Optical-Property-Enhancing Novel Near-Infrared Active Niosome Nanoformulation for Deep-Tissue Bioimaging

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ABSTRACT: Indocyanine green (ICG) is a clinically approved near-infrared (NIR) contrast agent used in medical diagnosis. However, ICG has not been used to its fullest for biomedical imaging applications due to its low fluorescence quantum yield, aqueous instability, concentration-dependent aggregation, and photo and thermal degradations, leading to quenching of its fluorescence emission. In the present study, a nanosized niosomal formulation, ICGNiosomes (ICGNios), is fabricated to encapsulate and protect ICG from degradation. Interestingly, compared to free ICG, the ICGNios exhibited higher fluorescence quantum yield and fluorescence emission with a bathochromic shift. Also, ICGNios nanoparticles are biocompatible, biodegradable, and readily uptaken by the cells. Furthermore, ICGNios show more enhanced fluorescence intensity through ∼1 cm thick chicken breast tissue compared to free ICG, which showed minimal emission through the same thickness of tissue. Our results suggest that ICGNios could offer a promising platform for deep-tissue NIR in vivo imaging to visualize inaccessible tissue microstructures for disease diagnosis and therapeutics.

INTRODUCTION

Near-infrared (NIR) fluorescence imaging has attracted tremendous attention in biomedical science as it greatly improves imaging performance due to negligible tissue autofluorescence, reduced light scattering, and absorption. Among various NIR-active fluorophores, indocyanine green (ICG) is one of the US Food and Drug Administration (FDA)- and European Medicines Agency (EMA)-approved chromophores used for clinical applications. However, ICG has not been used to the fullest for diagnostic and imaging applications due to its aqueous instability, rapid optical and thermal degradation, and concentration-dependent aggregation, leading to quenching of its fluorescence emission. Further, nonspecific binding of ICG to blood plasma proteins leads to a relatively short circulation half-life. To overcome these limitations, one of the promising approaches is to encapsulate ICG within a nanocarrier. With advancement in nanotechnology, various types of nanoparticles (NPs) such as metals, liposomes, polymers, and proteins have been developed to encapsulate ICG for biomedical applications.

Among various types of NPs, vesicular systems have become an important platform for pharmaceutical applications. These vesicular bilayers are made of amphiphilic molecules, which are capable of trapping hydrophilic and hydrophobic theranostic agents. Vesicular systems such as liposomes have short stability and a high cost of production. Therefore, finding an alternative vesicular system with similar properties as liposomes is greatly needed. Niosomes, synthesized from nonionic surfactants, are cost-effective vesicular systems with amphipathic characteristics, neutral charge, and self-assembly properties. Niosomes are colloidal nanocarriers made from nonionic surfactants and cholesterol. Nonionic surfactants have a hydrophilic head group connected to a hydrophobic tail and possess a neutral charge. Niosomes have an aqueous cavity similar to that of liposomes, but the neutral charge on nonionic surfactants makes them less toxic than liposomes. Additionally, cholesterol acts as a helper lipid that hinders the interaction of niosomes with proteins of the immune system and enhances their stability within body fluids. Moreover, the physical characteristics and the preparation method of unilamellar or multilamellar structures of niosomes are similar to those of liposomes. However, niosomes, compared to liposomes, have unique properties such as a longer storage stability, possess cost-effective raw materials, and can be loaded spontaneously with both hydrophilic and hydrophobic molecules. Interestingly,
cosmetic industries were the first to utilize niosomes as alternatives to phospholipid-based vesicles due to their low-cost production, enhanced stability, and easy storage.24,25 Later on, niosomes were also being assessed to deliver theranostic agents like genes, siRNA, and vaccines.26−30

In this study, NIR-active theranostic nanocarrier ICGNiosomes (ICGNios) were developed by encapsulating ICG within niosomes composed of nonionic surfactants (Tween 80 and Span 80) and cholesterol. These ICGNios are synthesized by hydration of the thin film of nonionic surfactants using DI water and ICG dissolved in DI water.

Figure 1. Schematic representation of the preparation of niosomes and ICGNios by the thin-film hydration method. Tween 80, Span 80, and cholesterol are dissolved in chloroform followed by the formation of a thin film by evaporating chloroform using rotary evaporation at 37 °C. Niosomes and ICGNios are fabricated by hydration of the thin film of nonionic surfactants using DI water and ICG dissolved in DI water.

Figure 2. Morphological characterization of niosomes and ICGNiosomes (ICGNios). (a) Scanning electron microscopy (SEM) image of niosomes, (b) SEM image of ICGNios, and (c) transmission electron microscopy (TEM) image of ICGNios. Scale bar is 200 nm. (d, e) Dynamic light scattering (DLS) measurement of niosomes and ICGNios depicting the hydrodynamic diameter (Dh) and the polydispersity index (PDI) of niosomes and ICGNios.
hydration technique, which is cost-effective and encapsulates ~62.5% of ICG. Spectral measurements of ICGNios show a peak shift toward longer wavelengths with enhancement in fluorescence emission. The ICGNios showed enhanced optical stability as compared to free ICG. Also, niosomes are biocompatible and could be used as nanocarriers for biomedical applications. The in vitro NIR imaging has shown enhanced uptake of ICGNios with improved fluorescence intensity compared to free ICG. Further, the ex vivo imaging of ~1 cm thick chicken breast tissue using ICGNios enabled the visualization of a clear microcellular structure compared to free ICG. Therefore, the fabricated novel ICGNios present a biocompatible, biodegradable, versatile, and cost-effective contrast agent for deep-tissue NIR imaging and could easily be transferred to clinics in the future.

### RESULTS AND DISCUSSION

Figure 1 shows the schematic representation of niosomes and the ICGNios fabrication process. Niosomes were prepared using the thin-film hydration technique. For this, Tween 80, Span 80, and cholesterol were mixed in the molar ratio of 0.4:0.4:0.2 and dissolved in chloroform. To obtain the thin film, the chloroform was dried using a rotary evaporator at 37 °C under an ultrahigh vacuum. This was followed by hydration of the thin film by 5 mL of deionized (DI) water for fabrication of empty niosomes, and for ICGNios, ICG was dissolved in DI water. After hydration, spherical-shaped niosomes and ICG-loaded niosomes were obtained, which were used for further studies.

#### Morphological Characterization of Niosomes and ICGNios.

The morphology and size of empty niosomes and ICGNios were characterized by field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM). The SEM image (Figure 2a) shows that the niosomes appear spherical, and the size is ~250 nm. However, both SEM and TEM images (Figure 2b,c) show that when ICG is encapsulated within the niosome NPs, they become more compact, and the size is reduced a bit to ~200 nm, whereas the shape remained spherical. This could be attributed to the strong binding of the lipophilic moieties of ICG with the lipophilic part of the niosomes, which might result in a reduction in the size of the ICGNios after encapsulating ICG. The hydrodynamic diameter (Dh) distribution of niosomes and ICGNios was studied by DLS measurements. The estimated average diameter of niosomes was ~262 nm with a polydispersity index (PDI) of 0.29. However, after loading with ICG, the average diameter of ICGNios reduced to 210 nm with a PDI of 0.27 (Figure 2d,e). These results suggest that electrostatic interaction between niosomes and ICG plays a significant role in reducing the size and PDI of ICGNios compared to niosomes, which is crucial for their use in biomedical applications.

The encapsulation efficiency (EE) of the niosomes signifies the efficacy of vesicles to load therapeutic agents. Therefore, the EE of ICG within ICGNios was estimated using eq. 1. For optimizing the EE of ICG, the ICGNios were prepared with different ICG concentrations: 50, 100, and 200 μM. For quantification of loaded ICG, the ICGNios NPs were solubilized and disintegrated using dimethyl sulfoxide (DMSO), and the absorption at 794 nm was measured. The EE of the ICGNios formulation using different concentrations of ICG is shown in Table 1. The loading efficiency of the niosomes seems to increase with the increasing concentration of ICG. Also, the highest EE (~62.5%) was obtained when niosomes were fabricated using 200 μM ICG and are used for further studies.

| Table 1. ICG Encapsulation Efficiency (EE) Using Different Concentrations of ICG for ICGNios Fabrication |
|---|---|---|
| s. no. | concentration of ICG used for ICGNios formulation (μM) | ICG EE (%) |
| 1. | 50 | 47.5 |
| 2. | 100 | 50 |
| 3. | 200 | 62.5 |

#### Biochemical Characterization of Free ICG and ICGNios.

The absorption and fluorescence emission properties of free ICG, niosomes, and ICGNios were studied to validate ICG encapsulation within the NPs. The absorption spectra revealed that the absorption of ICGNios were enhanced and showed ~20 nm bathochromic shift from 778 to 798 nm compared to free ICG (Figure 3a). This remarkable red shift (~20 nm) in the absorption spectrum of ICGNios suggests an active interaction of ICG with the niosomes. Essentially, the absorption peak shift of ICGNios to the longer wavelength (~798 nm) allows optical imaging in the NIR window that can penetrate deeper into tissues. On the other hand, empty niosomes do not show any absorption peak in this wavelength range. Further, to validate peak shifting in ICGNios, Gaussian peak fitting was performed in the major absorption spectra of free ICG and ICGNios. The peak position of the monomeric form of ICG was found at 782 and 801 nm for free ICG and ICGNios, respectively. However, the peak width of ICGNios (~42.3 nm) was not changed significantly compared to the free ICG (~39 nm). The fitted absorption spectra revealed that a red shift occurs in the ICGNios sample for both the monomeric and dimeric shoulder peaks of nanoencapsulated ICG compared to the free form of ICG (Figure 3b,c). The red shift in the absorbance of ICGNios compared to free ICG might be due to the formation of ICG J-aggregates (IJA) by the interaction of amphiphilic ICG with the niosomal membrane as reported previously with different ICG-loaded nanoparticles. Thus, the absorption peaks of ICGNios indicate that ICG is successfully encapsulated within niosomes in the form of IJA, which is an optically stable form. Once encapsulated, IJA remains stable in a J-aggregated state in different solvents and shows excellent optical properties, which is crucial for NIR imaging for diagnosis and treatment. Following the absorption assessments, the fluorescence emission measurements of free ICG, niosomes, and ICGNios were carried out. The free ICG shows maximum fluorescence emission (λmax) at 810 nm, while the λmax of ICGNios is shifted to 830 nm (Figure 3d). In addition to the fluorescence peak shift, an ~1.5-fold fluorescence enhancement is also observed in the case of ICGNios, as shown in Figure 3d. Here, all fluorescence spectra were smoothened using a Savitzky–Golay filter. This fluorescence enhancement might be due to the interaction between ICG molecules and surfactants, which reduces the random vibration and/or rotation of ICG molecules. In accordance with the enhancement of fluorescence emission of ICGNios as compared to free ICG, the quantum yield Φ of ICGNios was determined using eq ii. The Φ of ICGNios was estimated relative to that of free ICG (Φref = 0.016) in water. The measured Φ of ICGNios is 0.0202, which suggests that with the enhancement in fluorescence intensity, the ICGNios also improved the quantum yield of ICG. Also, the enhanced quantum yield suggests a higher-order arrangement of ICG molecules within the ICGNios, forming a stable structure by preventing free molecular vibrations and motions. Thus, an overall analysis
of the optical characteristics (absorption and emission) of samples confirms the encapsulation of ICG within ICGNios. The higher quantum yield and red shift at longer wavelengths of ICGNios are advantageous and could be used to improve the imaging capabilities for deep-tissue NIR bioimaging for disease diagnosis.

**Optical and Storage Stability of ICGNios.** Free ICG is an optically unstable NIR chromophore as it gets degraded when exposed to light. Hence, for the wide use of ICG for bioimaging or photothermal applications, its photostability should be preserved by encapsulating the ICG within the nanocarriers. Here, we encapsulated ICG within niosomes, and the photostability of the free ICG was compared with that of ICGNios by placing both samples in ambient light at room temperature for 48 h. The absorbance of the samples was recorded at different time intervals to check the photostability of free ICG and ICGNios. The result indicates that free ICG degraded about ∼90%, while ICGNios degraded only 25% over the 48 h time, as shown in Figure 4a. This result suggests that ICG encapsulated within niosomes is more optically stable as compared to free ICG. In addition to this, free ICG also becomes degraded over a period of time, even at low temperatures such as 4 °C. However, the rate of degradation would proportionately increase with the rise in temperature.33,40 The storage stability of ICG could also be increased by encapsulating it within nanocarriers. The storage stabilities of free ICG and ICGNios were studied after four...
weeks of storage at 4 °C in the dark. Interestingly, we found that ICGNios showed ~40% degradation after 30 days of storage at 4 °C in the dark condition and free ICG showed ~80% degradation in the same conditions (Figure 4b). Thus, the long-term stability of ICGNios is advantageous for its use in biomedical applications.

To further improve the optical stability and fluorescence property of the ICG, niosomes can be loaded with the ICG−albumin complex. The ICG−albumin interaction showed an improvement in the optical stability of ICG with the enhancement of fluorescence emission and quantum yield.41 Therefore, loading of the ICG−albumin complex within niosomes might further enhance the fluorescence quantum yield of ICG. Additionally, the noncovalent binding of albumin with ICG enhances the water solubility of ICG. As a result, ICG becomes encapsulated within the inner aqueous core of niosomes, which could further enhance the optical stability of ICG.42

**Biosafety of ICGNios.** The NPs used for different biomedical applications should be nontoxic and biodegradable. Therefore, the biosafety of ICGNios and niosomes was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-

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Figure 5. Biocompatibility and NIR imaging of niosomes, ICGNios, and free ICG in HeLa cells. (a) MTT assay of niosomes and ICGNios, where PC is the positive control. (b) Fluorescence imaging of HeLa cells; the DAPI staining nuclei are shown in blue color, and ICG fluorescence emission is displayed in red color. Scale bar is 20 μm. (c) Mean fluorescence emission intensity plot of the control, free ICG, and ICGNios.

Figure 6. *Ex vivo* deep-tissue enhanced NIR imaging of chicken breast tissue of ICGNios vs free ICG. (a) Picture of capillary tubes filled with water, free ICG, and ICGNios placed over a chicken tissue sample with the thickness of ~1 cm. (b) Bright-field and NIR bioimaging of CT filled with water, free ICG, and ICGNios (column 1 and 2) and chicken breast tissue with CT filled with water, free ICG, and ICGNios (columns 3 and 4). Scale bar is 100 μm. (c) Mean fluorescence intensity plot of chicken breast tissue placed beneath the CT filled with water, free ICG, and ICGNios.
trazolium bromide (MTT) assay. For this, different concentrations of niosomes and ICGNios (0.5, 2, 10, 20, 25, 50, and 100 μM) were used. Figure 5a shows that more than 80% of the cells were viable even when the cells were treated with the maximum concentration (100 μM) of ICGNios and niosomes. The HeLa cells without any treatment were used as a control, while the cells incubated with a solubilizing buffer were used as a positive control, which showed less than 5% cell viability. This result suggests that niosomes and ICGNios are safe to be used as nanocarriers for NIR bioimaging and other biomedical applications.

**In Vitro NIR Bioimaging Using ICGNios.** The NIR optical imaging was performed to determine the imaging ability of ICGNios in comparison to free ICG. For this, the HeLa cells were incubated with freshly prepared ICGNios and free ICG for 4 h. Cells incubated with media were used as a control. After four hours, the fluorescence images of control cells, ICG, and ICGNios-treated HeLa cells were recorded. The nuclei of cells were counterstained with DAPI to understand the intracellular distribution of ICG (Figure 5b). The NIR fluorescence imaging of HeLa cells shows that ICGNios are readily internalized within the cells to release ICG in the cytoplasm and emit higher fluorescence as compared to the free ICG-treated cells. Control cells did not show any NIR emission. This suggests that the ICGNios are efficient nanocarriers and can be used as contrast agent delivery vehicles for NIR imaging. Further, the mean fluorescence intensity (MFI) of fluorescence-emitting cells was quantified (Figure 5c). The MFI measurement supports the cellular uptake results (Figure 5b), where control cells showed negligible fluorescence intensity and ICGNios-treated cells showed ~1.5-fold higher NIR fluorescence emission than free ICG-treated cells (Figure 5c). These results suggest that the enhanced cellular uptake of ICG is due to the efficient binding of ICGNios to the cells, followed by the internalization and release of ICG in the cytoplasm. These findings indicate that ICGNios could be used as exogenous contrast agents for NIR fluorescence bioimaging.

**Ex Vivo Deep-Tissue NIR Imaging.** The fluorescence emission spectra of ICGNios showed an enhancement and a red shift compared to free ICG (Figure 3d). This property could be helpful in deep-tissue NIR bioimaging. For this purpose, ex vivo NIR bioimaging was done with chicken breast tissue of ~1 cm thickness. Capillary tubes (CTs) of 75 mm length and 1 mm inner diameter were filled with free ICG (10 μM), ICGNios, and water, separately. These CTs were then placed over the thick breast tissue sample for deep-tissue NIR bioimaging (Figure 6a). Ex vivo fluorescence images were recorded in reflectance geometry. Figure 6 shows the bright-field (BF) and NIR fluorescence images of CTs (filled with water, ICG, and ICGNios) and chicken breast tissue samples (placed with different CTs). The tissue portion placed with ICGNios-filled CT showed a higher fluorescence emission as compared to the tissue with ICG-filled CT (Figure 6b). Similarly, the NIR imaging of CT filled with ICGNios showed a significantly higher fluorescence intensity as compared to free ICG-filled CT (Figure 6b). However, the water-filled CT did not show any NIR fluorescence emission (Figure 6b). Further, the mean fluorescence intensity (MFI) of the tissue section with ICGNios-filled CT showed ~7-fold higher fluorescence intensity as compared to the tissue section with free ICG-filled CT (Figure 6c). This result suggests that the depth of tissue penetration is enhanced by ICGNios and can be detected across ~1 cm thick chicken breast tissue samples. Thus, this novel nanoformulation could offer a promising platform for deep-tissue NIR imaging to visualize inaccessible cellular microstructures for biomedical applications.

**CONCLUSIONS**

In this study, novel NIR-active niosomes (ICGNios) are fabricated using nonionic surfactants (Tween 80, Span 80) and cholesterol for NIR bioimaging. The ICGNios exhibited enhanced absorbance and fluorescence emission compared to free ICG. Additionally, the ICGNios displayed excellent photostability upon 48 h of light exposure. Moreover, the ICGNios showed only ~40% degradation after 30 days of storage at 4 °C compared to the free ICG, which showed ~80% degradation. Thus, ICGNios provides a simple approach to enhance the fluorescence quantum yield and optical stability of ICG by protecting it from photodegradation and improving the long-term storage stability. Further, the ICGNios are biocompatible and are safe for biomedical applications. Additionally, the NIR fluorescence of ICGNios could be detected through ~1 cm thick chicken breast tissue, which was not possible using a free form of ICG. Thus, the novel ICGNios with improved optical and storage stability of ICG are promising cost-effective platforms for deep-tissue NIR fluorescence bioimaging.

**MATERIALS AND METHODS**

**Materials.** ICG, Span 80, and Tween 80 were purchased from Sigma-Aldrich (St. Louis, MO). Cholesterol was purchased from LobaChemie (Mumbai, India), and chloroform was purchased from MP Biomedical LLC. The stock solution of ICG was prepared in deionized (DI) water (18.2 MΩ Millipore, Sartorius system) and stored at ~20 °C. DAPI (2-(4-Amidinophenyl) indole-6-carboxamide-dihydrochloride) was used for staining the nucleus and was procured from the Tokyo Chemical Industry (TCI, Shanghai, China). HeLa cells were procured from the National Centre for Cell Science (NCCS) Pune, India. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and 0.25% trypsin-1 mM ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Invitrogen).

**Niosome Preparation.** Nonionic surfactant mixtures consisting of Tween 80, Span 80, and cholesterol were used to prepare niosomes by a thin-film hydration method at a specified molar ratio of 0.4:0.4:0.2. In brief, surfactants and cholesterol were dissolved in 500 μL of chloroform in a 10 mL round-bottom flask. A rotary evaporator apparatus was used to evaporate the solvent at 37 °C under a high-vacuum atmosphere for thin-film formation and kept in a desiccator for removal of residual chloroform. The film was hydrated with DI water to achieve a final niosome concentration of 5 mM (Tween 80: 2 mM; Span 80: 2 mM; and cholesterol: 1 mM). For fabricating ICGNios, 50, 100, and 200 μM stock solutions of indocyanine green (ICG) were used for hydration of the thin film, which was done while stirring the mixture followed by a vigorous vortexing. The ICGNios were then pelleted down by centrifugation at 20 000g for 3 hours to remove unencapsulated ICG. The pellet containing ICGNios was resuspended in DI water for further use.

**Encapsulation Efficiency Estimation of ICG within Niosomes.** To determine the ICG encapsulation efficiency (EE) of niosomes, the ICGNios were centrifuged, and the pellet of ICGNios was resuspended in dimethyl sulfoxide (DMSO) for the disintegration of NPs to release ICG from niosomes. The
absorption of released ICG was taken by UV–vis spectrophotometry, and the concentration was calculated using the standard curve of free ICG in DMSO. The EE was calculated by eq i

\[
EE(\%) = \frac{\text{concentration of ICG in ICGNios disintegrated in DMSO}}{\text{total concentration of ICG used for ICGNios synthesis}} \times 100
\]  

(i)

Morphological Characterization. The morphologies of niosomes and ICGNios were assessed by a field emission scanning electron microscope (FESEM) (Supra-55, Carl Zeiss Sigma Series). For SEM imaging, samples were dropcast on a glass slide and dried overnight in a desiccator. Following drying, the samples were placed on carbon tape, mounted on an aluminum stub, and sputter-coated with gold by a direct current (DC) sputter coater (Q-150RES, Quorem). The operating voltage of the electron gun was kept between 2 and 5 kV with a working distance of ∼10 mm. SEM images were analyzed using Image J software.\(^{43}\) The accurate size of ICGNios was also determined by a transmission electron microscope (TEM) operating at 200 kV (Philips CM-200). Using TEM imaging, the sample was placed on a 400-mesh carbon-coated copper grid and was negatively stained using uranyl acetate (1%). The hydrodynamic diameters and polydispersity indexes (PDIs) of niosomes and ICGNios were studied by dynamic light scattering (DLS) spectroscopy (NanoPlus-3, Micromeritics) equipped with a diode laser of 660 nm at 25 °C in DI water. For the size measurements, the geometry of the scattering light collection was 175°.

Spectroscopic Investigation. The biochemical characterization of ICG encapsulation within niosomes was studied by absorption and emission analyses. The absorption measurements of all samples were performed using a UV–vis–NIR spectrophotometer (Lambda-35, PerkinElmer), and fluorescence spectra of the samples were recorded using a spectrofluorimeter (FL3-21, Jobin Yvon, Horiba). The samples were excited using a 400 W xenon lamp with an excitation wavelength (\(\lambda_{\text{ex}}\)) of 680 nm. The fluorescence was measured with excitation and emission slit widths of 5 and 2 nm, respectively. Further, the fluorescence spectral data were collected and analyzed using Origin 2021 software (OriginLab Corporation, Northampton, MA).

Fluorescence Quantum Yield Calculation (\(\Phi_f\)). For calculation of \(\Phi_f\), the test (ICGNios) and reference (Free ICG) samples with similar absorbance at the excitation wavelength were used as they are assumed to absorb the same number of photons.\(^{44}\) The absorbance spectra of all samples were recorded using a UV–vis–NIR spectrophotometer (Lambda-35, PerkinElmer), and fluorescence spectra of the samples were recorded using a spectrofluorimeter (FL3-21, Jobin Yvon, Horiba). For the relative \(\Phi_f\), the \(\lambda_{\text{ex}}\) and \(\lambda_{\text{em}}\) were 680 nm with the excitation/emission slit widths of 5 and 2 nm. The \(\Phi_f\) of the ICGNios was compared with free ICG based on the absorption and fluorescence spectra. The relative \(\Phi_f\) was calculated using eq ii\(^{45}\)

\[
\frac{\Phi_f}{\Phi_{\text{ref}}} = \left(\frac{A_{\text{ref}}}{A_{\text{S}}}\right) \times \left(\frac{F_S}{F_{\text{ref}}}\right) \times \left(\frac{\eta_s}{\eta_{\text{ref}}}\right)^2
\]

(ii)

where \(\Phi_f\) and \(\Phi_{\text{ref}}\) are the quantum yields of the test (ICGNios in water) and reference (Free ICG in water) samples, respectively; \(F_S\) and \(F_{\text{ref}}\) are the areas under the emission curve of the test and reference samples, respectively; \(A_S\) and \(A_{\text{ref}}\) denote absorbances of the test and reference samples at the excitation wavelength, respectively; and \(\eta_s\) and \(\eta_{\text{ref}}\) represent refractive indexes for the test and reference samples suspension media, respectively. The \(\Phi\) of free ICG and ICGNios in ultrapure water is determined by comparison with a freshly prepared ICG as a standard (\(\Phi_{\text{ref}} = 0.016\)).\(^{38}\)

Photostability Study. The photostability study of free ICG and ICGNios was performed by incubating samples at room temperature under the exposure of ambient light for 48 h. The experiment was performed by placing the samples under an ambient light (Philips fluorescent lamp) at a distance of 15 cm. Further, the absorbances of both ICG and ICGNios were measured at different time points (0, 2, 4, 12, 24, and 48 h) using a UV–vis–NIR spectrophotometer. The percentage of stable ICG was calculated using eq iii

\[
\% \text{ of stable ICG} = \frac{\text{absorbance of ICG at each time point}}{\text{absorbance of ICG at initial time point}} \times 100
\]  

(iii)

Storage Stability Study. For storage stability assessment, free ICG (5 μM) and ICGNios were kept at 4 °C in the dark for a period of 30 days. The absorbance of both samples was taken on 0 days and 30 days, and their stabilities in terms of percentage were calculated.

Cell Culture. The cervical cancer cell line (HeLa) was used for in vitro NIR imaging. HeLa cells were cultured in DMEM media supplemented with 10% FBS and 100 units/mL penicillin-streptomycin. HeLa cells were incubated at 37 °C supplemented with 5% carbon dioxide (CO₂). Cells were further subcultured when the cell density reached ∼80% confluency by detaching with 0.25% trypsin-EDTA.

MTT Assay. HeLa cells were seeded in 96-well plates at a density of 1 × 10⁵ cells/well and incubated for 24 h at 37 °C in a 5% CO₂ incubator. Different concentrations of empty niosomes and ICGNios (0.5–100 μM) were added to 96-well plates in triplicates and incubated for 4 h at 37 °C in a 5% CO₂ incubator. After incubation, 100 μL of MTT (0.5 mg/mL in PBS) was added to the wells and incubated for 4 h at 37 °C in a 5% CO₂ incubator. The supernatants were then removed, and 100 μL of DMSO was added to each well. Formazan formation was quantified by reading the absorbance of a wavelength of 570 nm using a microplate reader (Biotek, Winooski VT). Controls were considered as follows: positive control (PC) = untreated cells + MTT + solubilizing buffer (10% SDS in 0.1 N HCl) + DMSO and control = untreated cells + MTT. Further, cell viability was calculated by eq iv

\[
(\% \text{cell viability}) = \frac{\text{absorbance for treated cells}}{\text{absorbance for control cells}} \times 100
\]  

(iv)

In Vitro NIR Bioimaging Using Free ICG and ICGNios. The cellular uptake of ICGNios by cancerous cells (HeLa) was evaluated by NIR bioimaging. HeLa cells were seeded onto glass coverslips in a six-well plate with a cell density of ∼1 × 10⁵ cells/well and were incubated for 16 h at 37 °C for adherence. The next day, the medium was replaced with a fresh medium and further treated with ICGNios and free ICG for 4 h. For control (untreated cells), the cells were incubated with a plain medium for 4 h in an incubator at 37 °C with 5% CO₂ supply. Further, the medium was removed, and cells were washed with 1× PBS and fixed with 4% paraformaldehyde for 20 min. The cell nuclei were then stained with DAPI for 5 min. The slides were prepared by placing a coverslip onto mounting media for visualization. The
NIR imaging was done using a Nikon Eclipse Ti-U inverted microscope. The cells were visualized by mercury and xenon lamp excitations for DAPI and ICG, respectively. Fluorescence intensity mean values were analyzed using Image J software.

**Ex Vivo NIR Imaging for Tissue Depth Penetration.** Chicken breast tissue samples of ~1 cm thickness were used to assess fluorescence detection of ICG and ICGNios through the tissue. The capillary tubes (CT) with 1 mm inner diameter were filled with free ICG (10 μM), and ICGNios constituting an equivalent concentration of ICG were placed above the tissue sample. Bright-field and NIR images were captured using a Nikon Eclipse Ti-U inverted microscope with a 5× objective lens.

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### Notes

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