Erythrocyte-derived photo-theranostic agents: hybrid nano-vesicles containing indocyanine green for near infrared imaging and therapeutic applications

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Development of theranostic nano-constructs may enable diagnosis and treatment of diseases at high spatial resolution. Some key requirements for clinical translation of such constructs are that they must be non-toxic, non-immunogenic, biodegradable, with extended circulating lifetime. Cell-based structures, particularly those derived from erythrocytes, are promising candidate carrier systems to satisfy these requirements. One particular type of theranostic materials utilize light-sensitive agents that once photo-activated can provide diagnostic imaging capability, and elicit therapeutic effects. Here we demonstrate the first successful engineering of hybrid nano-scale constructs derived from membranes of hemoglobin-depleted erythrocytes that encapsulate the near infrared chromophore, indocyanine green. We show the utility of the constructs as photo-theranostic agents in fluorescence imaging and photothermal destruction of human cells. These erythrocyte-mimicking nano-structures can be derived autologously, and may have broad applications in personal nanomedicine ranging from imaging and photo-destruction of cancerous tissues to vascular abnormalities, and longitudinal evaluations of therapeutic interventions.

Transduction of light by specific molecules into other types of energy such as heat, chemical energy, and acoustic waves, has provided the basis for the development of photothermal therapy and photodynamic therapy (PDT) procedures, as well as diagnostic methods based on fluorescence and photoacoustic imaging. The use of near infrared (NIR) wavelengths (\(\lambda\)), particularly in the range of \(\approx 650–1450\) nm, is especially advantageous since in this "optically transparent window," light can penetrate relatively deeply into biological materials as there is minimal photons absorption by endogenous molecules.

Delivering NIR-transducing exogenous materials to a particular target of interest (e.g., abnormal vasculature, tumor mass) provides an approach to enhance the local optical absorption of the target. Once activated by NIR light, the exogenous material may be used as a photo-theranostic agent to enhance both the optical contrast of the target, and phototherapeutic efficacy, particularly in deeply seated targets.

A particular NIR-transducing molecule is Indocyanine Green (ICG) (\(\text{C}_{43}\text{H}_{47}\text{N}_{2}\text{NaO}_{6}\text{S}_{2}\); molecular weight \(\approx 775\) Da). It is composed of two polycyclic (benzoindotricarbocyanine) lipophilic moieties, linked with a polyene bridge. A sulfonate group, bound to the nitrogens of each polycyclic part, provides net negative charge and water solubility. Therefore, the entire molecular structure leads to amphiphilic characteristics of ICG.

ICG's medical applications began in late 1950's for measurements of cardiovascular output to characterize valvular and septal defects, and soon after it was used to assess hepatic function. It received supplemental approval by United Stated Food and Drug Administration (FDA) for ophthalmic angiography in 1975. In ophthalmological applications, ICG is recommended for imaging selective chorioretinal disorders including suspected polypoidal choroidal neovascularization (CNV), chronic central serous chorioretinopathy (CSC), and choroidal hemangioma. To-date, ICG remains the only NIR dye approved by FDA for cardiovacular measurements, liver function tests, and ophthalmological imaging.

In addition to its current clinical utility, ICG has been investigated for sentinel lymph node mapping in patients with melanoma, prostate, breast, and other types of cancer. Its utility in fluorescence-guided PDT of choroidal disease has also been reported. Additionally, ICG has been studied for potential phototherapeutic applications including treatment of CSC, cutaneous hypervascular malformations, and PDT of choroidal melanomas.
Despite usage in clinical medicine, ICG’s major drawbacks are its short half-life within plasma (≈2–4 minutes), and exclusive uptake by hepatic parenchymal cells followed by biliary excretion. Given its amphiphilic nature, ICG binds to various molecular species including albumin, and high- and low-density lipoproteins within the vasculature. Given these limitations, the potentials of this clinically-proven exogenous material as a theranostic agent for broader medical applications, which may require extended circulating lifetimes, remain limited.

Encapsulation has been investigated to shield ICG from non-specific interactions within the plasma, and extend its circulation time in the vasculature. Our group has reported that encapsulation of ICG into synthetic polymer-based nano-constructs enhances the contrast of in vivo fluorescent images from the heart and lungs for at least up to 90 minutes14, and delays maximal hepatic accumulation to at least 60 minutes after tail vein injection in mice15.

Recently, attention has been given to the use of biological materials as platforms for the delivery of therapeutic or imaging agents. We have reported the use of genome-depleted plant infecting brome mosaic virus doped with ICG as a nano-construct for NIR fluorescence and photoacoustic imaging16–17.

Mammalian cells, particularly erythrocytes, present another type of biological vectors for the delivery of therapeutic and imaging agents18–21. In relation to cell-based imaging, hemoglobin-depleted red blood cells (erythrocyte ghosts (EGs)) have been used to encapsulate iron oxide for potential magnetic resonance imaging (MRI) applications22–24. Gold nanoparticles have been incorporated into human erythrocytes to produce tracers with enhanced contrast for potential dynamic X-ray imaging of blood flow25. Fluorescein isothiocyanate, a non-NIR dye, has been successfully loaded into EGs having diameters of ≈5 µm, with the intention of developing such constructs as plasma analyte or extracellular pH sensors26–27. Flower et al. recently loaded ICG into micron-sized EGs, and utilized the constructs as a fluorescent analogue of erythrocytes to characterize the movement of erythrocytes in retinal capillaries and choriocapillaris of monkeys and rabbits28.

Here, we report the first proof-of-principle to demonstrate the successful engineering of ICG-loaded nano-sized vesicles derived from EGs, and their utility as photo-theranostic agents for fluorescence imaging and photothermal destruction of cells. We refer to these nano-vesicles as near infrared erythrocyte-mimicking transducers (NETs).

Results
We can manipulate the diameter distribution of the NETs, and subsequently, their photophysical properties by varying the extrusion procedures required to fabricate nano-sized constructs, and the time of the EGs exposure to the hypotonic solution containing ICG at a given concentration. With the exception of the transmission electron microscope (TEM) image of the NETs, all results presented herein are based on 20 initial extrusions of EGs through 400 nm diameter polycarbonate porous membranes, followed by 20 additional extrusions through 100 nm diameter membranes, subsequent addition of 215 µM ICG into the hypotonic buffer solution containing the suspension of the nano-sized EGs, and incubation for five minutes (see Supplemental Information for effects of incubation time on diameter distribution, absorption, and loading efficiency).

The measured peak (\(d_{\text{peak}}\)) and estimated mean (\(\bar{d}_{\text{mean}}\)) diameters of EGs are 80.77 nm and 95.26 nm, respectively (Figure 1a). When doped with ICG, \(d_{\text{peak}}\) and \(\bar{d}_{\text{mean}}\) increase to 134.78 nm and 124.61 nm, respectively. Only about 5% of ICG leaks from the NETs after one hour (see Supplemental Information).

Transmission electron microscope (TEM) imaging confirms the nano-sized diameter of the NETs (Figure 1b). NETs imaged by TEM were fabricated by six extrusions through 100 nm membranes, and using 215 µM ICG.

Absorption spectrum confirms the elimination of EG’s hemoglobin content as evidenced by the absence of the signature absorption peaks of oxygenized hemoglobin at 542 and 576 nm, and that of deoxygenized hemoglobin at 556 nm (Figure 2a). The UV absorption for both non-loaded EGs and NETs is attributed to membrane proteins. Doping the EGs with ICG endows the constructs with NIR absorption capability.

The absorption spectrum of free ICG consists of spectral peaks at 780 and 707 nm, corresponding to the monomeric and dimeric (H-like aggregate) forms of ICG (Figure 2a), consistent with the well-established spectra of diluted (µM level) ICG in distilled water5. In comparison to free ICG, the absorption spectrum of NETs has several differences: (1) increased absorbance values for \(\lambda > 600\) nm, explained by the fact that higher ICG concentration was used to fabricate the NETs; (2) elimination of the previously distinct absorption peak associated with the H-like aggregate form of free ICG at 707 nm; (3) broadened absorption spectrum over the 600–935 nm band; and (4) bathochromic (red) shift from 780 to 783 nm (with respect to the spectral peak associated with the monomer form of ICG). We attribute these differences to the presence of various conformational states of ICG, and induced changes in molecular dipoles and polarizability of ICG when encapsulated into these constructs (Figure 2b). We offer further explanations in the discussion section.

The spectral peak of fluorescent emission from NETs in response to photo-excitation at 650 nm is at 695 nm , and originates from the aggregated forms of ICG within the constructs (Figure 3a). Spectral peaks of fluorescence for free 3.22 µM ICG in response to 650 nm excitation are at 695 nm and 793 nm, and correspond to the H-like aggregate and monomeric forms of ICG, respectively. Fluorescence emission of free ICG at the higher concentration of 43 µM is quenched, and resembles that of the EGs, which do not contain ICG. We summarize the spectral features of the NETs fabricated under the protocol reported here in Table 1.

Human dermal microvascular endothelial (HDME) cells were incubated with NETs or free ICG for three hours, and subsequently washed twice with phosphate buffer saline (PBS) prior to fluorescent imaging induced by photo-excitation at 740 ± 55 nm spectral band provided by a Nikon Mercury/Xenon arc lamp. Co-registration of
the NIR fluorescent emission from the constructs, and the visible fluoresce emitted by staining the nuclei of the cells produced images that suggest the NETs were internalized and localized to the nuclei periphery (Figure 3b). The maximum NIR emission intensity from HDME cells incubated with free ICG (Figure 3b, left panel) was eight times lower than that from the cells incubated with NETs (Figure 3b, right panel). This result indicates that free ICG was not effectively uptaken by the HDME cells since most of it was removed after washing the cells. However, cell wash was not as effective in removing the NETs, indicating that the uptake of the NETs by the HDME cells was greater than ICG uptake.

Temperature measurements indicate that NETs, suspended in PBS, are capable of eliciting a photothermal response in response to laser irradiation at 808 nm and incident intensity \( I_0 \) of 19.7 W cm\(^{-2} \) for 200 s (Figure 4a); however, sustained laser irradiation was accompanied by a reduction in temperature. Since these temperature measurements were made 2.0 mm away from the laser irradiated spots, they actually underestimate the true temperature of the NETs or ICG in response to laser irradiation. Nevertheless, these suggest that ICG within the NETs can be photo-degraded (see Supplemental Information for the effect of cyclic laser irradiation on temperature). To further investigate the photostability of the NETs, we obtained the absorption spectra of the NETs following laser irradiation at 808 nm and \( I_0 = 19.7 \) W cm\(^{-2} \) for different radiant exposure times (60 s–200 s). There was a progressive reduction in the absorption of NETs over the 630–950 nm spectral band, and increased UV absorption at 213 and 245 nm with increasing radiant exposure time (Figure 4b).

HDME cells were incubated with NETs or free ICG (positive control) for three hours, and subsequently washed twice with PBS prior to laser irradiation at 808 nm laser irradiation with \( I_0 = 19.7 \) W cm\(^{-2} \) for 200 s. Cells incubated in PBS without exposure to NETs or ICG were used as the negative control population. Live/dead assays demonstrate that NETs are capable of mediating photothermal destruction of the HDME cells (Figure 4c). There was a statistically significant difference \( (p < 10^{-4}) \) between the fraction of photo-thermally destroyed HDME cells after three hours of incubation with NETs, and those incubated with free ICG or PBS.

**Figure 2** | Absorption spectra and a physical model of a NET containing ICG. (a), Absorption spectra corresponding to free (non-encapsulated) ICG (3.22 \( \mu \)M) dissolved in PBS, and EGs and NETs re-suspended in PBS after fabrication. (b), A physical model of a NET showing an ensemble of ICG monomers, ICG aggregates, and monomers and aggregates of ICG bound to membrane lipids and/or membrane proteins. For illustration purposes, we present three main membrane integral proteins of erythrocytes: Aquaporin, Band3 and Glycophorin.

**Figure 3** | Fluorescence spectra and fluorescent images of human dermal microvascular endothelial (HDME) cells incubated with ICG or NETs. (a), Normalized fluorescence spectra in response to 650 nm photo-excitation of free ICG (3.22 and 43 \( \mu \)M) dissolved in PBS, and EGs and NETs re-suspended in PBS. Emission spectra were smoothed using IGOR Pro software with second order binominal algorithm. (b), Fluorescent images of HDME cells after three hours of incubation in vascular cell basal medium containing 13 \( \mu \)M ICG (control) dissolved in PBS (left panel), or NETs (right panel) at 37 °C and 5% CO\(_2\) in dark. A Mercury/Xenon arc lamp was used for photo-excitation at 740 ± 35 nm. Cells nuclei were stained by DAPI, and falsely colored in blue using the ImageJ software. A filter transmitting \( \lambda > 780 \) nm was used to collect the emitted NIR fluorescent, falsely colored in red. Scale bars on both panels correspond to 10 \( \mu \)m. The scale bar, 0–54354, corresponds to the NIR fluorescent emission intensity for both panels. The inset on the left panel represents the same image shown on the panel at the scale of 0–6565.
The results presented in Figures 3b, 4c, and 4d collectively demonstrate the effectiveness of NETs as photo-theranostic agents for combined fluorescence imaging and photothermal destruction.

To evaluate the potential cytotoxicity of the NETs, we incubated the HDME cells for three and 24 hours in the culture medium containing the NETs at effective dosage \((D_{\text{eff}})=12 \text{ ml}^{-1}\) suspended in PBS (see Methods section for definition of \(D_{\text{eff}}\)). Nearly all the HDME cells remained viable after three hours of incubation with NETs at \(D_{\text{eff}}=12 \text{ ml}^{-1}\) (Figure 5a). After 24 hours of incubating the HDME cells with NETs at \(D_{\text{eff}}=12 \text{ ml}^{-1}\), nearly 80% of the cells remained viable. In comparison, there was more than 90% cell death following 24 hours of incubation with methanol as the positive control. Illustrative fluorescent image of the HDME cells demonstrate the viability of most of the cells after 24 hours of incubation with NETs at \(D_{\text{eff}}=12 \text{ ml}^{-1}\) (Figure 5b).

**Discussion**

Spectral broadening of the NETs absorption, and the elimination of a distinct dimeric (H-like aggregate) peak are indicative of different conformations of ICG. Specifically, when ICG is incorporated into the EGs, an ensemble of ICG conformational states comprised of ICG monomers, ICG aggregates, and monomers and aggregates of ICG bound to membrane lipids and/or membrane proteins can form following 24 hours of incubation with methanol as the positive control. Illustrative fluorescent image of the HDME cells demonstrate the viability of most of the cells after 24 hours of incubation with NETs at \(D_{\text{eff}}=12 \text{ ml}^{-1}\) (Figure 5b).

| Table 1 | Spectral Features of 3.2 \(\mu\)M free ICG, and NETs* in PBS |
| Feature | Wavelength (nm) |
|-----------------|-----------------|
| Absorption peak of monomers in free ICG | 780 |
| Absorption peak of ICG monomers in NETs | 783 |
| Absorption peak of dimers (H-like aggregates) in free ICG | 707 |
| Absorption peak of dimers (H-like aggregates) in NETs | Not distinct |
| Fluorescence peak of monomers in free ICG (650 nm excitation) | 793 |
| Fluorescence peak of monomers in NETs (650 nm excitation) | 790 |
| Fluorescence peak of dimers (H-like aggregates) in free ICG (650 nm excitation) | 695 |
| Fluorescence peak of dimers (H-like aggregates) in NETs (650 nm excitation) | 695 |

*The spectral features of the NETs correspond to the constructs fabricated by 20 initial extrusions of EGs through 400 nm diameter porous membranes followed by 20 additional extrusions through 100 nm diameter membranes, using 215 \(\mu\)M ICG in hypotonic buffer solution, and five minutes of incubating the EGs with ICG.

Figure 4 | Photothermal response of NETs, and NETs-mediated photothermal destruction of HDME cells. (a), Photothermal response of NETs suspended in PBS, 13 \(\mu\)M free ICG dissolved in PBS (positive control), and PBS solution (negative control) in response to laser irradiation at \(\lambda = 808 \text{ nm}\) with incident intensity \(I_o=19.7 \text{ W cm}^{-2}\). The volume of all samples was 120 \(\mu\)l. The free ICG solution and NETs suspension samples were prepared to have nearly the same absorbance value of 0.6 at 808 nm. Temperatures were measured using a thermistor placed 2 mm outside the irradiated spot. (b), absorption spectra of four different PBS-suspended NETs samples (each 120 \(\mu\)l) following laser irradiation for various durations (60, 90, 140, and 200 s) at 808 nm and \(I_o=19.7 \text{ W cm}^{-2}\). (c), Fluorescent images of HDME cells after three hours of incubation with PBS (negative control), 13 \(\mu\)M free ICG (positive control) and NETs, followed by laser irradiation (\(\lambda = 808 \text{ nm}, I_o=19.7 \text{ W cm}^{-2}\)). The radiant exposure time in all three samples was 200 s. The volume of NETs suspension or free ICG added to the cells was 200 \(\mu\)l with nearly the same absorbance value of 0.6 at 808 nm. Live cells were stained using Calcein, and falsely colored in green. Dead cells were distinguished using Ethidium homodimer-1 (EthD-1), and falsely colored in red (Scale bars = 10 \(\mu\)m). (d), Percentage of HDME cells photothermally destroyed by NETs as assessed by a fluorescence microplate reader. Three different spots (each spot diameter = 2.2 mm) were irradiated in each well, resulting in irradiation of \(\approx 80\%\) of cells. Each bar represents the mean fraction of the dead cells for three different wells. Error bars correspond to single standard deviations. There was a statistically significant difference in fraction of cells photothermally destroyed by NETs (identified by the asterisk) as compared to those incubated in PBS or free ICG \((p < 10^{-4})\).
which was more intense than that from the monomer form of ICG peak at 793 nm from the monomeric form of 3.22
650 nm excitation, we still observed a distinct fluorescence emission (Figure 3a). The 650 nm excitation wavelength does not correspond
with the emission intensity of 3.22 m
the monomer form of ICG in NETs, is diminished in comparison
demonstrate that the emission intensity at 790 nm, associated with
ICG29.

albumin, and attributed to the interaction between albumin and
of the bound monomer produces a transition from ground to
attraction that produces the lower excited state (E
phase arrangement of transition dipoles leads to an electrostatic

ensemble (free) ICG. The effective NIR absorption cross section of the
ensemble components, confined within the nano-scale volume of
Figure 2b). The excited energy levels associated with these ICG ensemble components, confined within the nano-scale volume of NETs, can be different than those associated with non-encapsulated (free) ICG. The effective NIR absorption cross section of the ensemble (σensemble), which is directly related to NIR absorbance of the NETs, can be expressed as:

$$σ_{ensemble}(λ) = f_M σ_M(λ) + f_A σ_A(λ) + \sum_{i=1}^{n} f_{M^i} σ_{M^i}(λ) + \sum_{i=1}^{n} f_{A^i} σ_{A^i}(λ)$$

where f_M and f_A are the respective fractions of the monomeric and aggregated forms of ICG in the ensemble; σ_M and σ_A are the absorption cross sections of the monomeric and aggregated forms of ICG respectively; f_{M^i} and f_{A^i} are the respective fractions of the ensemble comprised of the monomeric and aggregated forms of ICG bound to a given membrane-associated molecule (i); σ_{M^i} and σ_{A^i} are the absorption cross sections of the monomeric and aggregated forms of bound ICG, respectively.

The observed bathochromic shift of the monomer absorbance in NETs is indicative of the changes in molecular dipoles and polarizability of ICG. We use the exciton theory for molecular assemblies to explain the origin of this bathochromic shift. In accordance with this theory, the excitonic state of the ICG monomer bound to membrane lipids or proteins can split into two levels (E" and E'). The in-phase arrangement of transition dipoles leads to an electrostatic attraction that produces the lower excited state (E'). Photo-excitation of the bound monomer produces a transition from ground to E', resulting in a bathochromic shift for the bound ICG monomer. A similar bathochromic shift in the monomer absorbance is reported for ICG dissolved in aqueous solution containing human serum albumin, and attributed to the interaction between albumin and ICG.

Fluorescence spectra in response to photo-excitation at 650 nm demonstrate that the emission intensity at 790 nm, associated with the monomer form of ICG in NETs, is diminished in comparison with the emission intensity of 3.22 μM free ICG at 793 nm (Figure 3a). The 650 nm excitation wavelength does not correspond to the spectral peaks associated with the monomeric forms of ICG in its free form or in the NETs (Figure 2a). The fact that in response to 650 nm excitation, we still observed a distinct fluorescence emission peak at 793 nm from the monomeric form of 3.22 μM free ICG, which was more intense than that from the monomer form of ICG in NETs, suggests that the fraction of ICG in its aggregated form is greater than its monomeric fraction within the NETs. However, in the 3.2 μM free ICG solution, there were sufficient number of monomers to elicit fluorescence emission.

While fluorescence is quenched in the case of 43 μM free ICG, NETs are still fluorescent despite the fact that they were fabricated using 215 μM ICG (with loading efficiency of 30%) to give an effective loaded concentration of 64.5 μM distributed into the population of the constructs (Figure 3a). This result suggests that the presence of the erythrocyte-derived membrane, as an encapsulating shell, protected the entrapped ICG molecules within each NET from interactions with other such entrapped ICG molecules to ultimately prevent fluorescence quenching. While ICG molecules still form aggregates within the NETs, the effective concentration of such aggregates within the NETs fabricated under the protocols described herein, was not sufficient to induce fluorescence quenching that would otherwise take place at high (e.g., 43 μM) concentrations of free ICG in solution. Therefore, by loading the ICG into the NETs, higher ICG concentrations that could otherwise result in fluorescence quenching of its free form, may be used. In our future studies, we will characterize the effects of ICG concentration over a broad range on the resulting excitation-emission spectra of the NETs and fluorescence quantum yields.

Continuous laser irradiation at the specific parameters investigated here can result in photo-degradation of the NETs (Figure 4b). The progressive increase in absorbance values at the spectral peaks of 213 and 244 nm with increased radiant exposure time is indicative of the conformational changes in the membrane proteins of the NETs due to photothermal denaturation. Our observed progression in reduction of the NET’s NIR absorption with sustained laser irradiation can result from photo-addition to the alternating double bonds in the polyene bridge of the ICG molecule to produce a leuco form of the dye with converted sp² to sp³ carbon hybridization, and/or induce cleavage of the π-conjugation along the bridge while keeping the aromatic benzoindotricarbocyanine moieties intact. The observation that NETs were somewhat more susceptible to photo-degradation than free ICG (Figures 4b and S4) suggests that entrapment of ICG within the NETs may weaken the alternating double bonds along the polyene bridge, and/or alter its electronic localization and polarizability, a proposition consistent with the excitonic theory described earlier. Our results are in agreement with those reported by Holzer et al. where there were progressively increased optical transmissions (i.e., lower absorption) through various solvents (e.g., H2O, D2O, human plasma) containing

Figure 5: Cytotoxicity assessment of NETs. (a), Percentage of live cells post incubation with NETs for 3 hours (solid green bar) and 24 hours (dashed green bar). Cells incubated with culture medium for 24 hours without any additional reagent were used as the positive control. Cells incubated with 100 μl methanol for 24 hours were used as negative control. Each bar represents the mean fraction of the live cells for three different wells. Error bars correspond to single standard deviations. Statistical analysis of the results for NETs (3 hours) compared to positive control yielded no significant difference in cell viability. Cells treated with methanol (negative control) and NETs for 24 hours (identified by asterisks) yielded statistically significant viability results as compared to the positive control population (p < 10−3). Fraction of the viable cells treated with NETs for 24 hours was significantly higher than those treated with methanol (p < 10−4). (b), Fluorescent images of HDME cells 24 hours post incubation with NETs (right panel) and culture medium (positive control) (right panel). Live cells were stained using Calcein, and falsely colored in green. Dead cells were distinguished using Ethidium homodimer-1 (EthD-1), and falsely colored in red (Scale bars = 100 μm).
free ICG as the laser irradiation time increased34. For example, these investigators reported nearly 12% increase in transmission of 785 nm light through a sample containing 10 μM ICG dissolved in H2O after 600 s of photo-irradiation at this wavelength and I0 = 0.17 mW cm−2; transmission subsequently increased to about 25% after 2,400 s of irradiation37.

The major potential advantages of NETs as compared to other types of dye-loaded nano-constructs are their safety, and longevity within the circulation. As constructs that can be engineered autologously, NETs offer the potential of being biodegradable, and non-immunogenic photo-theranostic platforms for use in personalized nanomedicine. Flower et al. reported no immune or allergic responses, even after multiple injections of ICG-loaded EGs into mononanomedicine. As constructs that can be engineered autologously, NETs offer the potential of being biodegradable, and non-immunogenic photo-theranostic platforms for use in personalized nanomedicine. Flower et al. reported no immune or allergic responses, even after multiple injections of ICG-loaded EGs into monkeys and rabbits despite the fact human-derived erythrocytes were used to fabricate the constructs35.

The natural lifetime of erythrocytes in systemic circulation is on the order of 90–120 days. While further studies are needed to determine the biodistribution and vascular retention time of NETs, the reported circulation times of erythrocytes-based carrier systems exceed those of synthetic vectors, which are on the order of several hours (e.g., <10 hours for polyethylene glycol-liposomes with diameters in the range of 50–500 nm)32. Hu et al., investigated the circulation dynamics of nano-constructs (~80 nm diameter) composed of a poly (lactic-co-glycolic acid) core coated with erythrocyte-derived membranes in mice32. The investigators reported that the constructs were retained in blood for three days with circulation half-life of nearly 40 hours32,32.

In a clinical study, patients with acute lymphoblastic leukemia (ALL) were injected with erythrocytes encapsulating L-asparaginase (L-ASNase) to treat ALL. L-ASNase loaded erythrocytes were still detectable within the vasculature at 24 days following the first injection37. As compared to non-encapsulated L-ASNase, the investigators reported a reduction in the number and severity of allergic reactions in those patients receiving the L-ASNase loaded erythrocytes.

In another study, 20 patients with ulcerative colitis received autologously-derived erythrocytes encapsulating dexamethasone 21-P (Dex 21-P) as the therapeutic agent34. At 14 days post-infusion, there were still detectable levels of Dex 21-P within the plasma. There were no steroid-related adverse effects in patients treated with erythrocyte encapsulating Dex 21-P as compared to 8 of the 10 patients receiving oral steroids.

In summary, we have engineered a new type of optical nano-construct composed of erythrocyte-derived membranes encapsulating the organic NIR chromophore, ICG. To-date, only iron oxide nanoparticles are approved by the FDA for use in conjunction with MRI. There are currently no FDA-approved nano-constructs as contrast agents for use in optical imaging modalities, and furthermore, as a photo-theranostic material for combined optical imaging and phototherapy. Given the existing FDA-approved status of ICG and prior clinical studies with erythrocyte-based delivery systems, NETs present a promising photo-theranostic candidate for clinical translation. For example, the enhanced permeability and retention effect in tumors may provide the basis for the delivery of NETs into tumors. Since particles with diameters <200 nm are more effective for extravasation into tumors35, NETs may prove a suitable material for combined optical imaging and photothermal destruction of tumors. Other potential applications may include dynamic imaging and photo- destruction of vascular abnormalities, and longitudinal evaluations to assess therapeutic interventions.

**Methods**

**Fabrication of NETs.** The schematic of the NETs fabrication procedure is shown in Fig. S1 (Supplemental Information). We collected whole blood from mice through mini extruder. The natural lifetime of erythrocytes in systemic circulation is on the order of 90–120 days. While further studies are needed to determine the biodistribution and vascular retention time of NETs, the reported circulation times of erythrocytes-based carrier systems exceed those of synthetic vectors, which are on the order of several hours (e.g., <10 hours for polyethylene glycol-liposomes with diameters in the range of 50–500 nm)32. Hu et al., investigated the circulation dynamics of nano-constructs (~80 nm diameter) composed of a poly (lactic-co-glycolic acid) core coated with erythrocyte-derived membranes in mice32. The investigators reported that the constructs were retained in blood for three days with circulation half-life of nearly 40 hours32,32.

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**Characterization of NETs.** The hydrodynamic diameters of NETs suspended in PBS were measured by dynamic light scattering (Zetasizer NanoZS90, Malvern Instruments Ltd). To prepare samples for TEM, a drop of NETs suspension in PBS was placed on a carbon-coated grid for 10 minutes. The grid was rinsed twice with double distilled water. To stain the NETs, a drop of 1% uranyl acetate solution was deposited on the grid and dried in air. The grid was imaged using a Philips Tecnai 12 Transmission Electron Microscope. The absorption spectra of NETs suspended in 2.4 μl of PBS were obtained using a UV-Vis spectrophotometer (Jasco-V670 UV-Vis spectrophotometer) with optical pathlength of 1 cm. The fluorescence spectra of NETs suspended in 650 nm excitation with a 450 W xenon lamp were obtained using a fluorometer (Fluorolog-3 spectrometer, Horiba Jobin Yvon). We obtained the normalized fluorescence spectra \( \frac{\zeta}{\lambda} = \frac{I_0}{1 - 10^{-\alpha I}} \) (2)

where \( A \) and \( F \) are the wavelength-dependent absorbance, and intensity of the emitted fluorsecent light, respectively.

**Fluorescence microscopy imaging of human dermal microvascular endothelial (HDME) cells.** To illustrate the effectiveness of NETs as photo-theranostic agents, we utilized HDME cells (ATCC® as model cell systems for fluorescence imaging and photothermal destruction. We cultured the HDME cells in 96 well culture plates containing vascular cell basal medium supplemented with 5% Fetal Bovine Serum (FBS) and endothelial cell growth Kit-VEGF for 24 hours in advance of imaging to ensure adhesion and cell density growth to \( \sim 10^5 \) cells/ml. The cells were then incubated with complete vascular cell basal media containing NETs or 13 μl ICG (control) for three hours. We then washed the cells incubated with NETs or free ICG twice with PBS prior to imaging. The cells nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI). The fluorescence emission from DAPI in the range of 435–485 nm was collected in response to 360 ± 20 nm excitation. In response to photo- excitation by a Nikon Mercury/Xenon arc lamp providing light the 740 ± 35 nm spectral band, the NIR emission (>570 nm) transmitted through a long pass filter was captured using an electron multiplier gained CCD camera (Quant EM- CCD, Hamamatsu) at integration time of 0.1 s and gain of 1.0.

**Photothermal response and photostability of NETs, and photodestruction of HDME cells.** To investigate the photothermal response of the NETs, we used a continuous wave near-infrared 808 nm diode laser with irradiation spot diameter of 2.2 mm, and incident irradiance \( I_{0} = 19.7 \text{ W/cm}^2 \). NETs suspended in PBS, and ICG dissolved in PBS were prepared in concentrations that gave the same absorbance value at 808 nm for both samples. As the negative control sample, we irradiated the PBS solution at the same irradiation parameters as above. Same volume of each sample (120 μl) was irradiated, and temperature changes were measured using a negative temperature coefficient thermistor (20 KΩ, Vernier) connected to a Vernier LabQuest placed 2 mm outside the irradiation spot. We investigated the photostability of the NETs by collecting their absorption spectra following laser irradiation of 120 μl of the NEts suspensions for various radiation times (60, 90, 120, and 200 s) at 808 nm. Additionally, we performed a cyclic laser irradiation, and measured the resulting temperature response (Supplementary Information).

To investigate the photo-destructive capability of the NETs, we cultured the HDME cells in a 96 well plate as described above. On the following day, cells were incubated with 200 μl of NETs suspended in PBS, or 200 μl (13 μM) of free ICG dissolved in PBS, in separate wells for three hours in dark at 37°C supplied with 5% CO2. These levels of NETs or ICG were used to ensure the same absorbance value at 808 nm in both samples. As the negative control sample, we irradiated the PBS solution at the same irradiation parameters as above. Same volume of each sample (120 μl) was irradiated, and temperature changes were measured using a negative temperature coefficient thermistor (20 KΩ, Vernier) connected to a Vernier LabQuest placed 2 mm outside the irradiation spot.

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Cytotoxicity assessment of NETs. To evaluate the potential cytotoxicity of the NETs, we cultured the HDEMs in 96 well plates as described above. On the following day, cells were washed with PBS and then incubated with complete cell culture media containing NETs for three and 24 hours. The effective dosage (D_{50\text{eff}}) of the NETs for cytotoxicity experiments was 12 ml^{-1} where we define D_{50\text{eff}} as the absorbance of the NETs population (1.2 at 808 nm) suspended in 100 μl of PBS solution. Cells incubated with complete cell culture media for 24 hours without any additional reagents were used as the positive control population. Cells incubated with 100 μl methanol for 24 hours were used as the negative control population. Cells were washed twice with PBS post incubation with NETs, and stained using the live/dead assay described above.

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