Supporting Information

Vesicle Formation by L-Cysteine-Derived Unconventional Single-Tailed Amphiphiles in Water: A Fluorescence, Microscopy and Calorimetric Investigation

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Synthesis of Surfactants

The zwitterionic amphiphiles were synthesized by the Michael addition reaction of L-cysteine with poly(ethylene glycol) methyl ether methacrylate by thiol–ene “click” chemistry according to the reported method\(^1\) from our group. The details of the chemical identifications like FT-IR spectra, \(^1\)H and \(^{13}\)C NMR and the synthetic scheme have been presented below. From the NMR spectra it can be concluded that both the amphiphiles remain in neutral form in organic solvents; however, in D\(_2\)O solvent, the \(^{13}\)C peak of the COOH group shifts to 170.2 ppm, indicating the existence of the zwitterionic form in aqueous solution.

\[ \text{H}_3\text{N}^+\text{SH} + \text{OOC} \text{O} \text{CH}_3 \xrightarrow{\text{r.t., 6 h}} \text{TEA MeOH} \rightarrow \text{HOOC} \text{S O} \text{O} \text{CH}_3 \]

\( n=4, \text{mPEG}_{300}\text{-Cys} \)

\( n=23, \text{mPEG}_{1100}\text{-Cys} \)

Scheme S1. Synthetic scheme for the mPEG\(_{300}\text{-Cys} \) and mPEG\(_{1100}\text{-Cys} \) amphiphiles.
Chemical identification.

State: white solid, m.p. 170 °C (mPEG300-Cys) and 230 °C (mPEG1100-Cys). Yield ~80%, $[\alpha]_D^{25}$ (1%, H$_2$O) = +20.

FT-IR (KBr, cm$^{-1}$) The broad band centered at 3411 cm$^{-1}$ corresponds to O-H stretching for hydrogen-bonded COOH group. Absence of double bond is confirmed by absence of C-H stretching around 3000 cm$^{-1}$. Absence of the characteristic band of S-H stretch around 2550 cm$^{-1}$ confirms the absence of S-H. Strong peaks near 1734 cm$^{-1}$ and 1725 cm$^{-1}$ show the presence of C=O stretches of ester and carboxylic acid group, respectively. The absorption peak at 674 cm$^{-1}$ indicates the presence of C-S bond. $^1$H NMR (CDCl$_3$, δ ppm): 1.254 (COCH$_3$, t, 3H), 2.868 (COOHCH$_2$, d, 2H), 3.043 (SCH$_2$-CH, d, 2H), 3.203 (SCH$_2$CHCO, m, 1H), 3.381 (OCH$_3$, s, 3H), 3.558 (CO-OCH$_2$CH$_2$, t, 2H), 3.661 (long chain glycolic CH$_2$, m, 18H for mPEG$_{300}$-Cys and 104H for mPEG$_{1100}$-Cys), 3.729 (OCH$_3$ CH$_2$CH$_2$, t, 2H), 3.795(NH$_2$CHCH$_2$, t, 1H), 4.351 (CO-OCH$_2$-CH$_2$, dd, 2H). $^{13}$C NMR (D$_2$O, 1% DMSO, 100 MHz) δ (ppm) 175.1 (COOCH$_2$), 170.2 (COO-), 68.7, 67.3, 66.1, 61.5 (ether CH$_2$), 55.7 (OCH$_3$), 51.4(NCH CH$_2$) 37.7 (SCH$_2$CH), 32.2 (SCH$_2$ CH), 30.3 (SCH$_2$ CH$_2$NH$_2$), 13.9 (CH$_3$). $^{13}$C NMR (CDCl$_3$, 100 MHz) δ (ppm) 175.08 (COOCH$_2$), 172.3 (COOH), 71.8, 70.4, 69.0, 63.7 (ether CH$_2$), 59.7 (OCH$_3$), 53.4(NCH CH$_2$) 40.0 (SCH$_2$CH), 35.2 (SCH$_2$ CH), 33.4 (SCH$_2$ CH$_2$NH$_2$), 16.9 (CH$_3$).

Experimental Methods

Surface tension measurements. Surface tension ($\gamma$) measurements were performed on a GBX 3S (France) surface tensiometer using Du Nüoy ring method. The instrument was calibrated and checked by measuring the surface tension of Milli-Q water (18 MΩ) before each experiment. To a 10 mL phosphate buffer (pH 7.0) solution, aliquots were added in measured volume and $\gamma$ value was measured in each case. The solution was gently stirred and
allowed to equilibrate for 10 min before each measurement and repeated at least three times until the error was negligible. The temperature of the solution was controlled by a JULABO MC water-circulating bath with a temperature accuracy of ±0.1 °C.

**Steady-state fluorescence measurements.** The steady-state fluorescence measurements were performed either on a PerkinElmer LS-55 luminescence spectrometer equipped with a temperature-controlled cell holder or on a Horiba FL3-11 spectrophotometer. Again, a SPEX Fluorolog-3 (model no: FL3-11) spectrophotometer was used for recording fluorescence emission spectra of Py. NPN, C153, Py, and DPH were used as fluorescence probes to investigate the polarity as well as the viscosity of the microenvironment of the self-assemblies. Surfactant solutions of known concentrations were prepared in pH 7.0 and were incubated for about 30 min prior to measurement. For solution preparations in case of NPN probe study, a saturated solution of NPN in pH 7.0 buffer was used. The final concentration of Py, C153 and DPH were kept at 1µM. Py solutions were excited at 335 nm, and emission spectra were recorded in the wavelength range of 350-500 nm using excitation and emission slit widths of 2 and 5 nm, respectively. The solutions containing NPN were excited at 340 nm, and the emission was followed between 350 and 600 nm. For C153, the excitation wavelength was 420 nm and the emission spectra were recorded in the wavelength range of 430-700 nm. The slit width was set at 2.5 nm for excitation and 2.5–10 nm for the emission depending upon sample concentration for the steady-state fluorescence anisotropy measurement. Temperature controlled measurements were carried out by use of a Thermo Neslab RTE-7 circulating bath.

**Fluorescence anisotropy measurements.** A PerkinElmer LS-55 luminescence spectrometer was used to measure the steady state fluorescence anisotropy (r) of DPH in presence of the surfactants. The instrument is equipped with a polarization accessory that
uses the L-format instrumental configuration and a thermostating and magnetically stirred cell housing that allowed temperature control. The \( r \)-value was calculated employing the equation:

\[
 r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}} \tag{1}
\]

where \( I_{VV} \) and \( I_{VH} \) are the fluorescence intensities polarized parallel and perpendicular to the excitation light, and \( G (= I_{VV}/I_{VH}) \) is the instrumental grating factor. The software supplied by the manufacturer automatically determined the correction factor and anisotropy value. For each measurement, the anisotropy value was recorded over an integration time of 10 s. Five readings were recorded for each sample and the average value was accepted as the \( r \)-value. A stock solution of 1 mM DPH was prepared in super dry methanol. Aliquots of this stock solution were added to the surfactant solutions so that the final concentration of the probe was 1 \( \mu \)M. The anisotropy measurements were carried out at different surfactant concentrations in the temperature range 20–70 °C. Before measurement started, each solution was equilibrated for 10 minutes at the experimental temperature. The sample was excited at 350 nm and the emission intensity was followed at 450 nm using excitation and emission slits width of 2.5 nm and 2.5-7.0 nm, respectively. A 430 nm cut-off filter was placed in the emission beam to eliminate the effect of scattered radiation. The measurements started 30 min after sample preparation.

**Time-resolved fluorescence measurements.** Optical Building Blocks Corporation Easylife instrument was employed to measure the fluorescence lifetime of DPH probe. The light source was a 380 nm diode laser. The time-resolved decay curves were analyzed by
single exponential or biexponential iterative fitting program. The best fit was judged by the $\chi^2$ value (0.8-1.2) and by the randomness of residual plot.

**Determination of microviscosity.** The rigidity or fluidity of the microenvironment of the self-assemblies was measured by determination of the microviscosity ($\eta_m$) using DPH probe. The $\eta_m$ value was calculated from the fluorescence anisotropy ($r$) and lifetime ($\tau_f$) of DPH probe using Debye-Stokes-Einstein relation:\(^2\)

$$\eta_m = kT\tau_R/v_h$$  \hspace{1cm} (2)

where $v_h$ is the hydrodynamic volume (313 Å\(^3\))\(^3\) of the DPH molecule and $\tau_R$ is the rotational correlation time of the fluorophore. The $\tau_R$ value was calculated from Perrin’s equation:\(^2\)

$$\tau_R = \tau_f(r_o/r - 1)^{-1}$$  \hspace{1cm} (3)

where $r_o$ and $r$ are the steady-state fluorescence anisotropy values of DPH in a highly viscous solvent (0.362)\(^4\) and in surfactant solution, respectively.

**Dynamic light scattering.** The dynamic light scattering (DLS) measurements were performed with Zetasizer Nano ZS (Malvern Instrument Lab, Malvern, U.K.) light scattering spectrometer equipped with a He-Ne laser operated at 4 mW ($\lambda_o= 632.8$ nm) at 25 °C. The solution solution was filtered directly into the thoroughly cleaned scattering cell through a Millipore Millex syringe filter (Triton free, 0.22 µm). The sample was allowed to equilibrate inside the DLS optical system chamber for 10 min prior to the start of measurement. The scattering intensity was normally measured at $\theta = 173^\circ$ to the incident beam. The data acquisition was carried out for at least 15 counts and each experiment was repeated two or three times.
**Zeta potential measurements.** The surface zeta (ζ) potential of the vesicles were also measured using a Zetasizer Nano ZS (Malvern Instrument Laboratory, Malvern, U.K.) optical system equipped with an He-Ne laser operated at 4 mW (λo= 632.8 nm) at 25 °C. The measurements were done by taking different surfactant concentrations at 25 °C in pH 7.0 at 25 °C. An average of three successive measurements was noted for each sample.

**Transmission electron microscopy (TEM).** The morphology of the aggregates was investigated by a transmission electron microscope (FEI-TECNAI G2 20S-TWIN, FEI, USA) operating at an accelerating voltage of 80 kV. A 4 µL volume of surfactant solution was dropped on to a 400 mesh carbon-coated copper grid, and allowed to stand for 1 min. The excess solution was blotted with a piece of tissue paper followed by staining with 1% aqueous uranyl acetate solution. The specimens were kept in desiccators overnight before measurement. Each measurement was repeated at least twice to check the reproducibility.

**Isothermal titration calorimetry.** A microcalorimeter of Microcal iTC200, (made in U.S.A) was used for thermometric measurements. In a microsyringe of capacity 40 µL, 30 mM mPEG300-Cys and 5 mM mPEG1100-Cys were taken and added in multiple stages to pH 7.0 buffer kept in the calorimeter cell of capacity 200 µL under constant stirring conditions, and the stepwise thermograms of the heats of dilution of the surfactant solution were recorded. The stirring speed was fixed at 400 rpm and Milli-Q water was taken at reference cell. Each run was duplicated to check reproducibility. Enthalpy calculations were performed with the help of ITC software. All measurements were carried out at 25 °C.

**Dye entrapment studies.** For the dye entrapment study, 67 mg of mPEG300-Cys (to make 80 mM stock solution) with 100 µL of 2 mM methylene blue (MB) in methanol was mixed and dried slowly in a RB flask by use of a rotavapor to make a thin film. For complete evaporation of the solvent, the flask was kept overnight in desiccators. The film was then
rehydrated with a small amount of buffer sol for overnight. The rehydrated suspension was vortexed for 30 min followed by dilution with pH 7 buffer to attain 0.1 mM MB ($\lambda_{\text{max}} = 665$ nm). A 2 mL volume of the resulting solution was then loaded into a column packed with a pre-equilibrated Sephadex G-75 (25 cm height and 1.2 cm diameter) and eluted with pH 7.0 buffer. Vesicular suspensions eluted right after the void volume. The filtration was carried out until free MB was gel-filtrated. The eluent was collected in 2 mL fraction each. The absorbance for all the fractions was taken at 665 nm and plotted against the elution volume.

Another water-soluble drug, calcein (CAL) ($\lambda_{\text{max}} = 465$ nm) was encapsulated in the inner aqueous pool of the vesicles formed by 80 mM mPEG$_{300}$-Cys in the same way as described above. The CAL concentration taken was 20 µM. The free unentrapped calcein was separated from the entrapped CAL by using the same gel filtration column chromatography described above for MB. In this case, the pH-triggered release of the drug from the inner aqueous compartment was also performed at pH 3.0 by monitoring its steady-state fluorescence spectrum at different time intervals. The % release of the drug was calculated from the relative fluorescence intensities at 514 nm using the value of $(1 - I/I_0) \times 100\%$, where $I$ and $I_0$ are the fluorescence intensities of CAL at any time $t$ and at the start of the experiment (i.e. at pH 7.0 buffer), respectively.
Results:

**Figure S1.** Plots of surface tension ($\gamma$) versus log $C_s$ in pH 7.0 at 25 °C: (□) mPEG$_{300}$-Cys, (▲) mPEG$_{1100}$-Cys.

**Figure S2.** Representative fluorescence emission spectra of NPN in pH 7.0 buffer and in the presence of 20 mM mPEG$_{300}$-Cys and 5 mM mPEG$_{1100}$-Cys.
**Figure S3.** Variation of spectral shift ($\Delta \lambda$) of C153 as a function of C$_s$ at 25 °C; (○) mPEG$_{300}$ –Cys, (■) mPEG$_{1100}$–Cys.

**Figure S4.** Representative fluorescence emission spectra of C153 in pH 7.0 buffer and in the presence of 20 mM mPEG$_{300}$–Cys and 5 mM mPEG$_{1100}$–Cys.
Figure S5. Representative fluorescence emission spectra of Py in pH 7.0 buffer and in the presence of 5 mM mPEG300-Cys and 2 mM mPEG1100-Cys showing I₁ and I₃ bands.

Figure S6. Plots of fluorescence anisotropy (r) of DPH probe versus Cₛ at 25 °C; (○) for mPEG₃₀₀-Cys and (■) for mPEG₁₁₀₀-Cys.
Table S1. Fluorescence anisotropy ($r$), $\chi^2$, lifetime ($\tau_f$) and rotational correlation time ($\tau_R$) of DPH, and microviscosity ($\eta_m$) of the surfactant self-assemblies at 25 °C.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>$C_s$ (mM)</th>
<th>$r$  (± 0.001)</th>
<th>$\chi^2$</th>
<th>$\tau_f$ (± 0.1 ns)</th>
<th>$\tau_R$ (ns)</th>
<th>$\eta_m$ (mPa s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPEG$_{300}$-Cys</td>
<td>15</td>
<td>0.173</td>
<td>0.910</td>
<td>5.4</td>
<td>4.95</td>
<td>65</td>
</tr>
<tr>
<td>mPEG$_{1100}$-Cys</td>
<td>5</td>
<td>0.205</td>
<td>0.830</td>
<td>4.6</td>
<td>5.60</td>
<td>79</td>
</tr>
</tbody>
</table>

Figure S7. Plots of variation of change in enthalpy ($\Delta H$) versus $C_s$ at 25 °C using lower stock concentrations of (■) mPEG$_{300}$-Cys (5 mM) and (▲) mPEG$_{1100}$-Cys (2 mM).
**Figure S8.** TEM images of (a) 40 mM mPEG$_{300}$-Cys and (b) 10 mM mPEG$_{1100}$-Cys in pH7.0 buffer.

**Figure S9.** Size distribution profile of mPEG$_{300}$-Cys (15 mM) in pH 7.0 at 75 °C (inset: TEM micrographs of 15 mM mPEG$_{300}$-Cys at 75 °C).
Figure S10. Hydrodynamic size distributions in (A) 20 mM mPEG$_{300}$-Cys and (B) 5 mM mPEG$_{1100}$-Cys aqueous solutions at different pHs after 30 min of sample preparation. Figure S6. Plots of turbidity ($\tau = 100 - \%T$) versus aging time.

Figure S11. Plots of turbidity ($= 100 - \%T$) versus aging time.
**Figure S12.** Size distribution histograms of the surfactant solutions at pH 7.0 and 25 °C after 20 days of sample preparation.

**Table S2.** The ζ-potential values at different concentrations of the amphiphiles.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>C₅ (mM)</th>
<th>ζ-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPEG₃₀₀-Cys</td>
<td>5</td>
<td>-0.03</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>-1.86</td>
</tr>
<tr>
<td>mPEG₁₁₀₀-Cys</td>
<td>2</td>
<td>-0.08</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-1.11</td>
</tr>
</tbody>
</table>
Figure S13. Gel filtration profile of the separation of the dye entrapped (small peaks) vesicle of mPEG$_{300}$-Cys from the corresponding free dye (MB).

Figure S14. Fluorescence spectra of vesicle-entrapped calcein in 80 mM mPEG$_{300}$-Cys in pH 7.4 and 3.0.
Figure S15. Plots of release (%) of DPH with time (min) at 37 °C in pH 3.0 and pH 7.4.

REFERENCES

