**SUPPLEMENTARY INFORMATION**

# Pluronic micelles encapsulated Curcumin manifests apoptotic cell death and inhibits pro-inflammatory cytokines in Human breast adenocarcinoma cells

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**S1. Molecular structure of Pluronic block co-polymer**

Pluronic block co-polymers are amphiphilic in nature arranged in A-B-A tri-block structure: PEO-PPO-PEO and also known as "poloxamers". The core of micelles consists of hydrophobic poly-(propylene oxide) (PPO) and shell consists of hydrophilic poly-(ethylene oxide) (PEO).

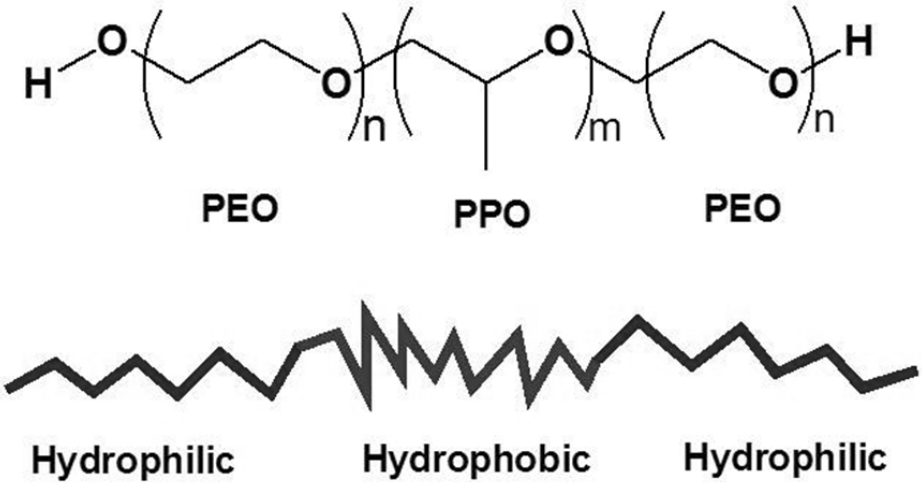


**FIGURE S1.** Molecular structure of Pluronic block copolymer.

**S2. Phase solubility of curcumin in Pluronic F127**

Phase solubility of curcumin was carried out on Shimadzu (UV-2450) UV–visible double beam spectrophotometer with matching pair of stopper fused silica cells of 1 cm optical path length. Various concentrations (1 wt%, 3 wt%, 5 wt%, 7 wt%, 9 wt%, 10 wt%) of Pluronic F127 were prepared in deionized water (10 mL). Thereafter, curcumin (w ≈ 10 mg, excess than normal) was added to an aliquot of copolymer solution. The system was slowly stirred at 37 °C (± 0.1 °C) for two days in a thermostatic bath. After that, 3 mL of supernatant were filtered (0.45 μm Millipore) to remove any non-solubilized curcumin. Aliquots of the filtered samples were diluted with methanol and the concentration of curcumin was monitored through UV-Visible spectroscopy at λmax of 425 nm. All measurements were made in triplicate.

Curcumin is known to insoluble in aqueous medium, but we observed that curcumin incorporated in various concentration of pluronic F127 shown abundant solubility. We observed that curcumin incorporated in Pluronic F127 shown high solubility due to formation of micelles.

The solubility of curcumin was constantly increased from 1 to 4.9 wt% of Pluronic F127 concentration and thereafter linear increase at > 5 wt% of Pluronic F127 was clearly noticed in phase solubility curve. The molecular characteristics of Pluronic F127 were shown in Table S1.

**Table S1.** Characteristics of Pluronic F127.

| **M /g·mol$^{-1}$** | **%EO** | **n EO** | **n PO** | **HLB** | **CP,°C (2.0 %w/v)** | **CMC, %w/v** | **HLB** |
|---|---|---|---|---|---|---|---|
| 12600 | 70 | 100 | 65 | 22 | >100° | 0.45[#] | 22 |

[#] measured using ST method @ 37°C

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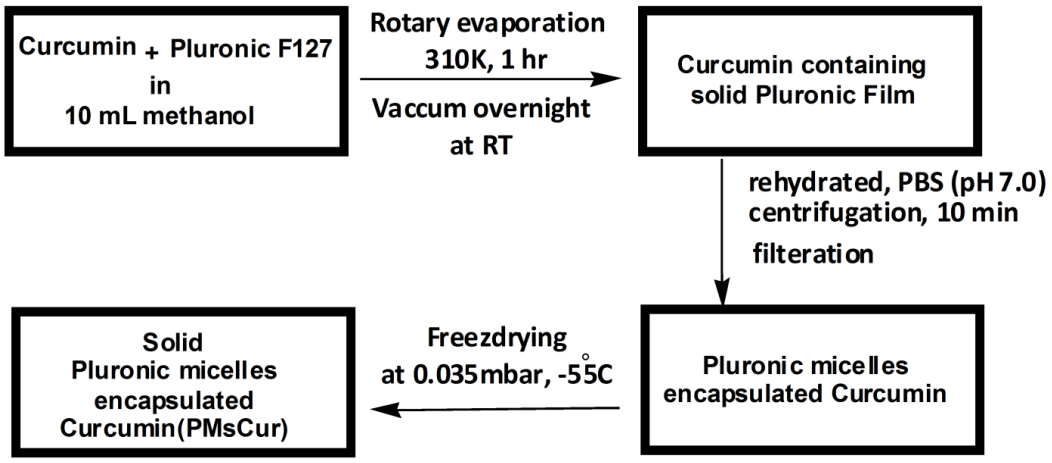
**S3. Synthesis of Pluronic micelles encapsulated curcumin (PMsCur)**

Briefly, the required amount of Pluronic F127 (fixed 5wt %) and curcumin (0.1wt%) in 10 mL of methanol was taken in two separate round-bottom flask. The solution was stirred for an hour and poured into the glass plate. The methanol was completely evaporated to obtain curcumin-containing solid polymer film. The residual methanol was removed in vacuum oven at room temperature for overnight. After that, the solid film was rehydrated with deionized water (pre-warmed at 37°C, pH 7.0) by extensive vortexing and sonication for 2 minutes to prepare Pluronic micelles encapsulated curcumin. Residual free curcumin was separated by centrifugation at 5000 rpm for 10 min and filtered using nylon syringe filter (0.2µM). Lyophilized PMsCur was obtained by freeze-drying ($N_2$ atm., 0.035 mbar pressure, -55°C temperature). The final product, PMsCur was kept in brown bottle with proper capping (Scheme 1).



**Scheme 1.**Schematic representation of Synthesis of Pluronic micelles encapsulated Curcumin (PMsCur).

Moreover, the incorporation efficiency and drug loading of curcumin within the Pluronic F127 micelles were 48.03 % and 41.51 % respectively, based on the calculations mentioned below.

**Percentage of Incorporation efficiency and drug loading:**

The percentage of curcumin incorporated in the synthesized Pluronic micelles encapsulated Curcumin (PMsCur) was determined as follows; 1.0 ml of the aqueous PMsCur solution was centrifuged for 30 min, and the amount of curcumin in the supernatant was measured spectrophotometrically at λ=425 nm. The drug loading and incorporation efficiency were calculated using the following formulas;

$$\text{Incorporation efficiency (\%)} = \frac{W_{loaded}}{W_{added}} \times 100$$

$$\text{Drug Loading (\%)} = \frac{W_{loaded}}{W_{total}} \times 100$$

Where, $W_{loaded}$ is the amount of curcumin entrapped into pluronic micelles, $W_{added}$ is the initially added curcumin, and $W_{total}$ represents the amount of both curcumin and Pluronic F127 in the PMsCur.

The aqueous solubility of curcumin in PMsCur was 0.021mg/mL, which was quite higher than the aqueous solubility of free curcumin. The properties of PMsCur were shown in Table S2. Further, storage stability of the freeze-dried formulation of PMsCur was tested for 3 months. The solubility of curcumin at specific time interval was monitored using the UV-Visible spectroscopy.

**Table S2.** Curcumin incorporation in PMsCur.

| PMsCur | Curcumin taken | Pluronic® F127 (*Wt. fixed 5wt% concn.*) | IE% | DL% | Solubility |
|---|---|---|---|---|---|
| | 1 mg | 50 mg | 48.03 | 41.51 | 0.021 mg/mL |

## S4. Characterization of PMsCur

### 1.1 Dynamic light scattering (DLS)

The average particle size (hydrodynamic diameter, Dh) and size distributions of aqueous solutions of 5 wt% Pluronic F127 and 1.0 wt% PMsCur was determined by DLS measurements using Horiba-Zetasizer, SZ-100 with a fixed scattering angle of $90^0$ at 37°C.

### 1.2 Small angle neutron scattering analysis (SANS)

The morphology and structural details of 5wt% Pluronic F127 and PMsCur in $D_2O$ were determined through Small angle neutron scattering measurements.SANS experiments were performed at the SANS diffractometer at Guide Tube Laboratory, Dhruva Reactor, Bhabha Atomic Research Centre, Mumbai, India. The mean incident neutron beam wavelength (λ) was 5.2 Å with a wavelength resolution (Δλ/λ) of approximately 15%. The scattered neutrons were detected in an angular range of 0.5–15° using a linear position sensitive detector (PSD). The scattered neutrons were measured for the scattering vector '*Q*' in the range of 0.015-0.3 $Å^{-1}$. The measured SANS data were corrected for the background, the empty cell contribution, and the transmission and were presented on an absolute scale using the standard protocols. All the data were recorded at 37°±0.1°C. In SANS, one measures the coherent differential scattering cross-section (dΣ/dΩ) per unit volume as a function of wave vector transfer *Q* (= 4π sin(θ/2)/λ, where λ is the wavelength of the incident neutrons and *θ* is the scattering angle). It provides information about the shape and size of the scattering particles in the length scale of 10-1000 Å. The mean wavelength of the monochromatized beam coming out from the neutron velocity selector is 5.26 Å with a spread of $\Delta\lambda/\lambda \sim 15\%$. The angular distribution of neutrons scattered by the sample is recorded using a 1m long one-dimensional $He^3$ position sensitive detector. The instrument covers a *Q*-range of 0.017–0.35 $Å^{-1}$. The temperature in all the measurements was kept fixed at 37°C.

The differential scattering cross-section per unit volume ($d\Sigma/d\Omega$) as measured for a system of monodisperse particles in a medium can be expressed as

$$\left(\frac{d\Sigma}{d\Omega}\right)(Q) = nV^2\left(\rho_p - \rho_s\right)^2 P(Q)S(Q) + B\ , \qquad (1)$$

Where, *n* denotes the number density of particles, $\rho_p$ and $\rho_s$ are, respectively, the scattering length densities of particle and solvent and *V* is the volume of the particle. *P*(*Q*) is the intraparticle structure factor and *S*(*Q*) is the interparticle structure factor. *B* is a constant term

representing incoherent background, which is mainly due to the hydrogen present in the sample. Intraparticle structure factor $P(Q)$ is decided by the shape and size of the particle and is the square of single-particle form factor $F(Q)$ as determined by

$$P(Q) = \langle |F(Q)|^2 \rangle. \quad (2)$$

For a spherical particle of radius $R$, $F(Q)$ is given by

$$F_p(Q) = 3\left[\frac{\sin(QR) - QR\cos(QR)}{(QR)^3}\right]. \quad (3)$$

In our measured systems the block copolymer micelles have been modeled as core-shell particles with different scattering length densities for core and shell. The structure of these micelles is described using a model consisting of non-interacting Gaussian PEO chains attached to the surface of the PPO core. The form factor of the micelles comprises four terms: the self-correlation of the core, the self-correlation of the chains, the cross term between core and chains, and the cross term between different chains. It is given by

$$F_{\mathrm{m}}(Q) = N_s^2 b_s^2 F_s(Q) + 2N_s b_c^2 F_c(Q) + 2N_s(2N_s - 1) b_c^2 F_{cc}(Q) + 4N_s^2 b_s b_c F_{sc}(Q) \quad (4)$$

Where, $b_s$ and $b_c$ are excess scattering length of the core and chain, respectively and $N_s$ is the aggregation number of the micelles. The subscript $s$ (= core) and $c$ (= chain) are used here. They can be calculated as $b_s = V_s(\rho_s - \rho_{solv})$ and $b_c = V_c(\rho_c - \rho_{solv})$, respectively, where $V_s$ and $V_c$ are the total volumes of a block in the core and in the corona. $\rho_s$ and $\rho_c$ are the corresponding scattering length densities and $\rho_{solv}$ is the scattering length density of the surrounding solvent ($D_2O$).

At higher polymer concentrations, an interaction between micelles takes place and a peak arising from the structure factor, $S(Q, R_{hs}, \phi)$ where $R_{hs}$ is hard sphere radius and $\phi$ is volume fraction of micelles, appears in the scattering intensity. $S(Q)$ describes the interaction between the particles present in the system and depends on the spatial distribution of micelles, is given by

$$S(Q) = 1 + 4\pi \mathrm{n} \int (g(r) - 1) \frac{\sin(Qr)}{Qr} \mathrm{r}^2 \mathrm{dr}, \quad (5)$$

Where, $g(r)$ is the radial distribution function describing the arrangement of the micelles. In the case of non-ionic micelles the interparticle interaction (direct correlation between two scattering objects) is obtained using Ornstein–Zernike equation with the Percus-Yevick approximation and employing hard sphere potential between micelles, and the analytical form of the structure factor is given as

$$S(Q) = \frac{1}{1 + 24\phi\ (G(x)/x)}\ , \qquad (6)$$

Where, $R_{hs}$ is the hard sphere micellar radius consisting of both the core and the shell which gives the physical size of the micelle, $\phi$ is the hard sphere volume fraction of the micelles in the solution, and $G$ is a function of $x = 2QR_{hs}$ and $\phi$.

In this equation, $G(x)$ is further defined as follows:

$$\begin{aligned} G(x) = {} & \frac{\alpha(\phi)}{x^2}(\sin x - x\cos x) + \frac{\beta(\phi)}{x^3}[2x\sin x + (2 - x^2)\cos x - 2] \\ & + \frac{\gamma(\phi)}{x^5}[-x^4\cos x + 4\{(3x^2 - 6)\cos x + (x^3 - 6x)\sin x + 6)\}] \end{aligned} \qquad (7)$$

Where,

$$\begin{aligned} \alpha(\phi) &= (1 + 2\phi)^2 / (1 - \phi)^4 \\ \beta(\phi) &= -6\phi(1 + \frac{\phi}{2})^2 / (1 - \phi)^2 \\ \gamma(\phi) &= \frac{\phi\alpha}{2} \end{aligned} \qquad (8)$$

In fitting the experimental scattering data, three unknown parameters, $R_c$, $R_{hs}$ and $\phi$ have been considered as fitting parameters in the analysis. The aggregation number of micelles, $N_{agg}$ can be calculated using the following expression, $N_{agg} = V_m/V_h$ where $V_m$ (= 4/3 $\pi R_c^3$) is the micellar volume and $V_h$ is the volume of hydrophobic part of the surfactant monomer.

The polydispersity in size distribution of particle is incorporated using the following integration

$$\frac{d\Sigma}{d\Omega}(Q) = \int \frac{d\Sigma}{d\Omega}(Q, R)\, f(R) dR + B\ , \qquad (9)$$

Where, $f(R)$ is the particle size distribution and usually accounted by a log-normal distribution as given by

$$f(R) = \frac{1}{\sqrt{2\pi}R\sigma} \exp\left[ -\frac{1}{2\sigma^2}\left( \ln\frac{R}{R_{med}} \right)^2 \right], \qquad (10)$$

Where, $R_{med}$ is the median value and $\sigma$ is the standard deviation (polydispersity) of the distribution. The mean radius ($R_m$) is given by $R_m = R_{med} \exp(\sigma^2/2)$.

The data have been analyzed by comparing the scattering from different models to the experimental data. Throughout the data analysis, corrections were made for instrumental

smearing, where the calculated scattering profiles smeared by the appropriate resolution function to compare with the measured data. The fitted parameters in the analysis were optimized using nonlinear least-square fitting program to the model scattering.

**Table S3** Fitted parameters obtained from the analysis of the SANS data of Pluronic®F127 and PMsCur in $D_2O$ at 37°C

| System | Core radius, $R_c$ (Å) | Polydispersity | Radius of gyration $R_g$, (Å) | Hard sphere radius $R_{hs}$, (Å) | Volume fraction $\phi$ | Aggregation number, $N_{agg}$ |
|---|---|---|---|---|---|---|
| **5% F127** | 55.1 | 0.36 | 21.6 | 101.1 | 0.17 | 112 |
| **PMsCur** | 58.2 | 0.35 | 21.6 | 102.9 | 0.03 | 130 |

### 1.3 UV-Visible spectroscopy (UV-VIS)

The UV-visible spectra of aqueous solutions of 1 wt% curcumin, Pluronic F127 and PMsCur were observed using UV–visible double beam spectrophotometer (UV-2450, Shimadzu, Japan) with matched pair of stoppered fused silica cells of 1 cm optical path length.

### 1.4 Solid state characterizations of PMsCur

#### 1.4.1 Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra of curcumin, Pluronic F127 and PMsCur were collected in order to examine the chemical structure of these compounds and possible changes after incorporation of curcumin. Samples were analyzed by spectrophotometer (FTIR-8400S, Shimadzu Co., Kyoto, Japan) using potassium bromide (KBr) pellet method. The spectra were obtained in the frequency range of 4000-400$cm^{-1}$ with a resolution of 4$cm^{-1}$**.**

#### 1.4.2 Powder X-ray diffraction spectroscopy (PXRD)

XRD study was performed to analyze the crystallographic structure of curcumin, Pluronic F127, and PMsCur. PXRD patterns were recorded with X-Ray diffractometer (Philips X' Pert MPD, USA) with a scan of 0.5°/s in the 2θ range of 2 – 50°.

### 1.4.3 Differential scanning calorimetry (DSC)

DSC measurements for the curcumin, Pluronic F127 and PMsCur were conducted on a Mettler-Toledo, 851e within 30-900°C at 10°C/min in continuous nitrogen flow for the evaluation of thermal behavior of curcumin and Pluronic F127 alone and in presence of each other.

### 1.4.4 Stability studies of PMsCur

To evaluate the physical stability of PMsCur, sample was stored at room temperature in the closed chamber and curcumin retention was monitored over three months after preparation of PMsCur and quantified using UV-Visible spectroscopy.

**S5 Antiproliferative effect of Pluronic F127, curcumin and PMsCur**

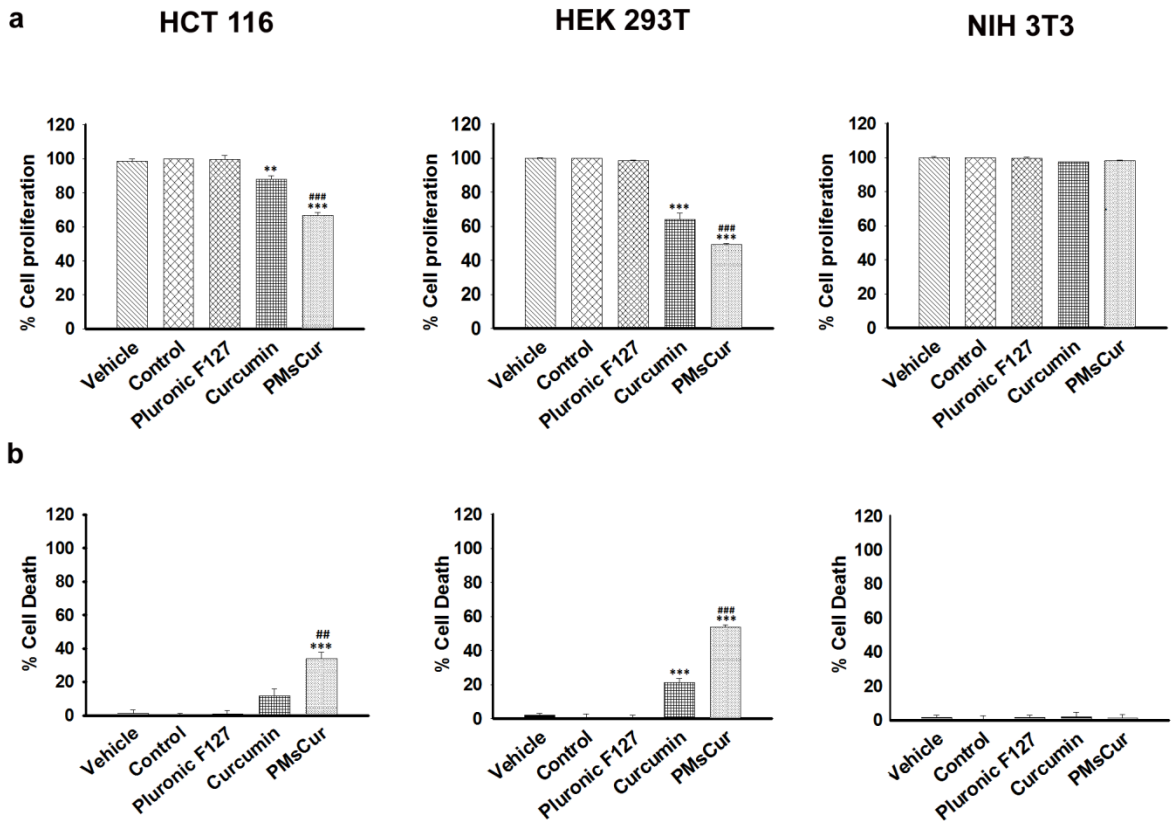



**FIGURE S5.** Antiproliferative effect of Pluronic F127, curcumin and PMsCur on HCT 116, HEK 293T and NIH 3T3 cells. Cells were treated with Pluronic F127 (364 μg/ml), curcumin (5.66 μg/ml) or PMsCur (364 μg/ml) for 24h. 0.02 % DMSO was taken as a vehicle control (Vehicle). (a) Evaluation of cell proliferation by MTT assay. The bar graphs represent the % of cell proliferation in control and treated cells. (b) Evaluation of cell death by Trypan blue assay. The bar graphs represent the percentage of cell death in control and treated cells. Error bars represent ±SEM of three independent experiments. Significance indicated as **p≤ 0.01, ***p≤ 0.001 between untreated cells and treated cells and $^{\#\#}$p≤ 0.01, $^{\#\#\#}$p ≤ 0.001 between free curcumin and PMsCur treated cells by performing one way ANOVA followed by Student Newman-Keuls multiple comparisons test. We have normalized the fluorescence and color of curcumin to avoid interference in measurements since curcumin is a colored and fluorescent molecule.