

Development of photostable near-infrared cyanine dyes†

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With the emerging interest in optical *in vivo* imaging, there is an increasing demand of photostable near-infrared (NIR) dyes. Herein we report the rational design of an amine tricarbocyanine structure with improved photostability (CyNA) and its combinatorial derivatization to render CyNA-414 as a NIR-fluorescent dye with stronger emission intensity and higher photostability than the NIR standard IndoCyanine Green (ICG).

Imaging in the NIR region (700–1000 nm) enables deeper tissue penetration with lower auto-fluorescence background than the visible range, being particularly suitable for *in vivo* studies.^{1–3} The rising interest in small animal optical *in vivo* imaging has accentuated the need for novel NIR fluorescent dyes that exhibit good photostability properties.⁴ Amongst NIR dyes, the tricarbocyanine scaffold (**1**) is synthetically accessible and displays enhanced fluorescent properties over other cyanine structures.^{5–8} The tricarbocyanine scaffold can be derivatized with different nucleophiles,⁹ but the low stability of the resulting compounds has remained as a significant drawback.¹⁰ Herein we report a rational design of the CyNA structure for improving the photostability of the tricarbocyanine core. The CyNA scaffold was derivatized in a combinatorial manner to identify CyNA-414 as a highly photostable NIR dye with superior fluorescent properties than the NIR standard ICG.

While the amine tricarbocyanine structure (CyN) has been described as a promising NIR scaffold for broad chemical derivatization,¹¹ the low photostability in aqueous media has hampered its biological application. Although the decomposition mechanism of some cyanine dyes has been studied,¹² no systematic study regarding the photostability of tricarbocyanine analogues in aqueous media has been reported to date. In order to examine such mechanism and surmount the stability limitations of tricarbocyanine dyes, we analyzed the decomposition products of one amine tricarbocyanine derivative (CyN-111). Upon light irradiation, CyN-111 quickly underwent the incorporation of a reactive singlet oxygen species to render CyN-111a, which subsequently generated the non-fluorescent CyN-111b and CyN-111c (Fig. 1 and Fig. S1–S3, ESI†, for

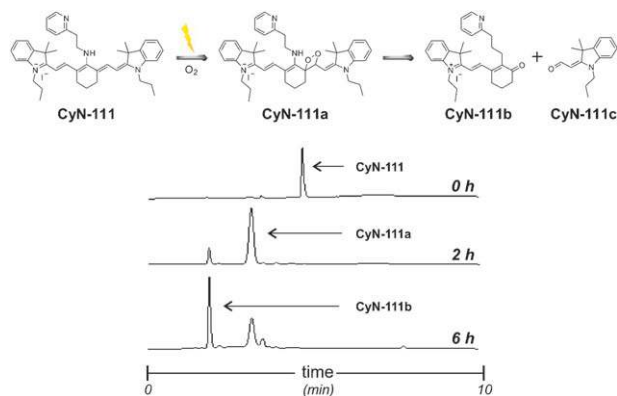
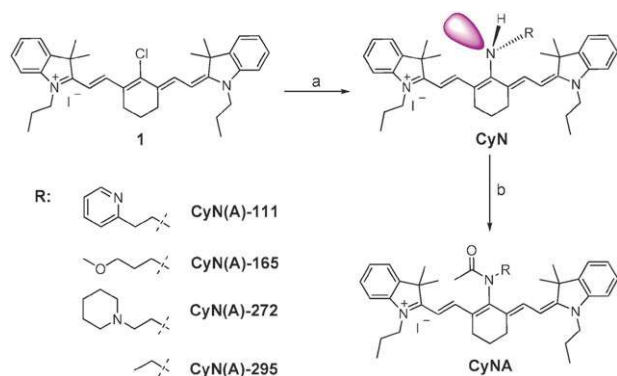


Fig. 1 Reverse-phase HPLC monitoring of CyN-111 decomposition in aqueous media (UV detection: 500 nm).

characterization). In view of these results, we hypothesized that the modification of the bridgehead nitrogen atom with an electron-withdrawing group would suppress the stabilization of the iminium intermediate that facilitates the incorporation of the reactive oxygen species. Hence, the removal of some electron density from the amine tricarbocyanine structure may minimize the photoinduced decomposition and improve its photostability.

To investigate this hypothesis, we modified 4 structurally diverse (including different-length aliphatic, aromatic, and heterocycle-containing amines) CyN compounds with an electron-withdrawing acetyl group to afford the corresponding acetylated compounds (CyNA) (Scheme 1).

The photostability properties of CyN and CyNA structures (CyN-111, CyN-165, CyN-272, CyN-295, and their CyNA counterparts) were evaluated by time-course fluorescence measurements in HEPES buffer, and compared to the starting material **1** (Fig. 2). Interestingly, the pseudo-first order rate



Scheme 1 Synthesis of amine tricarbocyanine derivatives. Reagents and conditions: (a) RNH₂, DIEA, CH₃CN, 80 °C, 10–60 min; (b) CH₃COCl, DIEA, CH₂Cl₂, 0 °C, 15 min.

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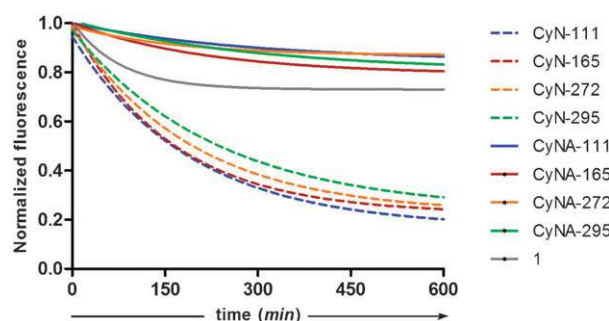


Fig. 2 Photostability evaluation of **1**, CyN and CyNA derivatives under a xenon flash lamp. Compounds were dissolved in HEPES buffer (10 mM, pH 7.4) containing 1% DMSO to a 10 μ M final concentration, and fluorescence measurements were recorded for 10 h at r.t. Values are represented as means for sequential measurements every 10 min and fitted to a non-linear regression, one-phase exponential decay.

constants (k) of the CyN compounds were 8 to 13-fold faster than those of the corresponding CyNA derivatives, indicating the significantly ameliorated photostability of the CyNA scaffold.

The improved photostability of the CyNA scaffold validated our initial hypothesis that the incorporation of an electron-withdrawing group could increase the photostability of the amine tricarbocyanine structure. Since the k values of the different CyNA compounds indicated some dependency on the amine chemical structure (Table 1), we expanded the derivatization of the CyNA scaffold to a broad range of 80 primary amines (Chart 1) so that we could identify the compounds with the best photostability properties. The fluorescence properties of the 80-member CyNA library were measured, with the absorption maximum wavelengths ranging from 802 nm to 806 nm, emission maximum wavelengths from 817 nm to 823 nm, and average quantum yields around 0.10 (Table S2, ESI \dagger). As a primary photostability evaluation, we analyzed the fluorescence decrease of the 80 CyNA compounds under a xenon lamp (for detailed data and procedures, see Table S2 \dagger). The average intensity decrease after 8 h due to decomposition was around 18%, and a subset with the most photostable compounds was examined under stronger irradiation in various buffer conditions. Among all CyNA compounds, CyNA-414 displayed the best photostability and fluorescence quantum yield (for detailed data and procedures, see Fig. S4 and S5 \dagger) and the lowest photobleaching rate ($2.5 \times 10^{-6} \text{ s}^{-1}$, Table S3 \dagger), hence it was selected for further examination.

Since CyNA-414 exhibited outstanding properties as an NIR dye, we compared it to the NIR standard ICG (Chart 2). ICG is the only NIR dye clinically approved to date,¹³ and has a very similar spectral profile (absorption–emission: 790–810 nm) to CyNA-414. However, ICG shows a low quantum yield and a poor stability in aqueous media that have hampered its use for many bioimaging applications.¹⁴

The comparative analysis of the fluorescence properties of CyNA-414 and ICG indicated that CyNA-414 emitted stronger NIR fluorescence intensity in aqueous media (Fig. S6, ESI \dagger) and exhibited a remarkably higher photostability than ICG

Table 1 Photobleaching rates of CyN and CyNA derivatives in aqueous media

| Compound | k/s^{-1} | $k_{\text{CyN}}/k_{\text{CyNA}}$ |
|----------|-----------------------|----------------------------------|
| CyN-111 | 40.4×10^{-6} | — |
| CyNA-111 | 3.41×10^{-6} | 12 |
| CyN-165 | 34.9×10^{-6} | — |
| CyNA-165 | 4.53×10^{-6} | 8 |
| CyN-272 | 35.2×10^{-6} | — |
| CyNA-272 | 2.67×10^{-6} | 13 |
| CyN-295 | 31.3×10^{-6} | — |
| CyNA-295 | 3.81×10^{-6} | 8 |

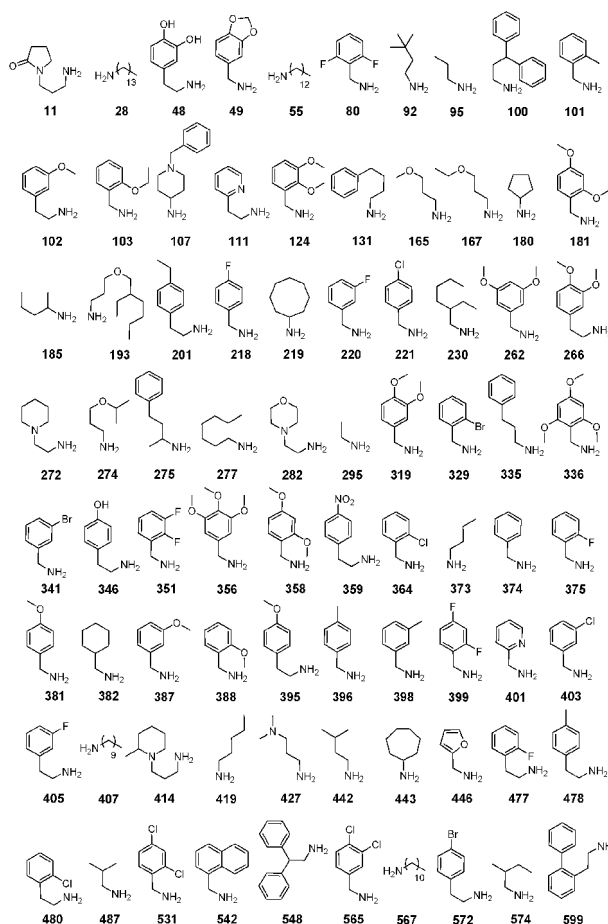


Chart 1 Amine building blocks of the CyNA library. \dagger

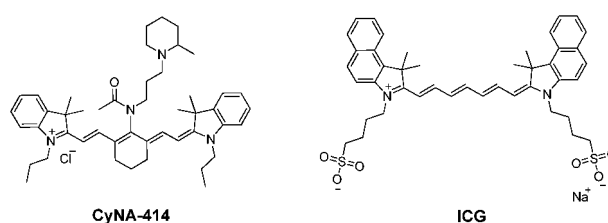


Chart 2 Chemical structures of CyNA-414 and ICG.

(Fig. 3). The superior photostability of the CyNA structure over the CyN scaffold was further corroborated by comparing the photobleaching profiles of CyNA-414 and CyN-414 (Fig. 3).

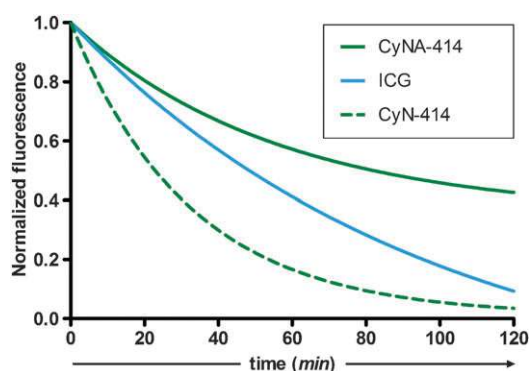


Fig. 3 Photostability analysis of CyNA-414, ICG and CyN-414 under a high-intensity ultraviolet lamp. Compounds were dissolved in PBS buffer (pH 7.3) containing 1% DMSO to a 10 μ M final concentration. Values are represented as means ($n = 6$) for sequential measurements every 10 min and fitted to a non-linear regression, one-phase exponential decay.

In summary, we designed a NIR fluorescent scaffold (CyNA) with excellent photostability properties. CyNA was designed after examining the decomposition mechanism of amine tricyanocyanine derivatives, whose poor photostability was largely improved upon incorporation of an electron-withdrawing acetyl group. The combinatorial derivatization of CyNA enabled the identification of CyNA-414 as a highly fluorescent NIR dye with significantly better photostability than the NIR standard ICG. CyNA-414 encloses a practical prospective for NIR *in vivo* imaging, and its biological application will be reported in due course.

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Notes and references

‡ Code numbers were randomly assigned in a non-consecutive order.

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