Supplementary Information file

Colorization of Optically Transparent Surfactants to Track Their Movement in Biphasic Systems Used for Differentiation of Nanomaterials

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1. Experimental

1.1. Materials

Table S1 Compounds that were used to prepare samples, their acronyms and vendors.

Compound	Acronym/formula	Vendor
2-bromobenzaldehyde	(2-Br)BA	Sigma Aldrich (USA)
2-nitrobenzaldehyde	(2-NO_2)BA	Sigma Aldrich (USA)
3-([3-Cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate	CHAPSO	Abcr (Germany)
3-hydroxybenzaldehyde	(3-OH)BA	Acros Organics (India)
3-methoxybenzaldehyde	(3-OMe)BA	Acros Organics (India)
4-hydroxybenzaldehyde	(4-OH)BA	Acros Organics (India)
Acetic acid (99.9%)	CH_3COOH	VWR Chemicals (France)
Dextran (70 000 Da)	DEX	PanReac AppliChem (Germany)
Ficoll 400	Ficoll	PanReac AppliChem (Germany)
Furfural	FUR	Thermo Scientific (Dominican Republic)
Lithocholic acid	LCA	Thermo Fisher (Italy)
Pluronic F68	F68	Sigma Aldrich (USA)
Pluronic L35	P35	Sigma Aldrich (USA)
Polye(ethylene glycol) (6 000 Da)	PEG	Alfa Aesar (Germany)
Signis SG65i SWCNTs	SWCNTs	Sigma Aldrich (USA)
Sodium chenodeoxycholate	SCHDC	Abcr (Germany)
Sodium cholate	SC	Alfa Aesar (New Zealand)
Sodium dehydrocholate	SDH	Abcr (Germany)
Sodium deoxycholate	SDC	Thermo Scientific (New Zealand)
Sodium dodecyl sulfate	SDS	Sigma Aldrich (USA)
Sodium hyodeoxycholate	SHDC	Abcr (Germany)
Sodium taurocholate hydrate	STC	Abcr (Germany)
Sodium taurodeoxycholate	STDC	Abcr (Germany)
Sucrose	$C_{12}H_{22}O_{11}$	Südzucker (Poland)
Sulfuric acid (98%)	H_2SO_4	Supelco (Belgium)
Triton X-100	TX	Acros Organics

Double-distilled water obtained from the Elix Millipore system was used for all experiments.

1.2. Preparation of SWCNT dispersions

SWCNTs (40 mg) and SC solution (40 mL; 2%, m/V) were introduced into a 50 mL vial and placed in an ice bath. Then, the mixture was sonicated (Hielscher UP200St, 200 min, 30 W) and centrifuged (Eppendorf Centrifuge 5804 R) at 18 °C at the Relative Centrifugal Force (RCF) of 15314 × g for 1.5 h to precipitate bundled SWCNTs. 80% of the upper volume of the supernatant was collected and used for further experiments. Optical absorption spectrum and PL map of as-made SWCNT dispersion are shown below (**Figure S1**).

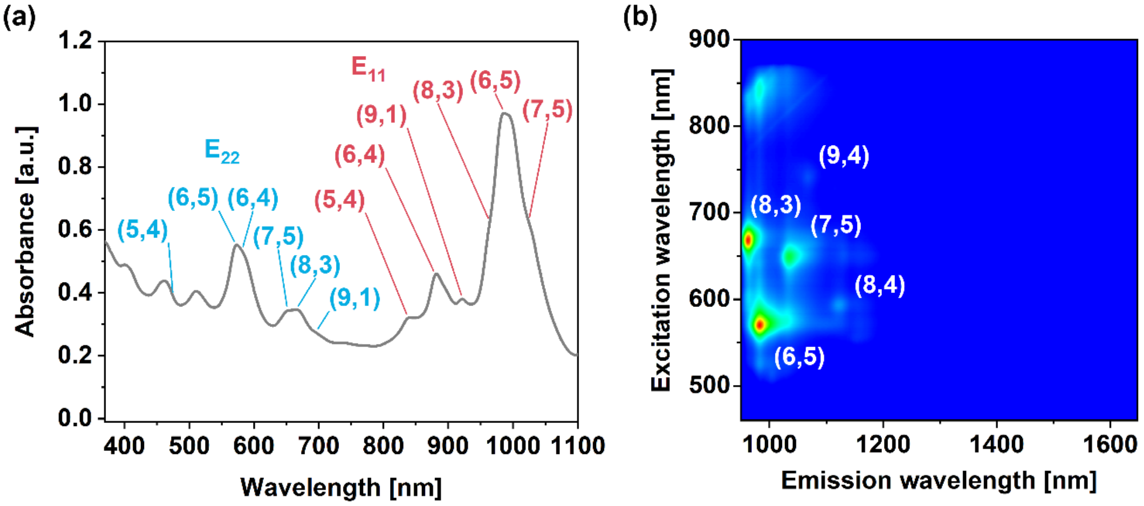

Figure S1 Characterization of the starting SWCNT material dispersed in 2% SC: (a) Optical absorption spectrum indicating key chiralities (metallic species were not assigned) and (b) photoluminescence excitation-emission map (some of SWCNT types from the optical absorption spectrum were not detected due to limited measurement range or low photoluminescence quantum yield of selected SWCNT chiralities).

1.3. ATPE protocol

Water, stock solutions of phase-forming polymers (DEX/Ficoll and PEG/P68/P35/P121) and other components (surfactants and SWCNT dispersion) were transferred to a centrifuge tube. The mixture was then homogenized by a Vortex mixer (about 10 s per sample) and centrifuged (Eppendorf Centrifuge 5804 R) for 3 minutes at 18 °C at an RCF of 2025 × g to promote the formation of a biphasic system. Top and bottom phases were collected by pipetting to avoid cross-contamination.

Concentrations of stock solutions employed for ATPE sample preparation are included **in Table S2**. Compositions of ATPE samples are described in **Table S4** (for DEX-PEG system including only SC addition), **Table S7** (for DEX-PEG system including TX and SC addition), **Table S10** (for DEX-PEG system including SDS and SC addition) and **Table S13** (for other ATPE systems including SC addition)

Table S2 Concentrations of stock solutions employed for ATPE sample preparation and determination of SC concentrations therein.

Compound	DEX	PEG	SC	TX100	SDS	Ficoll	P68	P35	P121
Concentration of stock solutions (w/w; water) [%]	20	50[1]	10[2]	2.5	10	20	25	25	25

[1] 25% where indicated

[2] 2% when used instead of SWCNT dispersion

1.4. Determination of binodal curve

In a clean and dry glass Nessler cylinder, a magnetic stirring bar was placed, after which the combined weight was recorded. Subsequently, a sufficient amount of DEX solution (70 kDa) was added, and the weight of the system was measured again. Afterward, PEG (6 kDa) was introduced dropwise while stirring until the solution became cloudy. The weight of the mixture and the glassware was re-registered. Next, water was slowly added until the solution was clear again, and the weight was acquired. This routine leading to a cloudy/transparent solution was repeated several times to collect more measurement points. Finally, based on the obtained data, the mass fractions of both polymers necessary to form the biphasic system was drawn in the form of a binodal curve (**Figure 2d**).

1.5. Optical characterization

Optical absorption spectra were measured with the Hitachi U2910 spectrophotometer were measured from 360 to 1100 nm using cuvettes made of polystyrene with the optical path length of 10 mm. A reference cuvette containing distilled water was put in the second measurement channel for analysis. Excitation-emission photoluminescence (PL) maps were measured with PhotonClaIR spectrophotometer across the specified wavelengths ranges: excitation (460–900 nm) and emission (948–1600 nm).

2. Determination of SC concentration in aqueous solution

2.1. The reaction procedure developed by Pettenkofer

In 1844, Pettenkofer reported that mixing sugar, concentrated sulfuric acid and ox bile resulted in the appearance of a violet color, reminiscent of potassium permanganate. Based on this observation, he proposed that this method could be used for detecting cholic acid. The procedure involves careful addition of concentrated sulfuric acid (approximately two-thirds the volume of the bile acid solution) to a small portion of the analyte, ensuring the temperature does not exceed 62.5°C. Subsequently, two or three drops of a cane sugar solution (prepared by dissolving one part sugar in four to five parts water) are added. The mixture is then shaken gently, and if the cholic acid is present, purple color appears[1]. We adopted this strategy to evaluate whether Pettenkofer's procedure could be used to detect sodium salt of cholic acid dissolved in water, which is commonly used to improve the partitioning of materials within the framework of the ATPE method. After combining 2 mL each of aqueous solutions of SC (2%) and sucrose (20%), 4 mL of concentrated sulfuric acid was slowly introduced down the wall of the glass cylinder. Due to the density difference, two phases were formed, and subsequently yellowish color started to appear at the interface **(Figure 2**). After a few minutes, the color at the interface turned purple indicating that Pettenkofer's test can be utilized to determine SC presence. In a reference experiment, wherein SC solution was replaced with water, the interface became black because of the formation of the products of sucrose decomposition.

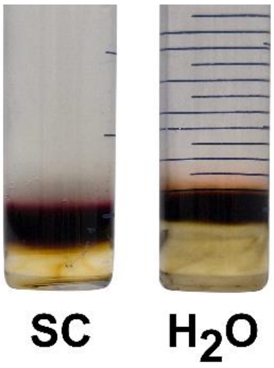

Figure S2 Picture of samples subjected to Pettenkofer's test in the presence/absence of SC.

2.2. Improved protocol reported in this work

According to the results described in the main text, the application of an aromatic aldehyde in the form of (4-OH)BA instead of sucrose produced the best results, so this reactant was used for the quantification of SC. In such a case, 200 µL of 1% (m/m) solution of (4-OH)BA in 70% acetic acid was mixed with 200 µL of 70% acetic acid and 100 µL of the analyzed cholate-containing sample in a glass container. The vial was then placed in a glycerin bath preheated to 70°C. Once the reagent mixture reached 70°C, 2.6 mL of sulfuric acid (1:1.3 v/v corresponding to 57% m/m) was added. The mixture was subsequently incubated at this temperature for 10 minutes, after which it was transferred to an ice bath to halt the reaction. After cooling, the mixture was stored in the dark to allow the reaction to develop, which led to more intense coloration of the sample, giving to higher absorbance values, thereby facilitating cholate detection. We noted that the 7 days of incubation time was sufficient.

To validate this approach, we carried out a series of six experiment series, each of which examined a number of SC concentration values under various ambient conditions. All of the calibration curves shown below had very good linearity with high goodness of fit, which approached unity **(Figure S3)**. Besides, all of them were practically analogous and no major deviation from the trend could be observed. As a result, the proposed approach may be used for quantitative analysis with confidence.

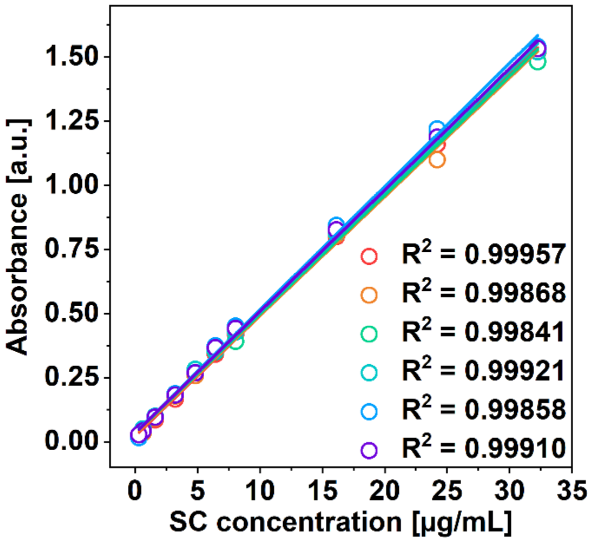

Figure S3 Intensity of optical absorption at the maximum of the peak located at ca. 567 nm as a function of SC concentration after 7-day reaction time.

3. Determination of SC concentration in DEX-PEG systems

Table S3 Compositions of ATPE samples prepared using the DEX-PEG system and SC.

Sample	DEX$_{aq.}$ (20%) [µL]	PEG$_{aq.}$ (50%) [µL]	SC$_{aq.}$ (10%) [µL]	SC$_{aq.}$ (2%) [µL]	1 mg/mL SWCNT dispersion in SC$_{aq.}$ (2%) [µL]	H$_2$O [µL]
A1	1350	540	-	-	-	2700
A2	1350	540	360	-	-	2340
A3	1350	540	360	225	-	2115
A4	1350	540	360	-	225	2115
B1	1380	1110	360	-	-	1740
B2	1380	1110	360	225		1515
C1	2760	550	360	-	-	920
C2	2760	550	360	225		695

The spectra registered for these samples, after subjecting them to the modified Pettenkofer procedure, can be found in **Figure 2a** and **Figure S4**. Based on the acquired spectral data and the calibration curve (**Figure S3**), the mass of SC in each phase was calculated (**Table S4**).

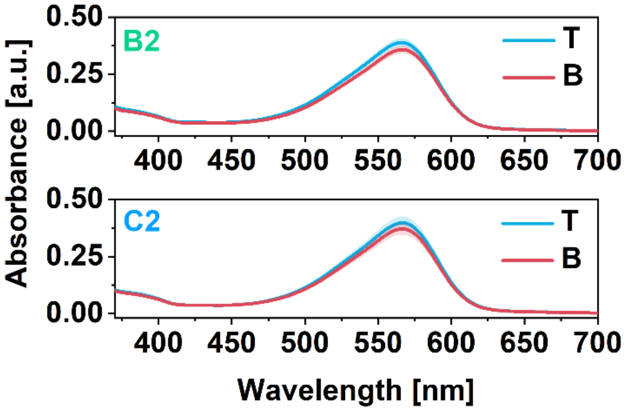

Figure S4 Optical absorption spectra of top and bottom phases of the samples prepared in the indicated conditions (**Table S3**) after subjecting them to the modified Pettenkofer procedure.

Table S4 Determined mass of SC in the top and bottom phases for the indicated samples using the calibration curve displayed in **Figure S3**. The discrepancy between the calculated and introduced mass of SC was used for the presentation of statistical uncertainty as shaded areas in **Figure 2e** and **Figure S4**.

Sample	**Mass of SC**				
	Mass of SC introduced into the sample [mg]	**Calculated mass of SC in top phase**	**Calculated mass of SC in bottom phase**	**Calculated mass of SC in both phases**	**Calculated mass/ introduced mass [%]**
A1	-	-	-	-	-
A2	36.00	25.64	11.52	37.16	+ 3%
A3	40.50	29.97	13.10	43.08	+ 6%
A4	40.50	30.28	13.40	43.68	+ 8%
B1	36.00	25.28	11.00	36.29	+ 1%
B2	40.50	29.86	12.18	42.04	+ 5%
C1	36.00	27.19	11.03	38.22	+ 6%
C2	40.50	30.66	12.69	43.35	+ 7%

4. Determination of SC concentration in DEX-PEG systems with TX-100

Analogous experiments were conducted in the DEX-PEG system operated with the addition of TX-100 as a counter-surfactant favoring the migration of SWCNTs to the top phase. The parameters used to carry out purification are provided in **Table S5**, whereas the corresponding optical absorption spectra and the mass of SC determined in the relevant phases is given in **Figure S5** and **Table S6**, respectively.

Table S5 Compositions of ATPE samples prepared using the DEX-PEG system as well as SC and TX-100.

Sample	**DEX$_{aq.}$ (20%) [µL]**	**PEG$_{aq.}$ (50%) [µL]**	**SC$_{aq.}$ (10%) [µL]**	**SC$_{aq.}$ (2%) [µL]**	**TX-100$_{aq.}$ (2.5%) [µL]**	**1 mg/mL SWCNT dispersion in SC$_{aq.}$ (2%) [µL]**	**H$_2$O [µL]**
D1	1350	540	-	-	650	-	2050
D2	1350	540	360	-	650	-	1690
D3	1350	540	360	225	650	-	1465
D4	1350	540	360	-	650	225	1465

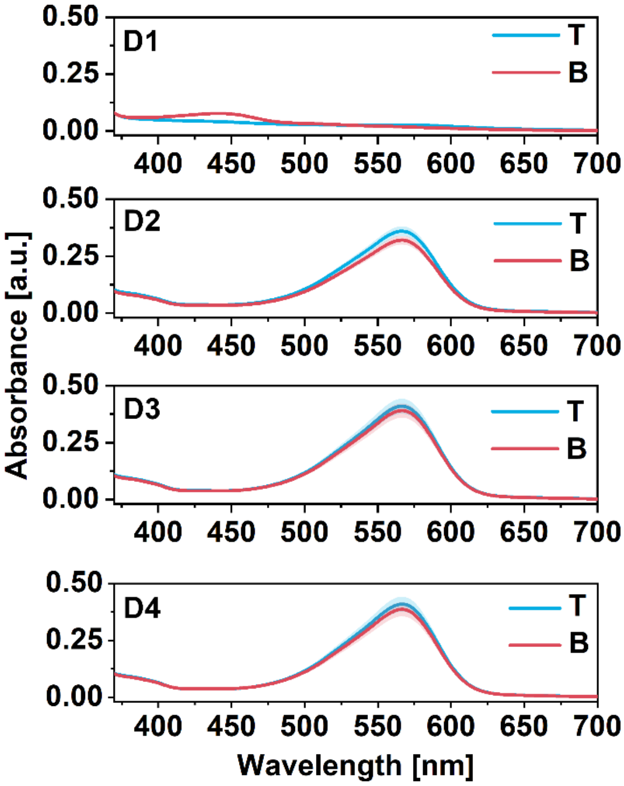

Figure S5 Optical absorption spectra of top and bottom phases of the samples prepared in the indicated conditions (**Table S5**) after subjecting them to the modified Pettenkofer procedure.

Table S6 Determined mass of SC in the top and bottom phases for the indicated samples using the calibration curve displayed in **Figure S3**. The discrepancy between the calculated and introduced mass of SC was used for the presentation of statistical uncertainty as shaded areas in **Figure S5**.

Sample	**Mass of SC**				
	Mass of SC introduced into the sample [mg]	**Calculated mass of SC in top phase**	**Calculated mass of SC in bottom phase**	**Calculated mass of SC in both phases**	**Calculated mass/ introduced mass [%]**
D1	-	-	-	-	-
D2	36.00	27.23	10.79	38.06	+ 6%
D3	40.50	30.76	13.03	43.79	+ 8%
D4	40.50	30.71	12.87	43.58	+ 8%

5. Determination of SC concentration in DEX-PEG systems with SDS

Similar experiments were conducted in the DEX-PEG system operated with the addition of SDS as a counter-surfactant favoring the migration of SWCNTs to the top phase. The parameters used to carry out purification are provided in **Table S7**, whereas the corresponding optical absorption spectra and the mass of SC determined in the relevant phases is given in **Figure S6** and **Table S8**, respectively.

Table S7 Compositions of ATPE samples prepared using the DEX-PEG system as well as SC and SDS.

Sample	**DEX$_{aq.}$ (20%) [µL]**	**PEG$_{aq.}$ (50%) [µL]**	**SC$_{aq.}$ (10%) [µL]**	**SDS$_{aq.}$ (10%) [µL]**	**1 mg/mL SWCNT dispersion in SC$_{aq.}$ (2%) [µL]**	**H_2O [µL]**
E1	1350	540	-	180	-	2520
E2	1350	540	360	180	-	2160
E3	1350	540	360	180	225	1935

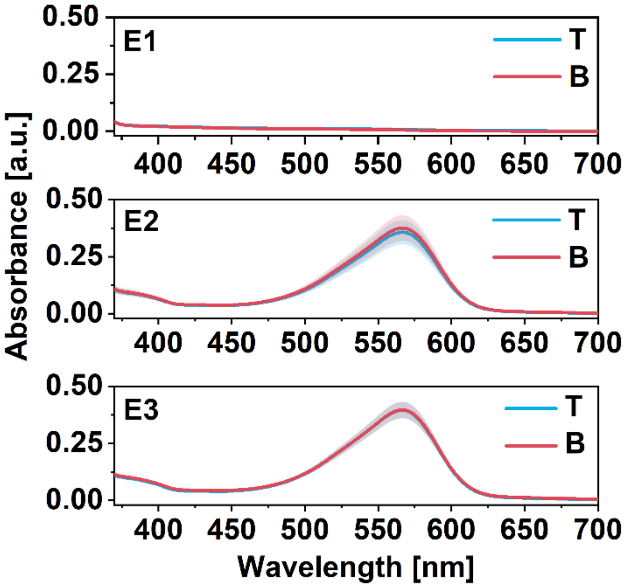

Figure S6 Optical absorption spectra of top and bottom phases of the samples prepared in the indicated conditions (**Table S7**) after subjecting them to the modified Pettenkofer procedure.

Table S8 Determined mass of SC in the top and bottom phases for the indicated samples using the calibration curve displayed in **Figure S3**. The discrepancy between the calculated and introduced mass of SC was used for the presentation of statistical uncertainty as shaded areas in **Figure S6**.

Sample	**Mass of SC**				
	Mass of SC introduced into the sample [mg]	**Calculated mass of SC in top phase**	**Calculated mass of SC in bottom phase**	**Calculated mass of SC in both phases**	**Calculated mass/ introduced mass [%]**
E1	-	-	-	-	-
E2	36.00	28.03	13.20	41.23	+ 15%
E3	40.50	30.51	13.66	44.17	+ 9%

6. Determination of SC concentration in other partitioning system

Analogous experiments were conducted in other partitioning systems wherein either PEG or DEX was substituted for another phase-forming polymer. The parameters used to carry out purification are provided in **Table S9**, whereas the corresponding optical absorption spectra and the mass of SC determined in the relevant phases is given in **Figure S7** and **Table S10**, respectively.

Table S9 Compositions of other system ATPE samples

Sample	DEX (20%) [µL]	PEG (25%) [µL]	Ficoll (20%) [µL]	PL68 (20%) [µL]	PL35 (20%) [µL]	SC (10%) [µL]	H_2O [µL]
F1	-	2000	2000	-	-	360	230
F2	2000	-	-	2000	-	360	230
F3	2000	-	-	-	2000	360	230

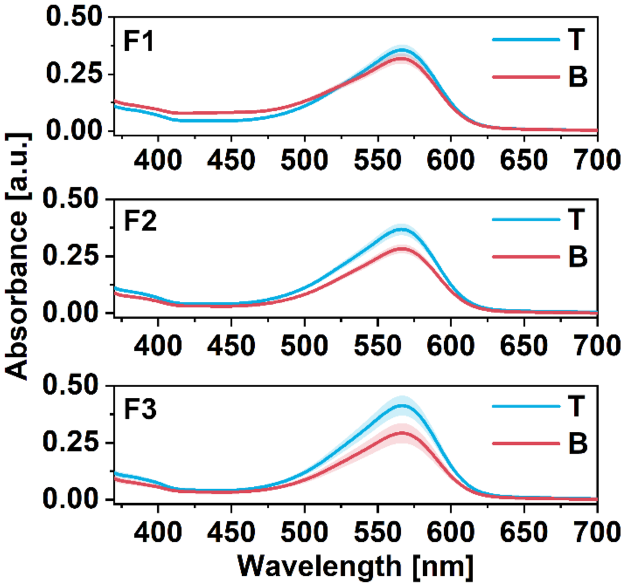

Figure S7 Optical absorption spectra of top and bottom phases of the samples prepared in the indicated conditions (**Table S9**) after subjecting them to the modified Pettenkofer procedure.

Table S3 Determined mass of SC in the top and bottom phases for the indicated samples using the calibration curve displayed in **Figure S3**. The discrepancy between the calculated and introduced mass of SC was used for the presentation of statistical uncertainty as shaded areas in **Figure S7**.

Sample	Mass of SC				
	Mass of SC introduced into the sample [mg]	Calculated mass of SC in top phase	Calculated mass of SC in bottom phase	Calculated mass of SC in both phases	Calculated mass/ introduced mass [%]
F1	36.00	28.97	9.71	38.68	+ 7%
F2	36.00	28.97	9.71	38.68	+ 7%
F3	36.00	31.61	9.68	41.29	+ 15%

7. Comparison of DEX-PEG and DEX-PL partitioning systems

Four extraction systems were prepared to investigate the difference in the capacity of SC to cause downward migration of SWCNTs. The optical absorption spectra along with photographs of the analyzed samples are provided in **Figures S8-S11**. The parameters used for the separations are given in captions accompanying the graphics.

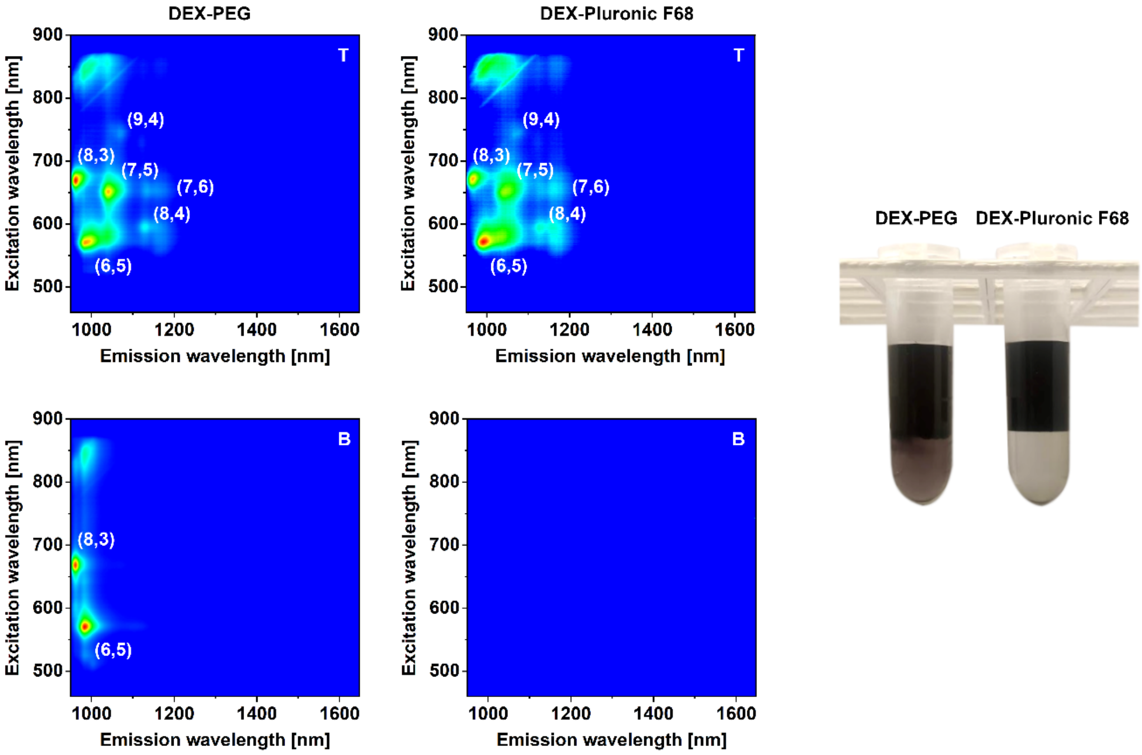

Figure S8 Photoluminescence excitation-emission maps and photographs of SWCNT samples sorted in DEX-PEG and DEX-Pluronic F68 systems. Extraction parameters: 360 µL of PEG or Pluronic F68 (25%), 450 µL of DEX (24%), 75 µL of SWCNT dispersion (in 2% SC solution), and 645 µL of H_2O.

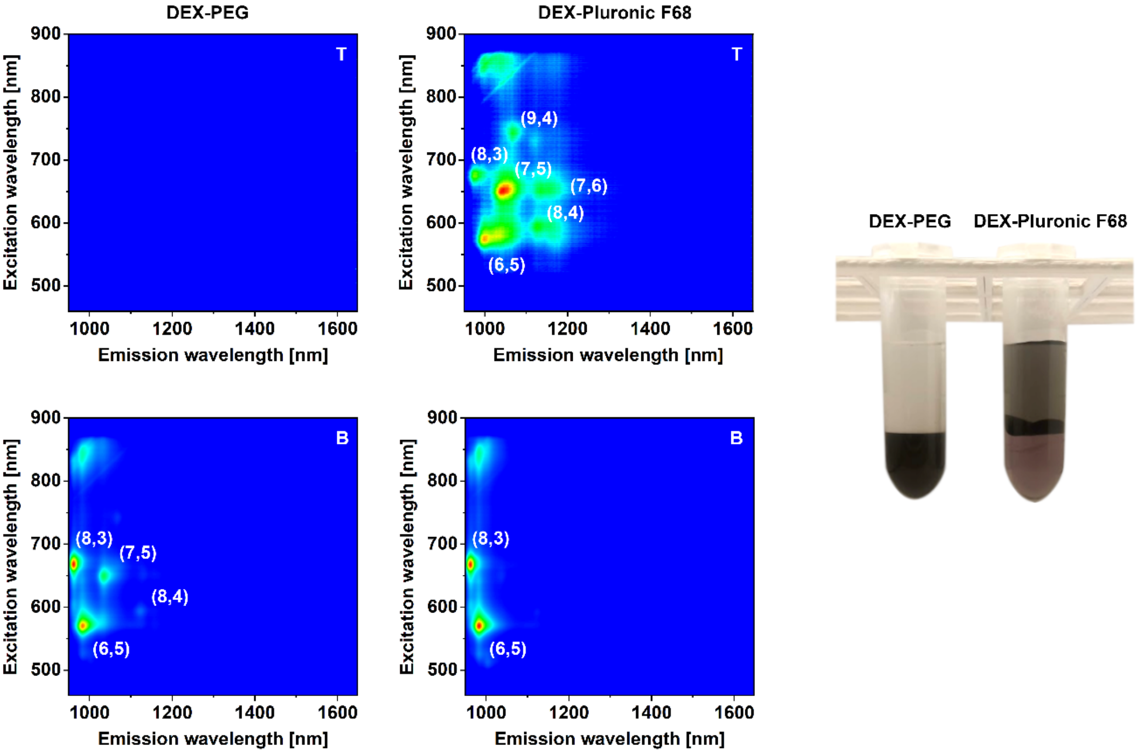

Figure S9 Photoluminescence excitation-emission maps and photographs of SWCNT samples sorted in DEX-PEG and DEX-Pluronic F68 systems. Extraction parameters: 360 µL of PEG or Pluronic F68 (25%), 450 µL of DEX (24%), 75 µL of SWCNT dispersion (in 2% SC solution), 120 µL of SC (10%), and 525 µL of H_2O.

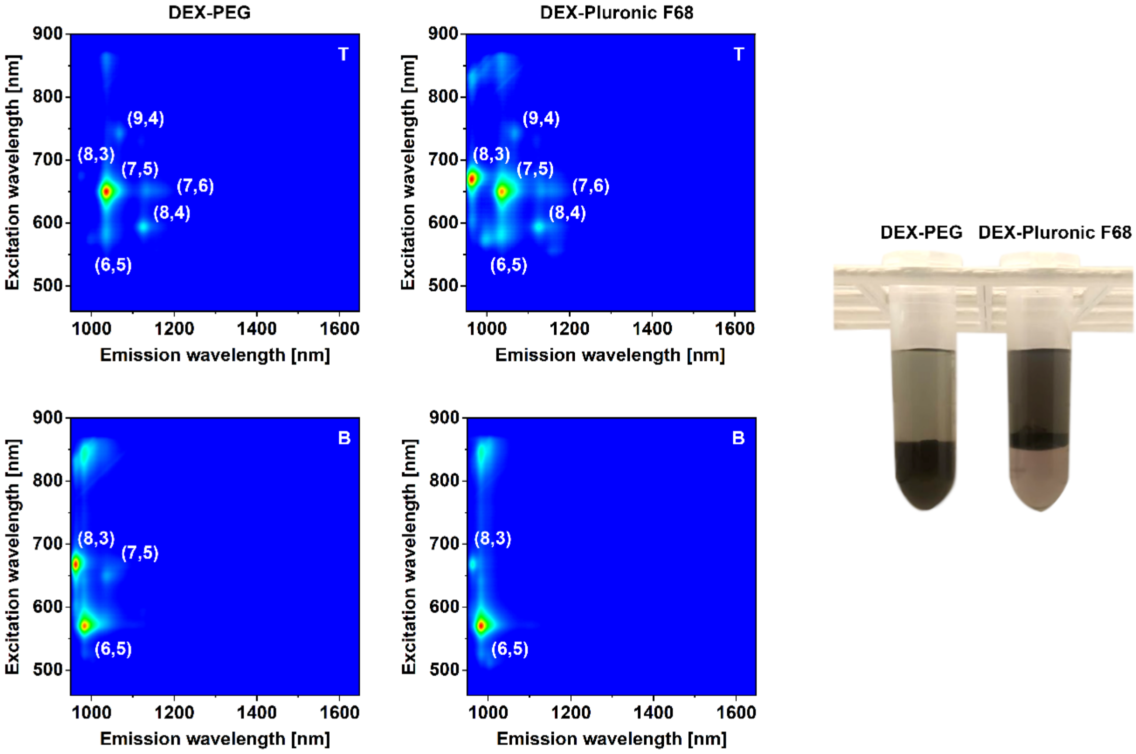

Figure S10 Photoluminescence excitation-emission maps and photographs of SWCNT samples sorted in DEX-PEG and DEX-Pluronic F68 systems. Extraction parameters: 360 µL of PEG or Pluronic F68 (25%), 450 µL of DEX (24%), 75 µL of SWCNT dispersion (in 2% SC solution), 120 µL of SC (10%), 35 µL of TX-100 (2.5%), and 490 µL of H_2O.

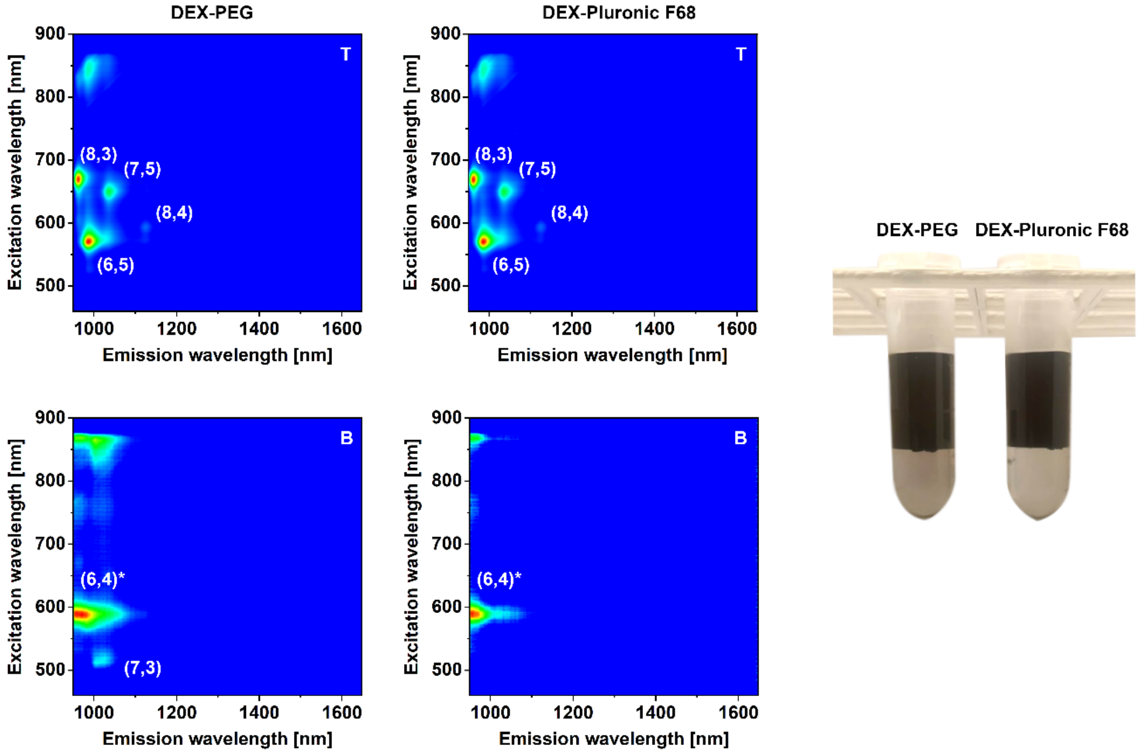

Figure S11 Photoluminescence excitation-emission maps and photographs of SWCNT samples sorted in DEX-PEG and DEX-Pluronic F68 systems. Extraction parameters: 360 μL of PEG or Pluronic F68 (25%), 450 μL of DEX (24%), 75 μL of SWCNT dispersion (in 2% SC solution), 120 μL of SC (10%), 200 μL of TX-100 (2.5%), and 325 μL of H_2O. Due to the limitations of the instrument, only the defect-induced E_{11}* peak in (6,4) SWCNTs is observed[2].

The presented results clearly show that the same amount of SC added into the biphasic system promotes the migration of SWCNTs to the bottom phase to a lower extent in the case of DEX-Pluronic partitioning systems. While we cannot eliminate from consideration the surface-active properties of Pluronic molecules, which may affect the SWCNT differentiation course, the lower amount of SC in the bottom phase, as shown and quantified in **Figure S7** and **Table S10** (**Samples F2 and F3**), respectively, can also hinder the downward extraction of SWCNTs.

8. Determination of SDS concentration

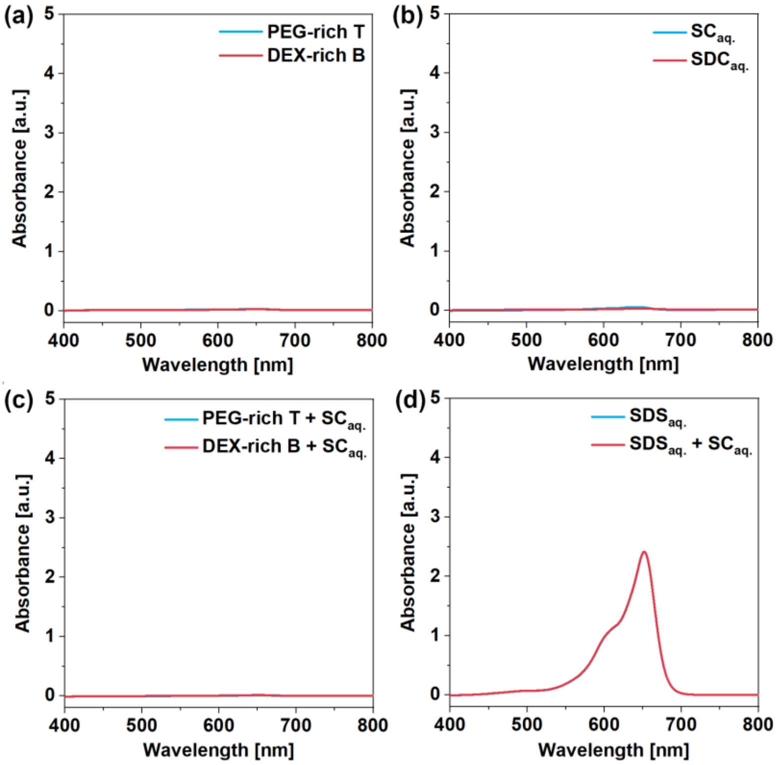

Figure S12 Optical absorption spectra of the $CHCl_3$-based phase interfaced with respective analytes mixed with MB under acidic conditions: (a) pure phase-forming components (PEG-rich top phase and DEX-rich bottom phase), (b) pure bile salt surfactant solutions (SC$_{aq.}$ and SDC$_{aq.}$), (c) phase-forming components (PEG-rich top phase and DEX-rich bottom phase) containing bile salt surfactant addition (SC$_{aq.}$), and (d) pure SDS$_{aq.}$ as well as SDS$_{aq.}$ mixed with SC$_{aq}$. Experimental conditions used for analysis are provided below.

3 mL of $CHCl_3$ were combined with 1.7 mL of H_2O, 200 µL of acidified MB solution, and 100 µL of the analyzed solution. The acidified solution of MB (50 mg/L) contained 61 µL of concentrated H_2SO_4 for each 10 mL of the solution. Upon introduction of the characterized solution, the whole mixture was vortexed and centrifuged to ensure proper homogenization and facilitate the phase separation, respectively. Chloroform-rich phase was then analyzed by optical absorption spectroscopy.

Procedures executed to obtain the samples for analysis:

- Figure S12a – PEG (25%, 540 µL), DEX (20%, 1 350 µL), H_2O (2 700 µL) were combined, vortexed and centrifuged. PEG-rich T and DEX-rich B phases were made.

- Figure S12b – SC and SDC were dissolved in water to generate 10% and 5% aqueous solutions, respectively.
- Figure S12c – PEG (25%, 540 μL), DEX (20%, 1 350 μL), H_2O (2 700 μL) were combined, vortexed and centrifuged. 50 μL of as-created top and bottom phases were collected and mixed with 250 μL of $SC_{aq.}$ (0.1%) and 1.7 mL H_2O. Both solutions were diluted 20× for analysis.
- Figure S12d – SC and SDS were dissolved in water to generate 0.05% aqueous solutions. SDS_{aq}. was analyzed as it and with an addition of an equal volume of SC_{aq}.

To monitor both SC and SDS concentrations in the biphasic system, PEG (25%, 540 μL), DEX (20%, 1 350 μL), SDS (10%, 270 μL), 1 mg/mL SWCNT dispersion in $SC_{aq.}$ (2%, 225 μL), SC (10%, 0 – 800 μL) and H_2O (1 405 – 2 205 μL, varied to keep the constant volume of the system equal to 4 590 μL) were combined, vortexed and centrifuged. PL excitation-emission maps of the samples are provided in Figure 5a. The content of SC and SDS given in Figure 5bc was determined using the aforementioned protocols, based on the respective optical absorption spectra generated (Figure S13). A small amount of SC detected at 0 μL SC added stems from the presence of SC-dispersed SWCNTs, which can liberate detectable SC molecules.

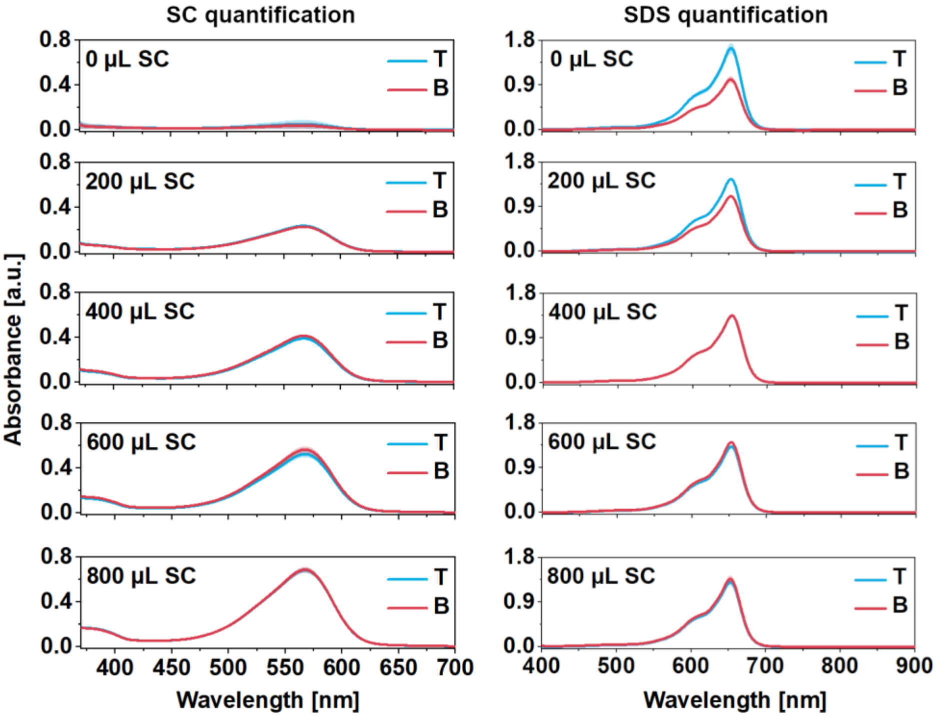

Figure S13 Optical absorption spectra of the indicated solutions.

9. References

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