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ORIGINAL PAPER

Early Detection of Breast Cancer: Synthesis and Characterization of Novel Target Specific NIR-Fluorescent Estrogen Conjugate for Molecular Optical Imaging

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Abstract Estrogen induced proliferation of existing mutant cells is widely understood to be the major risk determining factor in the development of breast cancer. Hence determination of the Estrogen Receptor[ER] status is of paramount importance. We have carried out the synthesis and characterization of a novel NIR fluorescent dye conjugate aimed at measuring ER+ve status in-vivo. The conjugate was synthesized by ester formation between 17-β estradiol and a cyanine dye namely: bis-1, 1-(4-sulfobutyl) indotricarbocyanine-5-carboxylic acid, sodium salt. The replacement of the sodium ion in the ester by a larger glucosammonium ion was found to enhance the hydrophilicity and reduce the toxic effect on cell lines. The excitation and emission peaks for the dye were recorded as 750 and 788 nm respectively; ideal for non-invasive optical imaging owing to minimal tissue attenuation and auto-fluorescence at these wavelengths. The dye **(NIRDC1)** has a significant drop in plasma-protein binding therefore leading to marked improvement in pharmacokinetic profile such as dye evacuation in comparison to ICG. In addition the dye showed enhanced fluorescence quantum yield, molar extinction coefficient and linearity in fluorescence relative to ICG. This dye can be potentially used as a target specific exogenous contrast agent in molecular optical imaging for early detection of breast cancer.

Keywords Breast cancer · Estrogen receptor status · NIR fluorescent dye · Estrogen conjugate · Fluorescence molecular optical imaging · NIRD1 conjugate (NIRDC1)

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Introduction

Many factors both genetic and hormonal have been cited to initiate and sustain breast cancer. The main estrogenic hormone, 17β-estradiol through the E2 action is mediated by transcriptional actions of the nuclear estrogen receptors, ERα and ERβ thus stimulating excessive cell growth in cancerous adenocarcinoma breast tissues [1]. This accounts for over 70% of all breast cancers and is termed ER+ve meaning that a significant number of cancer cells have ER present [23]. Thus the determination of ER status plays a crucial role in cancer prognosis, deciding the extent of cancer pathogenesis and treatment regime for breast cancer patients [1, 2, 3]. Unfortunately only invasive techniques such as immuno-histochemitry and steroid binding array studies which are based on collection of tissue samples through biopsy are present for ER status determination, which is a rather expensive and painful affair. This opens up a challenging proposition of developing non-invasive, possibly in-vivo diagnosis scheme for determination of

estrogen receptor status and other bio-molecular functions central to cancer pathogenesis. Surely, these developments will prove vital in not only early detection and hence early cure but also risk determination of cancer.

Non-invasive imaging with diffusive photon migration represents an intriguing avenue for extracting biological information from living subjects [4]. While light in the visible range is routinely used for intravital microscopy, imaging of deeper tissues requires the use of near-infrared (NIR) light (700–900 nm), as hemoglobin and water, the major absorbers of visible and infrared light, respectively, have their lowest attenuation and auto-fluorescence in this region (Fig. 1). Light photons can be used to measure different native parameters of tissue through which they travel, such as absorption, scattering, polarization, spectral characteristics, and fluorescence [9]. Exogenously added contrast agents would aid in the specificity and sensitivity of disease detection apart from contrast enhancement. It has been evident that fluorescence spectroscopy of the endogenous compounds can provide enhanced contrast as well as diagnostic information [11, 13, 26]. It has also been shown that exogenous dyes may offer the best contrast for optical imaging [18, 19, 20]. The benefit of specific targeting is apparent: high selectivity and affinity of receptor ligands enable the use of low (nanomolar) doses of the compounds, which result in a high signal to noise ratio. Tumor exhibit distinctive metabolite alternation, this ability to extract tissue functional information may offer a unique way to non-invasively differentiate between benign and malignant tumors [18, 19]. While a non target specific dye indocyanine green (ICG) has been used clinically for over 20 years with few side effects [5, 22] its use in designing targeted agents is limited primarily because of the non-availability of monoderivatized activated precursors.

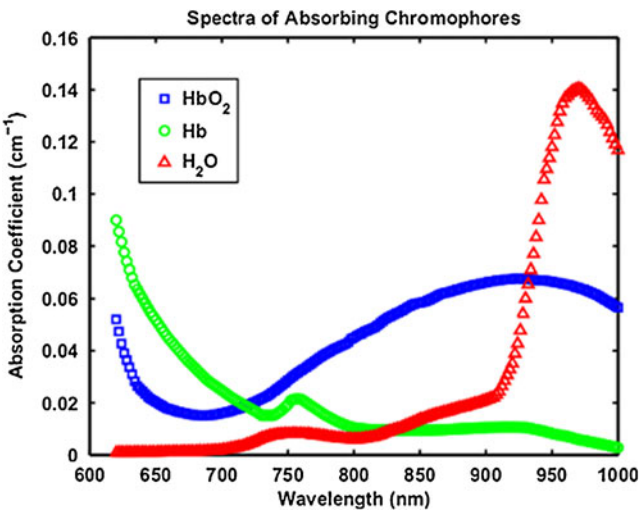


Fig. 1 Absorption of major chromophores in the NIR window

In the recent years different groups have illustrated and synthesized several new derivatives such as tricarbocyanines [7] and cyptates [8]. It has been studied that by structural modification of indocyanine green, a variety of novel cyanine dyes with different degrees of hydrophilicity and molecular weight can be generated. By systematic structural modifications on the exterior ring of bis-1,1-(4-sulfobutyl) indotricarbocyanine chromophore a class of new NIRF dyes can be obtained [27] (Fig. 2).

We have synthesized a novel functionalized Estrogen conjugate form carbocyanine dye. The ester functional groups present here are reactive toward primary amines and can be used as fluorescent probes. Additional disadvantages of ICG such as hydrophobicity, high albumin (plasma-protein) binding, nonlinear fluorescence, and other intrinsic properties [6, 24, 25] are also considerably reduced. This technique offers potential of non-invasive detection of the hormone receptor status *in-vivo* and helps in decreasing the load of unnecessary biopsies.

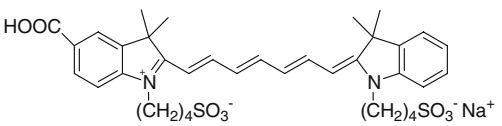
Materials and Methods

Base Chemicals and Reagents

All chemicals and solvents used for chemical reaction were reagent grade. Analytical grade solvents were used for chromatographic purification. Glutaconic aldehyde dianilide, 2,3,3-trimethyl-3H-indolenine, 1,4-butanesultone—Lancaster, Isopropyl methyl ketone—Merck, 17-beta estradiol-Sigma-Aldrich Ltd., Indion ion exchange resins were purchased from Indian Chemicals Ltd. Reactions were monitored by doing thin layer chromatography. All other reagents were purchased from Sigma Aldrich. 5-carboxy-1-(4-sulfobutyl)-2,3,3-trimethyl-3H-indolenine[10] and 1-(4-sulfobutyl)-2,3,3-trimethyl-3H-indolenine[12] were synthesized according to the literature[14, 15] and Bis-1,1′-(4-sulfobutyl)indotricarbo cyanine-5-carboxylic acid, sodium salt was synthesized according to the literature[1] (Fig. 3).

Tagging of 17-β Estradiol to NIRD1

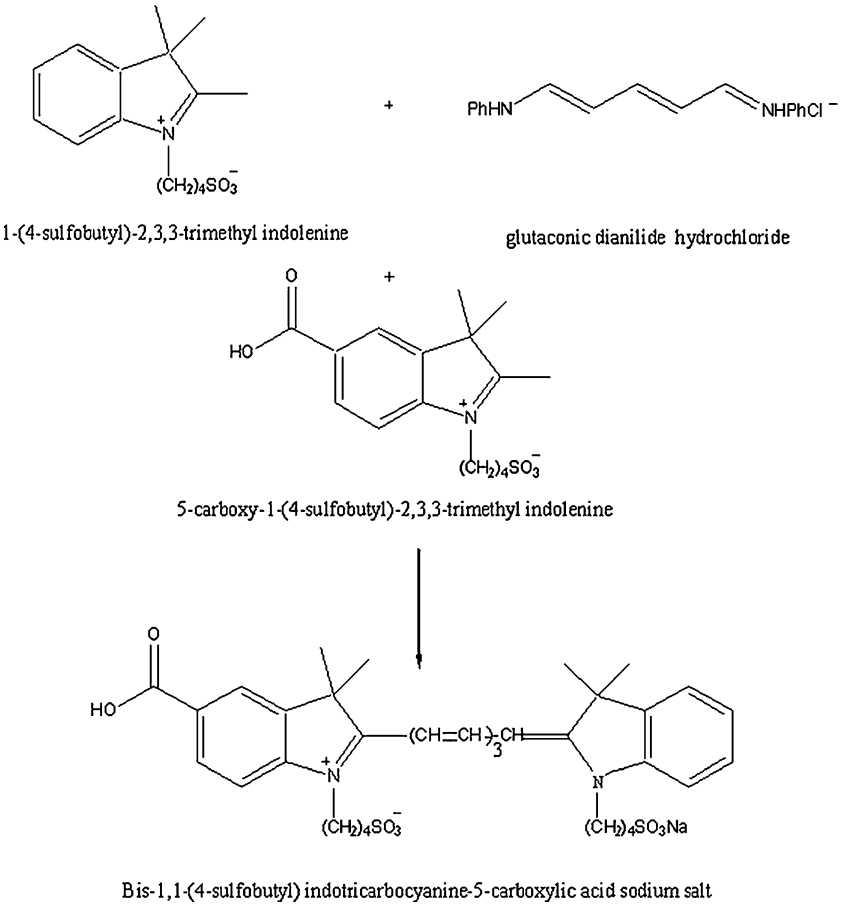
In a typical experiment, 10 mg of dye, 30 μL of Dicyclohexylcarbodiimide (DCC), 50 μL of 4-DimethylAminoPyridine



NIRD1

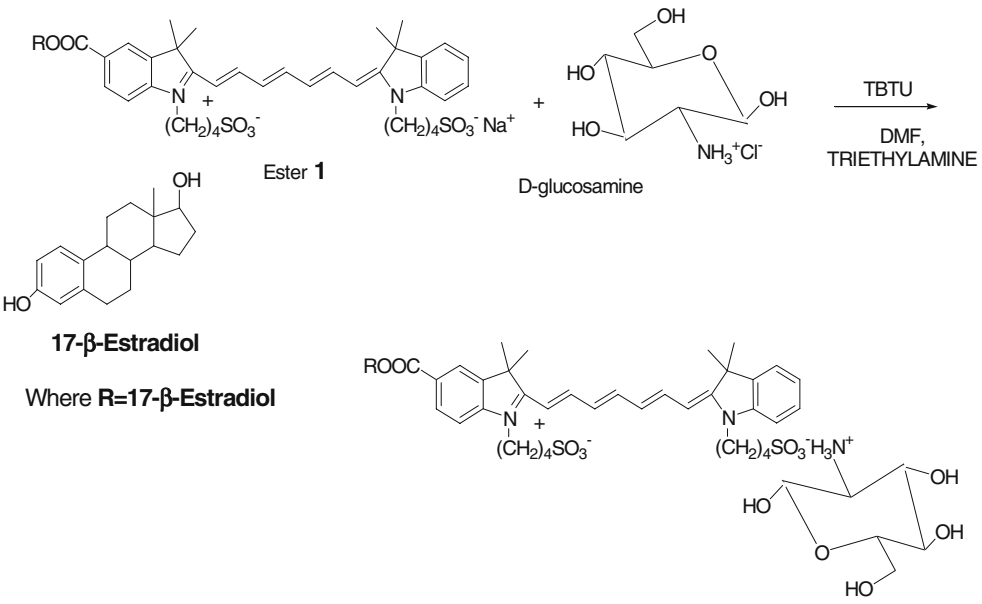
Fig. 2 Structure of NIRD1 flurochrome

Fig. 3 Synthesis of NIRD1 flurochrome



(DMAP), 22 mg of 17-β Estradiol and 0.5 mL of dry DMF were placed in a small round-bottomed flask under argon atmosphere. The mixture was stirred at room temperature for 3 h and the precipitated urea was then filtered. Residue was taken in CH_2Cl_2 and filtered further for any precipitated urea. This was further washed twice with 0.5N HCL and saturated $NaHCO_3$ solution and dried over $MgSO_4$ (anhydrous). The Solvent was removed by evaporation and the ester was isolated by distillation/recrystallization. Product obtained was then purified as NIRD1Conjugate. The result

Fig. 4 Complete reactions for synthesis NIRDC1



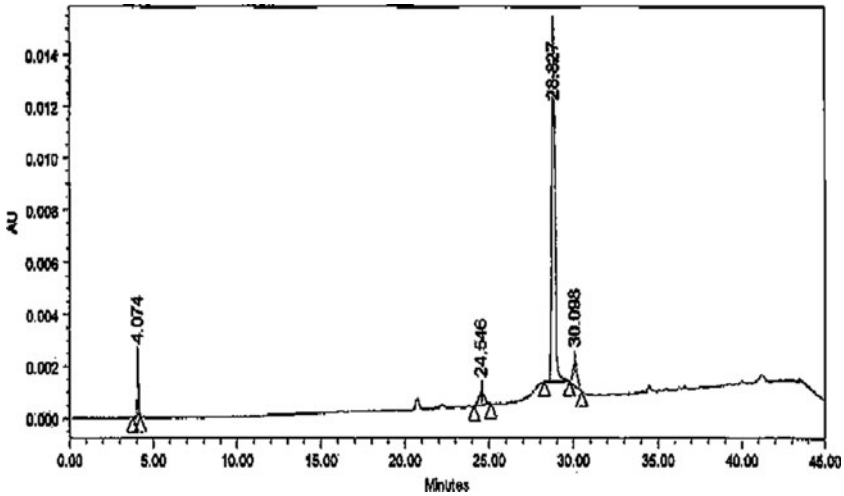


Fig. 5 HPLC traces after modification

indicated more than 95% of the dye being converted to active ester.

Replacement Na^+ Ion

A solution of Ester **1** (0.8 g, 1.1 mmol) in dry DMF (10 ml) containing triethylamine (0.25 g, 2.5 mmol) was cooled to 0°C. Into this solution 0-(benzotriazol-l-yl)-*N,N, N'N'*-tetramethyluronium tetrafluoroborate [TBTU] (0.38 g, 1.2 mmol) dissolved in DMF (2 mL) was added. After 20-30 min of stirring at 0°C a portion of 2 mmol of the D-glucosamine hydrochloride (0.43 g, 1.2 mmol) dissolved in DMF (2 mL) was added. The mixture was allowed to stir for 2 h at room temperature and the following compound with 67% yield was obtained (Fig. 4).

Purification and Spectroscopic Analysis

Purification of dyes was performed on a Inertsil ODS preparative HPLC instrument, using a C18-RP preparative column (flow rate=1 mL/min; eluant A, water with 0.1% TFA; eluant B, 90% of acetonitrile and 10% of eluant A; starting at 90% A for 5 min and then a linear gradient over 40 min to 50% A) [17]. The dual HPLC detector was set at 240 and 360 nm. Dye was collected, and solvent was removed by Speed-vacuum rotary as shown in Fig. 5. The absorbance spectra were measured on a JASCO V-570 spectrophotometer and the fluorescence studies were performed on Perkin Eimer LS-55 Luminiscent spectrometer. The ^1H NMR spectrum was recorded in a 400 MHz VARIAN NMR Spectrometer.

NMR Spectroscopy

^1HNMR ($CDCl_3$, 400 MHz) δ 1.62(m, 25H, 5xCH_3 and 2x $CH_2CH_2CH_2CH_2SO_3^-$)1.66–1.91(m, 12H,Estradiol;β, γ,ω, ϕ, σ andλ proton positions), 2.91(t, 4H, 2x $CH_2SO_3^-$), 3.37(m, 2H, benzylic protons), 4.2(t, 4H, 2x>NCH_2), 7.3 (t,2H,5′-H), 7.58(d,2H, 4′-H,7′-H), 7.72(m,2H, δ−H, β′−H), 7.89(d,1H, 6-H), 7.93(dd, 2H, 4-H), 7–8(10H, Aromatic), 8.07(s, 1H, phenolic) (Fig. 6).

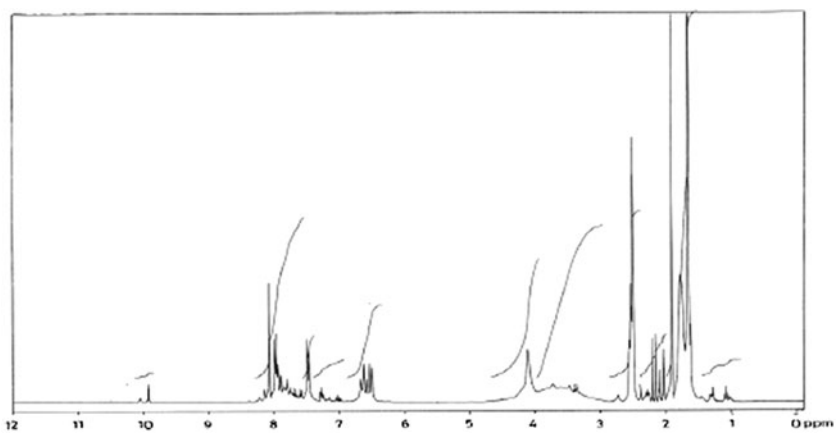


Fig. 6 ^1HNMR ($CDCl_3$, 400 MHz) data of NIRDC1

Table 1 Fluorescent properties of flurochrome prior to tagging (NIRD1)

Compound	Solvent		λ max, *em* (*nm*)	Stokes' Shift	ϕ^a
NIRD1	H_2O	750	788	38	0.067
	EtOH	753			
	DMSO	750			

[a] Based on ICG as standard (ϕ= 0.13)

Results

Spectral Properties of NIR Fluorochrome

The NIR Dye was purified by preparative HPLC as described above. The absorbance was measured individually in three dilutions of the stock solution in deionized water or in ethanol (95%) or in DMSO. The fluorescence emission maxima and intensities of the dyes were obtained using dilute solutions in water and exciting at both the main absorption peak as well as the short-wavelength shoulder of the main absorption peak as shown in (Table 1). The quantum yield for NIR Dye1 [NIRD1] was calculated relative to a standard solution of ICG, with quantum yield of 0.13.

Spectral Properties of NIRD1 Conjugate

The excitation and emission spectra of NIRD1Conjgate after the replacement of the ion is shown in (Fig. 7). NIRD1Conjugated dye exhibited a 38 nm Stokes' shift of the fluorescence maximum as shown in (Table 2). Quantum yield of the Ester was calculated both in DMSO and PBS and found to be 0.114 and 0.110 respectively. The individual data are given in (Table 2). The absorption and the fluorescence spectra taken in PBS is shown in the

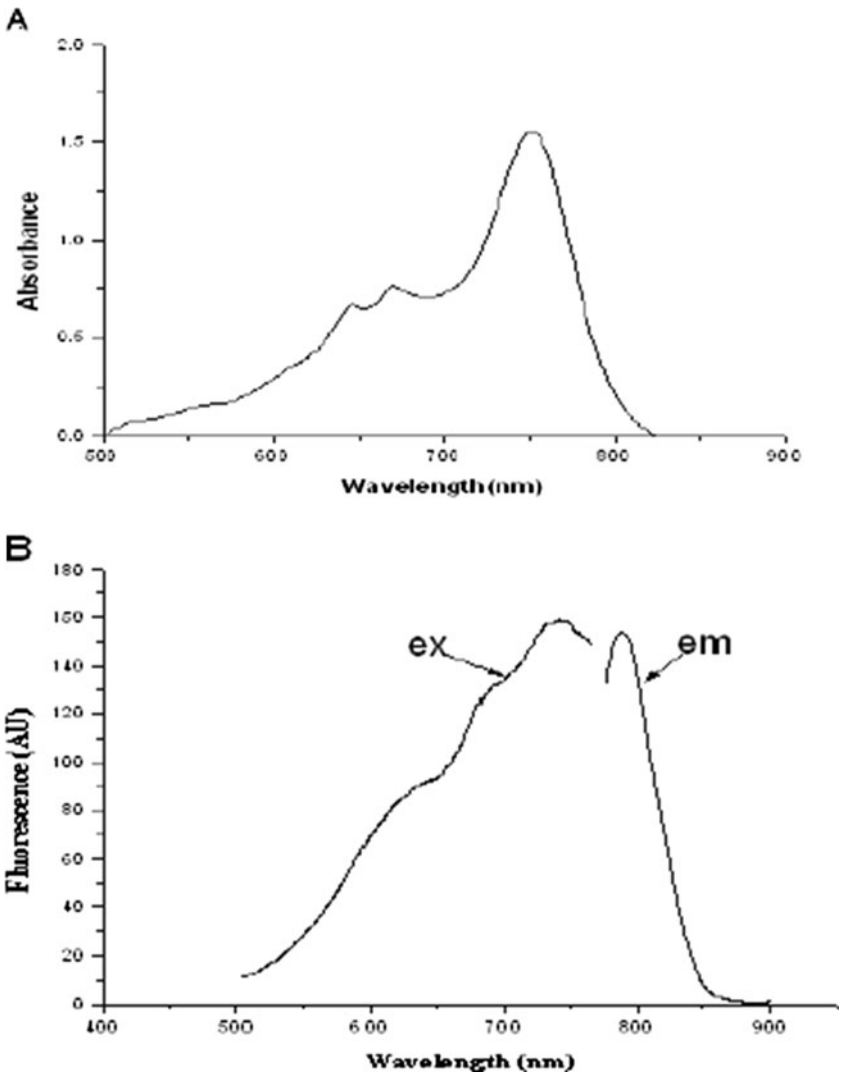


Fig. 7 Absorption (**a**) and Fluorescence (**b**) spectra of NIRD1Conjugate

Table 2 Fluorescent properties of NIRDC1

Based on ICG as standard (ϕ= 0.13)

Compound	Solvent	λ max, *abs* (*nm*)	λ max, *em* (*nm*)	Strokes' Shift	ϕ*
NIRD1Con.	DMSO	754	787	33	0.114
NIRD1Con.	PBS	750	788	38	0.110

(Fig. 7) (a) and (b) respectively. The fluorescence emission maxima and intensities of the dyes were obtained using dilute Solutions in DMSO and exciting at both the main absorption peak as well as the short-wavelength shoulder of the main absorption peak. In cases of NIRD1Conjugate the quantum yield was calculated relative to a standard solution of ICG with quantum yield of 0.13 (Table 2). The DyeConjugate exhibited higher molar absorption coefficients with relatively high values of symmetric chemical substitutions.

Absorption and Fluorescence Spectra

Discussion

The characteristics of the DYE1Conjuagte (Ester **1**) showed a considerable amount of rise in the quantum yield and hydrophilicity. ICG has a poor fluorescence quantum yield (0.012) described for solution in water [22], where as the synthesized ester **1** showed the quantum yield which was at the higher level and did not differ significantly between PBS and DMSO as shown in the (Table 2). The absorption maxima of the conjugate was shifted to a shorter wavelength of 750 as compared to ICG at 780. This we believe is due to the omission of the fused benzene rings of ICG. The increased fluorescence quantum yield of the hydrophilic derivatives reduces the risk of forming the fluorescence-quenched aggregation [16]. Apparently, the interaction within the dye and aggregation is markedly reduced by hydrophilic, non charged substituents. Eventually the change in quantum yield was only insignificant between the solvents used. The test for toxicity was carried out on cell lines MCF-7 and the Ester was found non toxic with 90% viability. At extracellular dye concentrations exceeding 6 mM in equilibrated cell suspensions, both mitochondrial and plasma membrane dye toxicity were observed. Viability and metabolic data demonstrated mutual reinforcement of therapeutic efficacy. The test results reported that both the Dye and the Conjugate were found nontoxic, thereby enabling us to carry out the in-vivo studies.

We are presently involved in testing the flurochrome tagged molecule for imaging of tumors in breast cancer. Thus we believe from these findings that there would be negligible changes in the fluorescence behavior when subjected to in-vivo situation and the fluorescent emission of the dye is expected to be detectable in high sensitivity above low tissue background [21].

Conclusion

Our effort to synthesize carbocyanine structurally related to indotricarbocyanine ICG allowed us to study the impact of chemical modification on the optical properties of these compounds. The hydrophilic substitution improved the optical properties of the conjugate as compared to that of the original dye. The conjugate showed an enhanced fluorescence quantum yields in physiological media, which we believe would definitely be useful in carrying out the in-vivo test. Thus the NIRD1Conjugate **(NIRDC1)** illustrated in this paper promises to facilitate optical imaging applications where intrinsic contrast between tumor and healthy tissue has to be elevated, predominantly to overcome the restrictions in spatial resolution and sensitivity.

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