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INTERNSHIP REPORT

Preparation of C8S3 with D_2O

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Introduction

This work evaluated the nanotube-forming efficiency (DWNTs) of the C8S3 molecule in the presence of deuterated water, D_2O. In fact, one of the problems in using water in nanotube formation is the formation of "bundles," which are concentric tubular structures, that occur several days after the nanotubes preparation.

C8S3 nanotubes were obtained via self-assembly of the amphiphilic cyanine dye 3,3-bis(2-sulfopropyl) -5,5'6,6'-tetrachloro-1,1'-dioctylbenzimidacarbocyanine (C8S3-Cl, M=902.8 g/mol) in D_2O via the "alcoholic route". For sample preparation, the C8S3 monomers was first dissolved in methanol (MeOH) to create a stock solution. Next, aggregation of monomers into nanotubes was induced by adding Milli-Q water to the stock solution. Addition of D_2O to the stock solution was accompanied by an immediate color change from orange to pink, indicating J-type aggregation. D_2O was added in two consecutive steps with a waiting time of 24hrs. Measurements were taken four days after sample preparation to see if bundles formation was slower with the use of D_2O.

Results and Discussions

Linear Absorption and Linear Dichroism (LD)

The linear absorption spectrum of DWNTs in D_2O four days after preparation is shown in Figure 1 (a). Five absorption peaks are visible: 560 nm, 570 nm, 580 nm, 589 nm and 599 nm. The peaks at 589 nm and 599 nm are associated with the outer and inner nanotube, respectively, while the higher energy absorptions are caused by other aggregates in solution.
To investigate the polarization dependence of the transitions, the spectrum of Linear Dichroism (LD) four days after preparation was taken and shown in Figure 1 (b).
LD resolves the absorption difference for linearly polarized excitation light parallel or perpendicular to the orientation axis of an anisotropic sample. Since the probability of absorbing a photon depends on the alignment of the transition dipole moments of the sample with respect to the incident light, the LD spectra provide information about the geometry of the system as well as the molecular structure [1]. To obtain quantitative information on transitions polarization, the Reduced Linear Dichroism (LDr) is used [1]:

$$LDr = \frac{A_{\parallel} - A_{\perp}}{\frac{1}{3}(A_{\parallel} - 2A_{\perp})}$$

The LDr spectrum of DWNTs in D_2O four days after preparation is shown in Figure 1 (c). On the condition that the nanotubes are flow-aligned, a positive LDr ($A_{\parallel} > A_{\perp}$) means an

excitonic transition is polarized parallel to the nanotube orientation axis, whereas a negative LDr ($A_{\parallel} < A_{\perp}$) means a transition is perpendicular to the orientation axis. Reduced linear dichroism of double-walled nanotubes show that the nanotubes align along the flow axis since the main excitonic transitions have large positive amplitudes. The outer tube absorption peak and inner tube absorption peak are transitions polarized parallel to the orientation axis of the nanotubes.

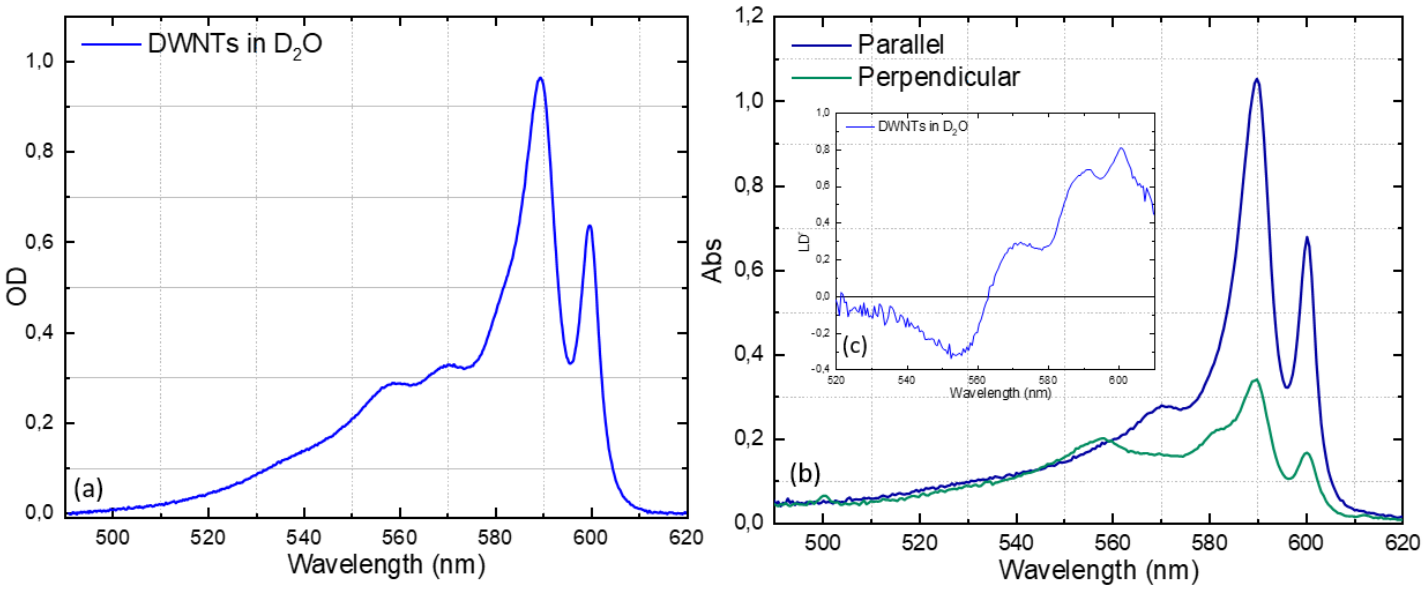

Figure 1: Isotropic linear absorption spectrum of DWNTs in D_2O four days after preparation (a); Polarized absorption spectra of DWNTs in D_2O four days after preparation of parallel polarized and perpendicular polarized light (b); Corresponding reduced linear dichroism LDr spectrum for the of DWNTs in D_2O four days after preparation (c).

Cryo-TEM imaging of DWNTs

Cryo-TEM imaging of the DWNTs was performed to relate the spectra to the structural data. Cryo-TEM image of DWNTs in D_2O four days after preparation is shown in Figure 2 (a). The averaged line profiles is shown in Figure 2 (b). The boundary distance b_{DW} is determined from the intersection of the Fresnel fringes with the baseline, while the dip-to-dip distance a_{in} is established by finding the inner minima of the line profile

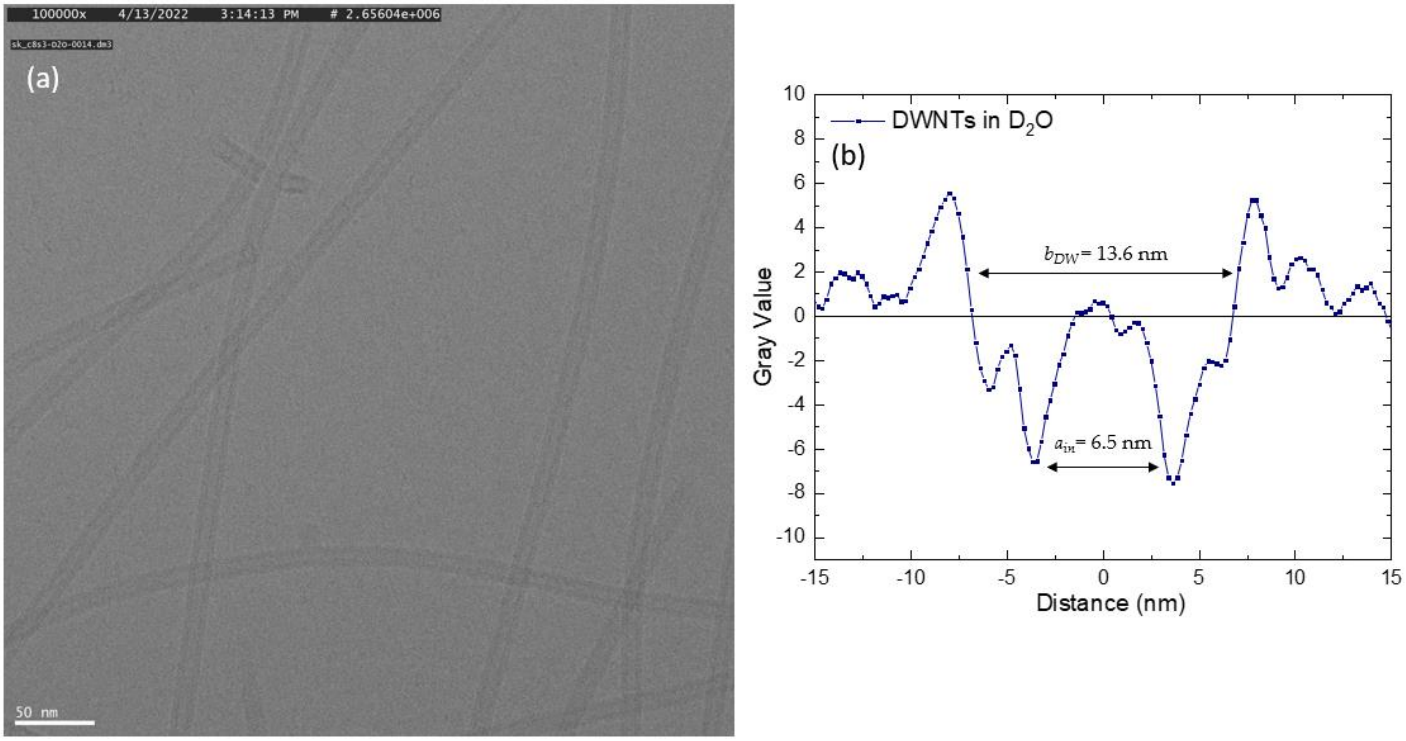

Figure 2: Cryo-TEM image of DWNTs in D_2O (a); Averaged line profiles of DWNTs. The line profiles are averaged over 30 TEM line profiles, each 22.5 nm in length.

Histograms of the boundary and dip-to-dip distances of the individual Cryo-TEM line profiles are shown in Figure 3, while Table 1 reports the different metrics for the DWNTs. The homogeneity of the DWNTs can be established by the distribution of boundary and dip-to-dip distances of a various Cryo-TEM line profiles.

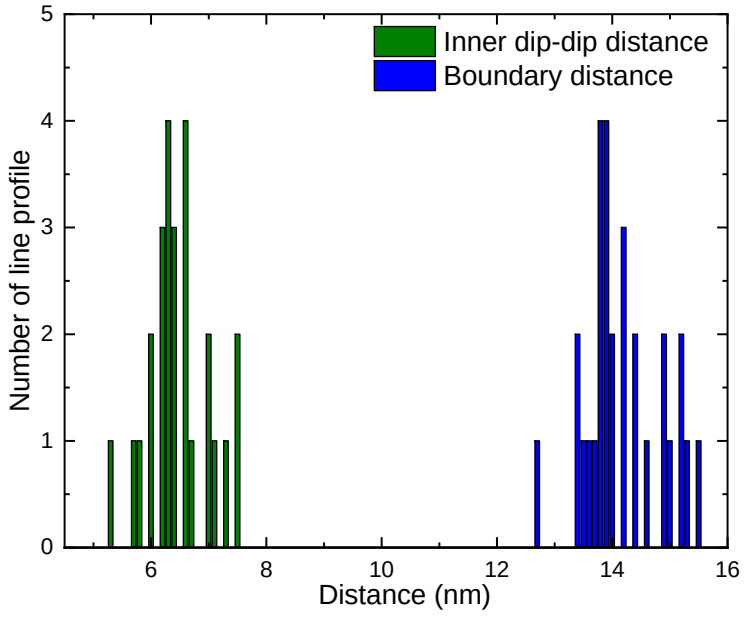

Figure 3: Histograms of boundary distances (blue) and inner dip-to-dip distances (green) of DWNTs in D_2O.

The boundary distance of DWNTs is 13.6 ± 0.6 nm, in agreement with the literature for DWNTs in H_2O, and the inner dip-to-dip distance is 6.5 ± 0.5 nm again in agreement with the literature.

	Mean	S.D.
Boundary distance $b_{DW,}$ nm	13.6	0.6
Inner dip-to-dip distance, a_{in}, nm	6.5	0.5

Table 1: Parameters of the distributions of boundary and dip-to-dip distances of DWNTs

Regarding the formation of bundles, Figure 4 shows Cryo-TEM images at different magnification of DWNTs and bundles. The amount of bundles within the solution is not very high considering that the sample is four days old. This explains the fact that no contribution from the budnles appeared in the absorption spectra; the amount of bundles was not enough to produce absorption.

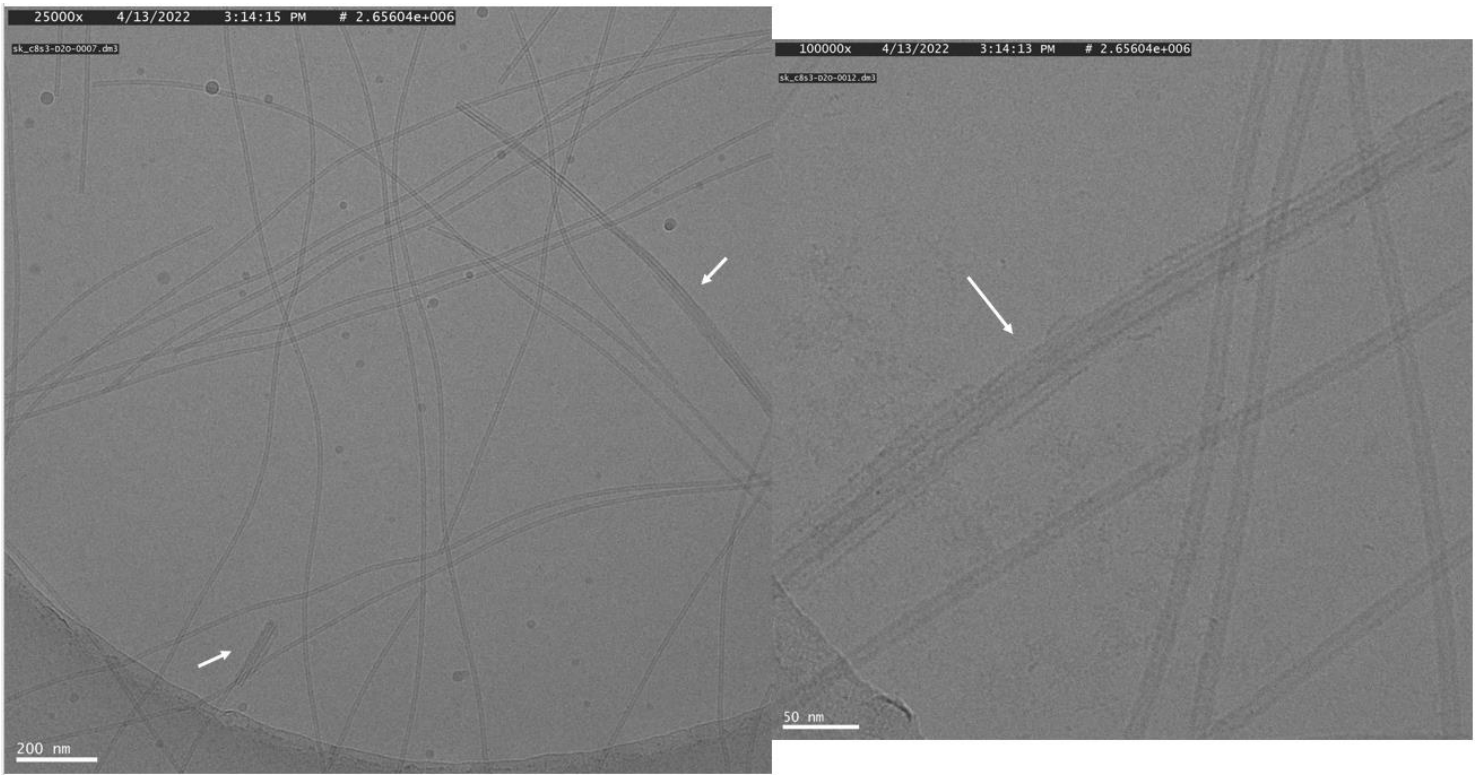

Figure 4: Cryo-TEM images of DWNTs and bundles in D_2O four days after preparation. White arrows indicate bundles.

Conclusions

By using optical spectroscopy and Cryo-TEM, it can be concluded that the use of D_2O improves the performance on DWNTs formation.

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2. Krishnaswamy, S.R., Gabrovski, I.A., Patmanidis, I. *et al.* Cryogenic TEM imaging of artificial light harvesting complexes outside equilibrium. *Sci Rep* **12,** 5552, 2022

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INTERNSHIP REPORT

Self-Assembly routes of amphiphilic cyanine dye (C8S3)

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Introduction

Self-assembled nanostructures arise when constituent building blocks spontaneously organize into ordered aggregates that exhibit different physical and chemical properties than the initial monomer molecules. Under certain conditions, self-assembly proceeds spontaneously toward a decrease in total free energy, leading to thermodynamically stable aggregates. Cyanine dyes are a family of molecules that form aggregates with different structures. In particular, they are known to form double-walled nanotubes a few nanometers long with interesting optical properties.

The self-assembly pathways of these nanotubes, however, still remains an open question, as well as the individual steps leading to the self-assembly of highly organized and ordered structures.

In fact: put the different parts of a car in a big box, and shake the whole, will you get a car? This image is often used to express what self-assembly can be able to reach. Spontaneous arrangement of small building blocks orderly structures are ever-present in living systems and are critical for nanoscale design, where human tools are powerless.

Self-assembly is extremely advantageous from a technological point of view because it is a spontaneous and reversible process with minimal waste and a wide domain of applications ranging from nucleation of inorganic particles, to formation of vesicles, nanotubes, etc. The car metaphor is rather used in publications and its purpose is to emphasize the novelty of nanotechnology and the break with conventional top-down fabrication techniques.

On the other hand, it conveys a kind of magical power to nanoscientists: access to the nanoscale also triggers the ambition to create artificial cells and unravel the mystery of the origin of life. That's why understanding the phenomenon of self-assembly in detail is a key point from the perspective of scientific progress.

The amphiphilic dye studied in this work is cyanine 3,3′-bis(2-sulfopropyl) -5,5′,6,6′-tetrachloro-1,1′-dioctyl-benzimida-carbocyanine (C8S3), and the chemical structure of a single C8S3 monomer can be divided into three parts: the hydrophilic heads, the cyanine dye chromophore (aromatic core) and the hydrophobic tails. The hydrophilic and hydrophobic regions of C8S3, when dispersed in a polar solvent such as water in the presence of methanol, align themselves in a bilayer-like structure in which the hydrophobic regions maintain minimal contact with water to form the double-walled, tubular J-aggregate structure: double-walled nanotubes, DWNTs, as shown in Figure 1.

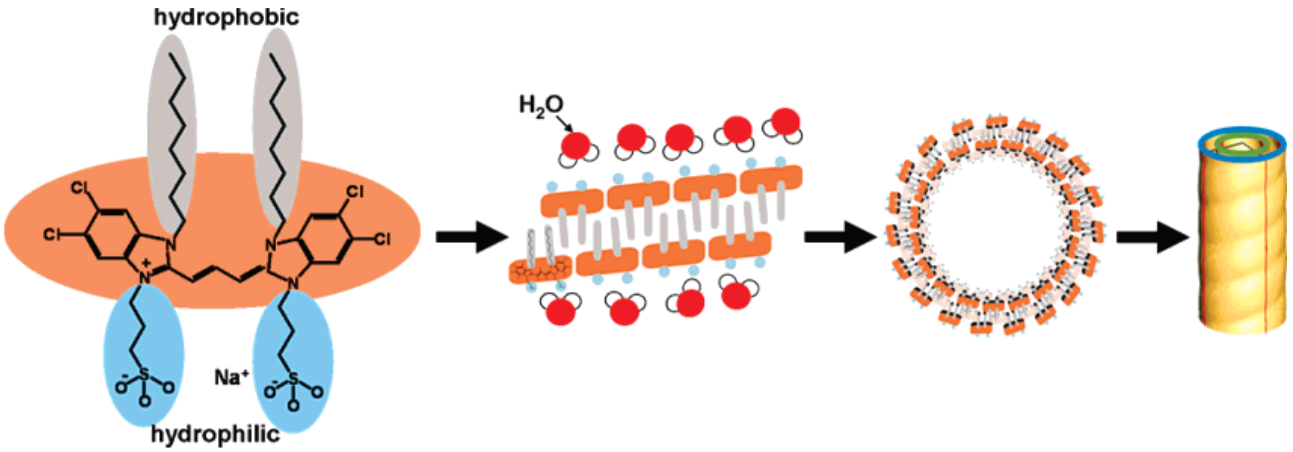


Figure 1: (Left to Right) Chemical structure of C8S3 cyanine dye; Sketch of DWNTs formation.

The driving force for the formation of C8S3 nanotubes is a combination of inter- and intra-molecular interactions. The hydrophobic effect determines the orientation of the polar groups toward the solvent within and around the nanotube and the nonpolar groups toward the interior of the nanotube, while electrostatics and π-π stacking create a network of interactions that regulates the position and placement of the aromatic nuclei with respect to each other. The high polarizability of the conjugate system generates powerful van der Waals forces (induced dipole interaction) and dense packing of the C8S3 monomers. In addition, the organized packing induces special excited states of the aromatic backbone electrons, called Frenkel excitons, which are responsible for the optical properties of the C8S3 nanotube and other cyanine dye aggregates in general.
The purpose of this work is to understand the early stages of the self-assembly process by evaluating the very first structures adopted during nanotube formation.

Sample preparation

C8S3 nanotubes were obtained via self-assembly of the amphiphilic cyanine dye 3,3-bis(2-sulfopropyl) - 5,5'6,6'-tetrachloro-1,1'-dioctylbenzimidacarbocyanine in water via the "alcoholic route". To measure "young" samples and follow them over time,a stock of C8S3 was first prepared in MeOH (2 mg dye/ 4 ml MeOH), then nanotubes were formed in the bottles at different ratios of stock : Milli-*Q* water (H_2O) and mixed vigorously. Addition of water to the stock solution was accompanied by an immediate color change from orange to pink, indicating J-type aggregation.

Results and Discussions

Steady-State Absorption

Consider a Stock: H_2O ratio of 1:1. Figure 2 shows the absorption spectrum of the first 9 minutes from the preparation of J aggregates. The spectrum shows a very pronounced peak at 520 nm, which can be associated with the monomer peak, and a slightly accentuated peak around 585 nm. No particular spectral changes are observed over time, indicating that at low amounts of water there are not enough water molecules to ensure a complete self-assembly process. This leads one to reflect on the nature of the single peak at 585 nm, which could be associated with that of the outer nanotube.

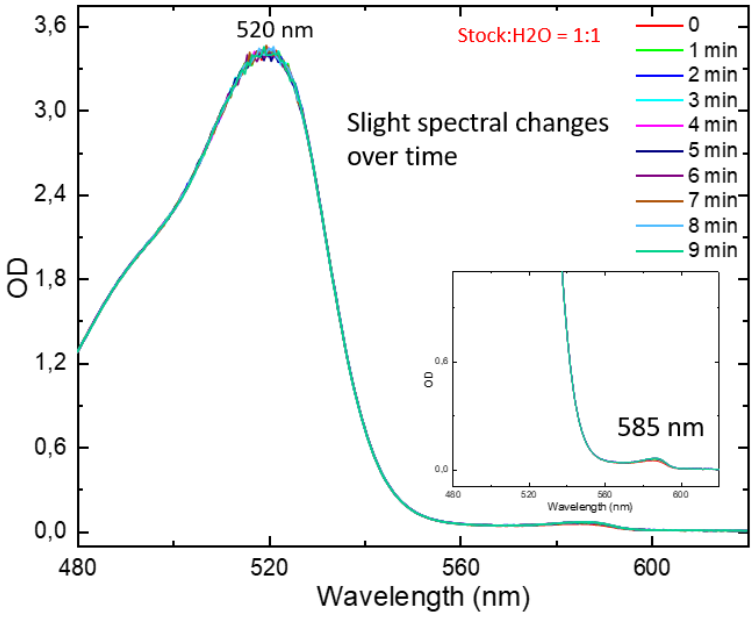


Figure 2: Linear absorption spectra of stock: H_2O = 1:1 over time.

A zoom on the peak of the outer tube reveals a slight change over time, not only in intensity but also in wavelength. This shows a red shift, that is, a shift to longer wavelengths, as shown in Figure 3.

The hydrophobic effect is dominant at the beginning of the self-assembly of C8S3 process, as the molecules immediately collapse into structures, in which the alkyl tails are hidden by water (like micelles), forming the first "outer" wall.

The only visible peak in the spectrum could indicate that the preferred packing mode of C8S3 molecules is already adopted at an early stage in the self-assembly process (nanotube) and that secondary structures emerge after a while.

Considering the fact that in the presence of the inner nanotube, absorption of the outer nanotube occurs at 590 nm, in the absence of interactions with the inner wall, the energy difference between the fundamental and excited states is larger; this results in a shift to shorter wavelengths (585 nm).

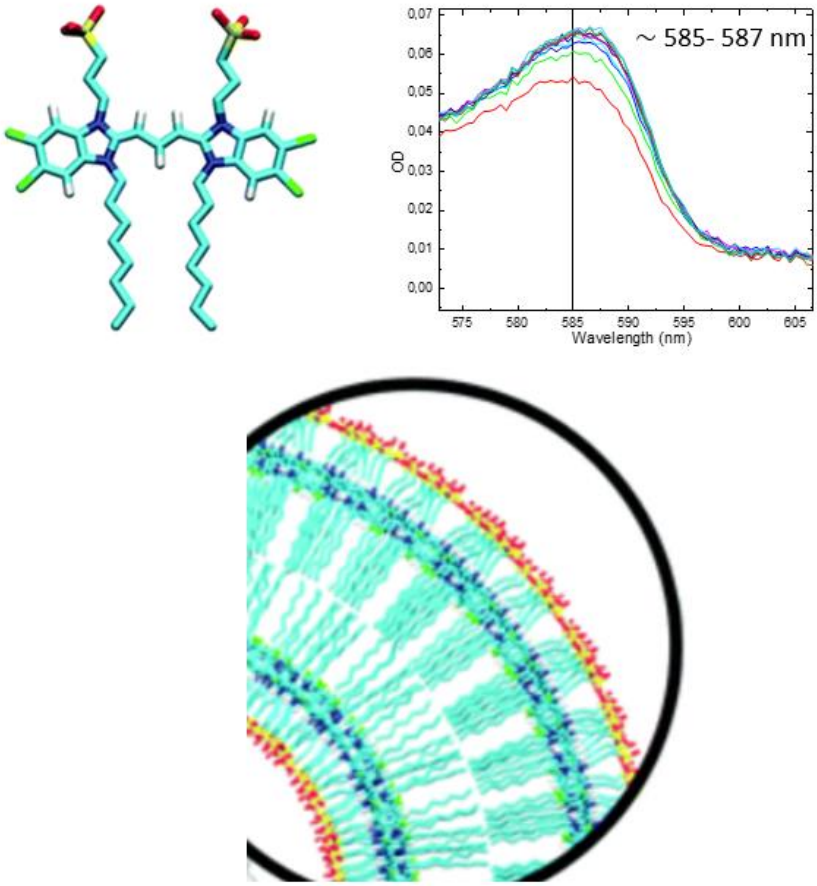


Figure 3: C8S3 monomer structure; 585-587 nm peak zoom; Nanotube double wall structure in which the apolar tails are arranged inward and the polar head is arranged toward the solvent.

Increasing the volume of water relative to stock (stock: H_2O = 1:1,5) the absorption spectrum (Figure 4) still shows the monomer peak at 520 nm strongly pronounced, as well as three peaks at 580 nm, 591 nm and 598 nm. The absorption peak at 591 nm is associated with the outer nanotube, that at 598 nm with the inner nanotube, while the higher energy transition at 580 nm is associated with another structure in equilibrium with that of the nanotubes. Again, no spectral change is observed in the first 15 minutes, however, in this case there is a sufficient amount of water molecules to ensure self-assembly, forming the double wall, but still a large amount of monomer present in solution.

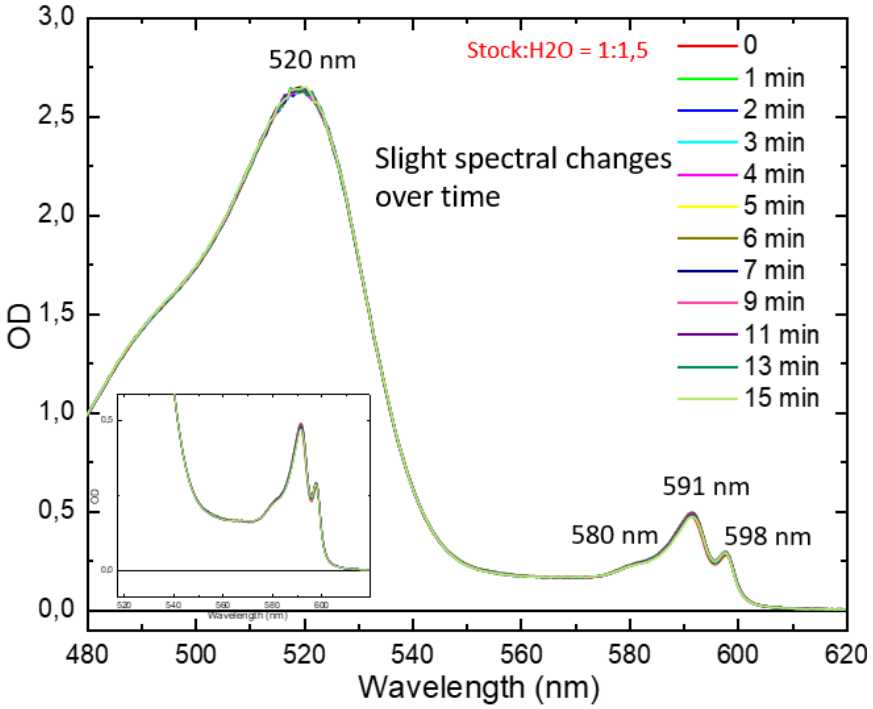


Figure 4: Linear absorption spectra of stock: H_2O = 1:1,5 over time.

By increasing the amount by volume of water, the absorption peaks of the inner nanotube, outer nanotube and other structures appear more resolved. Figure 5 (a) shows the absorption spectrum of the first 15 minutes of a stock: H_2O = 1: 2, while Figure 5 (b) shows that of the sample with stock: H_2O = 1: 2,5. Both samples show six absorption peaks at 520 nm, 556 nm, 567 nm, 580 nm, 589 nm and 598 nm. The monomer peak at 520 nm decreases with time while those of the aggregates increase with time. The peaks of the secondary structures become more and more defined with time, indicating that their formation takes place over more time.

Also at higher amounts of water (stock: H_2O = 1: 2,5), it can be seen that the intensity of the monomer peak is lower while the absorption intensity of the nanotube peaks is higher, indicating a larger amount of product.

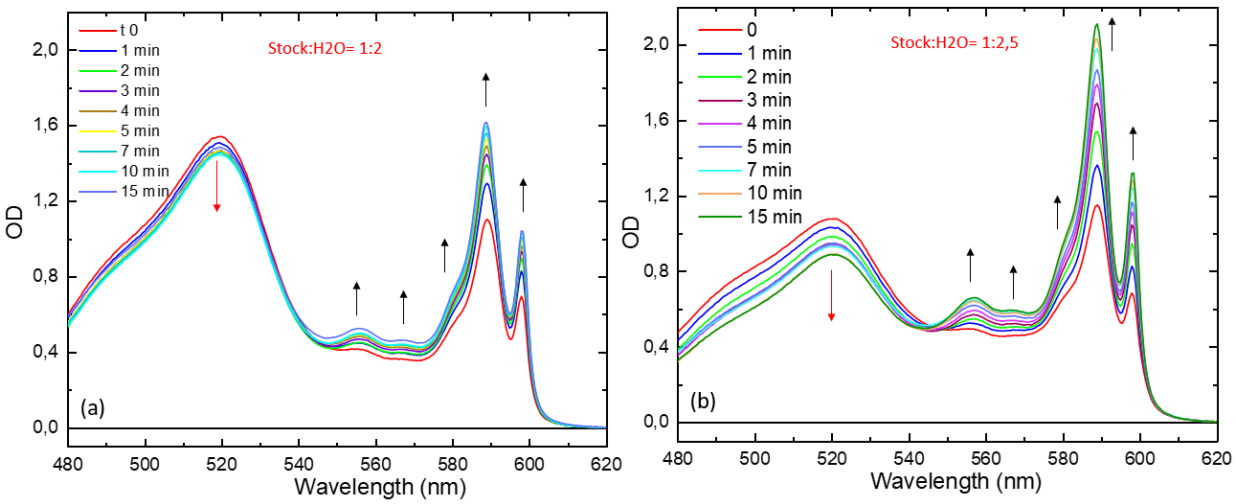


Figure 5: Linear absorption spectra of (a) stock: H_2O = 1:2 over time (b) stock: H_2O = 1:2,5 over time.

Figure 6 (a) shows the absorption spectrum over time of a stock: H_2O = 1:3 sample, while Figure 6 (b) shows the absorption spectrum of a stock: H_2O = 1: 5 sample. While a 1:3 sample produces well-defined and clear peaks, a 1:5 sample produces in addition to the monomer peak at 520 nm, a peak at 589 nm associated with the outer nanotube peak, and a weak shoulder at 599 nm associated with the inner nanotube. No further absorption peaks are visible until the first 4 minutes, when a shoulder at 580 nm begins to take place. The ratio of stock: H_2O = 1:3 seems to be the right compromise for good nanotube preparation, while increasing the volume amount of water seems to achieve the opposite effect. Probably in this case there are not enough monomer molecules to interact with water molecules.

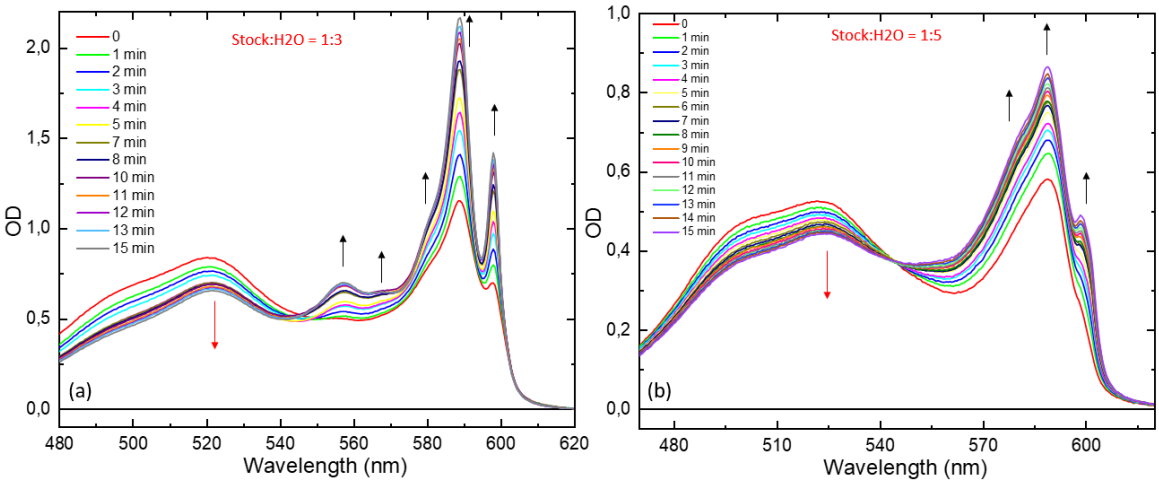


Figure 6: Linear absorption spectra of (a) stock: H_2O = 1:3 over time (b) stock: H_2O = 1:5 over time.

Once it found that the best stock/H_2O ratio for sample preparation is that of 1:3, the spectral evolution was followed for a large time period. Indeed, Figure 7 shows the evolution of the stock: H_2O = 1:3 sample from time zero (t_0) to three days after sample preparation. What can be seen is that not only does the monomer absorption peak continue to decrease with time, but the higher energy transitions, at 557 nm, 570 nm and 580 nm, become increasingly resolved. In addition, the peak of the outer nanotube seems to undergo a slight red shift after about 21 h, it in fact goes from 598 nm to 600 nm.

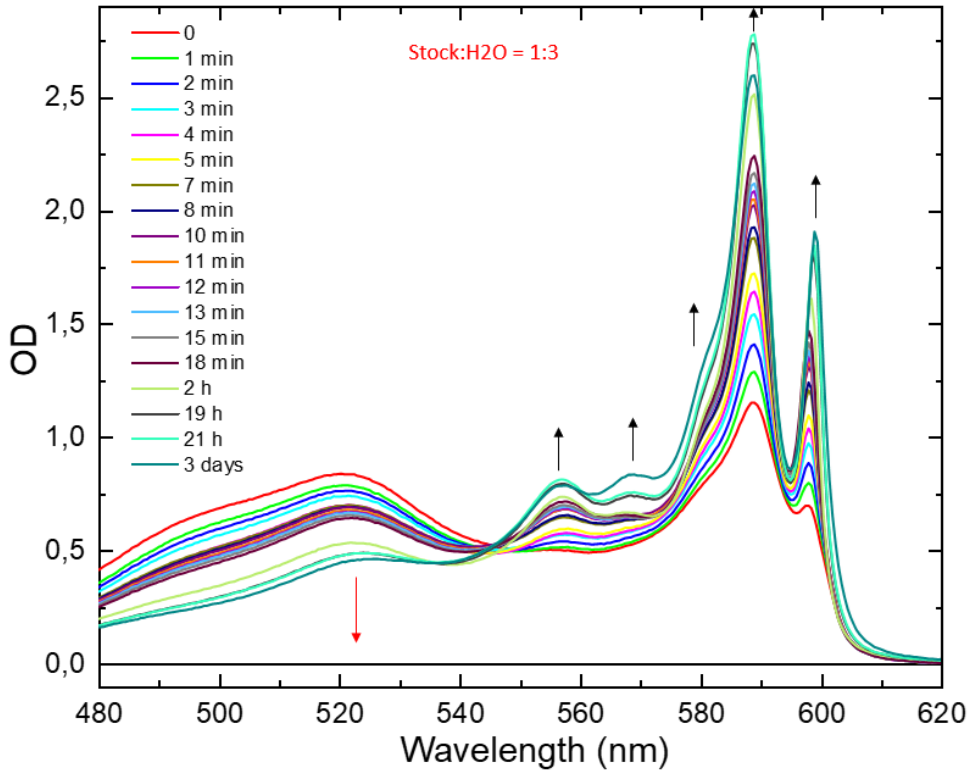


Figure 7: Linear absorption spectra of stock: H_2O = 1:3 over a long period time.

TEM imaging

To get a clearer view on the aggregates formed in the early stages of self-assembly, imaging by Transmission Electron Microscopy (TEM) was performed on a 1:1 (v/v) sample of stock: H_2O. The procedure for sample preparation involved the use of 1-2% uranyl acetate solution used as staining.

In electron microscopy, images are nothing more than magnified projections of the various densities proportional to the components in the section.

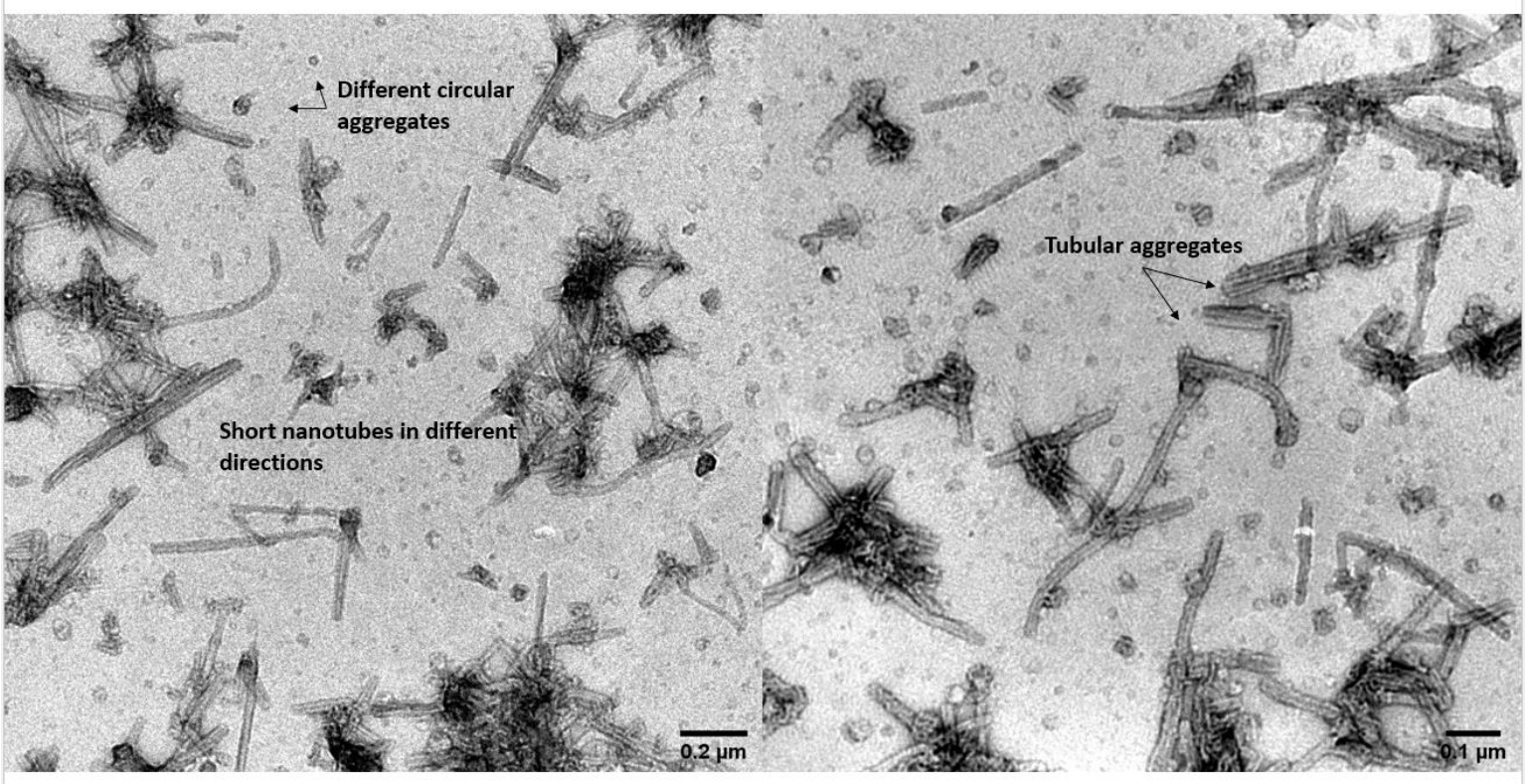
To achieve differential enhancement of densities in organic structures, differential contrast is required to produce sharp image definition.

In transmission electron microscopy (TEM), contrast is mainly produced by the scattering of electrons on the sample. Structures that strongly scatter electrons appear as dark areas while structures that scatter fewer electrons appear bright. To increase the contrast, electron dense dyes can be added to the sample.

Staining is usually performed with heavy metal salts commonly derived from uranium or tungsten. Heavy metal ions are used because they easily interact with the electron beam and produce phase contrast.

Moreover, the use of staining is necessary not only to increase contrast but also to "stop" molecules that would naturally move due to Brownian motion. In fact, staining by forming a film over the grid ensures proper visualization of the molecules.
However, this procedure does not prevent structures from changing over time, as is the case, for example, with Cryo-TEM, in which freezing the sample allows a real-time visualization of what is present in the sample at the same time it is frozen.
To minimize the change of structures over time, the 1:1 (v/v) sample was chosen, since spectroscopic measurements (Fig. 2) showed that it does not change rapidly with time.

Figure 8 shows TEM images of the 1:1 (v/v) stock: H2O sample in the early stages of nanotubes formation. This is further verified by the fact that the addition of H_2O to the stock leads to a color change of the solution from orange to light pink (Fig. 9), exactly as is the case with the standard preparation of DWNTs. The standard preparation for good formation of DWNTs requires a volume ratio of at least 1:2 (v/v).



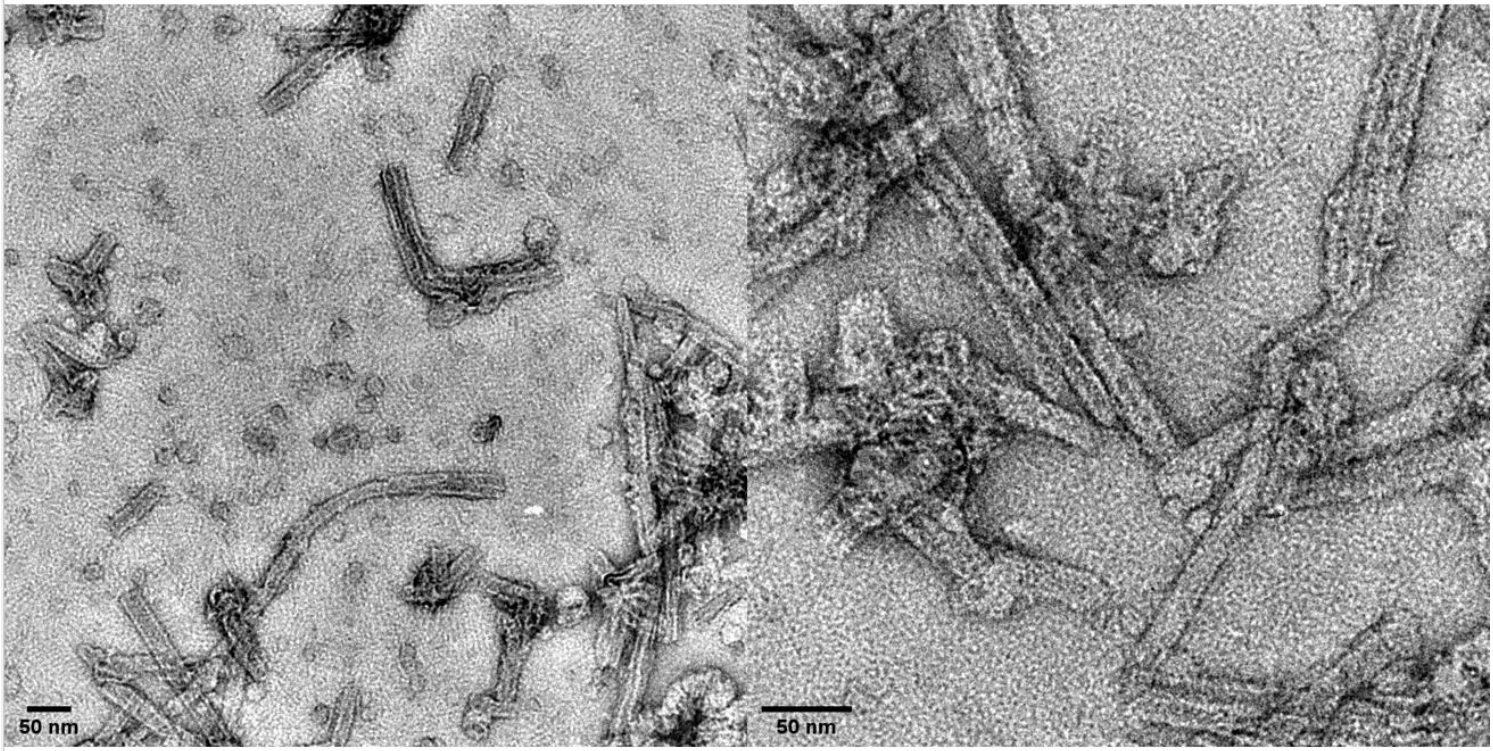


Figure 8: TEM images of a stock: H_2O = 1:1 sample in the initial stages of self-assembly. Images are at different magnification.

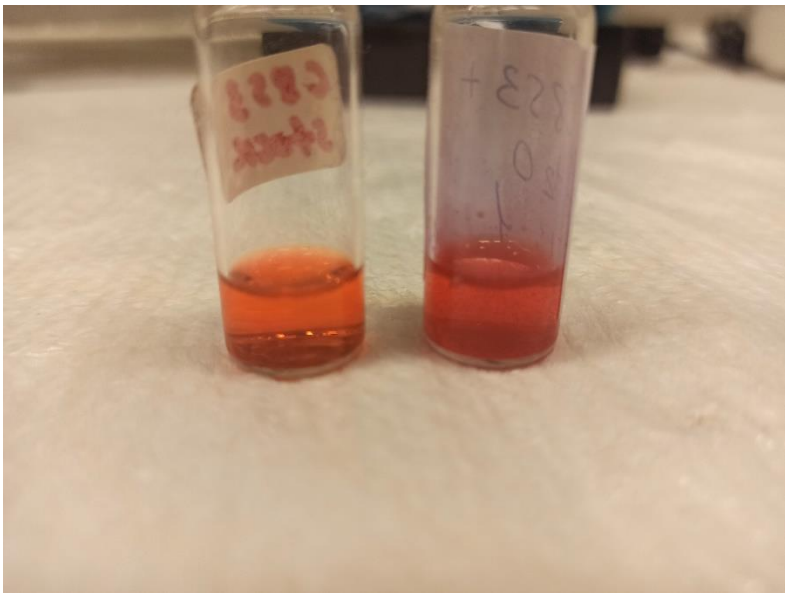


Figure 9: On the left is the orange-colored solution with the stock; on the right is the stock solution to which H_2O was added at a 1:1 (v/v) ratio, which changes color to pink.

The images show different aggregates in shape and size. Visible are some very short DWNTs, circular aggregates, some concentric tubular aggregates and some nanotubes without a highlighted double layer that could belong to a single nanotube structure.

Cryo-TEM images of the same sample could be made in the future to clarify the structures present.

Conclusions

This work concluded that the higher energy electronic transitions associated with other structures become increasingly clear and defined with time, indicating that nanotubular structures are the energetically favored ones in the early stages of self-assembly. This hypothesis can be confirmed by TEM images showing mostly short tubular structures.

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INTERNSHIP REPORT

Study of C8S4 aggregates

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Introduction

J aggregates are molecular self-assemblies with interesting excitonic properties, such as highly delocalized excitons, making them prime candidates for designing excitonic energy transfer systems [1]. J aggregates of the C8S3 molecule that form double-walled nanotubes (DWNTs) through hydrophobic and hydrophilic interactions have been studied for a long time [1] [2]. Here we evaluate the possibility of the C8S4 dye to form tubular J aggregates, like its relative C8S3. The C8S4 molecule is cyanine 1,1′- dioctyl-3,3′-di(4sulfobutyl) -5,5′,6,6′-tetrachloro-benzimidazolocarbocine; the molecular structure is shown in Figure 2 (a).

Preparation of the J aggregates did not involve the usual "alcohol route" as this produced a distinct phase separation, not allowing good use of the sample. For this reason, it was essential to use a solvent that would allow both the C8S4 monomer to be dissolved in it and allow the formation of aggregates through hydrophilic and hydrophobic interactions. The solvent chosen was dimethyl sulfoxide (DMSO), with a relative polarity (relative to water) of 0.444. It therefore is very polar, having a strongly polarized S-O bond.

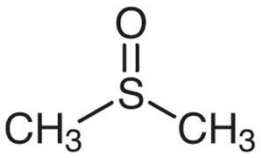

Figure 1: Molecular structure of DMSO

Once the stock solution was prepared with the monomer C8S4 in DMSO, aggregation of the monomers into aggregates was induced by adding Milli-Q water to the stock solution.

Results and Discussions

Linear Absorption

The absorption spectrum of the monomer C8S4 is shown in Figure 2 (b). One absorption peak is observed at 528 nm and one at 494 nm. After adding water, a red shift, caused by the formation of J aggregates, is observed, and an intense absorption peak is visible at 585 nm (Fig. 2 (c)). Also visible is a low absorption peak at 525 nm, and a shoulder at 595 nm. Aggregate formation is also accompanied by a color change of the solution from orange to pink, exactly what happens in nanotube formation with the C8S3 dye.

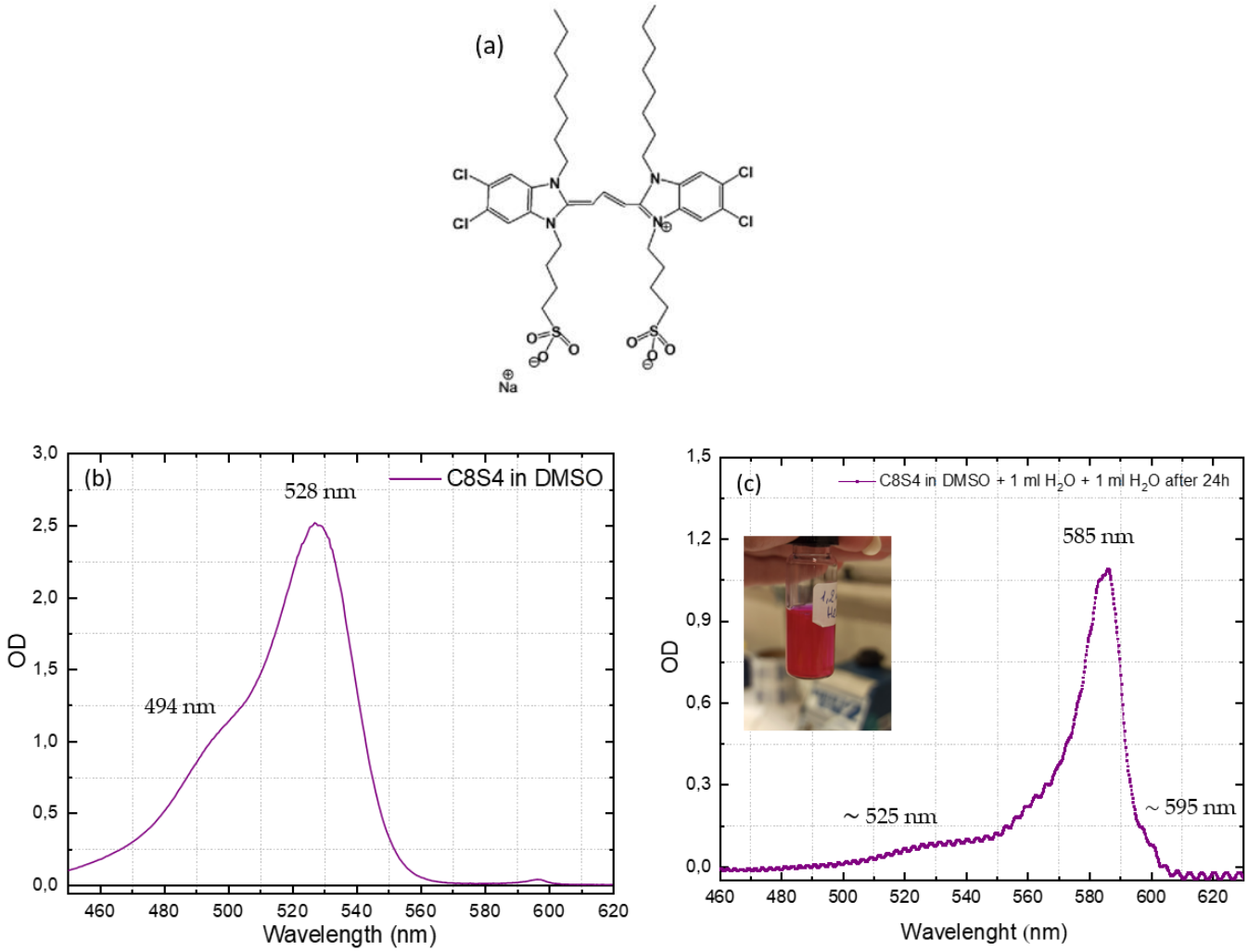

Figure 2: Molecular structure of C8S4 molecule (a) [3]; Absorption spectrum of C8S4 monomer dissolved in DMSO (b); Absorption spectrum of J-aggregates prepared by adding H_2O (c).

Cryo-TEM imaging of J-aggregates from C8S4

Cryo-TEM measurements are needed to evaluate the shape of the aggregates; however, DMSO is not a good solvent for microscope use. For this reason, it was decided to prepare the J-aggregates through the "direct route," which consists of dissolving the monomer directly in water, leave it stirring for 24 h and then add the same amount of water. This procedure ensures the formation of J aggregates and the complete dissolution of the monomer. The absorption spectrum of J aggregates prepared through "the direct route" is shown in Figure 3 (a). It appears broad and poorly resolved, however, at least three absorption peaks can be identified: the most intense at about 570 nm, a slight absorption at 620 nm, and a peak at about 500 nm. Cryo-TEM imaging showed the presence of small black dots and circular aggregates, attributable to micelle formation responsible for the absorption peaks at 500 and 570 nm (Fig. 3 (b)). In addition, indissolved particles were found (Fig. 3 (c)) that caused absorption at wavelength values greater than 600 nm.

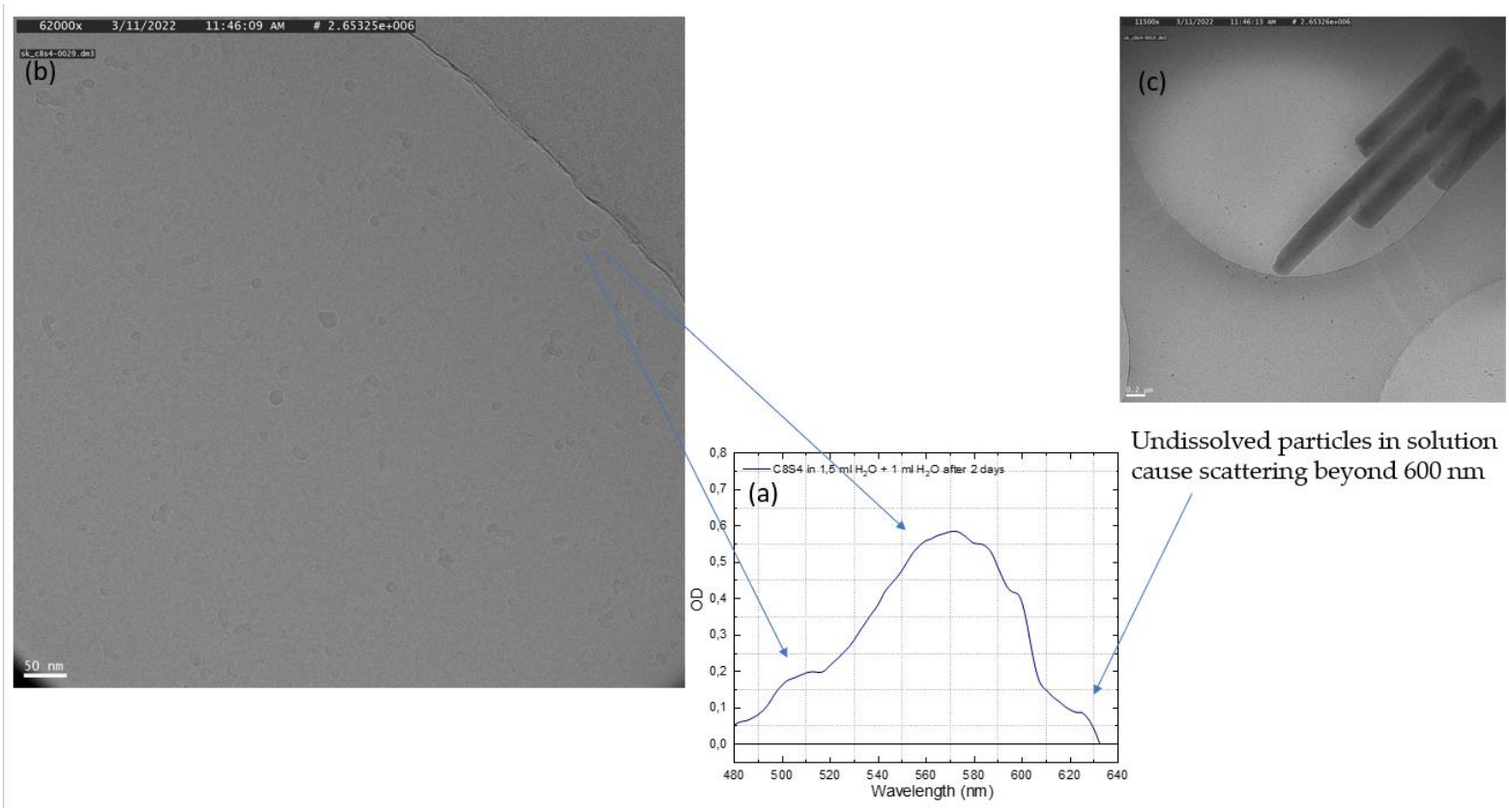

Figure 3: Linear absorption spectrum of J-aggregates prepared by the "direct route" (a); Cryo-TEM images showing the presence of circular aggregates (b); Cryo-TEM images showing the presence of undissolved particles in solution.

More Cryo-TEM images are shown in Figure 4.

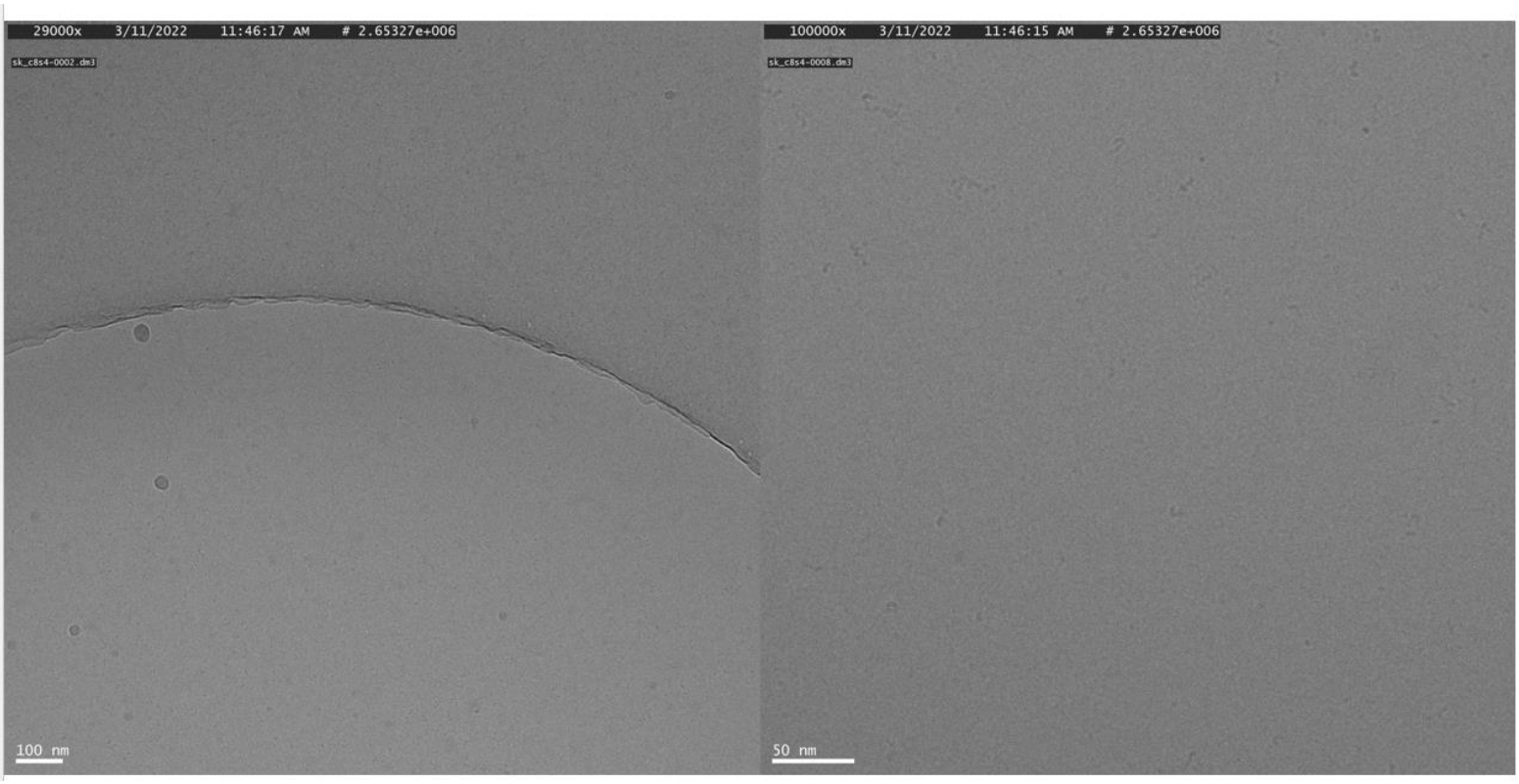

Figure 4: Cryo-TEM images of J-aggregates from C8S4 prepared by the "direct route". Black dots and larger circular aggregates are visible on the left. Black dots and other aggregates of different shapes are visible on the right.

Conclusions

By using Steady-State Absorption and Cryo-TEM, we can conclude that the cyanine dye C8S4 does not lead to the formation of double-walled tubular aggregates.

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Comparison of TEM images obtained by Negative Staining of Normal DWNTs and DWNTs with Fluorenone

- Negative Staining Procedure

A method to improve the contrast in the electron microscope is to stain the specimen with a heavy-metal salt.
Specific binding of the stain to certain chemical groups in the specimen leads to "positive staining". When the specimen is embedded in a thin layer of a stain, without any specific (preferential) binding this is called "negative staining".

The solution used for negative staining is: 1-2 % uranyl acetate, pH 3.5. The experimental procedure is schematically summarized in Figure 1.
Glow-discharge is used to make the surface of grids hydrophilic (Fig. 1 A). This improves the adsorption of particles and the spreading of the staining solution.
After diluting the sample, one takes the grid with the carbon film and pipettes about 5 µl of the sample to the grid (Fig.1 B), then blots up the excess liquid with a well-absorbent filter paper (Fig.1 C-D), next one adds 5 µl of staining solution to the grid (Fig.1 E), blots it up and leaves the grid in the air to dry (Fig.1 F).

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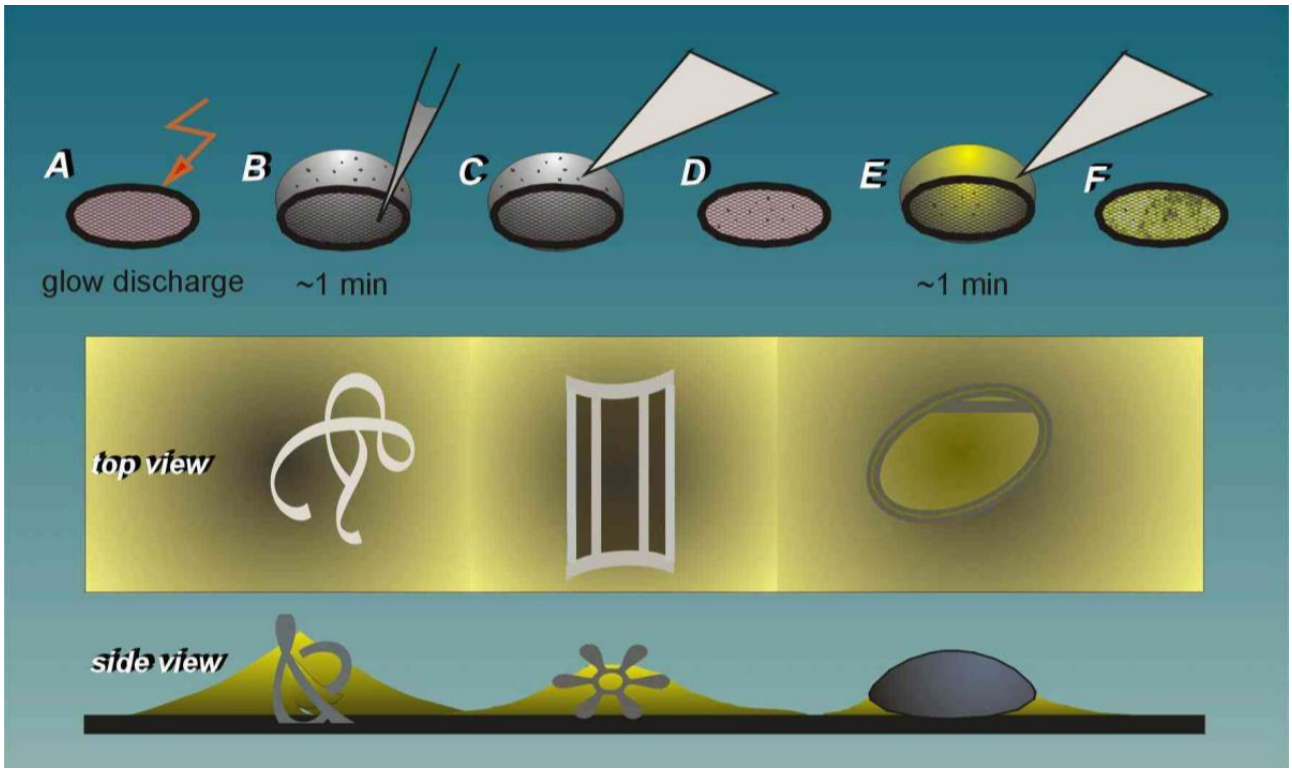


Figure 1: Experimental procedure for sample preparation with staining solution

- TEM Images

Normal DWNTs (without Fluorenone)

DWNTs with Fluorenone

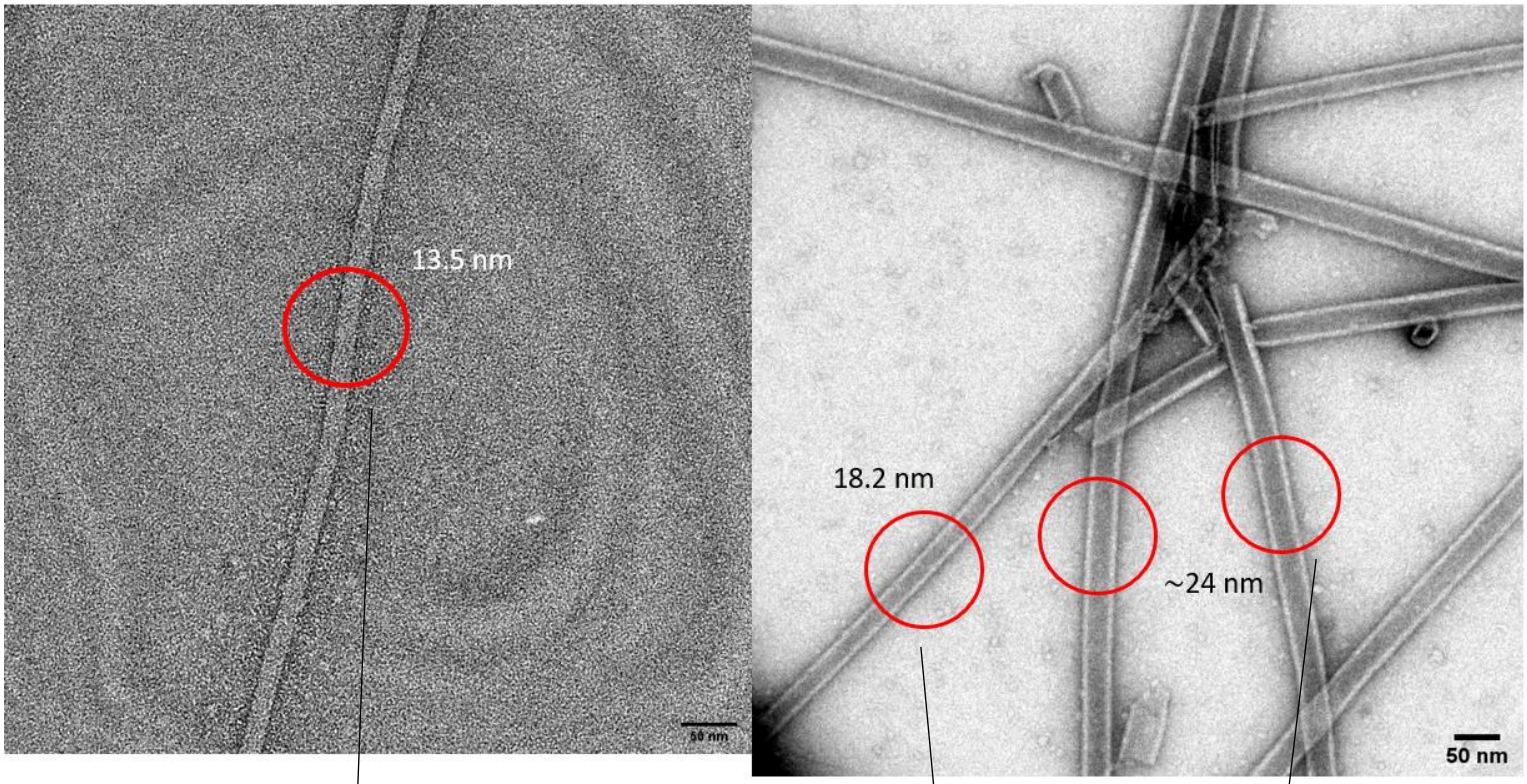
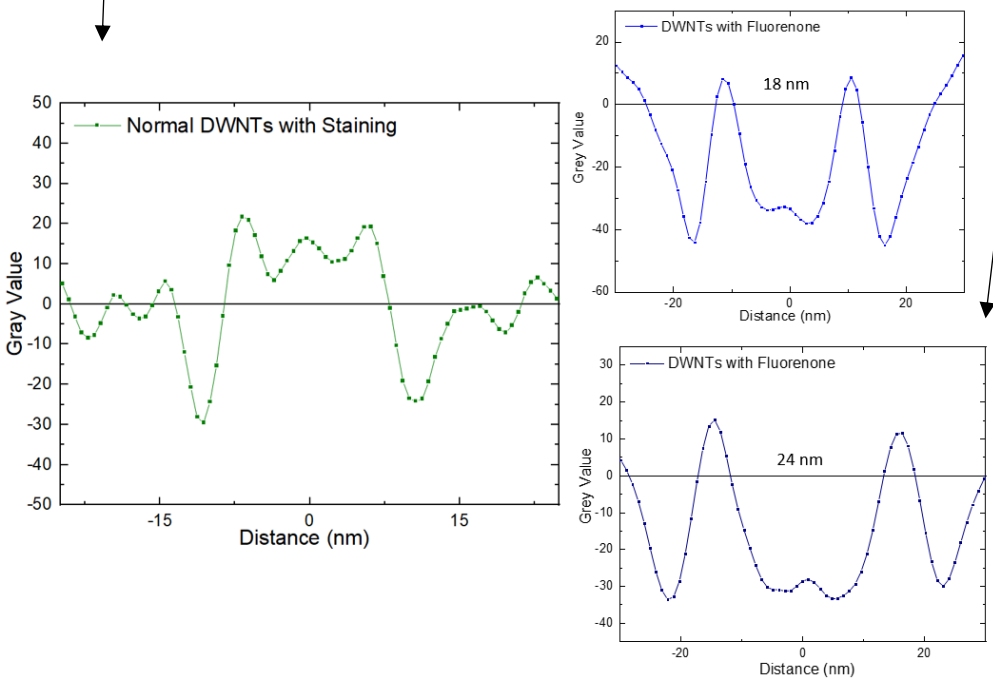


Figure 2: TEM images and averaged line profiles of DWNTs without and with Fluorenone



Through TEM profile analysis it is possible to determine of the size of the nanotubes: for DWNTs without fluorenone it is 13.5 nm, in agreement with Cryo-TEM (see Figure 3), while those with fluorenone are quite different, in fact there are both 18 nm and 24 nm nanotubes.

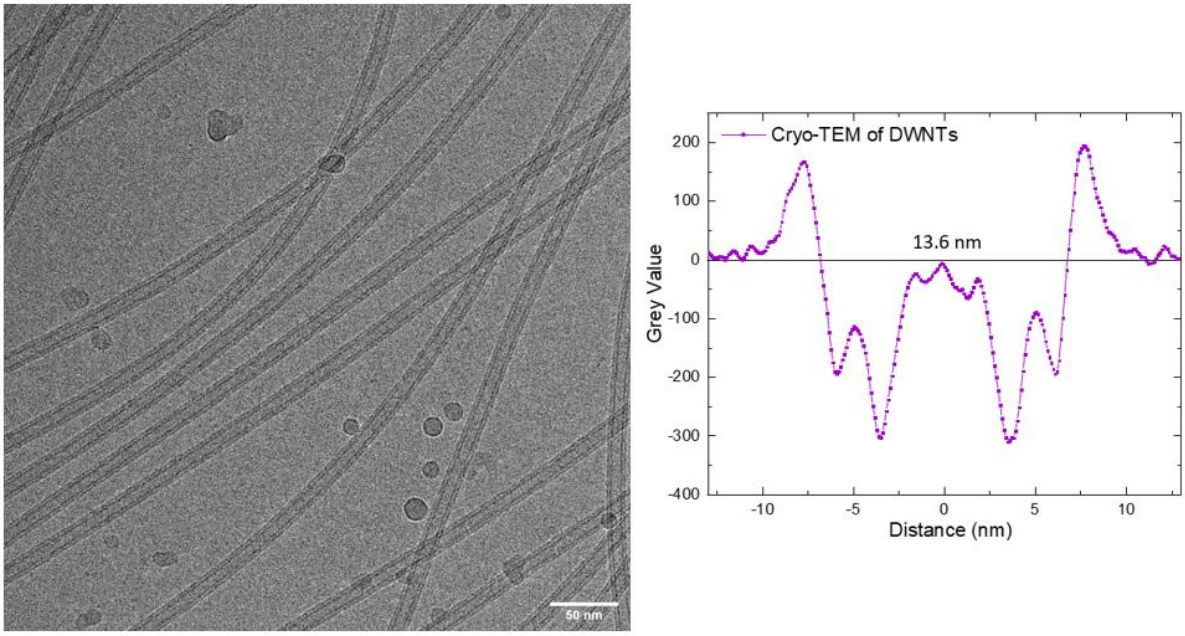


Figura 3: Cryo-TEM image and averaged line profiles of DWNTs.

Nanotubes (DWNTs) in the absence of fluorenone show no area more stained than others; the electron density of the inner part is the same as that of the grid, indicating that the solution has not penetrated inside them. On the other hand, nanotubes (DWNTs) in the presence of Fluorenone show a distinct presence of the stain within them, indicating that the staining solution was able to penetrate inside the nanotubes.