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Review Article

Shedding Light on Membrane Using Carbon Dots

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Carbon dots, a rising star of the carbon nanostructure family, have attracted considerable interest recently because of their luminescence quantum yield [1] and luminescence in the broad-spectrum range[2], which makes them useful for sensing[3] and imaging applications.[4] Another advantage of CDs compared to regular organic fluorophores is that they can be synthesized from readily available carbon precursors.[5] Depending on the precursor, one can tailor and tune the properties of CDs. In addition to that, they are environmentally friendly[6] and relatively less toxic compared to heavy metal-based inorganic quantum dots. The uniqueness of such fluorogenic nanoparticles also comes from their non-bleaching fluorescent properties once exposed to the high-intensity laser,[7] making them even more attractive for biological imaging and other imaging-related applications. CDs are also chemically stable and inert.[8]The abundance of several oxygen-containing functional groups[9] on the surface of CDs made it more attractive for tracking biomolecules in the cellular environment in a single particle resolution after attaching CDs onto their surface.[10] Significantly, as these nanoparticles are very tiny, once they are connected with biomolecules, they do not alter the native properties of biomolecules.

On the other hand, the membrane is one of the fascinating supramolecular aggregates of phospholipid that not only acts as a barrier between the interior and Outerror of the cell but also acts as a harbor for different kinds of reactions that are essential for existence, survival and proper functioning of the cell.[11] In addition, the interaction of the membrane with host molecules like viruses' bacteria, and drugs is one of the critical parameters that eventually determine the uptake of the host molecule, including the drug inside the cell.[12] Interaction of the host molecule at the cell surface requires a mechanism by which the cell converts environmental changes into internal signals, predominantly monitored by the membrane-associated proteins with specific receptors that bind with the host.[13] This triggers a series of bio-relevant processes like triggering the innate and adaptive immune response or activating G protein-coupled receptors, the primary drug targets. In cell biology, it is essential to understand how different molecules that constitute the membrane, including phospholipids and transmembrane proteins, are assembled on the membrane surface, real-time reorientation to produce primary cellular function and alteration of such essential functions in disease or infection conditions. To understand such a question, it is crucial to comprehend the microscopic orientation of the phospholipid molecule onto the cell surface, the heterogeneity on the membrane surface, especially the pinch point localisation and isolation of the liquid order and disorder phase of the membrane. CDs can be game changers fluorogenic nanoparticles with non-bleachable fluorescent properties to give a special resolution at the nanoscopic level to understand and reveal several biochemical and biophysical processes occurring across the membrane, especially the isolation of lipid rafts on the heterogeneous membrane surface.

Regarding synthetic strategies for synthesizing CDs, there are mainly two approaches: top-down and bottom-up.[14] Both directions have some merits and demerits, as depicted in Figure 2.

However, the bottom-up approach has engrossed enormous attention compared to the top-down approach because, in this approach, CDs can be synthesized using simple carbon precursors like amino acids,[15] carbohydrates[2], and other small organic

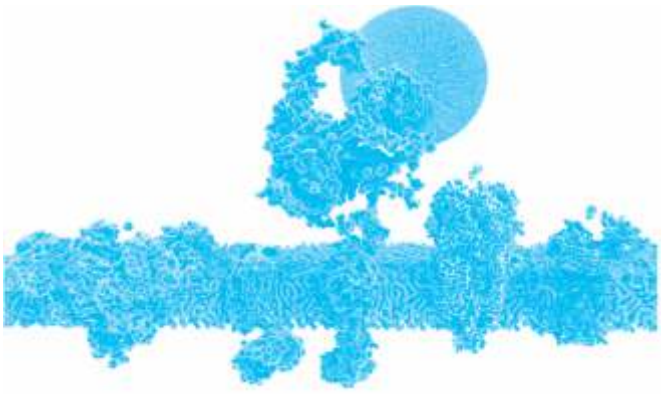


Figure 1: Schematic representation of the cell membrane.

molecules.[16] The major disadvantage of the bottom-up approach is the carbonization procedure and controlling the growth of the nucleation step that occurs after carbonization. Such uncontrollable nucleation steps can be avoided in the bottom-up approach using passivation techniques [16], as depicted in Scheme 1 as a representative example.[17] Additionally, some non-conventional ways of the system of carbon dots rather than top-down and bottom-up approaches have been reported recently.

We have designed and synthesized 6-O-acylated fatty acid ester of D-glucose by reacting D-glucose with O-O'-di-acylated tartaric acid anhydride, which had been subsequently used as a precursor for the synthesis of CDs using simple carbonization procedure as depicted in Figure 3A.[2] The advantage of this procedure is that during the synthesis of CDs, they are simultaneously in-situ self-passivated because of the presence of hydrocarbon chains in the structure's core that coats the graphitic core structure with hydrocarbon chains. The size distribution of such as-synthesized CDs can be confirmed by a high-resolution transmission electron microscopy (HR-TEM) study (Figure 3B), which showed that such as-synthesized CDs have a size distribution between 1.5 to 2.9 nm with an average population around 2.1 nm (Figure 3C).

XPS studies confirmed the abundance of several oxygen-containing functional groups on the surface of CDs. Because of polar oxygen-containing functional groups on the surface of the graphitic core structure, CDs, in addition to hydrocarbon chains, become amphiphilic. Such amphiphilic CDs could label the membrane without deforming the overall membrane structure and morphology. A rapid

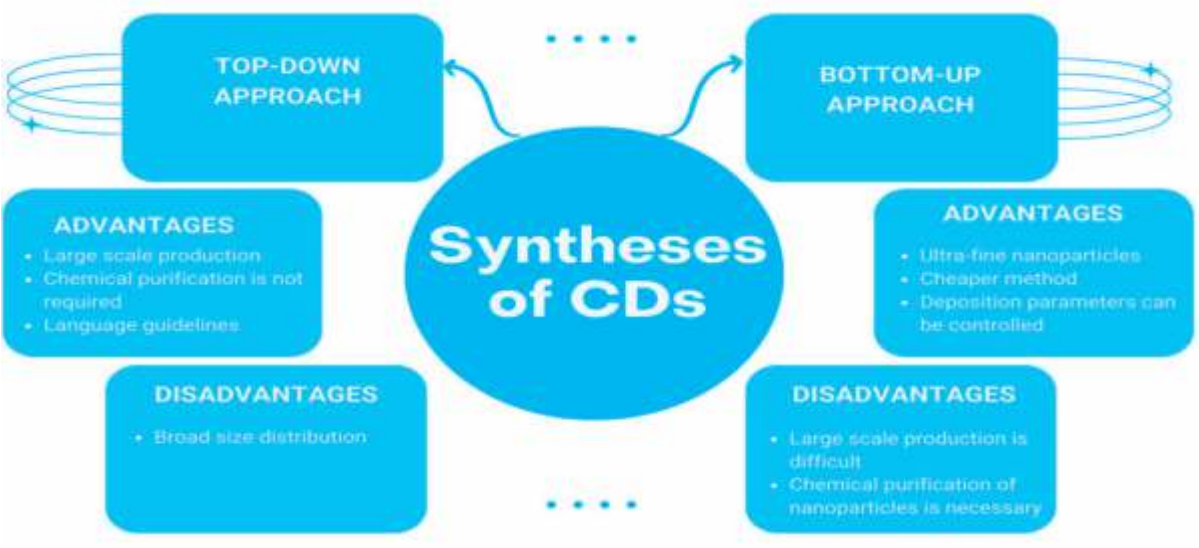
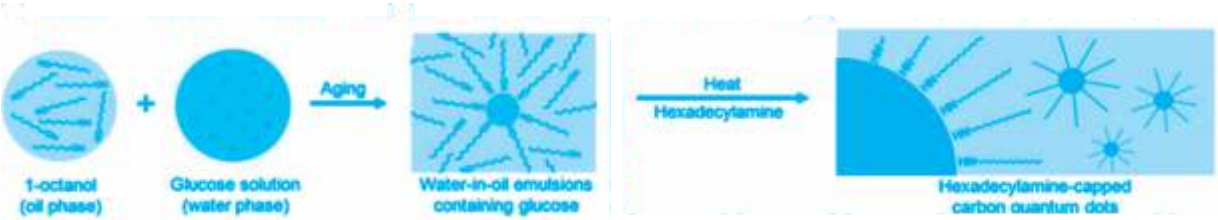


Figure 2: Top-down and bottom-up approaches, their advantages and disadvantages.



Scheme 1: Emulsion-mediated synthesis of CDs for controlling the nucleation step.[17]

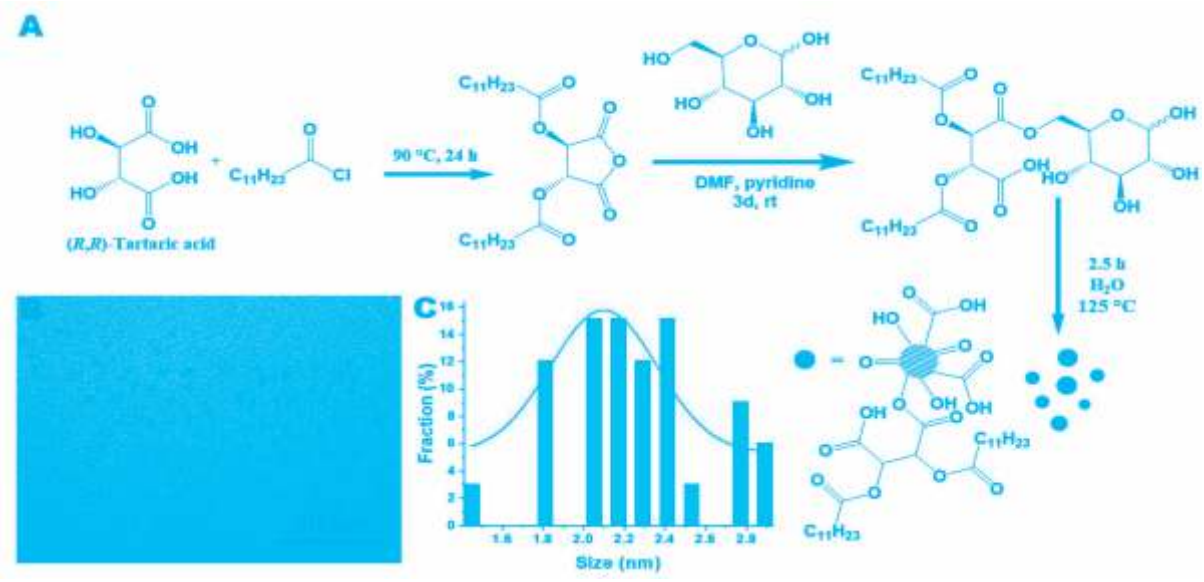


Figure 3: (A) Syntheses of CDs from glucose precursor, (B) HR-TEM image of the as-synthesized CDs, and (C) CD size distribution.

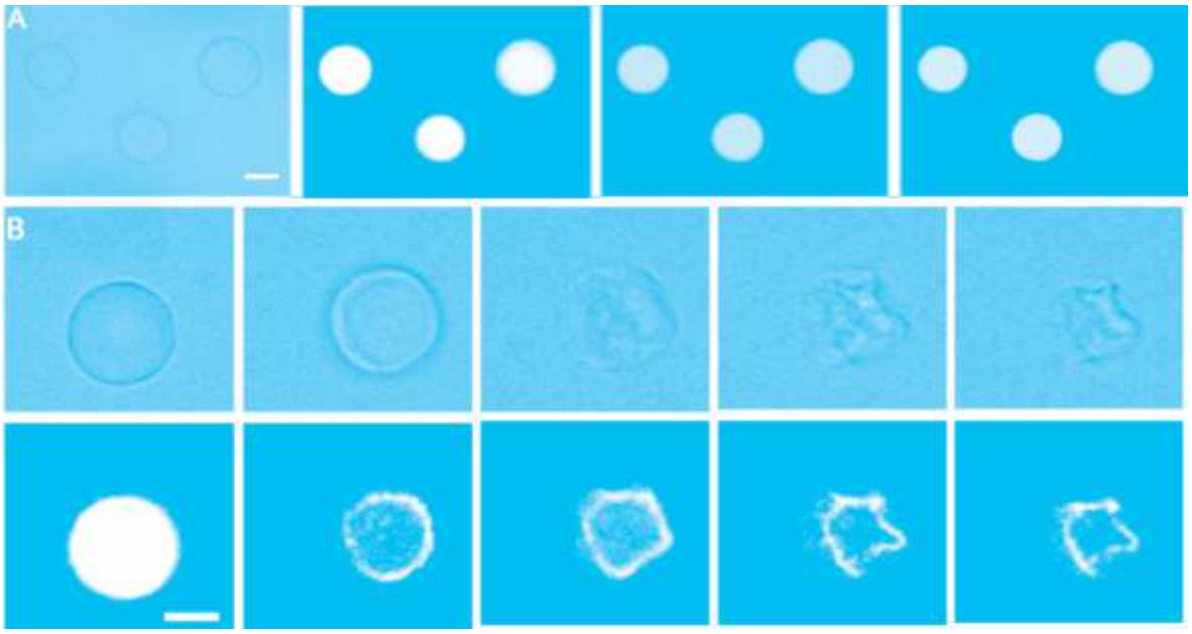


Figure 4: Fluorescence imaging of giant vesicles labeled with the amphiphilic carbon dots. (A) Bright-field microscopy (top left) and confocal fluorescence microscopy images were recorded upon excitation using a 440 nm emission filter EM 477/45 (green); excitation using a 488 nm emission filter EM 525/50 (magenta); excitation using a 514 nm emission filter EM 525/50 (orange). The scale bar corresponds to 10 mm. (B) Bright field (top) and fluorescent images (excitation at 440 nm) of giant vesicles labeled with the carbon dots following the addition of Aβ 1-40. From left: before addition (control),1 min after addition,10 min after addition,20 min after addition, and one hour after addition. The scale bar corresponds to 5 mm.

evaporation technique was used to prepare giant unilamellar vesicles (GUVs) comprising egg phosphatidylcholine (egg-PC), designed to mimic mammalian membranes. The confocal fluorescent microscopic image revealed a uniform distribution of the CDs on the membrane surface without changing the overall membrane structure and morphology (Figure 4A). Such labeled giant unilamellar vesicles comprising egg-PC can be used to visualize the membrane process in the presence of the host molecules.[2] Figure 4B depicts real-time dramatic visualization of the disruption of the membrane bilayer

after the addition of amyloid beta peptide monomer Aβ 1-42, a significant culprit and pathological hallmarks for Alzheimer's disease [18] on the surface of the membrane, which has considerable membrane recognition unit. After sitting on the membrane surface, the Aβ 1-42 rapidly undergoes misfolding and aggregation pathways, which are accelerated by the membrane platform, causing a dramatic deformation of the overall membrane structure and morphology.[2, 19]

A new question one needs to address is whether such CDs can be used to study the fluidity and dynamics of the membrane since it is one of the fundamental determinants of the overall cell membrane functionality.[20] Modulating membrane dynamical properties by membrane-active molecules contributes to various critical cellular processes, such as cell signaling, biomolecular recognition, and drug uptake.[21] We conjugated CDs to the head group of DMPC to obtain CDs-DMPC conjugate (Figure 5A), which was used as fluorescently tagged phospholipid dye to study the membrane's fluidity and dynamics.[22] The fluorescent recovery after photobleaching (FRAP) technique was used on glass-supported membranes to check the membrane fluidity. For the preparation of a glass-supported membrane, small unilamellar vesicles (SUVs) comprising 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and Carbon dots (CD)-1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) CDs-DMPC were incubated on a plasma-cleaned glass slide for 24 hrs. After the glass-supported membrane formed, it was characterized by an Atomic force microscopy (AFM) study, which shows the uniform distribution of the membrane bilayer on the glass surface without any patches and defects

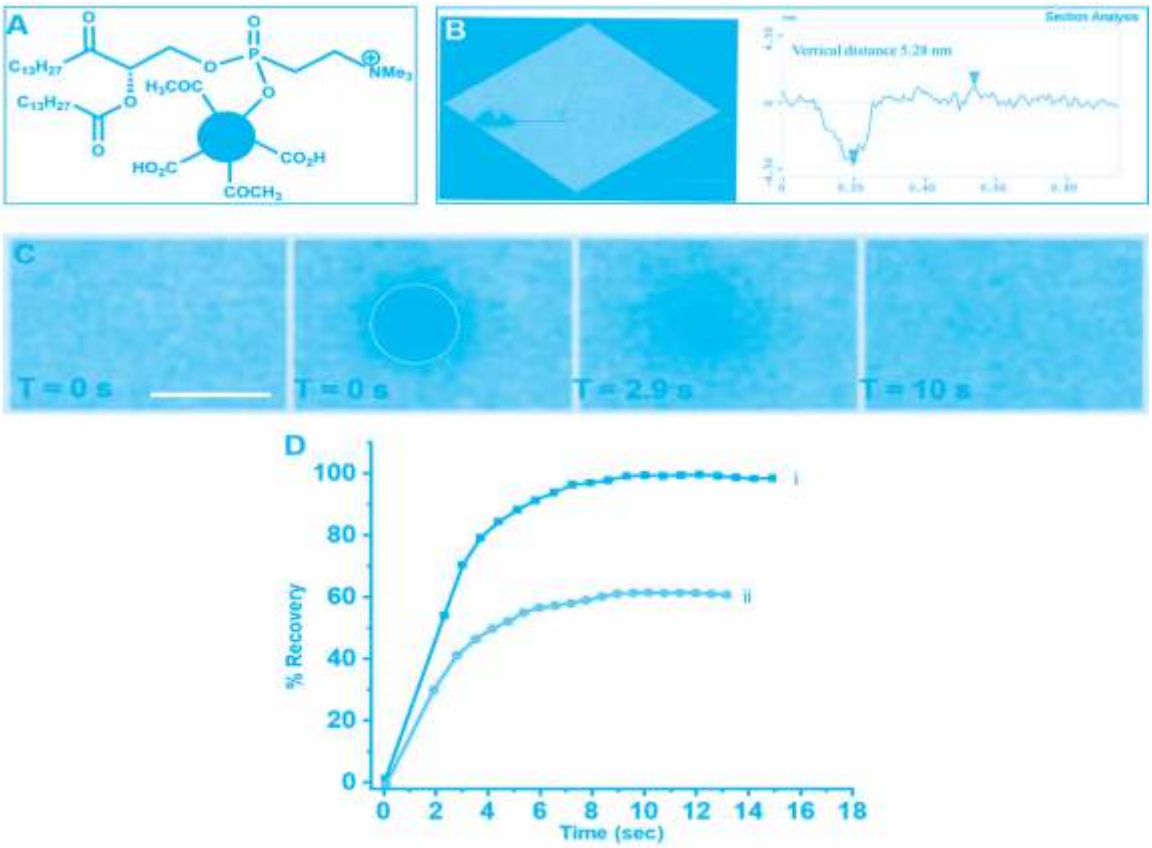


Figure 5: (A) Structure of CDs DMPC conjugate (CDs-DMPC); (B) AFM structure and the corresponding high profile of glass-supported membrane; The size of the AFM image is 1 μM × 1 μM.; (C) Confocal fluorescence microscopy images (excitation at 405 nm, emission filter EM 445/60) recorded at different times after photobleaching by a 405 nm laser (time immediately after laser irradiation is defined as T = 0 s). The circle indicates the bleached region. Scale bar is 5 mm; (D) Recovery curves recorded upon incubation of the glass-supported membrane with different molecules, (i) Control glass-supported membrane, (ii) polymyxin B.

(Figure 5B). Such uniform CDs-DMPC labeled bilayer was used for the FRAP study by choosing a circular region of interest (ROI).[23] Once the ROI was bleached with a high-intensity laser for a shorter period, the lipid molecules from all over two dimensions (2D) will come to make over the bleached portion (Figure 5C).

There will be a gradual increase in fluorescent intensity on the bleached circular ROI region. One can calculate the diffusion of the lipid molecules within the bilayer based on the recovery time. In the control glass-supported membrane, there was almost 100 % recovery after photobleaching, suggesting the homogeneity of the bilayer without patches and defects (Figures 5C and 5D). Once a host molecule with a significant membrane recognition unit binds with the membrane surface, it will cause a dramatic reduction in the recovery percentage. Figure 5D red curve depicts a substantial decrease in recovery percentage after incubation of the glass-supported membrane with polymyxin B, an antibiotic used to treat multidrug-resistant pathogens, due to the formation of lipid patches after insertion into the bilayer.[24]

Besides real-time imaging on membranes, the study of fluidity, and membrane dynamics, such fluorogenic CDs can be used for a pinch point discrimination of two distinct phases (liquid order, Lo and Lipid disordered, Ld) of the membrane using fluorescence lifetime imaging microscopy (FLIM).[25] The assorted membrane or lipid rafts hold many roles in biological events, including apoptosis, cell adhesion, numerous signal transduction pathways, synaptic transmission, organization of the cytoskeleton, and protein sorting.[25-26] Lipid rafts are useful for receptor activation, spatially organizing the plasma membrane, and intracellular trafficking.[25] Along with their impact on cellular functioning, the lipid rafts may also control the cellular entry of many viruses, bacteria, and toxins.[27] Based on the recent research on the composition of the lipids in the lipid raft region, it has been revealed that the lipid rafts region is cholesterol and sphingomyelin enriched 100-200 nm domains with a heterogenous and dynamic

Figure 6:Confocal fluorescent microscopic images of GUVs from (A) DOPC, (B) DPPC, (C) DOPC-DPPC-Chol (2:2:1) reconstructed from confocal slices (~0.4 ?m thick) with the LASX (Leica) software. Scale bar 5 µM.

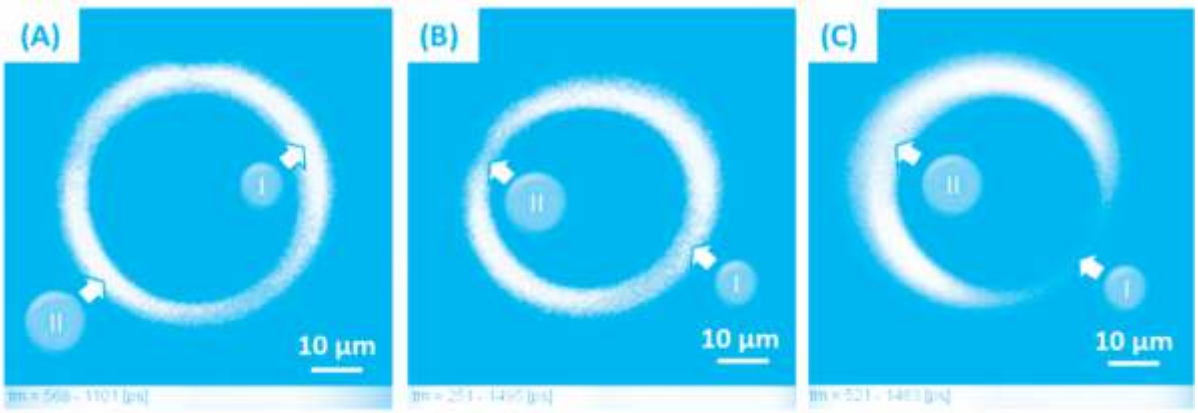


Figure 7: FLIM images of the CD-incorporated GUVs from (A) DOPC, (B) DPPC, (C) DOPC-DPPC-Chol (2:2:1). The excited state lifetimes at the two different marked regions (I and II) were noted in each case. The color codes for the lifetime distributions are given below each image. The samples were excited by a pulsed 488 nm laser at 25^{o}C.

Table 1: The determined lifetimes of the CDs at the two specified regions in each of the FLIM images of the GUVs in Figure 7.

Medium	Marked regions	τ_1 (ps)	τ_2 (ps)	τ_{av} (ps)
DOPC GUVs	I	359±8 (67)	1694±34 (33)	799±16
	II	344±7 (69)	1775±35 (31)	794±16
DPPC GUVs	I	799±16 (81)	2068±41 (19)	1036±21
	II	774±15 (82)	2307±46 (18)	1045±21
DOPC-DPPC-Chol GUVs	I	822±16 (85)	2335±47 (15)	1048±21
	II	412±8 (77)	2018±40 (23)	803±16

nature.[25] Hydrophobic CDs were prepared by a carbonization procedure using suitable carbon precursors for isolating lipid rafts using FLIM.[25] For the isolation of lipid rafts, giant unilamellar vesicles (GUVs) were prepared comprised of (i) DOPC, where only the Ld phase exists, (ii) DMPC, where only the Lo phase exists, and (iii) 2:2:1 mixture of DOPC, DPPC and cholesterol (Chol) where the coexistence of both Ld and Lo phase and cholesterol do tight intercalation with long and saturated acyl chain of the phospholipid forming lipid rafts on the Lo phase of the membrane. Such CDs' fluorescent intensity and lifetime are highly influenced by the fluidity and how tightly lipid molecules are packed inside the membrane. The fluidity of the membrane highly affects the fluorescent intensity of such CDs. There is an increase in the fluorescent intensity of CDs of the Ld phase of the membrane compared to the Lo phase. Most strikingly, in the case of GUV composting 2:2:1 mixture of DOPC, DPPC, and Chol, where Chol reorients into the Lo phase of the membrane forming rafts and hence there is a significant increase in fluorescent on the Lo phase of the membrane in comparison to the coexisted Ld phase of the same GUV.

Another interesting fact is that the Ld and Lo phases of the membrane highly influence the fluorescent lifetime of such CDs.[25] CDs localized in the Ld phase of the membrane have a higher lifetime than those comprising only the Ld phase (Table 1). By comparing the lifetime in FLIM, it is possible for a pinch point isolation of both the Ld and Lo phase of a membrane where both coexist, as depicted in Figure 7.[25]

Conclusion:

In summary, carbon dots can be synthesized from readily available carbon precursors, and based on the chosen precursor, one can tailor their luminescence and surface properties. Such synthesized carbon dots can be easily attached to the membrane surface to study the fluidity and dynamics of the membrane since they have relatively less bleaching properties than organic dyes; such fluorogenic nanoparticles can be used for real-time imaging of the membrane using fluorescence microscopy. However, the application of carbon dots for single-particle tracking is still lacking. Further study is needed to reveal the possible advantages of using such fluorogenic nanoparticles compared to Green Fluorescent Protein (GFP) tagged protein. Additionally, the potential toxicity of such fluorogenic nanoparticles needs to be studied intensively so that such carbon nanoparticles can be used in the healthcare sector.

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