

Nanoscale Chlorophyll-Liposome Composite (NCLC) Fluorescent Probe for In Vivo Bio-imaging

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Abstract Green technology for in vivo bioimaging is being developed for its biocompatibility and efficacy to serve as fluorescent probes in interior environment of the system. Herein, the preparation of nanoscale chlorophyll-liposome composite (NCLC) from the leaf extract of *Tridax procumbens* having near IR fluorescence is reported. The prepared NCLC has been characterized and confirmed using UV–vis spectroscopy, Fluorescence spectroscopy, Fourier transform infrared spectroscopy and Field emission-Scanning electron microscopic studies. Fluorescence microscopic images of *Artemia nauplii* and *Penaeus vannamei* after treatment with NCLC revealed its uptake evident from the appearance of red fluorescent granules in its alimentary canal which infers its possible utilization as an in vivo bioimaging tool (nanoprobes) on conjugation with specific antibodies for disease diagnosis in aquaculture.

Keywords Nanoscale chlorophyll-liposome composite · Fluorescent probe · In vivo bioimaging

Introduction

Fluorescence-based optical bio-imaging based on small organic molecules have become indispensable in modern biology as it provide dynamic and reliable information pertaining to the localization of the molecules of interest [1, 2].

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Numerous chemical and biological moieties play a significant role in the fabrication of fluorescent probes. Fluorescent probes are excellent sensors for biomolecules, being susceptible, instantaneous and capable of affording high spatial resolution via microscopic imaging. Suitable fluorescent probes are of great importance for bioimaging, however, their implications are vastly limited due to the lack of strategies in flexible designing as a bioimaging tool [3]. Fluorescent materials in a broad diversity including fluorescent proteins, organic dyes and inorganic nanoparticles have been employed as probes for various bio-imaging applications. More recently, probes based on nanomaterials [4, 5] and conjugated polymers [6] gained interest and have been applied for in vivo imaging. Nanomaterials as clusters exhibit fluorescence emission at different wavelengths. Metallic nanomaterials in the form of gold and silver nanoclusters have demonstrated trivial fluorescence emission which finds application in in vivo bioimaging [7, 8].

The preparation of biocompatible and cost-effective fluorescence probes is the need of the hour. Various methods have been adopted in the development of highly stable and fluorescent probes for in vivo imaging applications. Among the fluorescent probes, NIR fluorescent probes are attractive molecular tools because of their low autofluorescence interference, deep tissue penetration and minimal damage to the sample. In this concern, a unique approach has been developed toward NIR fluorescent probe with remarkably enhanced contrast. The probe has been developed with excellent photophysical properties with significantly enhanced image contrast and apparent brightness in biological imaging applications in comparison with traditional dyes [9]. Many fluorescent pigments are being used for bio-imaging. Among the natural pigments, chlorophyll, a cyclic tetrapyrrole-based molecule is structurally similar to porphyrin (a fluorescent dye) and has near IR fluorescence [10, 11]. It plays an important role in light-harvesting and transfer of electrons and energy in plants [12, 13]. The presence of chlorophyll a and b in almost all higher plants has been reported. As a food additive, chlorophyll is present in a variety of foods and beverages that are permitted to contain chlorophyll. Being recognized as a safe material having potent fluorescent properties, chlorophyll has been chosen as a candidate for in vivo bio-imaging studies. However, application of chlorophyll as an in vivo bio-imaging tool is greatly hampered due to its poor solubility in water. Hence, to enhance the water solubility of chlorophyll for in vivo bio-imaging applications, chlorophyll was encapsulated into nanoscale liposomes. Liposomes are small spherical vesicles made up of lipid bilayer that can serve as carriers for the delivery of various diagnostic and therapeutic drugs [14].

Tridax procumbens L. of family Asteraceae, is a common medicinal herb used by ethno-medical practitioners, [15]. It has been used in treating various ailments like wound healing, as anti-coagulant, anti-fungal agent and in the treatment of various infectious skin diseases [16]. Hence, the usage of this plant extract for the preparation of chlorophyll nanocomposites fluorescent probe could have great potential for bio-imaging due to their lack of toxicity. Therefore, in the present study plant extract of *Tridax procumbens* and egg lecithin, a lipid carrier, were used for the preparation of chlorophyll-liposome for bio-imaging of *Artemia nauplii* and *Penaeus vannamei* post larvae.

Materials and Methods

Materials

T. procumbens was collected from Sathyabama University campus (latitude 12.8731°N, longitude 80.2219°E), Chennai, and shade dried. Egg lecithin was prepared following standard protocol [17] and cholesterol was purchased from Sigma-Aldrich. All other chemicals used were of analytical grade.

Preparation of Extract and Chlorophyll Measurement

Dried leaves (10 g) of *T. procumbens* were added to 100 mL of ethanol for 12 h at room temperature. The extract was filtered and the filtrate was centrifuged at 5000 ×g to remove impurities. The supernatant was condensed using a rotary evaporator to obtain green crude chlorophyll. The concentration of chlorophyll content was measured based on the molecular structure of chlorophyll that each chlorophyll molecule contains one magnesium atom by Inductively Coupled Plasma Emission Mass Spectrometry (ICP-MS, Agilent 7700 series ASX500) and exact concentration of chlorophyll was calculated using the formula:

$$C = M_{(\text{Chlorophyll})} / M_{(\text{Magnesium})} \times c$$

where C, concentration of chlorophyll; c, concentration of magnesium; $M_{(\text{Chlorophyll})}$, molecular weight of chlorophyll a; $M_{(\text{Magnesium})}$, molecular weight of magnesium.

Preparation of Nanoscale Chlorophyll-Liposome Composites (NCLC)

NCLC was prepared following the method [18] with minor modifications. Briefly, 90 mg of egg lecithin, 45 mg of cholesterol, 0.5 mg/mL of crude chlorophyll dissolved in ethanol were mixed in a round bottomed flask with 1 mL of chloroform and condensed using rotary evaporator. The mixture was dried till the last traces of chloroform get evaporated. 2 mL of distilled water was added to the flask and gently shaken for 10 min and sonicated for 2 h. The resulting mixture was left at room temperature for 24 h until the free chlorophyll settles at the bottom of the flask. Thus a transparent upper suspension containing liposome-chlorophyll nanocomposites was obtained.

Characterization of NCLC

The optical absorbance of NCLC was obtained from the UV–vis spectra (UV-1800, Shimadzu) measured in the range of 300–1100 nm. Fluorescence spectroscopy (Shimadzu RF 530) was employed to study the emission properties of nanocomposites. Functional groups of NCLC were analyzed using FT-IR spectroscopy (Shimadzu, IR Affinity-1S). Surface morphology was studied using FE-SEM (Carl Zeiss SUPRA-55 microscope) by applying an electron accelerating voltage of 5 kV

for which the nanocomposite suspension was dropped over a carbon coated copper grid and examined after drying.

A. nauplii and *P. vannamei* Maintenance

A. nauplii cysts were collected from field experimental station, Jeppiaar salt pan, Chennai, India. The cysts were dried, allowed to hatch by decapsulation by adding sodium hypochlorite. Approximately, 2 g cysts were incubated in 1 L of filtered seawater and were incubated in aerated condition at 30 °C for 12 h under 1500 lux light illumination. Aeration was maintained by a small line extending to the bottom of the hatching device from an aquarium air pump. Under these conditions, *A. nauplii* cysts hatched within 12 h were used for analysis. *P. vannamei* post larvae were obtained from a private hatchery at Chennai and acclimatized to experimental conditions in our aquaculture facility lab. The shrimps were fed with live *A. nauplii* and used for in vivo bio-imaging studies.

Fluorescent Imaging of NCLC Using *A. franciscana* and *Penaeus vannamei* Post Larvae

A. nauplii and *P. vannamei* post larvae were treated with an appropriate concentration of NCLC for 15 min. After treatment, the organisms were examined under a fluorescence microscope (Leica DMI6000B).

Results and Discussion

Chlorophyll Measurement by ICP-MS and UV-Vis Spectroscopic Analysis

The final ethanol extract obtained from *T. procumbens* was dark green in colour (Fig. 1). The final concentration of chlorophyll in the extract was devised based on

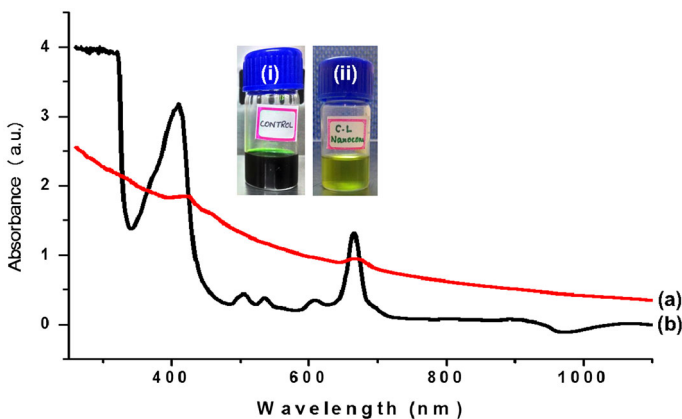


Fig. 1 Absorption spectrum of *a* chlorophyll (red line) and *b* NCLC (black line). Figure inset shows (i) chlorophyll and (ii) prepared NCLC (Color figure online)

the number of magnesium atoms present in the green crude solution which was found to be 0.525 $\mu\text{g}/\text{mL}$ detected by ICP-MS.

$$\begin{aligned}C &= M(\text{Chlorophyll})/M(\text{Magnesium}) \times c \\ &= 893.488/24.305 \times 0.525 \mu\text{g}/\text{mL} \\ &= 19.299 \mu\text{g}/\text{mL}.\end{aligned}$$

The resultant chlorophyll solution showed maximum absorbance at 422 and 644 nm that corresponds to the two band ranges i.e. 400 nm (blue) and 600–700 nm (red) shown in Fig. 1. The peaks that appeared at these ranges matches well with the absorption spectra obtained for chlorophyll a [19]. Likewise, the prepared NCLC was light green in colour and same pattern of absorption was observed. However, the higher absorption peak was observed for NCLC (Fig. 1). It indicated that the prepared NCLC did not affect the maximum absorption of chlorophyll.

The green photosynthetic pigment, chlorophyll, was immiscible and hard to be dispersed in water compared to NCLC which is soluble to a greater extent. It has been reported that the reason for the immiscible behavior of chlorophyll in water is due to its lipophilic nature in water [18]. Therefore, when chlorophyll was dispersed in water, the aqueous suspension happened to precipitate in some time. However, the aqueous suspension of NCLC remained stable, uniform and showed no visible aggregates for several days.

Fluorescence Spectroscopic Studies

From the fluorescence spectra (Fig. 2a & b) of chlorophyll and NCLC, it is evident that there is emission for all the absorption peaks observed and although there are three excitation peaks, the emission maximum was observed at 763 nm. Figure 2a & b shows the fluorescence spectra of chlorophyll extract and NCLC which shows a fluorescence intensity of 653 a.u. for chlorophyll extract and 339 a.u. for NCLC, which is two fold greater than that of chlorophyll extract itself. This suggests that the liposome coating did not evidently affect the fluorescence of chlorophyll which is an important criterion for its application in *in vivo* bio-imaging.

FTIR Spectroscopic Studies

FTIR spectra were observed at a range of 400–4000 cm^{-1} . FTIR spectrum peaks were observed at 3329, 2360, 1118 and 609 cm^{-1} . The peak at 3390 cm^{-1} shows the existence of strong $-\text{OH}$ group which is due to the presence of ethanol in the extract. The peak at 3390 cm^{-1} in chlorophyll extract has shifted to 3329 cm^{-1} in the NCLC indicating the involvement of $-\text{OH}$ or $-\text{COOH}$ group. The peak at 2945 cm^{-1} in chlorophyll extract has shifted to 2970 cm^{-1} which denotes the presence of asymmetric $-\text{CH}_2$ group in the reduction of metal salt. The peak at 1672 cm^{-1} has shifted to 1660 cm^{-1} which shows the involvement of amide I group (Fig. 3).

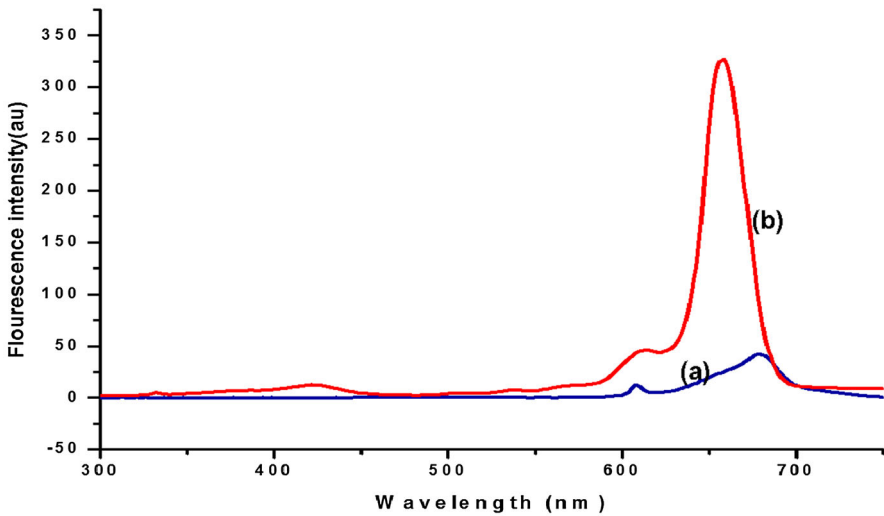


Fig. 2 Fluorescent spectra of *a* chlorophyll (*blue line*) and *b* NCLC (*red line*) (Color figure online)

FE-SEM Analysis of Liposome-Coated Chlorophyll Nanocomposites

Surface morphology of NCLC was investigated using FE-SEM and the images show particles that are well dispersed with different shapes such as a rod, triangle, cubes and few are irregular (Fig. 4). Similarly, an earlier report pointed out that liposome-coated chlorophyll nanocomposites were spherical in shape and have a

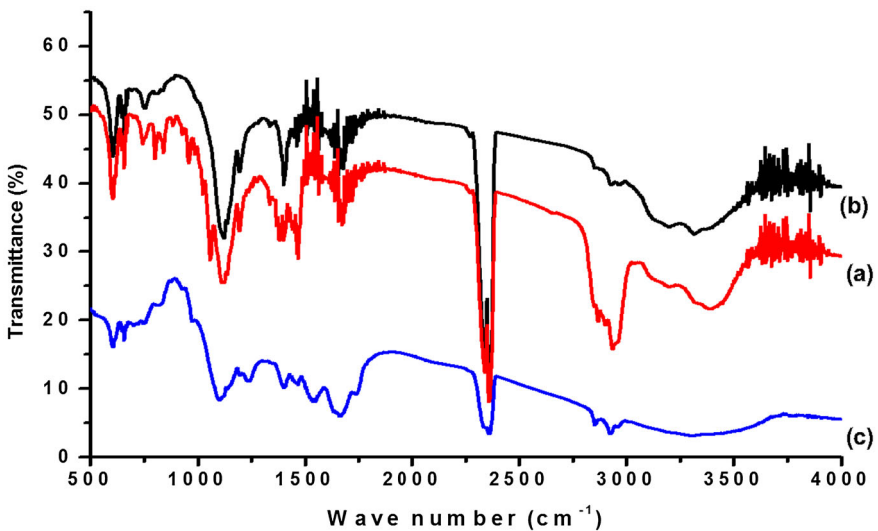


Fig. 3 FTIR spectrum of *a* chlorophyll (*red line*), *b* NCLC (*black line*) and *c* egg lecithin (*blue line*) (Color figure online)

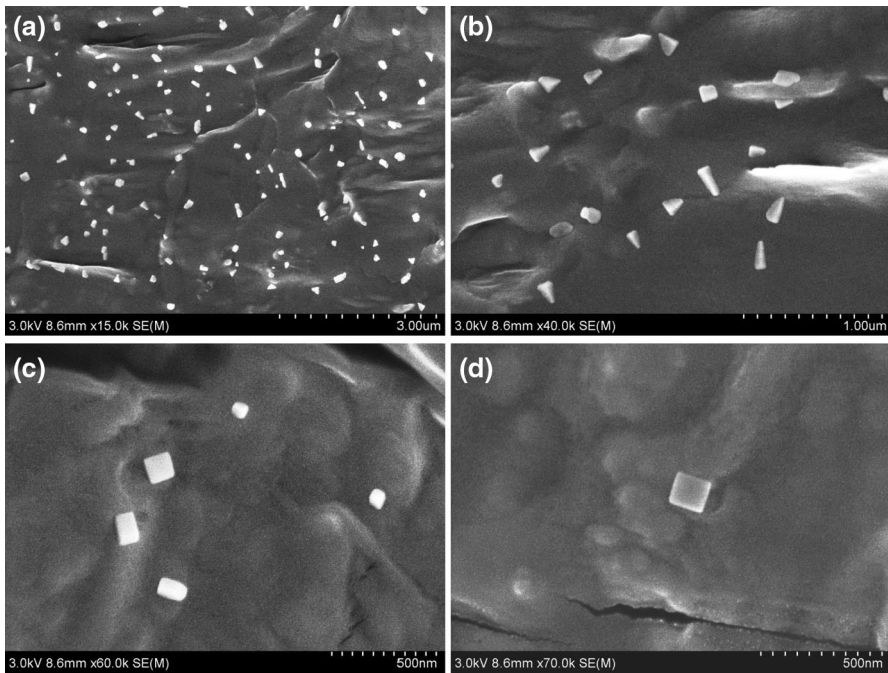


Fig. 4 Field Emission-Scanning Electron Microscopic (FE-SEM) images of NCLC

comparatively narrow size distribution with an average size of 21 nm [18]. Recently, synthesis of biocompatible and well dispersed spherical pluronic micelle-encapsulated red-photoluminescent chlorophyll derivative nanoparticles was carried out that sized around 15 nm [1].

Bio-imaging Studies

To investigate whether the fabricated NCLC shows sustained *in vivo* bio-imaging properties, its potential was examined in *A. nauplii* and *P. vannamei*. Figures 5 and 6 show the bright field and fluorescent images of the organisms, respectively, after administration of NCLC. From the figures, it could be observed that the nanocomposites emitted brighter near infrared fluorescence which is remarkably evident in the alimentary canal of *A. nauplii* and *P. vannamei* and was found to be distributed throughout the body of the organisms.

It could therefore be inferred that the synthesized NCLC could serve as a candidate for *in vivo* imaging in disease diagnosis and drug delivery applications in aquaculture. Few reports also suggest the efficacy of liposome chlorophyll nanocomposite for imaging in mice. The liposome-coated chlorophyll nanocomposites have been used to study the sentinel lymph node mapping *in vivo* in mice to visualize different sites through the skin and muscle [18]. Different nano red-photoluminescent chlorophyll derivatives (ZnChl-1, H2Chl-2, ZnChl-2) were synthesized via hydrophobic-hydrophilic interactions for cancer cell imaging which

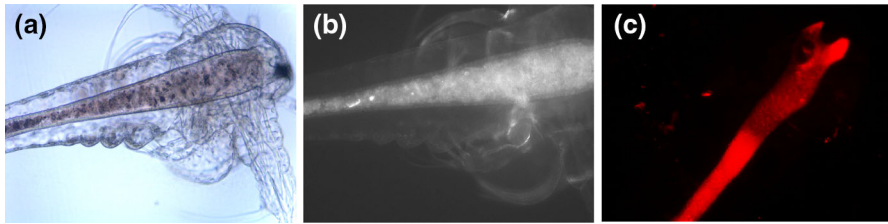


Fig. 5 a Bright field, b Dark field, and c Fluorescence images of *A. nauplii* treated with NCLC

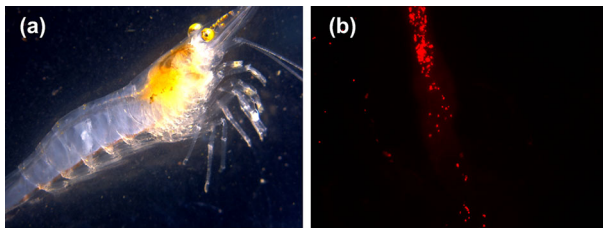


Fig. 6 a Bright field and b Fluorescence images of *P. vannamei* post larvae treated with NCLC

has been successfully demonstrated in HepG2 and A549 cancer cell lines [1]. On this basis, it is believed that the synthesized NCLC would serve as a platform for diagnostic and drug delivery applications in aquaculture.

Conclusion

Fluorescent nanoscale chlorophyll-liposome composite for bioimaging was prepared using the leaf extract of *T. procumbens*. The physico-chemical and structural features were characterized using various spectral and electron microscopic analysis. Bio-imaging of *A. nauplii* and *P. vannamei* treated with NCLC revealed bright red fluorescence inside the alimentary canal which infers its role as a nanoprobe whose efficacy could be improved by conjugating with *A. nauplii* specific ligands for disease diagnosis, drug delivery, etc. The developed NCLC can be applied in aquaculture for disease diagnosis, vaccine and drug delivery.

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