Cyanine Renaissance: Tailoring the Properties to Applications

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Abstract: In this account, we provide an overview of the applications that arose from the recently developed synthetic methodology that delivers heptamethine cyanines (Cy7) substituted at the central chain. The ability to easily introduce and manipulate various substituents in different substitution patterns along the cyanine chain enabled rational tailoring of the photophysical and photochemical properties. Exercising this control over the structure–property relationship proved to have a substantial impact in the field of cyanine dyes and was swiftly harnessed in a number of emerging applications in distinct areas, including fluorescent probes, biosensors, dye-sensitized upconversion nanoparticles, phototruncation of cyanines and photocages. While this method unlocked a number of new avenues, many synthetic challenges remain to be conquered in order to fully capitalize on the potential of cyanines, and we provide a short perspective that summarizes them at the end of this manuscript.

Keywords: Bioimaging ·Cyanine · Fluorescence · Near-infrared · Photocage · Zincke chemistry

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Introduction

Cyanine dyes are an important class of chromophores with a wide range of applications. These dyes display excellent photophysical properties, *i.e.* near-infrared absorption and unique chemical reactivity,[1] conferred by an odd-numbered polyene linker connecting two terminal nitrogen-containing heterocycles. In particular, heptamethine cyanine dyes (Cy7, Fig. 1) containing seven carbon atoms in the chain are highly appreciated for their strong absorption and emission maxima located in the center of the near-infrared (NIR) window (~800 nm),[2] making them indispensable fluorophores in modern science.[3] An important characteristic of cyanine dyes is the diversity and variability of their structure, allowing a design of complex and sophisticated molecular systems. As a result, this class of dyes has been technologically exploited throughout the past decades in various areas.

The medical significance of Cy7 derivatives is validated by Food and Drug Administration (FDA) approved indocyanine green (ICG) with many active clinical trials after over 60 years of its clinical use. Another promising derivative, IR800-CW, has been explored for a number of fluorescence-guided surgery applications.[4–6] Indeed, this attractive use of cyanine dyes arises from

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the combination of their fluorescent properties and conjugation with a biomolecule, such as an antibody. By detecting the fluorescence intensity of the complex, the message of the labeled object is transferred qualitatively or quantitatively in various protocols. In other applications such as chemosensors, systems that convert chemical stimuli into a response that can be easily detected, *e.g.* fluorescence, color change or an electronic signal,[7,8] pH changes,[9] interaction with glutathione[10] or reactive oxygen species[11–13] have been described, offering a temporal resolution and a rapid response.[14,15] Additionally, they have been utilized in single-molecule fluorescence microscopy and super resolution imaging,[16–18] photodynamic therapy,[19,20] NIR photouncaging,[21–24] gasotransmitter delivery,[25] and other technological applications, *i.e.* solar cells.[26,27] More recently, their derivatization also facilitated bioimaging in the NIR-II window,[28] alterations of the pharmacokinetic profile,[29,30] or enhanced singlet-oxygen sensitization properties.[20,31] All these applications rely strictly on the excellent photophysical properties or specific chemical reactivity of the polyene chain.[32] Nevertheless, modification of the Cy7 chain to manipulate their properties had been for a long time mostly out of reach and limited to some specific examples. In this account we will discuss the central role of the synthetic methodology based on Zincke chemistry, recently reported by some of us, that contributed to the birth of new applications across chemistry and biology fields (Fig. 1).[33]

Synthesis

Widespread utilization of cyanine dyes in bioapplications accentuated the importance of polyene substitution. Surprisingly, the initial strategies used to modify the central heptamethine chain had remained underdeveloped and limited for a long time. Most of the common methods start from the C4'-chloro-substituted cyanine **1a** and rely on an electron transfer-mediated $S_{RN}1$ reaction with N, O, or S nucleophiles[12,20,33–39] (**1b**) or palladium-catalyzed Suzuki[40] or Sonogashira[41] coupling reactions (**1c**), limited to a handful of coupling partners (Fig. 2A). The other option involves the synthesis of a custom substituted iminium,[42] which, however, requires harsh conditions, limiting its practical use.[43] Apart from an embedded cyclopentyl or cyclohexyl ring, no modifications at the C3' and C5' positions had been reported prior to the Zincke chemistry-based approach discussed herein. Therefore, essentially only the C4'-position modifications were available, imposing fundamental restrictions on the design of emerging Cy7 applications.

In the approach recently published by some of us in 2019, the notorious and over a century old Zincke chemistry was repurposed into a modern tool for cyanine synthesis.[33] The Zincke reaction has been traditionally used for the preparation of *N*-substituted pyridinium salts which are often poorly accessible *via* different pathways. In this transformation, an electron-deficient pyridinium salt **2** is first attacked by a nucleophile, providing a non-symmetrical open-chain Schiff base intermediate **3a**. At this stage an intramolecular cyclization can occur to provide **4**, usually driven by elevated temperature. Alternatively, **3a** can undergo an attack by another molecule of the nucleophile to create a symmetrical intermediate **3b**, highly reminiscent of those usually used in the synthesis of cyanines.

The latter pathway was leveraged to devise a synthetic strategy to Cy7 under mild conditions. The ability to essentially 'transfer' the substituents of a commercial or synthetically accessible pyridine into the C3', C4' and C5' positions of the central cyanine chain proved to be highly valuable. As a result, a whole library of Cy7 with different substituents along the chain and terminal heterocycles was prepared and characterized. Given their proximity to the frontier molecular orbitals of Cy7, it is not surprising that the substituents exhibit effects on the photophysical and photochemical properties in the range of 2–3 orders of magnitude.[44] For instance, the same substituent displays a complementary effect on the absorption maxima when placed in a different position on the chain, and in the same vein, substituents with different electronic properties in the same position exhibited opposite effects on the absorption maxima.[44] Afterwards, our group continued exploring further modifications of this method, such as omission of the auxiliary nucleophile,[21] or use of aprotic solvents, further increasing its utility towards wider scope of functional or solvolytically labile groups. Recently, Feringa and co-workers employed a modification of this method using a heterocycle containing an exocyclic double bond with no additional nucleophile or a base.[22] A related strategy has been published recently and it uses furfuryl alcohol as a source of carbon in the cyanine chain,[21] providing a compelling alternative utilizing a source of carbon readily available from green sources such as biomass. However, its major drawback lies in a low modularity of the substitution pattern.

The ability to exercise control over photophysical properties is highly attractive and since its inception, this synthetic approach has been leveraged in many fields of chemistry to reach otherwise
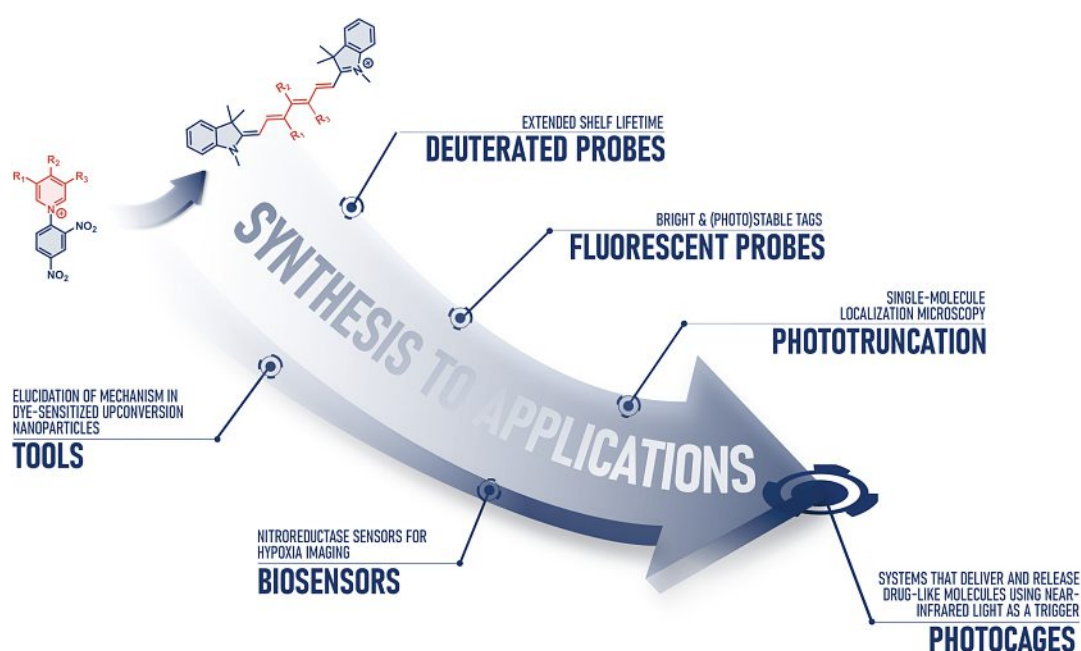

Fig. 1. The progress of applications in different fields drawing from the synthesis of cyanines based on Zincke chemistry.
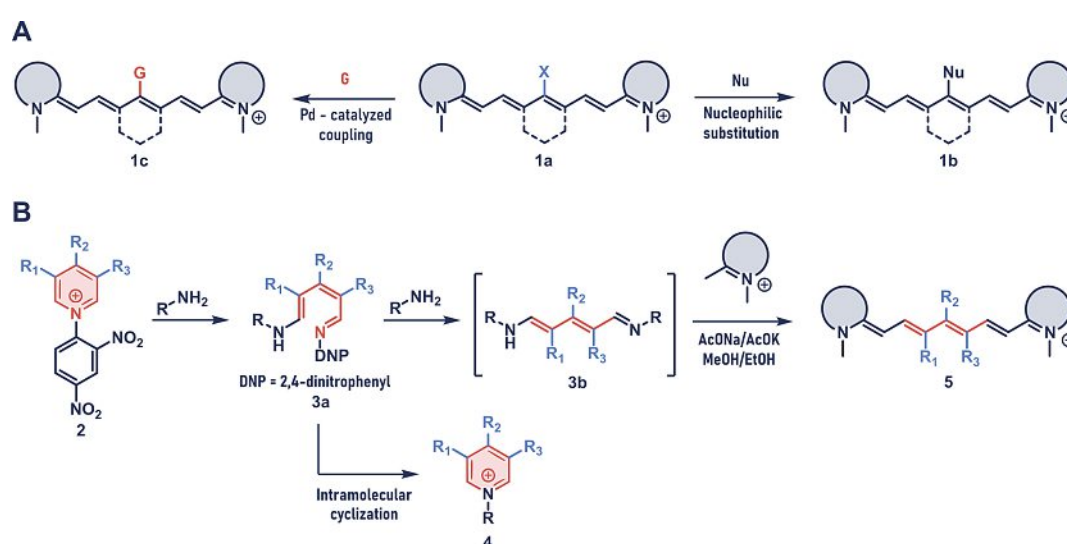
Fig. 2. (A) Approaches to the heptamethine chain modification using palladium-catalyzed coupling reactions or $S_{RN}1$ reactions with N, O, or S nucleophiles. (B) Strategy based on Zincke salts that involves the pyridinium ring opening, followed by incorporation of the substituted pyridine residue (red) into the cyanine scaffold.

inaccessible substitution patterns and properties. In the following chapters, we highlight some of the applications in more detail.

Fluorescent Probes

Dye instability, aggregation, and poor pharmacokinetics of fluorophores often limit their performance and scope of the applications. In an attempt to overcome these limitations, Smith and co-workers recently reported an intriguing molecular design, remotely inspired by the rotaxane chemistry, producing a sterically shielded and charge-balanced cyanine **s775z**.[45] The key design feature is a *meso*-aryl group that projects two shielding arms directly over each face of the linear heptamethine polyene (Fig. 3A,B).

This newly introduced fluorophore **s775z** was accessed from a Zincke salt containing a biaryl connection with sterically demanding substituents in the positions 2 and 6, showcasing the robustness of the method. In the paper, Smith and co-workers acknowledged the paramount importance of this approach for the development of their probes: "*The synthetic advance that allowed us to prepare linear and meso functionalized s775z (cyanine) was the newly reported methodology of Štacková and co-workers that involves ring opening of Zincke salts. The significant advantage gained by employing this innovative synthetic strategy is that the C-C link to the center of the heptamethine polyene is formed before the complete polyene is created and thus the C-C coupling reaction does not encounter high steric hindrance.*"[45]

The exceptional performance of **s775z** comprised high photostability compared to ICG and improved thermal stability in aqueous media (Fig. 3C). The authors showed that it can be stored at 4 °C virtually indefinitely. Another important aspect that often plagues application of fluorophores is aggregation in aqueous media, leading to hypsochromic shift of their absorption maxima and very low quantum yields of fluorescence. Remarkably, the shielded system displayed significantly lower aggregation in comparison to the non-shielded analogue. This observation opened the possibility to make densely labeled conjugates without stacking of the appended fluorophores and further increasing the signal to noise ratio throughout the imaging.

Another aspect addressed by Smith's design was pharmacokinetics, which has been a major concern for years, in particular for cyanines. Biodistribution studies performed on animal models showed that **s775z** significantly outperformed the FDA-approved ICG. After 2 h, the shielded system was almost completely cleared from the animal body, whereas ICG was cleared only from the blood stream, remaining accumulated in the intestines and liver (Fig. 3D). The shielding arms did not prevent the targeted ana-
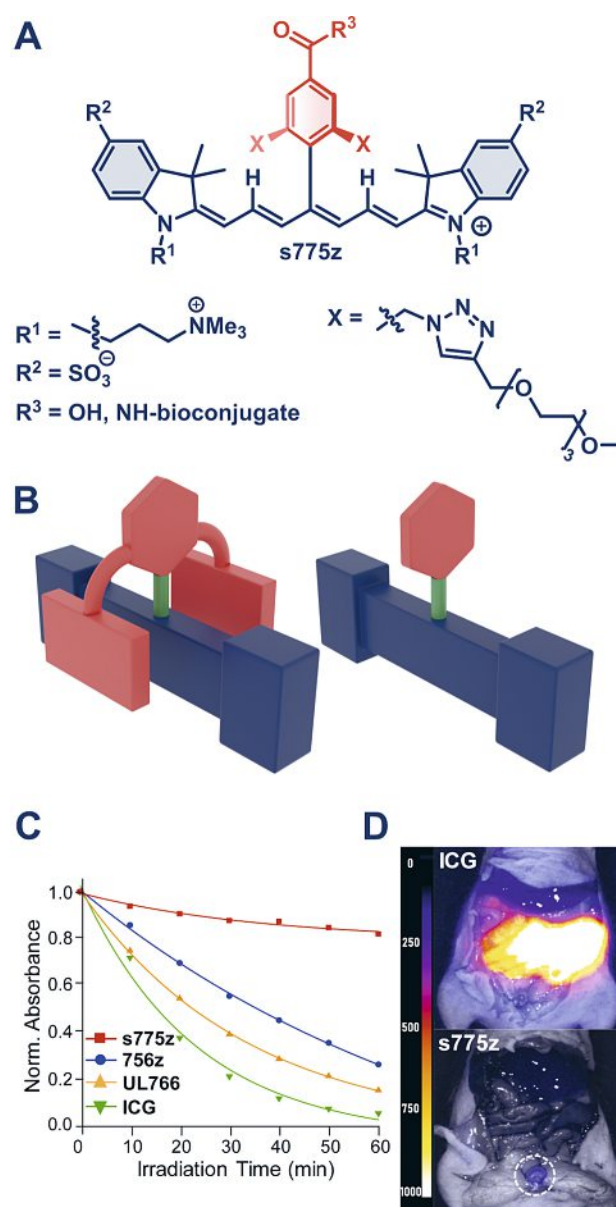

Fig. 3 (A) Structure of sterically shielded cyanine **s775z** and its derivatives. (B) Conceptual comparison of the sterically shielded heptamethine cyanine and the non-shielded analogue. (C) Comparison of the photostability of **s775z** with other cyanine derivatives. (D) Phantom mice injected with ICG and **s775z** irradiated with an *in vivo* imaging station.

logue of **s775z** from binding to cancer overexpressed cell-surface receptors, while simultaneously improving its cell uptake compared to the non-shielded analogue. In the *in vivo* imaging experiments, the shielded **s775z** (and its several peptide and antibody bioconjugates) was benchmarked against other Cy7 dyes and it displayed a remarkable combination of photophysical, physicochemical, and biodistribution properties that greatly enhance bioimaging performance.

Hypoxia Probes

Distinct differences between the level of oxygen in cancer tissue can be used to distinguish hypoxic and normoxic cancer tissue, which is an important clinical objective to surpass the challenges associated with cancer diagnosis and treatment.[46] Many nitro-containing molecules have been explored as '*turn on*' fluorescent probes *via* reduction by nitroreductases (NTRs).[47] While the field progressed with fluorescent probes across the visible region of the spectrum, hypoxia imaging in the NIR region had remained underdeveloped due to the lack of suitable probes. A recent work of Smith's group described a Cy7 dye bearing C4'-carboxylic ester group on the chain that is converted, *via* a self-immolative fragmentation mechanism, into C4'-caboxylate group (Fig. 4A).[48] This conversion is promoted by a biomarker NTR, an enzyme that catalyzes the reduction of nitro-substituted compounds using NADH or NADPH as the stoichiometric reducing agent, overexpressed in hypoxic cells. The probe took advantage of the substantial influence of C4'-ester group in **6** and C4'-carboxyl group in **7** on the photophysical properties of the cyanines – a ~40 nm blue shift in emission wavelength with concomitant ~20-fold increase of fluorescence intensity. These encouraging results convinced them to further optimize the motif for imaging of hypoxia in live models.

In the subsequent collaboration with us, Smith and co-workers explored the possibilities to red-shift both absorption and emission maxima of these probes.[49] In this quest, the first developed class of probes was a homologous pair inspired by ICG (Fig. 4B) which incorporated benzoindolines as the terminal heterocycles. Although this class of compounds exhibits a red-shift in both absorption and emission, strong self-aggregation in aqueous media was observed. This phenomenon decreased the NTR activity, prompting the design of a second class of derivatives with propargyloxy group located on the terminal indolenines. In this way, the self-aggregation was significantly reduced (especially in presence of ubiquitous serum albumin), while also retaining the desired red-shift of the absorption properties. Moreover, the second-generation probe was rapidly cleaved by NTR into the carboxylic cyanine dye, which exhibited enhanced photon output compared to the first-generation probes when excited at the optimal wavelength of 808 nm (isosbestic point of oxyhemoglobin and deoxyhemoglobin). Their performance was assessed also *in vitro* (Fig. 4C) and using phantom mice. Due to the ability of the NIR to penetrate deep into tissue, this work stimulates the use of cyanine dyes in NIR fluorescence imaging of hypoxia in living subjects, and potential clinical applications such as fluorescence guided surgery.

Cyanine Phototruncation

The advance in optical microscopy techniques has come as far as single-molecule localization microscopy (SMLM), which is a powerful imaging tool that considerably improves spatial resolution over standard, diffraction-limited microscopy techniques, providing images of biological structures at the molecular scale. In this field, rhodamines have played an important role, but sometimes artifacts that alter the optical properties of the probe molecule have been identified, obstructing their application in microscopy analysis.[50–52]

In the light of the recently reported phototruncation reaction which leads to a two-carbon shortening of the central chain (Fig.
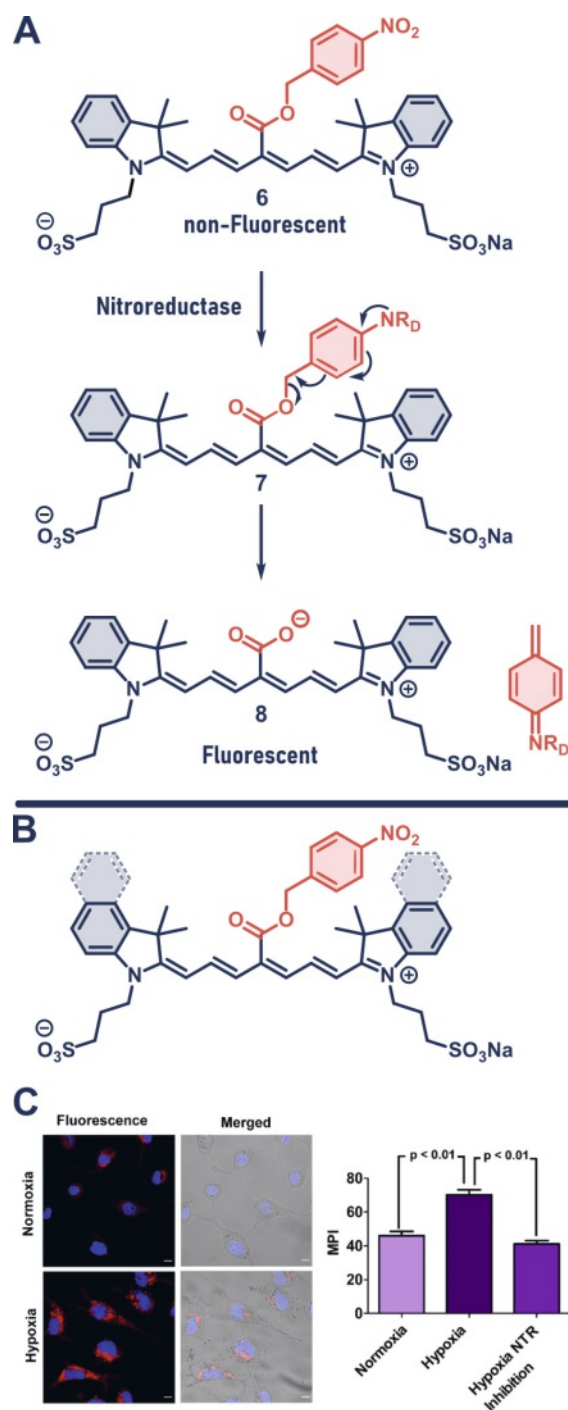

Fig. 4. (A) Mechanism of bioresponsive NIR fluorescent Cy7 probe based on self-immolative cleavage, resulting in a 'turn on' of the fluorescence. (B) Chemical structure of heptamethine cyanine hypoxia probes. (C) Microscopy study of A549 cells treated with the probe in normoxic or hypoxic conditions.

5A) and concomitant hypsochromic shift of absorption (Fig. 5B), cyanine dyes are emerging as the perfect candidates for SMLM. The hypsochromic photoconversion of cyanine was initially evaluated from the perspective of potential 'photoblueing' artifacts in multicolor experiments.[53,54] However, Schnermann and co-workers have followed up with a comprehensive mechanistic study in the quest to harness this phenomenon for practical purposes. In this study, an intramolecular rearrangement that leads to the phototruncation of cyanine (Fig. 5C), and a formal excision of a two carbon fragment from the central chain was identified, albeit with very low chemical yields (<2%).[55] They further ad-

dressed the dependency on reactive oxygen species and observed that hydrogen peroxide, superoxide, and the hydroxy radical, did not induce the phototransformation, whereas $^1O_2$ generated either from an external source or through photosensitization caused phototruncation. Evaluating a number of solvents, they concluded that the reaction is highly solvent dependent. The authors also evaluated the role of water in the photoreaction, observing that the reaction does not occur in non-aqueous media, regardless of presence of $^1O_2$. The phototruncation revealed high-pH dependency, thus they proposed that hydroxide is central to the intramolecular rearrangement of the photooxidized intermediate. After screening over 300 conditions, exploring a range of additives and buffers at various pH levels, they identified the most optimal neutral conditions (10 mM histidine, pH = 7.4) with a significant improvement in yield (4%). Subsequent microscopy experiments in microtubules demonstrated that phototruncation-SMLM with heptamethine cyanines can be carried out in cells, however, the imaging quality was limited by the poor photoconversion yield.

With the aim to overcome this limitation, Schnermann and co-workers set out to assess the effects of structure and substitution of the central chain on the chemical yield of phototruncation in their second work.[56] Screening a series of C3', C4', and C5'-substituted cyanines, they identified C3'-OMe-substituted cyanine as a promising derivative that led to 8-fold enhancement under physiological conditions. Taking advantage of this, the authors developed phototruncation-assisted cell tracking (PACT) and applied it to evaluate the migration of immune cells into tumor-draining lymphatics (TDLNs). Through intratumoral injection of a 3'-OMe-variant of the cell tracking DiR dye, the authors demonstrated that the system efficiently tracks immune cell migration into TDLNs. The effect of irradiation on 3'-OMe-DiR or DiR labeled cells was assessed by fluorescence imaging (Fig. 5D). Significantly higher fluorescence in the Cy5 channel was observed in cells stained with 3'-OMe-DiR compared to the parent unsubstituted derivative. A remarkable aspect of this work lies in the demonstration that the cyanine chain phototruncation can be performed *in vivo*, facilitating irradiation-dependent cell tracking experiments without genetic modification. It is worth mentioning that these results were enabled by the access to different chromophore substitution and its role on the chemical reactivity.

Cyanine-based Photocages

The development of photocages that operate in the near-infrared region of electromagnetic spectrum represents a major challenge in contemporary photochemistry, mainly due to the low energy of the excitation light and fast deactivation of the excited states *via* non-radiative pathways.[57] We recently reported, in parallel with the group of Feringa, the development of cyanine-based photocages that utilize this synthetic strategy and efficiently release carboxylic acids in aqueous media when irradiated with NIR light up to 820 nm.[21,22]

The design of these photocages drew heavily from the cyanine synthesis based on the Zincke chemistry. Using this approach, we were able to prepare photocages in three steps from cheap starting materials (Fig. 6A) and even upscale the protocol to a multigram scale. We encountered two major obstacles during the initial attempts to synthesize these photocages. First, we observed that the Zincke salts are deprotonated at the methylene bridge by the acetate used as a base followed by unidentified side reactions. Introduction of a methyl substituent at this position resolved this issue. Furthermore, the absence of aniline additive and use of ethanol as a less nucleophilic solvent proved to be critical to achieve good yields, providing evidence that the ring-opening of Zincke salts and the subsequent reaction cascade can proceed also in basic conditions without any auxiliary nucleophiles. We speculated that the use of aniline and methanol led to degradation pathways, such as transesterification and aminolysis of the ester group, that decreased the chemical yields. No need for an additional linker and the direct connection of the cargo directly to a central position of cyanine chromophore is a major advantage of these photocages. As a consequence, a significant reduction of the release process complexity was achieved in comparison with the previously published systems.[21] At this point, we must emphasize that the success of these photocages was directly contingent on the preceding development of synthetic methodology, because none of the classical approaches, *e.g.* transition metal-catalyzed chemistry, were successful in providing the target photocages.

One of the crucial experiments performed to shed light on the mechanism was irradiation of a cyanine **13a** in the presence and absence of oxygen followed by ^{19}F NMR spectroscopy (Fig. 6B). The photouncaging process involved in the release of cargo molecules was shown to be based on well-established photooxidative fragmentation of the cyanine chromophore.[21] The quantum efficiencies of uncaging were also found to be concentration dependent, further supporting the oxidative path, and in absolute numbers comparable to the first generation of BODIPY photocages, while operating at wavelengths red-shifted by 100 nm.[58]

In vitro experiments with the free carboxylate-bearing coumarin **14c** demonstrated that the uncaged acid was not readily uptaken by cells presumably due to its anionic nature at physi-
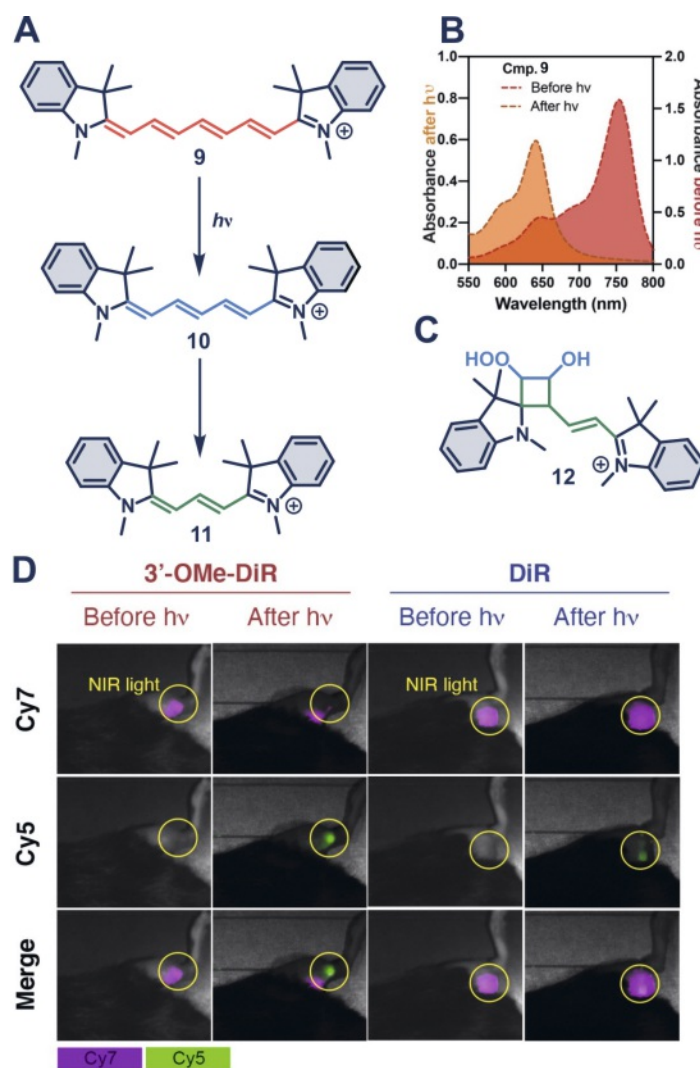

Fig. 5. (A) Cyanine phototruncation cascade converting Cy7 to Cy3. (B) Absorption spectra of 3'-OMe-Cy before and after irradiation at 730 nm. (C) Proposed intermediate of the phototruncation. (D) Fluorescence microscopy of MC38 tumors, after injection of 3'-OMe-DiR or control DiR. Cy5 and Cy7 fluorescence is shown in green and magenta, respectively.

ological pH. However, when incorporated within the cage **13c**, coumarin **14c** could be delivered and released inside live HeLa cells. Microscopy imaging of the cells treated with **13c** exhibited decrease of the fluorescence in red channel attributed to cyanine during NIR-light irradiation, accompanied by a concomitant increase of the fluorescence attributed to the uncaged **14c** in the blue channel (Fig. 6C). We consider this demonstration that the photocages can be used to modulate pharmacokinetic properties of drug-like molecules to be a rather intriguing hallmark of this study. This, in our opinion, overlooked and undervalued feature of photocages holds the potential to join high temporal and spatial control as their main benefits because it provides the option to revisit previously unsuccessful drugs or even design new molecules that are exempt from some of the common design rules.

Nevertheless, these new photocages work only in the presence of oxygen, so it remains our ultimate goal to transform them into single NIR photon-activated photoheterolytic systems. Until we reach this objective, they constitute a powerful tool that can seek potential application, for instance in the treatment of tumors[23] and traumatic brain injuries.[59]

Other Applications

As mentioned previously, ICG is a FDA-approved NIR fluorescent dye and widely used for various imaging and diagnostic procedures, for example, lymph nodes, or cancerous tissue before, during and after surgery; or visualizing blood flow in tissues during ophthalmic angiography.[60–63] Despite that, ICG utilization suffers from its rapid photodegradation in aqueous media which results in non-fluorescent products. Studies of its stability showed that only 20% of ICG remains intact after 24 h in aqueous solutions, whereas *in vivo* studies describe full loss of vasculature image when employing solutions older than 24 h.[64,65] The complex degradation pathways of ICG have been extensively investigated. The presence of the amphiphilic molecular structure promotes self-aggregation, with consequent changes in the photophysical properties of the dye. The combined action of light and molecular oxygen enables chemical degradation (Fig. 7) facilitated by the attack of singlet oxygen along the cyanine chain and production of non-emissive fragments **16** and **17**.[66,67] Recent studies unveil dimerization as a second degradation pathway to **18**, although not significant at the most used concentration (2.5 nm/mL).[68,69] The third, minor pathway that may occur is phototruncation of the Cy7 into Cy5, which can be also harnessed for additional benefits (*vide infra*).

Smith and co-workers explored the hypothesis of improving the ICG stability by the virtue of substituting certain hydrogen atoms for the heavier deuterium atoms, effectively taking advantage of the kinetic isotope effect.[70] The authors conducted a systematic study with ICG analogues deuterated along the central chain, d_5-ICG and d_7-ICG **15**, synthetized from a deuterated pyridine by the Zincke salt approach. d_5-ICG and d_7-ICG displayed improved aqueous photostability compared to the parent ICG. Additionally, they designed a set of experiments that simulate the storage conditions before the administration, exposure of the aqueous solution to ambient light and evaluated the stability over the time. They found that d_5-ICG in aqueous media is converted to the corresponding dimer slower than ICG by a factor of 3.1. Experiments on phantom mice with freshly prepared or stored solutions showed no difference in image intensities for the phantoms containing freshly prepared ICG or d_5-ICG. On the contrary, imaging experiments with stock solution of ICG that had been stored in the dark for 3 days displayed 80% of the image intensity of the heart region compared to that of d_5-ICG. The extended storage of aqueous deuterated ICG represents enhancement from the convenience standpoint for many biomedical imaging and diagnostic procedures, including fluorescence-guided surgeries
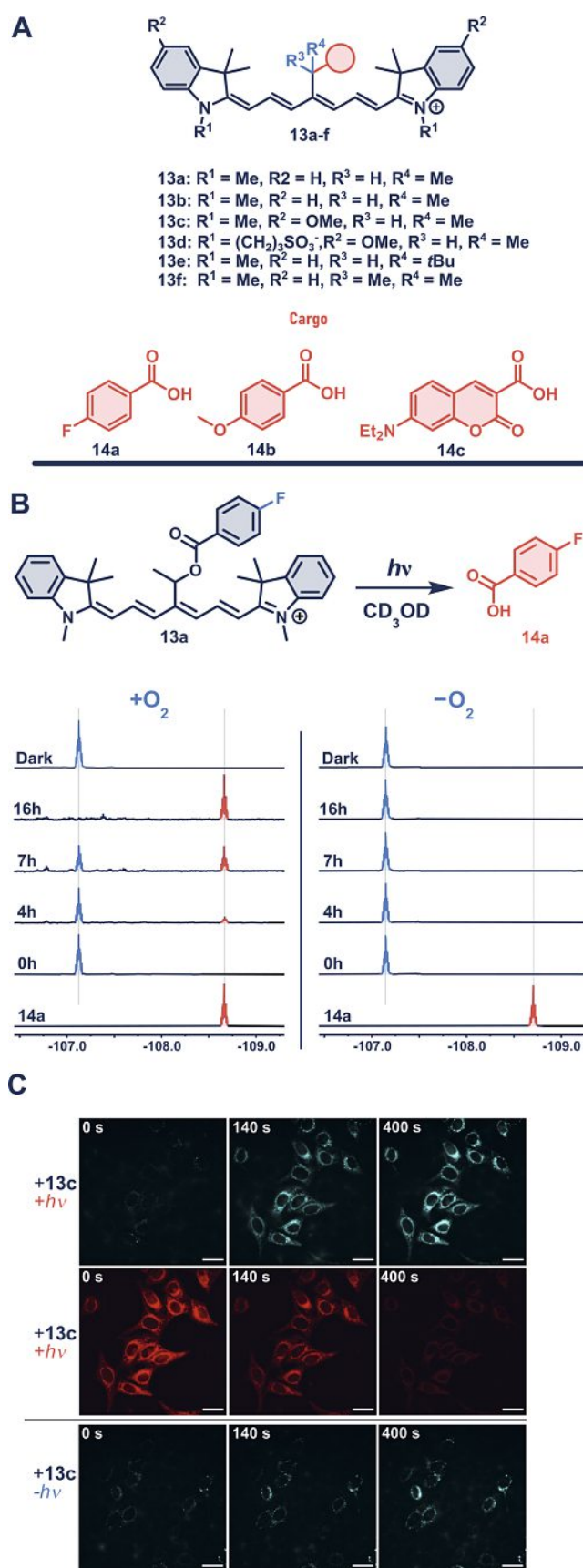

Fig. 6. (A) Chemical structure of the photocages. Cargo molecules **14a** in **13a,d–f**; **14b** in **13b** and **14c** in **13c**. (B) Release of 4-fluorobenzoic acid upon NIR irradiation followed by ¹⁹F NMR spectroscopy in the presence (left) and absence (right) of oxygen. (C) Fluorescence microscopy of HeLa cells treated with **13c** and irradiated with NIR light or kept in the dark; blue and red channel.
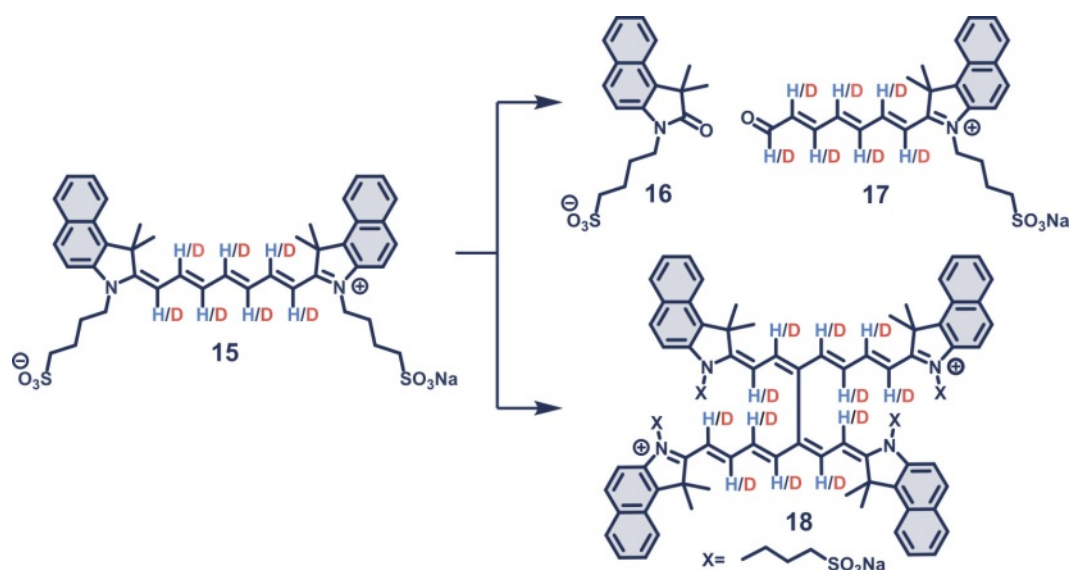
Fig. 7. Degradation pathways for aqueous ICG in the presence of air and light – oxidative alkene cleavage (top) and oxidative dimerization (bottom).

that require repeated doses from the same stock solution over the operation period.

Lanthanide-doped upconversion nanoparticles (UCNPs), capable of converting NIR excitation into visible and ultraviolet emission, have been successfully exploited in different fields, such as fluorescent microscopy, deep-tissue bioimaging, light-triggered drug delivery and solar energy harvesting.[71–73] The common low upconversion quantum yields can be improved by coating the external shell of the nanoparticles with organic dyes that operate as antennae.[74] The excitation light energy harvested by the dyes is then transferred *via* Förster resonance energy transfer (FRET) to the Ln^{3+} ions incorporated within the UCNPs. The commonly used Yb^{3+} sensitizers possess ~10 times lower optical absorption in comparison to organic dyes which, in addition, possess wider absorption bands and much higher molar absorption coefficients. As a result, the broad and customizable absorption properties of NIR cyanines dyes can overcome these limitations and enable flexible tuning of the excitation wavelengths of UCNPs. In collaboration with some of us, Turshatov and co-workers studied ultrasmall $NaGdF_4$:Yb^{3+},Er^{3+} UCNPs decorated with heptamethine cyanine dyes through non-covalent interaction of the carboxylate or sulfonate groups with the surface of the nanoparticle.[75] In order to provide further insights into the mechanism and its nuances, they explored six different Cy7s, with absorption between 740 nm and 792 nm. These cyanines contained functional groups to facilitate different non-covalent binding modes with UCNPs and substituents at the chain responsible for varying efficiencies of the intersystem crossing. The best performing dye displayed a substantial, up to 680-fold, enhancement of upconversion luminescence intensity. Experiments in the absence of oxygen, where the triplet excited state is not quenched, showed a significantly higher upconversion efficacy compared to oxygenated solution, confirming the role of triplet excited state in the mechanism of energy transfer from cyanines to Yb^{3+}. One of the major challenges to convert upconversion nanotechnology into real world applications remains to enhance the low emission efficiency of UCNPs and cyanine dyes. Thanks to the structural flexibility of Cy7 dyes provided by Zincke-based synthesis, this approach demonstrates a strong potential in this field.

Can the Renaissance Continue?

The use of the Zincke salt chemistry aided unleashing the potential of Cy7 dyes that was previously restricted by the available synthetic methodology. Several intriguing applications have spawned already in the three years since its inception. However, a number of shortcomings of this approach still remain to be addressed and harbor future investigations.

Unfortunately, the state-of-the-art still does not deliver the complete freedom to fully customize the cyanine scaffold at each individual site. The most glaring issue is the inability to modify the positions C1', C2', C6' and C7'. The modifications in the position C1' and C7' will presumably have to come from the terminal heterocycle intermediates or a posterior modification of the cyanine structure. Although the C2' and C6' positions might not be necessarily out of reach for Zincke chemistry, the main issue currently stems from the poor reactivity of 2'-substituted pyridines that prohibits the Zincke salts formation even at drastic conditions, and their unclear reactivity in the subsequent reaction cascade that leads to cyanine formation.

While the asymmetric cyanines bearing two different terminal heterocycles can usually be forgone in favor of simpler designs, such asymmetric scaffolds can be invaluable in certain applications. Their synthesis, however, is a long-standing issue in the field that often relies only on statistical distributions, and the purification is hindered by the presence of the symmetric byproducts with similar separation properties. More importantly, these protocols do not allow customization of the central chain. In this regard, we are convinced that Zincke salts hold strong potential due to the intrinsic asymmetry contained within intermediate **3a** formed upon the ring opening of **2**. Devising a way to harness this asymmetric information would unlock access to cyanines desymmetrized at the heterocycles containing also a derivatized central chain.

Other potential shortcomings include the use of basic conditions in combination with an auxiliary nucleophile and methanol as a protic nucleophilic solvent. While this is generally not an issue, it can represent a limitation for less stable substrates. We encountered this issue ourselves in the synthesis of the photocages. Although it has been eventually surpassed by omitting the nucleophile and change of the solvent, this solution might not be always sufficient, especially for less activated substrates. For obvious incompatibility reasons, the synthetic protocol is also limited to substituents that do not contain unprotected primary or secondary amines, and one can imagine that the electron-deficient Zincke salts might be also susceptible to reductive side reactions.

Witnessing the power that just three substitution positions (C3', C4', C5') hold over the applications of cyanines, we can only fantasize about the possibilities that would arise from the ability to control each of the seven positions, especially if allowed to freely

mix and match with terminal heterocycles. We are convinced that the aforementioned aspects warrant the development of innovative and even milder synthetic protocols, and they represent substantial challenges that are waiting to be conquered by synthetic chemists in the upcoming future.

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