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ZW800-PEG: A Renal Clearable Zwitterionic Near-Infrared Fluorophore for Potential Clinical Translation

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Abstract

Near-infrared (NIR) fluorescence imaging has advanced medical imaging and image-guided interventions during the past three decades. Despite tremendous advances in imaging devices, surprisingly only a few dyes are currently available in the clinic. Previous fluorophores, ZW800-1A and ZW800-1C, significantly improved the poor performance of the FDA-approved indocyanine green. However, ZW800-1A is not stable in serum and ZW800-1C induces severe stacking in aqueous media. To solve such dilemmas, ZW800-PEG was designed by introducing

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Conflict of interest

The authors declare no conflict of interest.

a flexible yet stable thiol PEG linker. ZW800-PEG shows high solubility in both aqueous and organic solvents, thus improving renal clearance with minimal binding to serum proteins during systemic circulation. The sulfide group on the meso position of the heptamethine core improves serum stability and physicochemical properties including the maximum emission wavelength shift to 800 nm, enabling the use of ZW800-PEG for image-guided interventions and augmenting photothermal therapy.

Keywords

minimal toxicity; photothermal therapy; renal clearance; serum stability; zwitterionic fluorophores

Near-infrared (NIR) light has great potential in image-guided interventions with distinct advantages including deep penetration, low absorption, and reduced scattering in tissue, thus providing a superior signal to background ratio (SBR).^[1–7] Currently, indocyanine green (ICG) is the only US FDA-approved heptamethine NIR fluorophore and is used in various image-guided interventions in the clinic.^[8–10] However, ICG binds heavily to serum proteins due to its high $\log D$ (7.88 at pH 7.4) and unbalanced surface charges, resulting in a short blood half-life and high liver uptake, as well as lacking the proper functional groups for further conjugation with targeting molecules.^[11] We recently reported two serum-free zwitterionic NIR fluorophores, ZW800-1A^[8, 12] and ZW800-1C,^[13, 14] by introducing sulfonate groups and quaternary ammonium cations (quats) on both indole rings, yielding $\log D$ values of -3.35 and -2.80 , respectively. The surface charge of these hydrophilic fluorophores is designed to be balanced geometrically, resulting in minimal background tissue uptake and rapid renal clearance with low plasma protein binding.^[8, 15, 16] Additionally, the introduction of a carboxylate moiety on the mesocarbon linker enables further conjugation with targeting ligands including small molecules,^[17–19] peptides,^[20] proteins,^[21, 22] and nanoparticles.^[23, 24]

Despite these advantages, challenges still remain for the clinical use of zwitterionic fluorophores. The ether bond on the meso carbon of ZW800-1A's heptamethine core results in instability in serum during systemic circulation, an increased risk of being attacked by amine-bearing molecules during conjugation, or being hydrolyzed in basic conditions.^[13] On the other hand, ZW800-1C containing a central carbon-carbon (C–C) bond instead of the vulnerable ether linkage shows robust serum stability, but the rigid C–C bond induces π – π stacking in aqueous solvents, resulting in a strong dimer peak (*H*-aggregation) with an impaired extinction coefficient.^[10, 13, 25] In this study, we report an innovative zwitterionic NIR fluorophore ZW800-PEG by substituting the mesocarbon of the heptamethine skeleton with a flexible and stable thiol PEG linker to improve serum stability and optical properties.

As depicted in Figure 1, ZW800-PEG is synthesized by the conjugation of the chloro-substituted ZW800 (Compound **9**) with 3-(2-(2-mercaptoethoxy)ethoxy)propanoic acid (thiol PEG). This substitution requires milder and greener reaction conditions (60 °C, 40 min) compared with those of ZW800-1A (65 °C, 6 h)^[8] or ZW800-1C (105 °C, 48 h)^[13] (Supporting Information, Figure S1). All three zwitterionic fluorophores possess two sulfonates and two quats on both sides of the indole rings which maintain a balanced surface

charge. The purity of ZW800-PEG after solvent purification was found to be greater than 90.5% on the UV channel (254 nm) and 95.2% on the NIR channel (750 nm) without further purification by column chromatography. To produce an ultrapure compound, we revisited the synthetic procedures of the ZW800 heptamethine core and optimized each intermediate step as detailed in Supplementary Methods. Briefly, to minimize the amount of methylated side products which carry forward into subsequent steps, the reaction time for compound **5** was reduced from 48 h to 24 h at 110°C under a nitrogen atmosphere. This mild condition resulted in the major impurity, methylated compound **5**, to account for < 1.0% of the chromatogram's area when monitored at 254 nm (Supporting Information, Figure S2). Next, the precipitation procedure of compound **8**, comprised of a mixture of two forms (dianiline, 323.13 Da and aldehyde, 247.08 Da) was improved by using water instead of *n*-hexane (Class 2) and methyl tertiary butyl ether (Class 3). This new method is more concise and environmentally friendly, and the overall purity was improved from 81.9% to 91.8% (Supporting Information, Figure S3). In the following step, to improve the purity of compound **9**, the molar ratio of compound **5** to compound **8** was adjusted to be slightly higher than 2:1. Compound **9** (yield: 88.0%; purity: 93.2%) was obtained by thoroughly washing and precipitating the crude product using solvents, which controlled the amount of the methylated side product (MW: 727.25) to be < 1.2% (Supporting Information, Figure S4). After substitution of a thiol-PEG linker (**10**) on the meso-chloride, the final product, compound **11**, was obtained with 85.6% yield and 90.50% purity with solvent precipitation and washing. To prepare clinically available samples, however, we further applied preparative chromatography and produced ZW800-PEG with > 95.0% purity at 254 nm UV channel (Supporting Information, Figure S5). The chemical structure of ZW800-PEG was further confirmed by NMR (Supporting Information, Figure S6) and LC-MS (Supporting Information, Figure S7).

The 2D and 3D fine-built and energy-minimized structures of ZW fluorophores were calculated by MarvinSketch (ChemAxon, Budapest, Hungary) and compared in Figure 1. The flexible ether linkage in ZW800-1A induces the twisting of the sulfonates and quats while folding the linker forward and downward, while ZW800-1C displays a relatively rigid confirmation of heptamethine backbone with the linker facing upward. Interestingly, the flexible yet stable thiol PEG linker makes ZW800-PEG have a twisted backbone with the thiol-PEG linker straight upward.

Physicochemical properties of ZW800-PEG were calculated by JChem (ChemAxon) and compared with ZW800-1A and ZW800-1C in Table 1. The hydrophobicity ($\log D$) and polarity (topological polar surface area; TPSA) of fluorophores play important roles in in vivo performance. Apart from the common characteristics of surface charges and similar molecular weights, ZW800-PEG gives the lowest theoretical $\log D$ value of -4.70 at pH 7.4 that may be attributed to the hydrophilic characteristic of the thiol-PEG linker, while ZW800-1C provides the highest theoretical $\log D$ value (-2.80 at pH 7.4) due to the hydrophobic C–C bond. ZW800-PEG also offers the highest theoretical TPSA value ($179.24 \text{ \AA}^2$), suggesting a highly polar surface and reduced cell permeability. Moreover, ZW800-PEG contains 23 rotatable bonds implying high flexibility compared with ZW800-1A and ZW800-1C, of which the numbers of rotatable bonds are 18 and 17, respectively.

As shown in Table 2, the C–C bond in ZW800-1C resulted in poor solubility in both aqueous and organic solvents.^[13] Owing to the thiol PEG linker restructured on the meso-carbon, ZW800-PEG improved its solubility in most solvents tested including water, phosphate-buffered saline (PBS), fetal bovine saline (FBS), dimethyl sulfoxide (DMSO), methanol (MeOH), and ethanol (EtOH), which increased its usage in various reaction conditions (Supporting Information, Table S1). The substitution of linkers also affected the optophysical properties of zwitterionic fluorophores. Although all three ZW compounds show similar optical properties, ZW800-PEG shows the most dramatic red shifted absorbance and fluorescence emission in all three solvents compared with ZW800-1A and ZW800-1C (Figure 2a). Such red shift in fluorescence emission > 800 nm leads to a better coverage of ZW800-PEG in the conventional NIR fluorescence imaging instruments used in the OR, of which optics are mostly optimized to the optical property of the only FDA-approved NIR fluorophore ICG.^[1] All other optical properties of zwitterionic fluorophores and ICG in various solvents are summarized in Table 2.

Despite the superior optical properties and water solubility, ZW800-1A suffers from instability in serum due to the electron transfer-mediated $S_{RN}1$ reaction with nucleophilic phenoxypropionic acid.^[8] By connecting a phenyl linker directly to the heptamethine core through the palladium-catalyzed Suzuki reaction, ZW800-1C improved its overall chemical stability due to the reduced electrophilicity of the polymethine bridge.^[13] On the other hand, the thiol linker in ZW800-PEG lowers the electron deficiency of the heptamethine bridge compared with the ether linkage in ZW800-1A, thus improving its chemical stability in serum-containing media.

As shown in Figure 2b, ZW800-PEG exhibited 93.78% of serum stability at 24 h post-incubation in 10% FBS supplemented with 50 mM HEPES buffer, pH 7.4 at 37°C. We further tested the plasma protein binding (PPB) of ZW800-PEG because protein binding significantly effects not only the pharmacokinetic profile, involving the longevity of retention time and clearance patterns, but also the bioavailability for targeting tissues.^[26] The innovative skeleton commonly exploited by ZW fluorophores, sulfonate groups introduced to position 5 on both indole groups and alkylated quats, significantly lowers the PPB down to 20%, while ICG shows > 90% of PPB (Figure 2c). Notably, ZW800-PEG exhibits a low PPB around 9% due to the flexible PEG linker at the meso position, which is comparable to ZW800-1A showing the lowest PPB (ca. 5%). Furthermore, ZW800-PEG is stable in a broad range of pH, including at a strong acidic condition (e.g., pH 1.0) as well as at a slightly basic condition (pH 9.0), allowing further amide chemistry reactions with various targeting ligands. Next, the optostability of ZW800-PEG was measured in terms of concentrations under the excitation by a laser diode and a reflectance imaging system in PBS and FBS (Figure 2e). Quenching started at 5 μ M of ZW800-PEG in both solvents after irradiation of a laser diode at 770 nm excitation with a 90° geometry between the light excitation and detection. Under the 0° geometry intraoperative imaging system (FLARE), the working concentration shifted to a range of 20 to 30 μ M, a pattern which is similar to our previous findings of ZW800-1A^[8] and ZW800-1C.^[13] Moreover, the tendency of fluorescence emission in FBS is close to that in PBS, suggesting limited impacts of serum proteins on the quenching pattern of ZW800-PEG and its consequent stability during in vivo imaging. We further measured the fluorescence tail emission of ZW fluorophores in

the NIR-II window (Ex 808 nm, Em 1070 LP). Interestingly, all three ZW fluorophores presented decent NIR-II signals in various solvents (Supporting Information, Figure S8). Among the tested, ZW800-PEG outperforms, especially in aqueous solvents, representing potential clinical applications for non-invasive tissue imaging.^[27–29]

To find out the effect of different linkers on the electron geometry of ZW fluorophores, we used density functional theory (DFT) calculations by Gaussian16 software (Wallingford, CT). As shown in Figure 3, ZW800-1A, ZW800-1C, and ZW800-PEG were tested at the optimally tuned B3LYP/6-31G (d) level.^[28, 29] The thiol linker in ZW800-PEG contributes to the HOMO and LUMO orbitals, localized mainly on the heptamethine core, thus its HOMO-LUMO energy levels are lower than that of ZW800-1A and ZW800-1C. Consequently, ZW800-PEG absorbs light around 778–806 nm in the tested solvents similar to ICG, and fluorescence emission was between 807–834 nm (Table 2). While the calculation results of ZW800-PEG and ZW800-1A agree with the experimental results, ZW800-1C shows a medium level energy gap according to the calculation but displays the shortest maximum absorbance wavelength experimentally. Such a difference might be attributed to the *H*-aggregation (π – π stacking) phenomenon of ZW800-1C that moves the absorbance spectrum towards shorter wavelengths.^[10]

Owing to the decent fluorescence quantum yield of ZW800-PEG, we hypothesized that the incident photons are absorbed and converted into heat energy,^[30] thus potentially making ZW800-PEG a promising photothermal therapy (PTT) agent.^[30, 31] In vitro temperature changes were measured on test tubes containing different fluorophores upon laser irradiation at 1 Wcm^{–2}.

Figure 4a illustrates concentration-dependent temperature changes of NIR fluorophores in 500 μ L PBS. PBS was used as control. While all the tested fluorophores displayed elevated temperatures proportional to the concentration, ZW800-PEG reveals the most significant temperature change upon 808 nm laser irradiation for 1 min. This indicates strong evidence of heat release during laser irradiation. Then, we fixed the concentration of fluorophores to 25 μ M in PBS and irradiated the 808 nm laser for 5 min to see the effect of irradiation time (Figure 4b). Interestingly, ZW800-PEG exhibited the steepest temperature increase among all the tested NIR fluorophores within the first 2 min, whereas the others share a similar pattern of slow but sustainable temperature change over 5 min of irradiation. Extension of the laser irradiation for more than 2 min can cause gradual decomposition of ZW800-PEG, which may be due to the generation of reactive singlet oxygen species, followed by degradation of the polymethine bridge.^[32] This finding also suggests the exothermal ability of ZW800-PEG under laser irradiation.

Then, we revealed the photocytotoxicity of NIR fluorophores in human lung cancer H-23 cells by exposing them to an 808 nm laser at 160 mWcm^{–2} for 3 min (Figure 4c). According to the results of the one-way analysis of variance (ANOVA) test, there is no statistical significance between the control and the laser irradiation groups, implying limited cytotoxicity induced by only laser exposure. The ZW800-PEG incubated group interestingly displays a significantly lower cell viability upon the laser treatment compared with the rest of fluorophore-treated groups (** $p < 0.01$). To address the concern of possible safety issues,

cytotoxicity was evaluated on another human cell line, prior to animal study. 25 μM of each fluorophore was incubated in H-1793 with CCK-8 solution (Dojindo, CK04) for 4 h, and cell viability was measured using a plate reader. Notably, ZW800-PEG exhibited about 100% cell viability similar to the other ZW fluorophores (Figure 4d; $p = \text{n.s.}$) over the concentration range up to 100 μM (Figure 4e; $p = \text{n.s.}$). Owing to the zwitterionic properties, interestingly, all three ZW fluorophores show neither association with the cellular membrane nor cellular uptake at the normal cell binding assay condition (2 μM , 1 h). However, they started to show some degree of cellular uptake at a 25 μM concentration and after 24 h incubation (Supporting Information, Figure S9).

Finally, to evaluate *in vivo* biodistribution and clearance, 25 nmol of ZW800-PEG was injected intravenously into CD-1 mice and imaged under the FLARE imaging system at 4 h post-injection (Figure 5). The biodistribution pattern of ZW800-PEG in the thoracic cavity, abdominal cavity, and resected organs was very similar to those of ZW800-1A^[8] with rapid renal clearance and thus low background tissue uptake (Figure 5a). In comparison, ZW800-1C shows slight uptake in the gallbladder (arrow), lungs (Lu), and intestine (In), due to its hydrophobic character brought by its C–C bond linker ($*p < 0.05$; Figure 5b).^[13] Blood samples were taken over the period of intraoperative imaging to determine *in vivo* distribution and the clearance rate of ZW800-PEG in mice. Similar to the biodistribution test, 50 nmol of ZW800-PEG was injected intravenously into CD-1 mice and blood samples were collected at the designated time points up to 4 h post-injection. As depicted in Figure 5c and d, ZW800-PEG was cleared rapidly from the bloodstream to the kidneys and bladder compared to ZW800-1A and ZW800-1C. ZW800-PEG also has the shortest half-life values as well as the smallest area under curve (AUC), the largest volume of distribution, and the fastest clearance rate, resulting in higher urinary excretion ($85 \pm 1.9\% \text{ID}$) than the others.

In summary, renal clearable and serum binding free ZW800-PEG was designed and synthesized using a thiol-PEG linker substituted on the meso-carbon of the heptamethine core. While preserving the most significant advantages of our previously reported zwitterionic NIR fluorophores, ZW800-1A and ZW800-1C, ZW800-PEG compensates for the shortcomings of both. ZW800-PEG yields red-shifted wavelengths of maximum absorbance and emitted fluorescence comparable to that of ICG; making it one of the optophysically stable fluorophores with strong promise for clinical translation. ZW800-PEG significantly improved chemical, physical, and optical stability in physiological conditions, removing the obstacle in biological applications of ZW fluorophores. Compared with ZW800-1C, ZW800-PEG eludes the aggregation phenomenon in aqueous media, has improved solubility in both organic and aqueous solvents, and minimizes serum binding effects and nonspecific uptake in the lungs and intestines. Moreover, ZW800-PEG demonstrates its potential in photothermal therapy *in vitro*. Ongoing studies are focused on using ZW800-PEG as a potential PTT agent in animals by exploiting its enhanced serum stability and optimal optical properties as well as its ability to conjugate with targeting ligands including small molecules, peptides, proteins, and nanoparticles for various biomedical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Synthesis of ZW800-1A, ZW800-1C, and ZW800-PEG from Compound 9. Red arrows indicate the substitution of meso-chloride with oxygen (ZW800-1A), carbon (ZW800-1C), or sulfur (ZW800-PEG) atom. *3D structures were generated by JChem Plugin (ChemAxon).

Figure 2.

Optophysical properties of zwitterionic fluorophores. a) Absorbance (Abs) and fluorescence (Fl) spectra of ZW800-1A, ZW800-1C, and ZW800-PEG were measured in 10% FBS solution. Dotted square indicates the fluorescence emission range of ICG, which is currently employed in clinics. b) Physiological stability of each fluorophore in 10% FBS supplemented with 50 mM HEPES, pH 7.4 at 37°C over 24 h. c) Plasma protein binding assay of zwitterionic fluorophores compared with ICG incubated in whole mouse serum for 4 h. d) pH stability of ZW800-PEG in various buffer solutions. e) Photostability of ZW800-PEG in buffers. Quenching patterns of ZW800-PEG in terms of concentrations measured in the 90° geometry using a 770 nm NIR laser diode light source (quenching by laser) or in the 0° geometry using the FLARE imaging system after incubating fluorophores in PBS and 10% FBS. Photobleaching curves were obtained by incubating different concentrations of ZW800-PEG in 200 μ L of 5% BSA for 3 h. Data are expressed as mean $\pm$ S.D. ($n = 3$).

Figure 3. Illustration of HOMOs and LUMOs energy levels of ZW800-1A, ZW800-1C, and ZW800-PEG based on density functional theory calculations. The HOMO and LUMO energy levels were plotted based on the optimized S0 and S1 geometries using Gaussian 16 at B3-LYP/6-31G(d).

Figure 4.

Photothermal and cytotoxic effects of zwitterionic fluorophores. ICG was used as the control for PTT non-cell assay. a) Photothermal effect of each fluorophore in 500 μL tubing exposed to an 808 nm laser at 1.0 Wcm^{-2} for 1 min. b) Temperature change over the laser irradiation time of each fluorophore at 25 μM exposed to an 808 nm laser at 1.0 Wcm^{-2} . Inset: In vitro thermal images of ZW800-PEG (25 μM) and PBS (500 μL) after exposure to 808 nm laser (1.0 Wcm^{-2}). The white number indicates the real temperature at the center of the solution. c) Photothermal in vitro cell study. Cell viability (%) of each fluorophore at 25 μM was measured after exposure to an 808 nm laser at 160 mWcm^{-2} for 3 min on H-23 cell line. Control indicates no treatment. d) CCK-8 assay of zwitterionic fluorophores at 25 μM for cell viability on H-1793 cell line. e) MTT assay of ZW800-PEG with different concentrations on H-1793 cell line. Data are expressed as mean $\pm$ S.D. ($n=3$). $**p < 0.01$; $n.s.$ =not significant.

Figure 5.

In vivo performance of zwitterionic fluorophores. a) Biodistribution patterns of ZW800-1A, ZW800-1C, and ZW800-PEG (25 nmol) in CD-1 mice at 4 h post-retroorbital injection. Abbreviations used are: AW: abdominal wall; Bl: bladder; Du: duodenum; In: intestine; Ki: kidneys; Li: liver; Lu: lungs; Pa: pancreas; Sp: spleen. Arrow=gallbladder; Scale bars=1 cm. b) Signal-to-background ratio (SBR) of resected organs against muscle was measured using ImageJ analysis. * $p < 0.05$ ($n=3$, mean $\pm$ S.D.). c) Blood curves and d) pharmacokinetic parameters obtained from CD-1 mice retro-orbitally injected with 50 nmol of each fluorophore.

Table 1:

Chemical properties of ZW800-1A, ZW800-1C, and ZW800-PEG.

Chemical property	ZW800-1A	ZW800-1C	ZW800-PEG
Molecular weight [Da]	944.24	928.24	971.30
Charges (+/-)	+3/-3	+3/-3	+3/-3
LogD, pH 7.4	-3.35	-2.80	-4.75
TPSA [$\text{\AA}^2$]	167.18	157.95	179.24
HBA/HBD	10/1	8/1	11/1
Rotatable bonds	18	17	23

Table 2:Solubility and optical properties of ZW800-1A, ZW800-1C and ZW800-PEG in DMSO, PBS, and FBS.^[a]

NIR	Solvent	Soly [mg mL ⁻¹]	λ_{Abs} [nm]	λ_{Em} [nm]	SS [nm]	ϵ [M ⁻¹ cm ⁻¹]	QY (ϕ , %)
ZW800-1A ^[12]	DMSO	33.0	792	814	22	256 000	26.5
	PBS	84.0	770	788	18	246 000	13.5
	FBS	86.0	772	788	16	249 000	15.1
ZW800-1C ^[13]	DMSO	0.5	779	798	19	225 000	33.2
	PBS (<i>d/m</i>) [*]	6.8	678/753	772	19	123 000/76 000	20.0
	FBS (<i>d/m</i>) [*]	7.0	680/754	776	22	95 000/111 000	23.9
ZW800-PEG	DMSO	140.0	806	834	28	200 000	15.4
	PBS	79.0	779	807	28	199 000	6.5
	FBS	53.0	778	808	30	210 000	5.9
ICG ^[8]	DMSO	10.0	798	825	27	121 000	13.0
	PBS	0.5	778	802	24	58 000	2.6
	FBS	2.5	795	823	28	121 000	10.1

^[a]Soly, solubility; λ , wavelength; SS, Stokes shift; QY, quantum yield. PBS, phosphate-buffered saline, pH 7.4; FBS, 30% fetal bovine serum supplemented with 50 mM HEPES, pH 7.4; DMSO, dimethyl sulfoxide.

^{*}
d, dimer; *m*, monomer.