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Near-infrared lipophilic fluorophores for tracing tissue growth

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Abstract

Longitudinal monitoring of cell migration, division and differentiation is of paramount importance in cell-based medical treatment. However, currently available optical techniques for tracing cell growth and tissue development are limited in applications due to genetic modification, toxicity and inaccurate detection when utilizing the visible spectrum. We have developed lipophilic near-infrared (NIR) fluorophores with high optical properties and a low background signal that allows longitudinal monitoring of cell proliferation and differentiation. Intracellular labeling efficacy was highly dependent on the physicochemical properties of fluorophores such as lipophilicity, charge, polar surface area and rotational bonds. Among the series of NIR cyanine fluorophores, ESNF **13** showed high solubility in aqueous buffer, high membrane penetration, low cytotoxicity and a long-term signal maintainability with a high signal intensity. This study will guide tissue engineers in designing long-term cell trafficking agents with better physicochemical and optical properties.

1. Introduction

Clinical trials based on regenerative medicine and cell-based therapy have been intensively explored over the last decade [1, 9, 15, 20, 21, 23]. A critical issue for the successful therapeutic application of regenerative medicine is a longitudinal assessment of engrafted cell survival, integration and proliferation during tissue regeneration [12, 2, 26, 13]. Conventional methods such as histological evaluation could lead to inaccurate results due to their nonconsecutive single-time-point information and compel the sacrifice of numerous animals for analysis [22, 26]. Noninvasive and dynamic molecular imaging with a high temporal and spatial resolution is essential for successful long-term cell-based therapy. Reporter gene transfection and direct fluorescence cell labeling are currently available methods for cell trafficking and monitoring of tissue growth [14, 27, 28]. Challenges remain, however, in translating these techniques to the clinic. Bioluminescence imaging using reporter genes requires transplantation of genetically modified cells and have limited clinical applications, whereas direct cell labeling using contrast agents such as magnetic nanoparticles, radionuclides or visible fluorophores suffers from signal dilution with each cell division and tissue growth [18, 25, 24]. Furthermore, high scattering and absorbance in mammalian tissues and their emissions in the visible spectrum is another limitation of optical fluorescence imaging [26, 11].

To overcome these limitations, near-infrared (NIR) fluorescence imaging has been used recently to monitor cell fate *in vitro* and *in vivo* [19, 10, 8]. NIR fluorophores, with wavelengths ranging from 700–900 nm, have excellent optical properties and are highly stable in serum and the body, which would be beneficial to track the fate of implanted cells *in vivo* [11, 7, 16]. In this study, we synthesized a series of lipophilic NIR fluorophores for long-term cell trafficking. Using NIR fluorescence-labeled chondrocytes and myoblasts, we investigated the effect of physicochemical properties such as lipophilicity, charges, polar surface area and rotational bonds on the initial cell membrane permeability and extended cellular retention during the cell proliferation and differentiation.

2. Experimental details

2.1. Chemicals and reagents

All chemicals and solvents were of American Chemical Society grade or HPLC purity. Sigma-Aldrich (Saint Louis, MO) or TCI America (Waltham, MA) is the commercial source for the starting materials utilized in the presented synthesis and the reagents were used without purification. The ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance (400 MHz) spectrometer using $\text{DMSO}-d_6$ or $\text{MeOD}-d_4$ containing tetramethylsilane (TMS) as an internal calibration standard. UV–Vis/NIR absorption spectra were recorded on a Varian Cary 50 spectrophotometer. High-resolution accurate mass spectra (HRMS) were obtained at the Georgia State University Mass Spectrometry Facility using a Waters Q-TOF micro (ESI-Q-TOF) mass spectrometer.

2.2. Synthesis of lipophilic pentamethine fluorophores (ESNF; endocrine-specific NIR fluorophore)

1,3,3-Trimethyl-2-((1*E*,3*E*,5*E*)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3*H*-indol-1-ium iodide *ESNF 10*: Yield 64%; ^1H NMR (400 MHz, $\text{MeOD}-d_4$): δ 1.71 (s, 12H), 3.63 (s, 6H), 6.28 (d, J = 16 Hz, 2H), 6.65 (t, J = 12 Hz, 1H), 7.24 (t, J = 8 Hz, 2H), 7.29 (d, J = 8 Hz, 2H), 7.39 (t, J = 8 Hz, 2H), 7.48 (d, J = 8 Hz, 2H), 8.25 (t, J = 12 Hz, 2H); ^{13}C NMR (100 MHz, $\text{MeOD}-d_4$): δ 27.90, 31.71, 50.50, 104.44, 111.83, 123.31, 126.20, 129.71, 142.56, 144.29, 155.52, 175.28. TOF HRMS m/z (M^+) calculated for $[\text{C}_{27}\text{H}_{31}\text{N}_2]^+$ 383.2487, found 383.2474.

1-Butyl-2-((1*E*,3*E*,5*E*)-5-(1-butyl-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3*H*-indol-1-ium iodide *ESNF 11*: Yield: 48%, M.P. > 260°C, ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 0.91 (t, J = 8 Hz, 6H), 1.37 (q, J = 8 Hz, 4H), 1.66 (s, 16H), 4.08 (s, 4H), 6.30 (d, J = 16 Hz, 2H), 6.59 (t, J = 12 Hz, 1H), 7.23 (s, 2H), 7.38 (s, = 4H), 7.60 (d, J = 8 Hz, 2H), 8.33 (t, J = 12 Hz, 2H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 13.39, 19.11, 26.80, 28.73, 42.84, 48.52, 102.81, 110.74, 122.09, 124.31, 125.25, 128.07, 140.75, 141.65, 153.65, 172.26. TOF HRMS m/z (M^+) calculated for $[\text{C}_{33}\text{H}_{43}\text{N}_2]^+$ 467.7074 found 468.4592.

2-((1*E*,3*E*,5*E*)-5-(3,3-Dimethyl-1-(3-phenylpropyl) indolin-2-ylidene) penta-1, 3-dien-1-yl)-3, 3-dimethyl-1-(3-phenylpropyl)-3*H*-indol-1-ium iodide *ESNF 12*: Yield 49%, M.P. 185–187 °C, ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 1.68 (s, 12H), 2.01 (t, J = 8 Hz, 4H), 2.74 (t, J = 8 Hz, 4H), 4.15 (t, J = 8 Hz, 4H), 6.075 (d, J = 12 Hz, 2H), 6.46 (t, J = 12 Hz, 1H), 7.40–7.24 (m, 16H), 7.62 (d, J = 8 Hz, 2H), 8.33 (t, J = 12 Hz, 2H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 27.60, 29.16, 32.58, 43.55, 49.40, 103.58, 111.47, 122.93, 125.19, 125.19, 125.97, 128.71, 128.91, 141.43, 141.43, 142.46, 154.54, 173.11. TOF HRMS m/z (M^+) calculated for $[\text{C}_{43}\text{H}_{47}\text{N}_2]^+$ 591.3734, found 591.2740.

5-Methoxy-2-((1*E*,3*E*,5*E*)-5-(5-methoxy-1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3*H*-indol-1-ium iodide *ESNF 13*: Yield 75%, MP 228–230 °C, ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 1.66 (s, 12H), 3.56 (s, 3H), 3.81 (s, 6H), 6.165 (d, J = 12 Hz, 2H),

6.46 (t, $J = 12$ Hz, 1H), 6.95 (d, $J = 8$ Hz, 2H), 7.30-7.28 (m, 4H), 8.23 (t, $J = 12$ Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 26.48, 30.62, 48.45, 55.29, 102.06, 108.43, 111.02, 112.84, 123.73, 135.80, 142.15, 151.94, 156.98, 171.55. TOF HRMS m/z (M^+) calculated for $\text{C}_{29}\text{H}_{35}\text{N}_2\text{O}_2$ 443.2699 found 443.2692.

3-Ethyl-2-((1*E*,3*E*,5*E*)-5-(3-ethyl-1,1-dimethyl-1*H*-benzo[*e*]indol-2(3*H*)-ylidene)penta-1,3-dien-1-yl)-1,1-dimethyl-1*H*-benzo[*e*]indol-3-ium iodide *ESNF 14*: Yield 79%, MP 266-268°C, ^1H NMR (400 MHz, DMSO- d_6): δ 1.333 (t, $J = 8$ Hz, 6H), 1.962 (s, 12H), 4.297 (t, $J = 8$ Hz, 4H), 6.37 (d, $J = 12$ Hz, 2H), 6.637 (t, $J = 12$ Hz, 7.51 (t, $J = 8$ Hz, 1H), Hz, 2H), 6.68 (t, $J = 8$ Hz, 2H), 7.74 (d, $J = 8$ Hz, 2H), 8.08 (t, $J = 8$ Hz, 4H), 8.25 (d, $J = 8$ Hz, 8.46 (t, $J = 12$ Hz, 2H), Hz, 2H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 11.96, 26.17, 48.04, 50.19, 102.05, 110.90, 121.58, 124.20, 125.07, 127.11, 127.19, 129.39, 129.83, 130.76, 132.72, 138.77, 152.54, 172.71. TOF HRMS m/z (M^+) calculated for $\text{C}_{37}\text{H}_{39}\text{N}_2$ 511.3113, found 511.3098.

4-(2-((1*E*,3*E*,5*E*)-5-(1,1-dimethyl-3-(4-sulfonatobutyl)-1*H*-benzo[*e*]indol-2(3*H*)-ylidene)penta-1,3-dienyl)-1,1-dimethyl-1*H*-benzo[*e*]indolium-3-yl)butane-1-sulfonate *ESNF 15*: Yield 76%, MP > 250°C, ^1H NMR (400 MHz, DMSO- d_6): δ 1.80 (m, $J = 12$ Hz, 9H), 2.00 (s, 12H), 4.2 (q, 4H), 6.4 (d, $J = 16$ Hz, 2H), 6.7 (t, $J = 12$ Hz, 1H), 7.50 (t, $J = 8$ Hz, 2H), 7.70 (t, $J = 4$ Hz, 2H), 8.10 (q, 4H), 8.35 (d, $J = 8$ Hz, 2H), 8.50 (t, $J = 12$ Hz, 2H).

2.3. LC-MS spectroscopy

The purity of all compounds was measured using a Waters liquid chromatography–mass spectrometry (LC-MS) system consisting of a 1525 binary HPLC pump with a manual 7725*i* Rheodyne injector, a 996 Photodiode Array (PDA) detector and a 2475 multiwavelength fluorescence detector. The column eluate was divided in two using a flow splitter (Upchurch Scientific). A portion of the eluate flowed into an ELSD (Richards Scientific), while the rest flowed into a Micromass LCT ESI-TOF spectrometer (Waters) equipped with a Symmetry(R) C18 (4.6 × 150 mm, 5 μm) reverse-phase HPLC column. For mass spectrometry, mobile phase was solvent A = 0.1% formic acid (FA) in water and solvent B = CH_3CN with 95% A for 5 min and a linear gradient from 5% to 40% CH_3CN (from A to B for 30 min) at a flow rate of 1 mL min $^{-1}$, capillary voltage was –3317 V and sample cone voltage was –50 V.

2.4. Optical property measurements

All optical measurements were performed at 37°C in phosphate buffered saline (PBS), pH 7.4 or 100% fetal bovine serum (FBS) buffered with 50 mM HEPES, pH 7.4 [4-6]. Absorbance and fluorescence emission spectra of the series of NIR fluorophores were measured using fiberoptic HR2000 absorbance (200–1100 nm) and USB2000FL fluorescence (350–1000 nm) spectrometers (Ocean Optics, Dunedin, FL). NIR excitation was provided by a 655 nm red laser pointer (Opcom Inc., Xiamen, China) set to 5 mW and coupled through a 300 μm core diameter, NA 0.22 fiber (Fiberguide Industries, Stirling, NJ). For fluorescence quantum yield (QY) measurements, oxazine 725 in ethylene glycol (QY = 19%) was used as a calibration standard, under conditions of matched absorbance at 655 nm. *In silico* calculations of physicochemical properties such as the partition coefficient (logD at pH 7.4), pKa, total polar surface area (TPSA), H-bond donors/acceptors and rotatable bonds were calculated using Marvin and JChem calculator plugins (ChemAxon, Budapest, Hungary).

2.5. Chondrocyte isolation

Thin-layered cartilage slices were harvested under sterile conditions from the knee joints of a 35 kg Yorkshire pig within 1 h after death. The obtained cartilage tissue was washed three

times with sterile physiological saline and cut into 2–3 mm pieces. The cartilage was washed three times with 2% penicillin-streptomycin (P/S) and incubated with collagenase type II (2 mg mL⁻¹; Worthington-Biochem, Lakewood, NJ) in serum-free media for 24 h at 37 °C in a 5% CO₂ incubator. After incubation the undigested cartilage fragments were removed using a 100 mm filter. The remnant solution was centrifuged at 1200 rpm, the spin-downed isolated chondrocytes were washed with physiological saline and suspended in warm Dulbecco's modified Eagle's medium/nutrient F-12 Ham (DMEM/F12; Mediatech, Herndon, VA), supplemented with 10% FBS and 1% P/S.

2.6. Live cell labeling and imaging

C2C12 mouse myoblast cell line was a kind gift from Drs Peter Kang and Soochan Bae (BIDMC). The cultured chondrocytes of passage 1–2 and myoblasts were seeded onto 24-well plates (5×10^4 cells per well) and incubated at 37 °C in a humidified 5% CO₂ incubator in DMEM/F12, supplemented with 10% FBS and 1% P/S. When the cells reached about 50% confluence, the seeded cells were rinsed twice with PBS and the NIR fluorophore was added to each well at a concentration of 2 and 10 μ M and incubated for 30–60 min at 37 °C in a humidified 5% CO₂ incubator. The myoblast cells were differentiated using 2% FBS or 2% horse serum in culture media. In order to improve image contrast, cells were washed once with phenol red free media prior to imaging. The cells were observed every 2–3 days on a 4-channel NIR fluorescence microscope over a month. The excitation and emission filters used for microscopy were 650 ± 22 and 710 ± 25 nm.

2.7. Cell viability assay

The cell toxicity and proliferation were assessed by a modified 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide (MTT, Sigma-Aldrich) assay. The chondrocytes were seeded onto 24-well plates (3×10^4 cells per well). To test cytotoxicity depending on the fluorophore type and concentration, cells were treated with 2 and 10 μ M of each NIR fluorophore ($n = 6$) for 30 min and cultured at 24 h post-treatment. On each assay time point, the incubation cell media were replaced with 1 mL of fresh media. 100 μ L of MTT solution (5 mg mL⁻¹ stock in PBS) was added to each well and incubated for 4 h at 37 °C in a humidified 5% CO₂ incubator. Cell media were eliminated carefully, and the formed crystals were re-dissolved in 1 mL of DMSO and plated into 96-well microtiter plates for measuring the absorption intensity at 590 nm using a microplate reader (E-max, Molecular Device, USA). The data were presented by dividing by dye-untreated control group. The statistical significance was determined by one-way analysis of variance (ANOVA).

3. Results and discussion

3.1. Synthesis of NIR fluorophores

As presented in figure 1, 4-methoxyphenyl hydrazine was allowed to react with 3-methyl-2-butanone in boiling acetic acid to form the cyclic indolenine **2**. Commercially obtained compound **1** and the synthesized compound **2** were alkylated in anhydrous acetonitrile utilizing alkyl halides to furnish the corresponding heterocyclic salts **4–7**, which were condensed to produce the final pentamethine cyanines ESNF **10–13** with various substituents. Using the similar synthetic strategy, the benz[e]indolenine heterocyclic structure was subjected to alkylation using alkyl halides or butane sulfone to afford ESNF **14** and **15**.

3.2. Quantitative analysis of NIR fluorophores

To eliminate the unknown factors and causes of cell death coming from the product's impurities during the cell viability and binding test, we purified each NIR fluorophore

carefully and the purity was analyzed using a Waters LC-MS system coupled with PDA, ELSD and TOF-MS to give the quantitative information of target compounds and impurities. As shown in figure 2, single-peaked consistency detected by PDA and ELSD reflected high purity of all the compounds (see section 2).

3.3. Physicochemical properties of NIR fluorophores

As summarized in table 1, physicochemical properties of synthesized NIR fluorophores were calculated by Marvin and JChem software to estimate the biological activity and bioavailability. The lipophilicity and cell permeability of NIR fluorophores can be expected from the parameters such as logD at pH 7.4, pKa, TPSA, H-bond donors/acceptors and rotatable bonds. To identify how the lipophilicity affects reciprocal interactions between fluorophores and cells, the NIR fluorophores were designed from the pharmacophore structure of ESNF **10** (logD; 3.56). Substitution of the ESNF pharmacophore with butyl (ESNF **11**; logD 6.20), phenyl propyl (ESNF **12**; logD 8.47), or benzo[e]indolenine ring (ESNF **14**; logD 6.25) resulted in significantly increased logD values, while methoxy group substitution on the indolium kept the value low (ESNF **13**; logD 3.24). It is important to note that ESNF **10-14** are all positively charged (+1) and have similar TPSA values and that they only vary in their lipophilicity depending on the side chains. It is well known that a negatively charged and/or highly polar molecule is less favorable in binding with a cell surface and tends to be poor in permeating cell membrane [3, 17]; therefore, ESNF **15** was designed to include 2 sulfonate groups resulting in a net negative charge (−1), high TPSA value (120.65 Å²) and increased rotatable bonds (13).

3.4. Optical properties of NIR fluorophores

As shown in figure 3, all of the fluorophores show maximum absorption in the range of 650 to 700 nm wavelength and maximum fluorescence emission spectra greater than 660 nm in both aqueous solutions, and their NIR range optical properties would eliminate cellular autofluorescence and increase accuracy in monitoring living cells [11]. Since all fluorophores are lipophilic, they are more soluble in FBS rather than in PBS due to the support of serum proteins. ESNF **10**, **13** and **15** can also be dissolved in PBS because their logD values are relatively lower than those of ESNF **11**, **12** and **14**. Therefore, all fluorophores showed highly increased QY in FBS, which indicates the importance of solubility for the best optical performance.

3.5. Intracellular trafficking of NIR fluorophores

Chondrocytes isolated from a 35 kg Yorkshire pig were treated with the series of NIR fluorophores at a concentration of 2 μ M to investigate their intracellular affinity. As shown in figure 4(A), ESNF **10** and **11** showed weak signals compared to other fluorophores, while ESNF **15** was not taken up by the cells and no visible intracellular signal was observed. The most lipophilic fluorophores, ESNF **12** and **14**, showed relatively high membrane permeability into chondrocytes, but initial cell death was observed during incubation at 37 °C. ESNF **13** presented the most effective cell permeability where it showed the brightest signal. A major concern when using a fluorescent marker is the dilution of labeling in each mitotic cycle. The fluorescence signals inside cells were maintained during the proliferation process because an excess amount of fluorophores was taken up into cells at the initial phase, i.e. partial quenching effect and the number of fluorophores in the cytoplasm was distributed to newly divided cells during proliferation. Around day 6-9 of cultivation, chondrocytes stopped mitosis because of contact inhibition by cell confluence and started to differentiate. The fluorophores retained inside cells during differentiation, and signals were maintained through day 35 without losing significant intensities. To confirm the labeling efficacy, as shown in figure 4(B), we treated C2C12 mouse myoblast cells with the series of

NIR fluorophores and tracked the fluorescence intensities inside cells. A very similar pattern was observed over the time course of observation, where ESNF **13** integrated into the cells most effectively and retained in the cytoplasm during proliferation and differentiation.

3.6. Preliminary cytotoxicity of NIR fluorophores

Figure 5(A) shows color images of chondrocytes treated with a higher concentration of ESNFs (10 μ M) to see the initial membrane permeability and washout effect. As shown in figure 5(B), the viability of chondrocytes was assessed by the MTT assay. Yellow MTT agents were taken up into the chondrocytes by mitochondrial enzymes during incubation, and the purple crystals formed at 24 h after cultivation were analyzed by a microplate reader. Most of the cells were viable when treated with NIR fluorophores at a concentration of 2 μ M, while notable cell death was observed from the group treated with 10 μ M of NIR fluorophores (ESNF **11**, **12** and **14**). ESNF **13** and **15** showed no significant difference in live cell numbers while showing significant difference in cell permeability. ESNF **13** preferably penetrated into the cytoplasm, while ESNF **15** exhibited almost no membrane permeation into chondrocytes or myoblasts because of the high TPSA (120.65 Å² in table 1). Since the concentration of NIR fluorophores affects cytotoxicity remarkably, but the maximum concentrated cells are required for a long-term signal trafficking, all NIR fluorophores need to be tested individually to find the maximum tolerable concentration in each cell line.

4. Conclusions

In this study, we developed a series of lipophilic NIR cyanine fluorophores with high optical properties and low background outside cells that allows longitudinal monitoring of cell proliferation and differentiation. Unlike other complex tracking techniques, the NIR fluorophores require facile and simple procedures for intracellular trafficking. Among the series of NIR fluorophores, ESNF **13** having median values of lipophilicity and TPSA showed low cytotoxicity with the highest signal intensity for a long period of time. However, a system to quantitatively measure the distribution of the NIR signal by cell division needs to be established to explain the detailed mechanism. Further studies will be needed to test the efficacy of these fluorophores *in vivo* with the optimization of the chemical structure and concentration, which will be a powerful tool in tissue engineering and regenerative medicine.

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Figure 1.

Chemical scheme for near-infrared fluorescence emitting pentamethine cyanine fluorophores.

Figure 2. LC-MS analysis of NIR fluorophores. Inset = Chemical structure and ESI TOF mass spectrum (+) of the target compound. PDA, photodiode array; ELSD, evaporative light scatter detection; ESI TOF, electrospray time-of-flight.

(A)

Dye	Charge (+/-)	Solubility		Optical Properties* in PBS				Optical Properties* in FBS			
		PBS	FBS	Abs _{max}	Em _{max}	ϵ (M ⁻¹ cm ⁻¹)	QY (%)	Abs _{max}	Em _{max}	ϵ (M ⁻¹ cm ⁻¹)	QY (%)
ESNF 10	+1/0	○	○	640	660	187,000	17.2	648	670	147,000	41.6
ESNF 11	+1/0	×	○	641	664	132,000	15.6	645	665	147,000	45.3
ESNF 12	+1/0	×	○	645	662	45,000	3.1	657	673	95,000	46.0
ESNF 13	+1/0	△	○	660	702	117,000	4.9	674	700	109,000	10.4
ESNF 14	+1/0	×	○	677	700	78,000	1.2	696	717	183,000	21.5
ESNF 15	+1/-2	△	○	680	702	135,000	4.4	699	718	210,000	20.1

*Optical properties were measured after dissolving ESNFs in PBS, pH 7.4 or 100% FBS supplemented with 50 mM HEPES, pH 7.4 at a concentration of 1-5 μ M. ϵ = extinction coefficient. For QY measurements, oxazine 725 in ethylene glycol (QY = 19%) was used as a calibration standard under conditions of matched absorbance at 655 nm. ○, soluble; △, semi-soluble; and ×, insoluble.

(B)

Figure 3.

(A) Optical properties of NIR fluorophores in PBS and FBS. (B) Absorbance (solid line) and fluorescence emission (dotted line) spectra of ESNFs in 100% FBS, pH 7.4. After purification each compound was diluted to a concentration of 1–5 μ M and subjected to absorbance spectrometry, fluorescence spectrometry and quantum yield (QY) measurements.

Figure 4.

Live cell imaging of NIR fluorophores in chondrocytes (A) and C2C12 mouse myoblast cells (B). Shown are phase contrast and NIR images of each cell line tested at a concentration of 2 μ M. Scale bars = 100 μ m. All NIR fluorescence images have identical exposure and normalizations.

Figure 5.

Cell viability assay of NIR fluorophores using chondrocytes. (A) Color images of chondrocytes were obtained 30 min (top) and 1 day (bottom) post-treatment of ESNF at a concentration of 10 μ M. (B) Cell viability was plotted 1 day post-treatment of ESNFs at a concentration of 2 μ M (left) or 10 μ M (right). Each error bar represents the average measurement from six replicates. * denotes statistically significant difference in cell viability at $P < 0.05$, compared to untreated control as measured by the MTT assay. Scale bars = 100 μ m.

Table 1

In silico physicochemical properties of NIR fluorophores calculated using Marvin and JChem calculator plugins (ChemAxon, Budapest, Hungary). LogD = partition coefficient at pH 7.4, TPSA = total polar surface area.

Structure	Formula	Mol Weight	LogD, pH 7.4	Strongest acidic pKa	Strongest basic pKa	TPSA	H bond donors	H bond acceptors	Rotatable bonds
C27H31N2	383.55	3.56		1.94	6.25	0	1	3
C33H43N2	467.71	6.20		2.59	6.25	0	1	9
C43H47N2	591.85	8.47		2.63	6.25	0	1	11
C29H35N2O2	443.60	3.24		2.57	24.71	0	3	5
C37H39N2	511.72	6.25		2.35	6.25	0	1	5
C41H45N2O6S2	725.94	4.38	-0.94	2.66	120.65	0	7	13