same scale bar (20 μm). Reproduced with permission from Ref. 56. Copyright 2015 Wiley-VCH.

Figure 11.

Panel I: Schematic of the targeted theranostic platinum (IV) prodrug with a built-In aggregation-induced emission light-up apoptosis sensor for non-invasive *in situ* early evaluation of its therapeutic responses. Panel II: (A–D) Real-time CLSM images displaying the apoptotic progress of TPS-DEVD-Pt-cRGD stained U87-MG cells upon treatment with TPS-DEVD-Pt-cRGD for 6 h. Nuclei were live stained with DRAQ5. (G–J) Corresponding fluorescence/nucleus overlay images of (A–D), respectively. All images share the same scale bar (20 μ m). Adapted with permission from Ref. 66. Copyright 2014 American Chemical Society.

Table 1Drug releasing behaviours between different DOX formulations^[58]

Nano Formulations	Categories	Drug release	Releasing site	Releasing speed	Carriers destination	Drug location
THyD NPs	Hydrazone bond	Yes	Lysosomes	Delayed release	Cytoplasm	Nucleus
TAmd NPs	Amide bond	No	–	–	–	–
TD NPs	Electrostatic force	Yes	Lysosomes	Fast	Cytoplasm	Nucleus
Free DOX	–	–	–	–	–	Nucleus