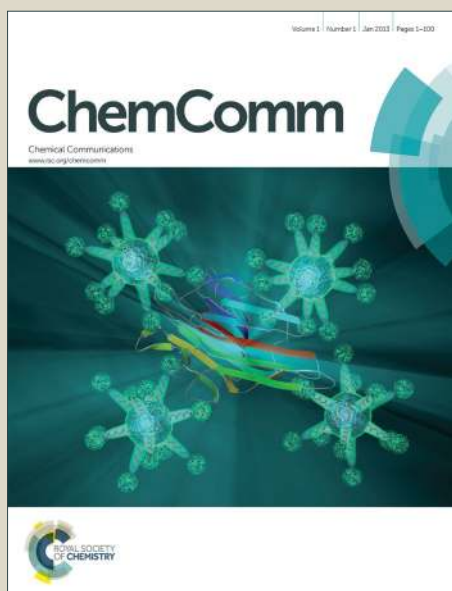


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## COMMUNICATION

# The Development of a Highly Photostable and Chemically Stable Zwitterionic Near-Infrared Dye for Imaging Applications

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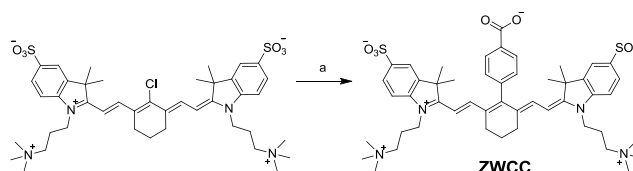
**A novel zwitterionic near-infrared (NIR) dye, ZWCC, has been designed and synthesized. It shows significantly enhanced photostability and chemical stability compared to the existing zwitterionic NIR dye. In addition, the feasibility of labeling ZWCC with biological ligands was investigated and used in live cell imaging applications.**

Near-infrared (NIR) fluorophores have attracted substantial attention in various chemical and biological studies due to their great advantages for *in vivo* imaging.<sup>1-3</sup> Firstly, NIR wavelengths ranging from 700 nm to 900 nm can penetrate into deeper tissues. Secondly, the low auto-fluorescence background of NIR fluorophores is essential for *in vivo* images. In view of rising interests in small animal optical *in vivo* imaging, there have been high demands for the design and synthesis of novel NIR fluorophore with better photophysical properties and chemical properties.

Till now, the famous and extensively used NIR fluorophore dye, indocyanine green (ICG), has good clinical availability and safety properties.<sup>3, 4</sup> However, its low fluorescence quantum yield, short fluorescence lifetime, poor photostability, bad chemical stability, as well as the lack of reaction sites to target ligands have hindered its application in chemical and life sciences.<sup>5</sup> One recently developed zwitterionic NIR fluorophore **ZW800-1** shows improved *in vitro* and *in vivo* performance.<sup>6, 7</sup> In brief, the NIR fluorescent small molecule **ZW800-1** can be rapidly cleared by kidneys, thereby exhibiting low background fluorescence due to less non-specific binding to normal tissues and organs. Moreover, **ZW800-1** contains one carboxylic acid group, which can be modified by functional groups for covalent conjugation to target ligands through a stable amide bond.<sup>8</sup> However, every coin has two sides, the additional carboxylic acid, which is the reactive site for targeting ligands, was introduced by replacing chloro of meso-chloro cyclohexenyl moiety with alkoxy group via ether linkage formation.<sup>9-12</sup> Consequently, this molecular assembly becomes chemically less stable. When there is a strong nucleophilic group present, the reactive site containing ether linkage in cyanine dyes may undergo nucleophilic substitution at the oxygen position via a S<sub>NR</sub>1 mechanistic pathway. Therefore, the possibility of using the **ZW800-1** dye in a harsh environment becomes limited.

Previously, Lee et al. reported the palladium-catalyzed C-C coupling reactions of chloro-substituted carbocyanines.<sup>13</sup> Inspired by

this approach, we designed and synthesized a novel zwitterionic near-infrared fluorophore which contains a C-C cross-coupling reaction of hydrophilic chloro-substituted heptamethine cyanines with arylboronic acids. The new NIR zwitterionic C-C bond formed cross-coupling product, which we named as **ZWCC**, is not only devoid of chemically vulnerable ether linkage, but also has improved photostable characteristics. Here, we present the design and systematic study of the spectroscopic properties of this new zwitterionic NIR fluorophore (Scheme 1). Its photostability and chemical stability, as well as its application in live cell imaging are evaluated.



Scheme 1. General synthetic scheme. Reagents and conditions: a: 4-boronobenzoic acid, Pd(PPh<sub>3</sub>)<sub>4</sub>, K<sub>2</sub>CO<sub>3</sub>, H<sub>2</sub>O, 100°C, microwave 1 h.

Spectra properties of the newly synthesized dye **ZWCC** were first tested in DMSO and compared with those of **ICG** and **ZW800-1** (Fig. 1 and Table 1). The absorption spectra of these three dyes show a characteristic band in the range of 780 -800 nm, while, the fluorescent spectra of the three compounds show some difference in emission wavelength and fluorescence intensity. Based on the same concentration, both **ZW800-1** and **ZWCC** show stronger fluorescence intensity compared to **ICG**. This is a good achievement since fluorescence intensity plays an important role in practical applications, especially in *in vivo* imaging, where higher fluorescence intensity can significantly lower the detection limit.

To further analyze and compare **ZWCC** with **ICG**, we characterized the absorption and emission spectra of these two dyes in different solvent system, such as DMSO, PBS, H<sub>2</sub>O and MeOH. The data was summarized in Fig. S1 and Table S1. The absorption spectra of **ICG** show significant absorption peak around 780 nm in all solvent systems and significant aggregation in H<sub>2</sub>O. For **ZWCC**, the absorption spectra are closely matched in PBS and H<sub>2</sub>O, with a clear new absorption peak at 680 nm. However, the absorption

## COMMUNICATION

spectrum in DMSO shows a single peak with significant bathochromic shift, as compared to that measured in PBS.

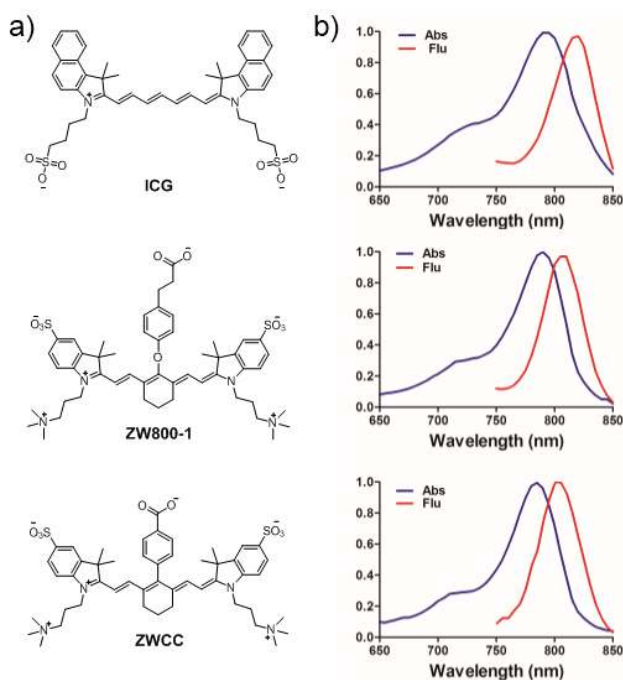


Fig. 1 (a) Chemical structures of **ICG**, **ZW800-1** and **ZWCC**; (b) Normalized absorbance and fluorescence spectra of each fluorophore (5  $\mu\text{M}$  in DMSO,  $\lambda_{\text{ex}} = 730 \text{ nm}$ ).

Table 1. Comparative spectral properties of **ICG**, **ZW800-1** and **ZWCC** in DMSO.

Compound	$\lambda_{\text{abs}}$ (nm)	Log $\epsilon_{\text{max}}$	$\lambda_{\text{em}}$ (nm)	$\Phi^{\text{a}}$	$\Delta\lambda^{\text{b}}$
<b>ICG</b>	793	4.22	816	0.13	23
<b>ZW800-1</b>	788	5.39	807	0.36	19
<b>ZWCC</b>	784	5.39	806	0.44	22

<sup>a</sup> Fluorescence quantum yields were determined using **ICG** ( $\Phi_f = 0.13$  in DMSO) as a standard.<sup>14</sup> <sup>b</sup> Stokes shifts of **ICG**, **ZW800-1** and **ZWCC**.

The aggregation tendency of **ZWCC** is evident from its absorption spectra taken in  $\text{H}_2\text{O}$  and PBS. From the area-normalized absorption spectra (equaling the concentration-weighted absorbances for different dye concentrations) shown in Fig. S2, we can see that **ZWCC** aggregates in  $\text{H}_2\text{O}$  even at concentration as low as  $0.5 \mu\text{M}$ .<sup>15</sup> And increasing the concentrations of **ZWCC** will lead to the typically blue shift in absorption, which indicate that **ZWCC** forms the commonly observed H-type aggregation as displayed by the vast majority of cyanine-type fluorescent dyes.<sup>15-18</sup> The formation of non-fluorescent H-type aggregation is supported by the excitation spectra in different solvent (Fig. S3).<sup>19, 20</sup> Even the absorption spectra of **ZWCC** in PBS,  $\text{H}_2\text{O}$  and MeOH show clear blue-shifted absorption bands, all the excitation spectra are similar to the excitation spectra in DMSO, in which **ZWCC** does not show aggregation signal.

Quantum yield ( $\Phi_f$ ) is also a key parameter to evaluate the practical application of dyes. Table S1 compared the quantum yield of **ZWCC** and **ICG**, where it shows that the quantum yields of **ZWCC** in relatively different solvents are much larger than the values of **ICG** due to the good solubility of **ZWCC** in aqueous phase, which will further benefit its biological applications.

A comparative analysis for the photostability of **ICG**, **ZW800-1** and **ZWCC** dyes was carried out by performing the time-course

fluorescence measurements in PBS buffer.<sup>21, 22</sup> First, fluorescence intensities of these dyes are examined under a strong UV lamp (UVP Blak-Ray1B-100AP high intensity mercury lamp, 100 W, 365 nm). By exposing these three compounds to a strong UV lamp and monitoring their fluorescence intensities, we observed that the average fluorescence intensity decrease for **ICG** and **ZW800-1** were around 85% and 30% due to decomposition, respectively, whereas the newly designed **ZWCC** did not show any observed decrease after 1 h irradiation (Fig. 2).

Based on the comparison, it can be concluded that **ZWCC** exhibited a remarkably higher photostability than **ICG** and **ZW800-1**. We hypothesize that the incorporation of a rigid cyclohexenyl ring in the polymethine chain increases the dye's photostability compared to cyanine dyes with an open polymethine chain. In addition, the robust C-C bond also plays an important role for enhancing the photostability of **ZWCC**.

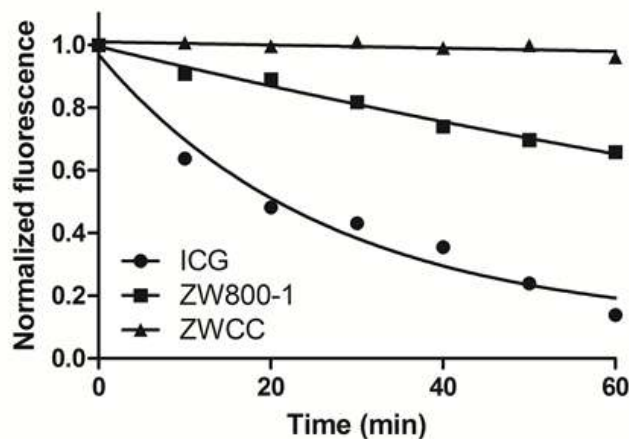


Fig. 2 Photostability evaluation of **ICG**, **ZW800-1** and **ZWCC** derivatives under strong UV irradiation. Compounds were dissolved in PBS buffer (pH 7.4) containing 1% DMSO to a  $10 \mu\text{M}$  final concentration, and fluorescence measurements were recorded for 1 h at rt. Values are represented as means for sequential measurement every 10 min and fitted to a non-linear regression, one-phase exponential decay.

Besides the photostability of NIR dyes, chemical stability is also an important factor when considering further chemical modification with functional groups. First, we tested the stability of **ZWCC** under certain biological environments, like pH5 and pH10, and the results showed that **ZWCC** is stable even after 20 h incubation (Fig. S4). Both **ZW800-1** and **ZWCC** provide one carboxylic acid for further modification. We evaluated the chemical stability of **ZW800-1** and **ZWCC** by examining the nucleophilic reaction between the dyes and primary amine (Fig. S5). The HPLC-MS results show that under the same reaction condition, amide bond formation product was clearly certified for **ZWCC** with high conversion yield. On the contrary, for **ZW800-1**, only byproduct (central oxygen position replaced with amine) formation was observed (Fig. S6). These results demonstrate that the robust C-C bond provides an easier approach for modification at the single carboxylic acid linker position, leading to the superior chemical stability of **ZWCC** even under harsh environment.

Signal-to-noise (S/N) ratio is a significant criterion for bioimaging. To evaluate the S/N ratio of **ZWCC**, **ZWCC** was injected to BALB/c nude mouse and the animal was imaged. It is clear from the images that **ZWCC** was distributed to organs including liver, pancreas, kidneys, spleen, intestine and fat pad. Subsequently, the signal quickly disappeared from the liver, pancreas, spleen, intestine and fat pad. Within 1 h, fluorescence signal has mostly cleared from the whole body and only very weak

signal remained to be observed in the kidneys and bladder, which indicated the low background of **ZWCC** (Fig. S7).

To further demonstrate the feasibility of labeling biological ligands, we conjugated the new zwitterionic NIR fluorophore **ZWCC** to a cyclic peptide consisting of cyclo (RGDyK) (cRGD), which shows specifically binding to integrin  $\alpha\text{v}\beta\text{3}$ . Integrins are expressed on a wide variety of cells to mediate cell-extracellular matrix interactions and the integrin receptor,  $\alpha\text{v}\beta\text{3}$ , in particular is an angiogenic marker expressed in most regions of tumors.<sup>23</sup> Several potent small molecules containing the arginine-glycine-aspartate (RGD) sequence have been found to block integrin function and inhibit tumor angiogenesis.<sup>24</sup> These promising results have suggested that integrin receptors are important targets for drug delivery and therapy, as well as molecular imaging.

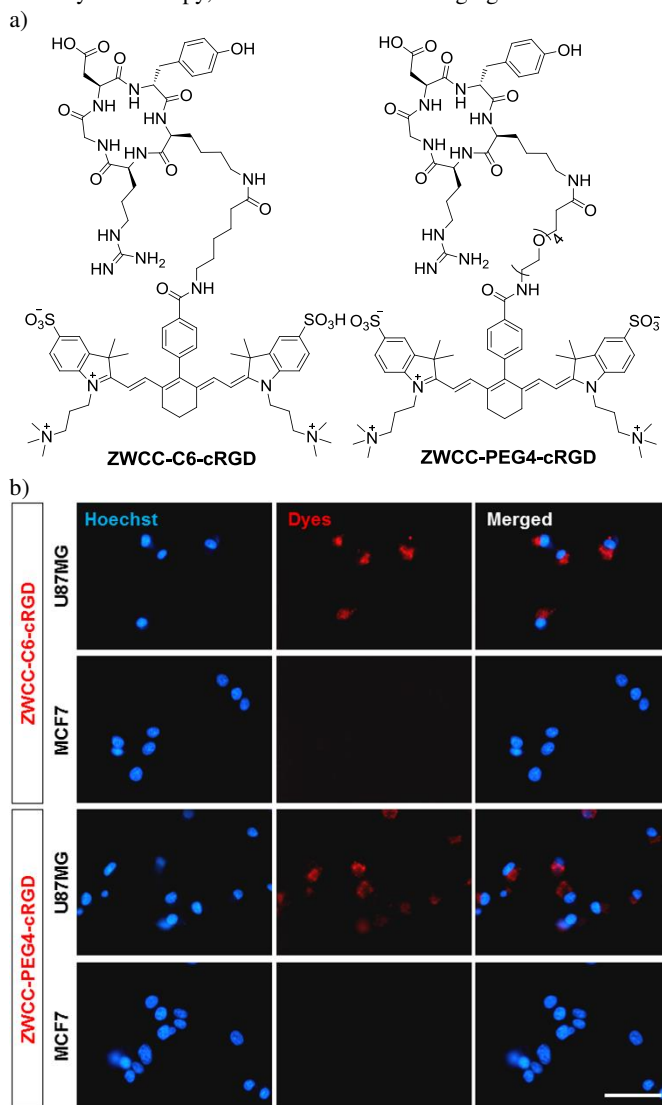


Fig. 3 Structures (a) and fluorescence cell imaging of **ZWCC**-linker-cRGD (b). Red colour is from **ZWCC** compounds (middle panels) and blue colour is from Hoechst stain (left panels) for nuclei visualization. Right panels show merged images of the two. Images were captured by 40x objective. Scale bar is 50  $\mu\text{m}$ .

We synthesized two cRGD-conjugated **ZWCC** compounds as possible probes for monitoring tumor angiogenesis. We evaluated integrin-targeting and cellular uptake of cRGD-conjugated **ZWCC** compounds by fluorescence imaging analysis using  $\alpha\text{v}\beta\text{3}$  integrin-positive U87MG cells and  $\alpha\text{v}\beta\text{3}$  integrin-negative MCF7 cells. As shown in Fig. 3, both **ZWCC-C6-cRGD** and **ZWCC-PEG4-cRGD**

were internalized by U87MG cells during the incubation period. As a negative control, incubating all three integrin-targeted **ZWCC** compounds did not yield any signals in  $\alpha\text{v}\beta\text{3}$  integrin-negative MCF7 cells. Similarly, no signal was observed when cells were treated with **ZWCC** alone (data not shown). In addition, the fluorescent signal from **ZWCC**-linker-cRGD could be effectively inhibited when U87MG cells were pre-treated with free cRGD peptide, indicating the specificity of **ZWCC**-linker-cRGD binding to integrin  $\alpha\text{v}\beta\text{3}$  (Fig. S8).

In summary, we have developed a novel zwitterionic NIR dye, **ZWCC**, which shows significantly enhanced photostability as well as chemically stable properties. Also, based on their functional carboxylic acid group, we designed and constructed two cRGD-modified NIR probes which demonstrated specific integrin-targeting in live cells. Taken together, we anticipate that the newly developed zwitterionic NIR dye, **ZWCC**, will offer a better and improved option in the field of NIR imaging.

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## Notes and references

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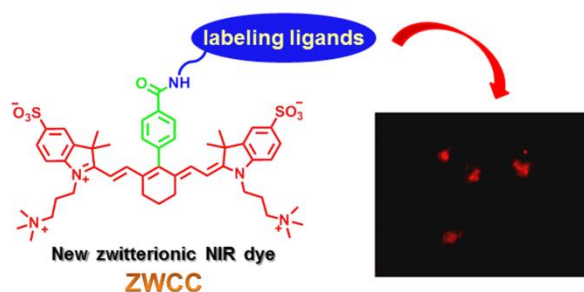
† Electronic Supplementary Information (ESI) available: Experimental details, spectra, cell image and HPLC-MS. See DOI: 10.1039/c000000x/

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## TOC



**ZWCC**, a new zwitterionic NIR dye with high photostability and enhanced chemical stability, can be easily functionalized for biological applications.