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Novel self-assembled lithocholic acid nanoparticles for drug delivery in cancer†

Cite this: DOI: 10.1039/c3ra42994f

Received 15th June 2013
Accepted 15th August 2013

DOI: 10.1039/c3ra42994f

www.rsc.org/advances

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Novel versatile self-assembled nanoparticles were developed from biocompatible, biodegradable lithocholic acid derivatives. These nanoparticles can incorporate different cytotoxic drugs (paclitaxel and doxorubicin) and PI3K signalling inhibitor (PI103). Drugs were released from the nanoparticles in a slow, sustained manner at acidic pH. The drug loaded nanoparticles were internalized through lysosomal compartments and induced cell death in HeLa cervical cancer cells.

Cancer is a devastating disease killing almost 10 million people per year.[1] Current cancer treatment is relying on using toxic chemotherapeutic drugs, which often kill healthy cells causing off-target toxic side effects to the patients. To address this, nanotechnology based platforms have emerged as revolutionizing strategies in cancer therapeutics.[2] Nanovectors can offer many advantages over free drugs by (a) protecting the drugs from premature degradation and interaction with the biological systems, (b) enhancing accumulation of drugs in specific tissues by active targeting or enhanced permeability and retention effect (EPR), (c) improving the pharmacokinetic/pharmacodynamics of the drugs and (d) increasing intracellular penetration.[3] Several nanovectors including polymer–drug conjugates, carbon nanotubes, micelles, dendrimers, liposomes, nanoshells and polymeric nanoparticles have extensively explored for drug delivery and diagnosis in cancer.[3,4] However, very few nanovector-based drugs are currently on the market.[3] So there is clearly a need to develop novel nanovectors for drug delivery in cancer. For rapid and successful clinical translation, the nanovectors should be developed from biocompatible, biodegradable, well characterized and easily functionalized materials. Moreover, the nanovectors should self-assemble into sub 200 nm particles with diverse drug loading to accumulate into tumor by EPR effect.[5] Finally, the nanovectors should release the active drugs in a controlled, slow and sustained manner. To address this we focus on developing novel nanovectors from lithocholic acid (LA). LA is one of the most common naturally occurring secondary bile acids biosynthesized from cholesterol by bacterial modification of intermediate chenodeoxycholic acid in colon and stored in liver and gall bladder for lipid digestion, absorption, transport and excretion.[6] Due to their versatile structural properties, bile acids and their derivatives have shown great potential for host–guest chemistry, biomimetics, molecular recognition, drug delivery and nanotechnology.[7] Moreover, lithocholic acid has structural similarity with cholesterol (an important component of cell membrane) (Fig. 1c) and can self-assemble to develop supramolecular nanostructures.[7c] Inspired by the unique amphiphilicity, high structural rigidity, biocompatibility and biodegradability, herein we report the engineering of novel, self-assembled lithocholic acid nanoparticles. These nanoparticles are mono dispersed having sub 200 nm size ideal for tumor homing by EPR effect. Furthermore, these nanoparticles can incorporate diverse clinically approved cytotoxic drugs (doxorubicin and paclitaxel) and PI3-kinase inhibitor (PI103) and release the active drugs effectively in a controlled manner at pH = 5.5. Finally, these drug loaded nanoparticles internalized into the lysosomal compartments by endocytosis and induced cell death in a cervical cancer cell line to show their potential in cancer therapeutics. To the best of our knowledge, this is the first example of lithocholic acid based self-assembled nanoparticle synthesis as a versatile platform for drug delivery in cancer.

To show the versatility of LA based nanoparticles, we chose two cytotoxic drugs (doxorubicin and paclitaxel) which are extensively used in clinics for the treatment of different types of malignancies including breast, ovarian, prostate, cervical and lung cancer. Doxorubicin is a highly potent anti-cancer drug

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† Electronic supplementary information (ESI) available: Experimental details and further characterization data are available. See DOI: 10.1039/c3ra42994f

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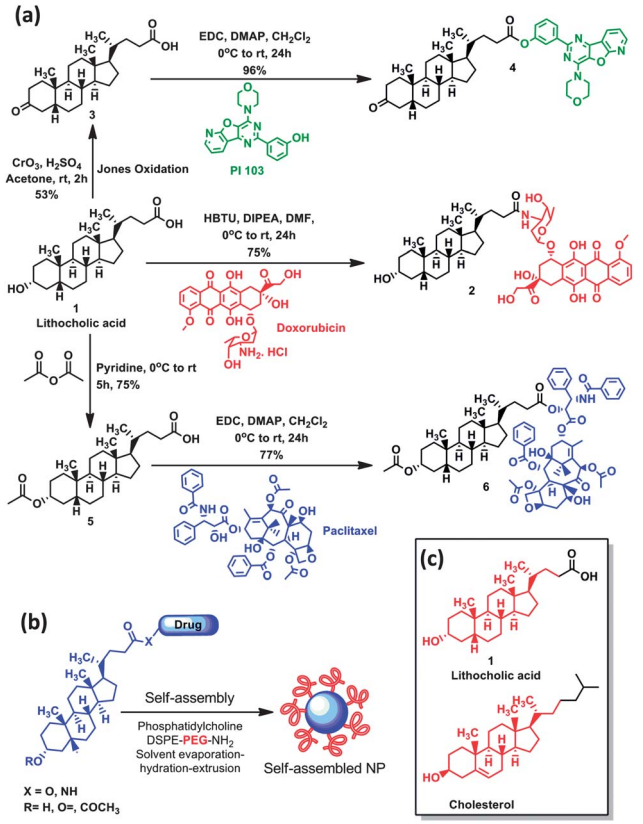

Fig. 1 Synthesis of drug loaded self-assembled lithocholic acid nanoparticles. (a) Synthesis of lithocholic acid–drug conjugates. (b) Schematic representation of self-assembled nanoparticle synthesis from lithocholic acid–drug conjugates. (c) Structure of lithocholic acid and cholesterol. Structural similarity is shown in red.

works by intercalating with the DNA as well as inhibiting the action of topoisomerase II enzyme.[8] However, doxorubicin's clinical use is limited as it may induce cardiotoxicity including congestive heart failure, dilated cardiomyopathy and death.[9] To reduce its toxic side effects, several doxorubicin nanoformulations including antibody-targeted liposome, polymeric nanoparticles, gold nanoparticles and fullerene have been studied extensively.[10] Currently, two FDA approved doxorubicin nanoformulations (Myocet® and Doxil®) are available in the market. However, both of them demonstrated side effects including congestive heart failure, myelosuppression, thrombocytopenia and palmer-planter erythrodysesthesia (PPE) or hand-foot syndrome due to their premature fast burst release of the physically encapsulated chemotherapeutic drugs.[11] Moreover, due to non-pegylated formulation, Myocet® rapidly cleared from the body leading to it being less effective.[3] On the other hand, paclitaxel is a widely used chemotherapeutic drug, exhibits its activity by binding and stabilizing microtubules, thus inhibiting cell division.[12] Paclitaxel is highly hydrophobic in nature, causing poor water solubility and leading to its clinical formulation using toxic Cremophor EL and ethanol. To address this, several nanovectors have been explored to deliver paclitaxel specifically into tumors.[13] However, the nanoformulations lead to limited improvement in water solubility, poor release of drugs. Recently, albumin bound nanoparticle formulation of paclitaxel (Abraxane®) has been approved by FDA for metastatic breast cancer treatment, although it leads to several side effects like sensory neuropathy, heart attack, nausea, infection, hair loss and so on.[14] To overcome the off-target side effects of cytotoxic drugs, kinase inhibitors have emerged as novel personalized cancer therapeutics.[15] Phosphatidyl-inositol-3-kinase (PI3K) signalling is one of the most frequently mutated signalling pathways in almost 30% of human cancers.[16] We chose PI103, a PI3K inhibitor, currently in pre-clinical study.[17] PI103 is a dual inhibitor for Akt and mammalian target of rapamycin (mTOR).[18] However, PI103 is poorly water soluble, metabolizes quickly and shows dose dependent insulin resistance.[19] To address the above mentioned drawbacks of cytotoxic drugs and PI3K inhibitors in clinics, there is clearly a need to develop novel versatile nanovector to deliver different cytotoxic drugs as well as kinase inhibitors specifically to the tumor to avoid off-target toxicities leading to side effects to the patients.

Doxorubicin was conjugated directly with carboxylic acid end of LA (**1**, Fig. 1a) by amide linkage using HBTU as coupling reagent to obtain LA–doxorubicin conjugate (**2**) in 75% yield. To conjugate PI103, secondary alcohol of LA (**1**) was oxidized by Jones oxidation using CrO_3–H_2SO_4 in acetone to obtain corresponding keto-lithocholic acid (**3**) in 53% yield. PI103 was then conjugated with **3** by phenolic ester linkage by using EDC as coupling reagent in presence of catalytic amount of DMAP at 0 °C to room temperature for 24 h to obtain keto-lithocholic acid-PI103 conjugate (**4**) in 96% yield. Finally, to conjugate paclitaxel, the free secondary –OH group in LA was first protected by using acetic anhydride in presence of pyridine as base to obtain acetyl protected LA (**5**) in 75% yield. Paclitaxel was then conjugated with acetyl LA (**5**) by ester linkage at 2′ OH position to afford **6** in 77% yields.

Self-assembled nanoparticles were engineered by solvent evaporation–hydration–extrusion method from LA–drug conjugates (**2**, **4** or **6**), phosphatidylcholine (PC) and DSPE-PEG in 1 : 2 : 0.2 weight ratio (Fig. 1b).[20] PC is a naturally occurring component of cellular membrane, hence biocompatible, biodegradable and non-toxic. DSPE-PEG was used to pegylate the surface of the nanoparticle to avoid clearance by reticuloendothelial system (RES) and ensure the long circulation half-life in blood.[21] Drug loading in the nanoparticle was determined by UV-Vis spectroscopy at characteristics λ_{max} = 480 nm, 292 nm and 277 nm for doxorubicin, PI103 and paclitaxel respectively (Fig. S1, ESI†). The drug loading was found to be =66.20 ± 7.8 μg mL^{-1} (121.96 ± 14.4 μM, loading efficiency = 22%), 306.9 ± 32.5 μg mL^{-1} (359.40 ± 38.2 μM, loading efficiency = 90%) and 34.15 ± 2.3 μg mL^{-1} (98.17 ± 6.5 μM, loading efficiency = 14%) (mean ± SEM, n = 3) for doxorubicin, paclitaxel and PI103 respectively (Fig. S2a and b, ESI†). The hydrodynamic diameter of the drug loaded nanoparticles was determined by dynamic light scattering (DLS) (Fig. 2a–c and S2c, ESI†) and found to be 99.3 ± 0.2 nm, 130.2 ± 1.8 nm and 116.4 ± 1.2 nm (mean ± SEM, n = 3) for PI103-NP, paclitaxel-NP and dox-NP respectively. Size, shape and morphology of the nanoparticles were also determined by atomic force microscopy (AFM) (Fig. 2d–f and S3, ESI†) and field-emission scanning

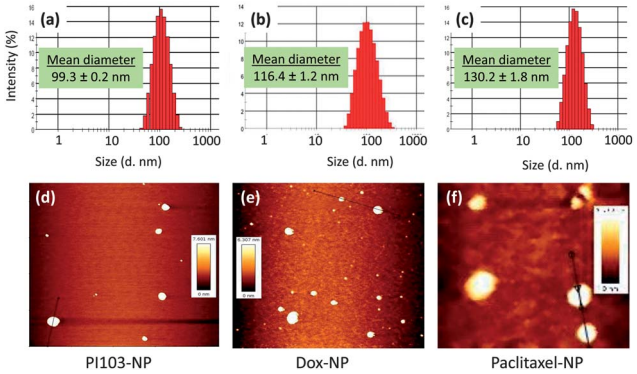

Fig. 2 Characterization of lithocholic acid NPs. (a–c) Size distribution of PI103-NP, doxorubicin-NP and paclitaxel-NP respectively by dynamic light scattering (DLS). (d–f) AFM images of PI103-NP, doxorubicin-NP and paclitaxel-NP respectively.

electron microscopy (FE-SEM) (Fig. S4, ESI†). DLS, AFM and FE-SEM data provided convincing evidence of self-assembly of different LA–drug conjugates into sub 200 nm particles for homing into tumor by EPR effect. To understand the role of lithocholic acid in nanoparticle formation, we synthesized drug encapsulated nanoparticles in the same solvent evaporation–hydration–extrusion method using only PC and DSPE-PEG in 2 : 0.2 ratio without any lithocholic acid. The nanoparticles formed were evaluated using DLS for size distribution. Unfortunately, this formulation produced particles having mean hydrodynamic diameter = 377.34 ± 3.3 nm, 286.88 ± 9.9 nm and 270. 30 ± 2.7 nm (mean ± SEM, $n = 3$) for PI103-NP, dox-NP and paclitaxel-NP respectively (Fig. S5, ESI†) with broad distributions. This clearly demonstrated that lithocholic acid is essential to self-assemble the particles in sub 200 nm size.

To deliver the drugs successfully in cancer, the nanoparticles should be stable enough in blood circulation over a period of time to home into the tumor using EPR effect. To evaluate the stability in physiological conditions, we incubated different drug loaded nanoparticles in fetal bovine serum (FBS) at 37 °C for 5 days and monitored their properties by size and polydispersity index (PDI) using DLS. The size of the paclitaxel-NP changed from 129.63 ± 0.08 nm to 132.67 ± 0.32 nm whereas PDI value changed from 0.193 ± 0.0 to 0.166 ± 0.01 over 5 days (Fig. S6, ESI†). On the other hand, size of dox-NP increased from 122.36 ± 0.67 nm to 158.90 ± 0.5 nm, whereas PDI value changed from 0.180 ± 0.0 to 0.411 ± 0.0 over 5 days. However, PI103-NP showed huge increase in size from 88.17 ± 0.69 nm to 416.00 ± 18.66 nm and PDI value from 0.219 ± 0.0 to 1.0 ± 0.0 in 4 days (mean ± SEM, $n = 3$). From this stability experiment it is clear that paclitaxel-NP and dox-NP are stable over 5 days, however PI103-NP is stable over 3 days in serum, which is sufficient enough time to accumulate into the tumor by EPR effect.

To be successful in clinics, the nanoparticles should release the drugs in a slow and sustained manner over a long period of time. To evaluate the release profile of different drugs, we used dialysis method[20b] by incubating the drug loaded NPs at pH 5.5 buffer at 37 °C which mimics the lysosomal compartment inside the cells. As a control we incubated the drug loaded NPs in pH 7.4 buffer, which mimics the physiological pH. PI103-NP exhibited 76.5 ± 7.4% (mean ± SEM, $n = 3$) PI103 release at pH 5.5, whereas, 63.7 ± 9.8% (mean ± SEM, $n = 3$) PI103 was release at pH 7.4 at 112 h (Fig. 3a). On the other hand, doxorubicin-NP demonstrated 84.5 ± 7.2% (mean ± SEM, $n = 3$) release of doxorubicin at 96 h (Fig. 3b). However, only 34.5 ± 1.7% (mean ± SEM, $n = 3$) doxorubicin was released from the nanoparticles even after 120 h. Finally, 83.0 ± 7.7% (mean ± SEM, $n = 3$) paclitaxel was released from self-assembled nanoparticles at pH 5.5, whereas, only 49.8 ± 1.1% (mean ± SEM, $n = 3$) paclitaxel was released at pH 7.4 after 120 h (Fig. 3c). We rationalized that amide linkage in conjugate **2** is more prone to hydrolysis in acidic pH (pH = 5.5) than neutral pH (pH = 7.4) leading to increased release of active doxorubicin at pH = 5.5. On the other hand, phenolic ester in conjugate **4** is labile in neutral pH leading to comparable release of PI103 in acid and neutral pH. Finally, the aliphatic ester bond in conjugate **6** is more labile in pH = 5.5 compared to pH = 7.4 leading to increased paclitaxel release in acidic pH. We also observed that the lithocholic acid–drug conjugates **2**, **4** and **6** are completely converted to the free drugs by using MALDI-TOF (Fig. S7–S9, ESI†). The release profile data clearly showed that different drugs were released from the nanoparticles in a slow and sustained manner at pH 5.5, which would lead the constant drug level in the body while the drug loaded nanoparticles being administered. Furthermore, higher amount of drug released in pH = 5.5 compared to pH = 7.4 clearly indicates that more drugs will be released in acidic lysosomal compartments of the tumor cells than premature drug release in the blood circulation.

To ensure the effect of our novel nanoparticles in cancer, we evaluated the *in vitro* efficacy of drug loaded lithocholic acid-NPs in HeLa cervical cancer cell line by cell viability assay using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) at 24 h and 48 h post-incubation. At 24 h, PI103-NP showed HeLa cell death with IC_{50} = 9.4 μM compared to free PI103 with IC_{50} = 4.78 μM (Fig. S10, ESI†). On the other hand, dox-NP induced HeLa cell death having IC_{50} = 7.5 μM compared to free doxorubicin with IC_{50} = 4.0 μM. Finally, paclitaxel-NP demonstrated IC_{50} = 19.2 μM whereas free paclitaxel induced cell death in IC_{50} = 0.1 μM. Interestingly, at 48 h, PI103-NP induced HeLa cell death having IC_{50} = 2.54 μM compared to IC_{50} = 2.39 μM for free PI103 (Fig. 4a). PI103-NP induced 73.33

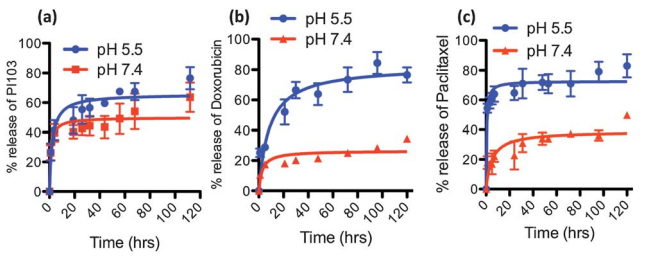

Fig. 3 Release profile of drugs from lithocholic acid-NPs at 37 °C at different pH. (a) Release profile of PI103 over 112 h. (b) Release profile of doxorubicin over 120 h (c) release profile of paclitaxel over 120 h.

± 5.1% (mean ± SEM, $n = 3$) cell death compared to 92.38 ± 2.6% (mean ± SEM, $n = 3$) cell death induced by free PI103 at 20 μM concentration. On the other hand, doxorubicin-NP demonstrated $IC_{50} = 1.32$ μM by inducing 77.83 ± 7.0% (mean ± SEM, $n = 3$) cell death, whereas free doxorubicin showed $IC_{50} = 0.17$ μM by killing 87.55 ± 3.9% (mean ± SEM, $n = 3$) cells (Fig. 4b) at highest concentration (20 μM). Finally, paclitaxel-NP exhibited $IC_{50} = 8.88$ μM by inducing 68.74 ± 2.7% (mean ± SEM, $n = 3$) cell death, whereas free paclitaxel showed $IC_{50} = 0.11$ μM by inducing 94.98 ± 0.3% (mean ± SEM, $n = 3$) cell death at 20 μM concentration (Fig. 4c). It is evident from this time dependent cytotoxicity assay that different drug loaded nanoparticles are more effective at 48 h compared to 24 h. It is expected that drug loaded nanoparticles would exhibit lower efficacy than the free drugs as nanoparticles would release the active drugs in a slow and sustained manner over long period of time, whereas free drugs would induce toxicity very quickly. However, different drug loaded nanoparticles showed dose dependent cytotoxicity in HeLa cells at 24 h and 48 h which showed their efficacy as cancer chemotherapy.

We further investigated the mechanism of cellular internalization of these novel lithocholic acid based NPs. We treated the HeLa cells with LA–doxorubicin-NPs and visualized their time dependent internalization by confocal laser scanning microscopy (CLSM). LysoSensor™ Green DND-153 and Hoechst 33342 dye were used to stain low pH lysosomal compartments (shown in green) and nucleus (shown in blue) respectively. We treated the cells with red fluorescent LA–dox-NPs or free doxorubicin in 2 μg mL^{-1} concentrations for 1 h, 3 h and 6 h time points to understand the temporal internalization of the nanoparticles compared to free doxorubicin. As shown in Fig. 5, after incubation with LA–dox-NP, the red and green fluorescence co-localize (yellow regions) with each other in a time dependent manner. At 6 h, it is clear from the CLSM images, that LA–dox-NPs home into the low pH lysosomal compartments by endocytosis mechanism. In contrast, after incubation with free doxorubicin, the red and green fluorescence did not co-localize (Fig. S11, ESI†) even after 6 h. However, blue and red fluorescence co-localize (purple regions) with each other within 3 h, which clearly indicates that free doxorubicin internalized through diffusion pathway and accumulated into the nucleus. From this CLSM images, it is evident that LA–dox-NPs were internalized into the HeLa cells through low pH lysosomal compartments in a time dependent manner over 6 h.

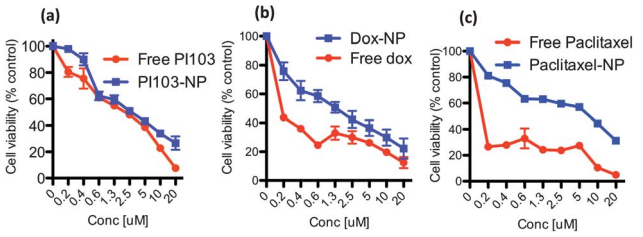

Fig. 4 (a–c) *In vitro* dose-dependent cytotoxicity assay of PI103-NP, doxorubicin-NP and paclitaxel-NP against HeLa cells at 48 h.

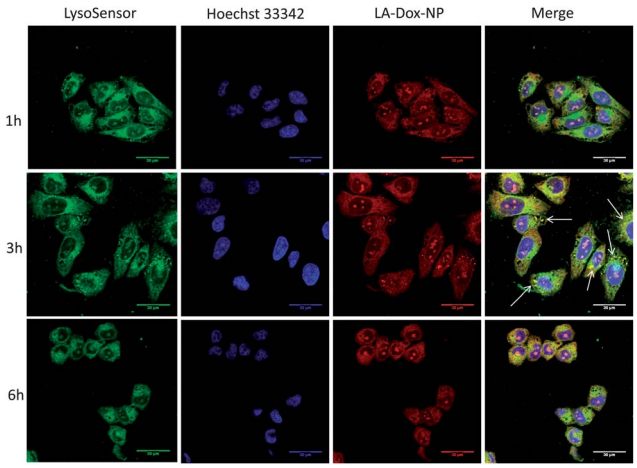

Fig. 5 Internalization of LA–doxorubicin-NPs in HeLa cells in 1 h, 3 h and 6 h time points. Lysosomal compartments and nucleus were stained by LysoSensor™ Green DND-153 (green) and Hoechst 33342 (blue) respectively. The merged images are showing the co-localization of LA–doxorubicin-NPs in the lysosomal compartments in a time dependent manner. Scale bar = 30 μm.

Conclusions

In conclusion, we successfully developed novel, versatile, self-assembled lithocholic acid nanoparticles which can hold diverse drugs (paclitaxel, doxorubicin and PI103) to deploy them inside the tumor. These nanoparticles exhibited slow and sustained drug release over a period of time at pH = 5.5. Finally, these nanoparticles internalized through lysosomal compartments by endocytosis to induce cytotoxicity in HeLa cervical cancer cells to show their future potential in cancer treatment. These nanoparticles can also be surface decorated by different tumor targeting moieties like antibodies, aptamers, cell penetrating peptides or cell surface receptor targeting peptides for tissue specific delivery of cytotoxic drugs or signalling inhibitors. We envision that lithocholic acid nanoparticles can serve as non-toxic, non-immunogenic versatile platform to conjugate diverse array of cytotoxic drugs or signalling inhibitors for temporal targeting of cancer and will be successfully translated to the clinics to provide better quality of life to the cancer patients.

Acknowledgements

We sincerely thank IISER-Pune, Department of Biotechnology (DBT) (Ramalingaswami Fellowship) and Science and Engineering Research Board (SERB) (Fast Track Scheme for Young Scientist) for financial support. We thank Department of Science and Technology (DST) Nanoscience unit for providing AFM facility. We thank Dr Shouvik Datta for capturing FE-SEM images. We also thank Dr Geetanjali Tomar and Ms Aishwarya Sivakumar from Institute of Bioinformatics and Biotechnology (IBB), University of Pune for providing tissue culture facility for doing MTT assay. Finally, we thank Mr Vijay Vittal from IISER Pune for helping in capturing CLSM images.

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