Mixed Pluronics/phosphatidylcholine self-assemblies for CUR drug for anticancer evaluations

5.1: Introduction

Phospholipid-based systems have substantially impacted drug delivery applications in recent years [1-3]. The most important advantage of this liposomal system is that phospholipids are compatible with human membranes both inside and outside the human skin (external membrane). The cell membrane comprises a double layer of phospholipid amphiphilic molecules with their hydrocarbon chains aligned inwards to create the lipophilic phase. Their polar heads are oriented outwards to create the inner and outer hydrophilic boundaries that face the surrounding aqueous environment. According to liposome research, phospholipids are the only class of excipients that provide distinct benefits for a surface-active component because they are nontoxic, well-tolerated parenterally, and have excellent biocompatibility [4,5]. On the other hand, phospholipid liposome structures are micelles that might be used to solubilize a high amount of a lipophilic drug [6,7]. The stability of liposomes incorporating lipophilic drugs inside the bilayer may be changed in terms of interactions between the drug and phospholipid derivatives [8].

When phospholipids and phospholipid derivatives like PC are mixed with the right surfactants, they form mixed micellar structures, including large vesicles as well as small spherical micelles [9,10]. Various biocompatible polymeric surfactants are applied in the pharma industry to increase the solubility(in aqueous media), stability, and bioavailability of poorly water-soluble drugs. Pluronic® block copolymers are one class of polymeric surfactant made of Pluronics and are approved as therapeutic excipients by the FDA for pharmaceutical purposes. Pluronics have been extensively explored for their nonionic surfactant properties and are used in various biological applications [11-14]. In water, below CMT and CMC, the Pluronics dissolve and reside as unimers. Above CMT and CMC, they form micelles with a hydrophobic PPO core bounded by a hydrophilic PEO shell [15]. These block copolymers micellize into various morphological structures, relying on external parameters such as pH, temperature, or concentrations. In aqueous media, Pluronic micelles are identified as a core-shell assembly, in which the hydrophobic PPO core can act as a drug encapsulating site, generating a space for the incorporation of poorly water-soluble drugs through physical or chemical interaction [16-18]. The outer PEO shell and PPO inner core also have an influence on the drug release action [19,20]. They have the ability to get away from a nonspecific

capture by the RES, because the PEO shell minimizes the interaction with proteins and their cellular adhesion [21,22]. Many research groups are investigating the self-assembly of mixed micellar systems constructed of PC and Pluronics, and many encouraging outcomes have been obtained in those systems **[**10, 23**]**. However, no formulation has been effectively introduced into the pharma drug market. When a lipophilic PC is combined with a Pluronic, small vesicles, mixed micelles, or mixed dispersions may occur. In general, the Pluronic mixed micelles formation shows the sizes are usually less than 30 nm **[**24**]**. Versatile Pluronic micelles have been used as drug delivery carriers due to their tailorable structures, properties, and functionalities. Depending on the characteristics of the micelles, the mixed PC/Pluronic micelles show superior performance in improved colloidal stability, prolonged circulation, and targeted and triggered release [25]. In our study, we investigated the structural changes of mixed micellar systems comprised of three different components: PC, Pluronic F127, and Pluronic P123. Due to the mixing of the PC, mixed F127/P123 micelles exhibit a diameter that can be greater than that of conventional micelles **[**26-28]. Here, we explore the Pluronic induced microstructural changes in PC vesicles for a better effect of dilution, which is minimal in the assemblies for the administration of various drugs. The microstructure of aggregates of PC and Pluronic F127/P123 is investigated by appreciative techniques like DLS, SANS, rheology, and TEM. From SANS and TEM studies, it's clear that by adding Pluronics, the PC structure changed from vesicle to spherical micelles.

For centuries CUR has been used in the Asian continent, specifically in India, to treat various disorders due to its antioxidant, anti-inflammatory, antiseptic, antimicrobial, anticoagulant, wound healing, and anti-carcinogenic properties [29]. In animals and humans, models have clearly displayed the safety of CUR at a higher dosage of 8 g/day [30]. Still, its medicinal uses are limited due to its lower water solubility, fast hydrolysis in basic medium, slight instability, and lipophilic properties, resulting in pharmacokinetic drawbacks such as poor absorption, below par bioavailability, substantial metabolism, and rapid elimination [30,31]. It's still very important to make CUR more soluble and stable so that it can be taken orally.

In view of the benefits of mixed PC/Pluronic micellar systems for pharma uses, the solubilization of CUR in a mixed PC-Pluronic F127/P123 (PCFP) micellar system is evaluated. ^{1}H NMR measurements have been performed to find the location of CUR drug in

the mixed PCFP micelles. The *in-vitro* release and adequate stability of CUR from these mixed PCFP micellar systems have been assessed. In a physiological environment, the antioxidant activity of free CUR and CUR-loaded PCFP micellar systems has been observed for their better therapeutic efficacy. *In-vitro* cell proliferation and cell death assays of mixed PCFP micellar solutions on the MCF-7 cells have been assessed for enhanced anticarcinogenic effects of CUR with these newer micellar systems.

5.2: Experimental Section

5.2.1: Preparation of mixed PC/Pluronic micellar systems

All the PC/Pluronic micellar solutions were prepared using the thin-film hydration method developed by Shaikh et al. [32], which found success in the investigations of such systems in bio-applications.

"Stock solutions of Pluronic with a concentration of 10% were prepared in cold conditions using deionized water and equilibrated for more than 3 hrs at RT (30±0.5°C). The F127, P123, and mixed F127/P123 micellar solutions of different weight fractions were prepared using the respective stock solutions and kept at RT for 2 hrs before use. In order to make the required number of round-bottom flasks, the fixed amount of PC was dissolved in chloroform and kept overnight under a vacuum for complete evaporation of the solvent. The dry PC film formed in the round bottom of the flask was again resuspended with the above prepared Pluronic micellar solutions. All of these samples were properly sonicated for the effective dispersion of PC molecules in the solutions.**"**

The compositions of the prepared mixed PC/Pluronic micellar systems, along with their code names, are reported in Table 5.1.

Table 5.1: Composition and code-names of mixed PC/Pluronics micellar systems

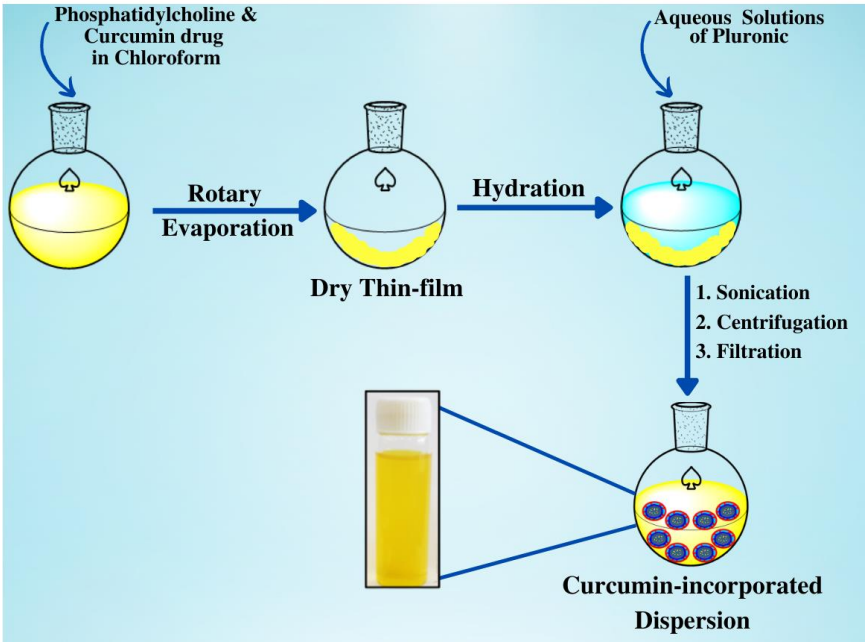
System	PC, (%w/v)	Pluronic, (%w/v)		Composition, (%) X_{PC} :$X_{Pluronic}$	Code name of the system
		F127	P123		
Mixed PC and F127	1.0	0.5	--	1.0 : 0.5	PCF (1)
	1.0	2.0	--	1.0 : 2.0	PCF (2)
	1.0	5.0	--	1.0 : 5.0	PCF (3)
Mixed PC and P123	1.0	--	0.5	1.0 : 0.5	PCP (1)
	1.0	--	2.0	1.0 : 2.0	PCP (2)
	1.0	--	5.0	1.0 : 5.0	PCP (3)
Mixed PC and F127/P123	1.0	0.25	0.25	1.0 : 0.5	PCFP (1)
	1.0	1.0	1.0	1.0 : 2.0	PCFP (2)
	1.0	2.5	2.5	1.0 : 5.0	PCFP (3)

5.2.2: Preparation of CUR-loaded mixed PC/Pluronic micellar systems

All the CUR-loaded mixed PC/Pluronic micellar solutions were also prepared using the thin-film hydration method developed by Shaikh et al. [32].

"For the preparation of CUR-loaded mixed PC/Pluronic micellar systems, the fixed amount of CUR was taken into the various prepared mixed PC/Pluronic micellar solutions mentioned above. The free CUR drug was removed by centrifugation (5 min), followed by filtration through a 0.22µm membrane filter."

The yellow color dispersion of CUR-loaded mixed PC/Pluronic micellar solutions was obtained (Scheme 5.1).



Scheme 5.1: Presentation of loading of CUR in the mixed micellar systems through thin film method.

5.2.3 Characterization methods

Mixed micellar size, zeta potential, and morphological transition of PCFP were confirmed using DLS, SANS, Rheology and TEM. The location of CUR in the mixed PCFP micelle was determined by using ^{1}H NMR spectroscopy. CUR stability in mixed micellar formulation investigated using UV-Vis spectroscopy techniques. The methology of the procedures of the all-mentioned investigation techniques are shown in Chapter 2.

5.2.4 In-vitro CUR release study

A dialysis membrane bag approach reported by Zhao et al. [33] was used to study the in vitro release of CUR. CUR release from a representative mixed PC/F127/P123 (PCFP) micellar solution in PBS with pH 7.4 was measured and reported in the same way as Zhao et al [33].

"The pre-prepared dialysis tube was filled with the predetermined volume of PCFP micellar solution. A 1000 mL glass beaker holding 500 mL of freshly prepared release medium was used to suspend the tube. The experiment was conducted in a water bath with a temperature control of 37±0.5°C. It was taken out from the release media at a predetermined time interval. Each time, a fresh batch of release medium was used to replace the used release medium. A UV-Vis spectrophotometer was used to measure the absorbance at a wavelength of 425 nm in the aliquot solutions and determine the amount of CUR dissolved (Shimadzu, UV-2450). There were three copies of each experiment. The experiment was conducted in the darkness. The release profile of CUR was obtained by plotting the % cumulative drug released from each micellar solution against time. Under the same conditions as CUR-loaded micelles, the release profile of CUR drug (100g/mL in propylene glycol) was measured.**"**

5.2.5 In-vitro antioxidant activity

Here, the in-vitro antioxidant activities of our samples are measured using the DPPH scavenging method developed by Shaikh et al. [32], which was highly recognized in the antioxidant study of CUR.

."The antioxidant activities of free CUR and CUR-loaded mixed PCFP micellar solution of different concentrations of 0.05, 0.1, 0.4, 0.8, 1.0, 2.0, and 3.0 µg/mL were measured by their ability to scavenge the DPPH radicals. The sample solutions were vigorously mixed and kept for half an hour in the dark at 30±0.5°C for incubation. The color intensity of the sample changed by the scavenging of free radicals from DPPH and it was measured by a spectrophotometer at λ=517 nm. The scavenging capacity of the tested samples was calculated by comparison of the sample color with the control. The experiments were carried out in triplicates. The percentage of inhibition was calculated using the following formula:

$$\% \text{ inhibition} = (X_0 - X_1)/X_0 \times 100$$

Where $\mathbf{X_0}$= control and $\mathbf{X_1}$= sample present

The result was evaluated in terms of IC_{50} values, which is the necessary amount of antioxidants to decrease the initial concentration of DPPH by 50%.

The IC_{50} value is calculated by plotting the % inhibition and concentration curve and finding the analyte concentration at 50% inhibition of DPPH.**"**

5.2.6 In-vitro cell proliferation assay

The mixed PCFP micellar solutions with and without CUR were freshly prepared by dissolving 130 µg/mL of PCFP in DPBS. MCF-7 cells were cultured in a DMEM culture medium supplemented with 1 % PSN and 10% FBS at 37±0.5°C in a humidified environment of 5% CO_2. MCF-7 cells were treated for 24 h with blank PCFP micellar solution and CUR-loaded PCFP micellar solution with various concentrations (0.25, 0.5, 1, 2.5, and 5 µg/mL). Untreated cells in the DMEM medium were taken as the control.

The MTT assay was used to determine the IC_{50} (half-maximal inhibitory concentration) of mixed PCFP micellar solutions [34]. Briefly, $1.0x10^4$ MCF-7 cells were treated with blank PCFP and CUR-incorporated PCFP at concentrations for 24 h. Thereafter, the cells were cleaned thoroughly with DPBS and treated with MTT (5.0 mg/mL) in a dark condition for four h at 37±0.5°C. After completion of the incubation time, MTT solution was removed, and DMSO was added to each well. The absorbance was measured at λ=570 nm with a reference wavelength of 650 nm using a multimode microplate reader (SpectraMax M2e, USA). The IC_{50} value was calculated, and data were represented as a percentage of cell proliferation.

5.2.7. In-vitro cell viability assay

The cell viability was assessed through trypan blue dye exclusion technique **[35]**. In brief, MCF-7 cells were incubated with different concentrations of blank PCFP and CUR-loaded PCFP micellar solutions for 24 h. The treated cells were collected, washed, and resuspended in DPBS. Thereafter, the cell suspension was mixed at a ratio of 1:1 with 0.4% Trypan blue and incubated for 2-3 minutes. The number of viable cells was counted, and the percentage of cell death was calculated using the formula:

[% Cell death= (Number of dead cells/Total number of cells) ×100].

5.3: Results and discussion

5.3.1. Aggregation behavior of mixed PC/Pluronic micellar solutions

According to thesis report of Shaikh et al. [32], the aggregation behaviour of mixed PC/Pluronic micellar solutions was characterised for further investigation in successful biological applications as better nanocarriers.

"Figure 1(a) shows the mean hydrodynamic diameters (Dh) of various PCF, PCP, and PCFP solutions. The DLS stacks, i.e., each solution's intensity profile, are presented in Figure 5.1(b). Here, the concentrations of F127, P123, and their mixture were taken above their respective CMC values reported in the literature [36]. The large bilayer vesicles of PC with a diameter of 250.2 nm were found in the absence of Pluronics. The PC vesicle sizes are comparable to previously reported values [37-39]. The mean diameter of PC vesicles decreased with the addition of F127, P123, and mixed F127/P123 micellar solutions to a total 5 %w/v concentration regime. It might be attributed to the solubilization of the vesicle bilayer of PC with the formation of mixed micelles of Pluronics [40]. Similar results were found when many nonionic surfactants were added to the water [41-43]. Table 5.2 also clearly shows that the PC vesicles with F127, i.e., PCF micellar solutions, had high D_h values compared to those containing P123 (PCP micellar solutions). This might be because F127 has a considerable molecular weight and a high percentage of PEO compared to P123 (12600 $g.mol^{-1}$ versus 5750 $g.mol^{-1}$). All the studied vesicles had PDI values of less than 1.0, indicating a homogeneous size distribution. The DLS stacks of all the studied solutions also clarify that the intense narrow peaks of PC vesicles were shifted towards broader peaks in the added Pluronic solutions (Figure 5.1(b)). Results of mixed PCFP micellar solutions found lower D_h values in comparison to PCF and PCP solutions. It is known that the mixed F127/P123 micelles are thermodynamically stable due to the tight hydrophobic interactions created by P123 and kinetically stable due to the long hydrophilic chain of F127 [44]. On the other hand, these mixed F127/P123 micellar solutions perform better at changing large PC vesicles into small spherical micelles.**"**[32]

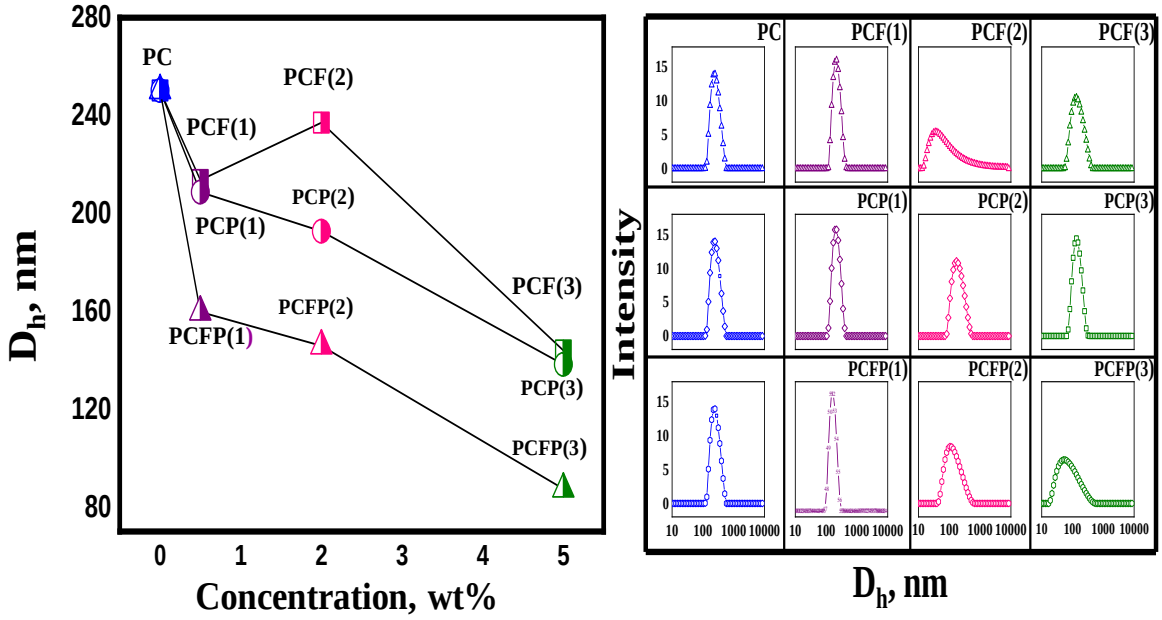


Figure 5.1: (a) Graph of D_h vs. concentration of PCF, PCP, and PCFP mixed micellar solutions (b) DLS stacks graph of PCF, PCP, and PCFP mixed micellar solutions (*at 30±0.5°C*).

Table 5.2: D_h and PDI values of mixed PC/Pluronic micellar systems(*at 30±0.5°C*).

Mixed PC/Pluronic system	D_h (nm)	PDI
PC	250.20	0.442
PCF (1)	213.60	0.334
PCF (2)	237.10	0.531
PCF (3)	144.00	0.439
PCP (1)	208.70	0.403
PCP (2)	192.70	0.732
PCP (3)	138.30	0.360
PCFP (1)	159.50	0.588
PCFP (2)	145.90	0.792
PCFP 3 (3)	87.40	0.256

"SANS measurements have been performed to understand the association mechanism of the formation of mixed PC-Pluronic F127/P123 micellar solutions. SANS is an important soft-condensed matter characterization technique that has been widely used to investigate

micelles and their interactions with PC vesicles [45,46]. Figure 5.2 depicts the experimentally fitted SANS curves of PC, PCFs, PCPs, and PCFPs solutions in D_2O at 30±0.5°C. The fitted morphological parameters like mean core radius (R_c), hard-sphere radius (R_{hs}), the volume fraction of micelles (ϕ), vesicle size (R_v), thickness of vesicle are listed in Table 5.3. It was noticed that the addition of Pluronic micelles very much influenced the structure of PC vesicles. The addition of F127 leads to a decrease in the thickness of PC vesicles compared to usual. At higher concentrations, PCF (3) has shown the change into spherical micelles with anRc of 56.1Å. Other P123 initially slightly increased the PC vesicle size due to its high adsorption at surfaces and then changed to spherical micelles at 2 %w/v concentration [PCP(2]. Highly hydrophobic P123 more effectively converted large PC vesicles into spherical micelles at low concentrations"[32].

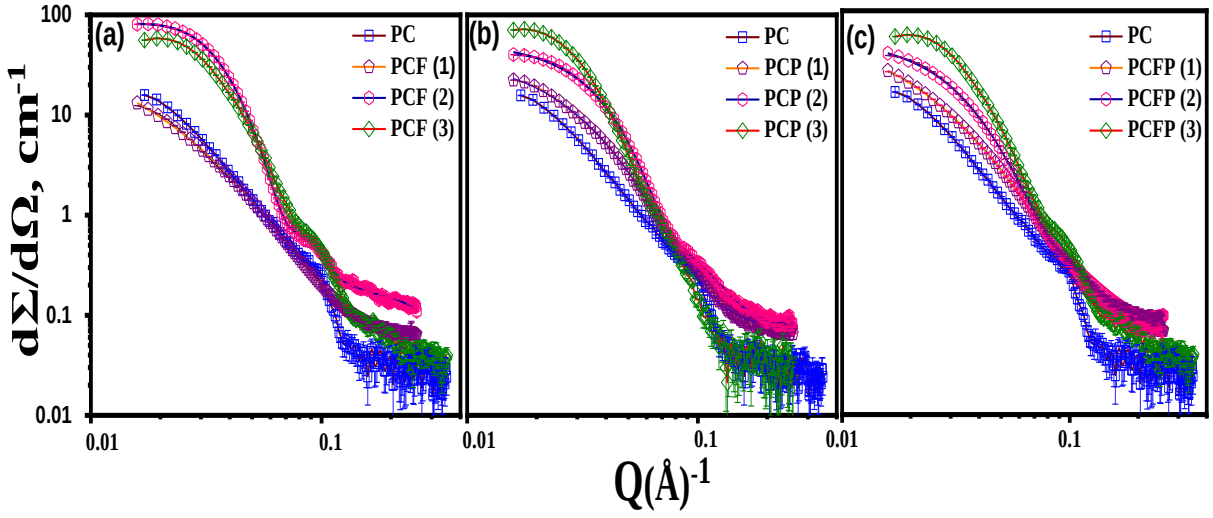


Figure 5.2: Experimental SANS pattern for mixed (a) PCFs (b) PCPs, and (c) PCFPs micellar solutions in D_2O (*at 30±0.5°C*).

Table 5.3: SANS fitted parameters of mixed PC/Pluronic micellar systems (*at 30±0.5°C*).

Mixed micellar system	R_c (Core radius) (Å)	R_{hs} (Hard sphere radius) (Å)	ϕ Volume fraction	N_{agg} (Aggregation number)	Morphology
PC		R_v = 210.0 Å t_v = 38.10 Å			Vesicles
PCF (1)		R_v =280.0 Å t_v= 34.0 Å			Unilamellar vesicles
PCF (2)		R_v=285.0 Å t_v= 35.6 Å			Unilamellar vesicles
PCF (3)	56.10	85.90	0.130	110.0	Spherical micelles
PCP (1)		R_v=285.0 Å t_v= 39.10 Å			Unilamellar vesicles
PCP (2)	58.30	–	–	–	Spherical micelles
PCP (3)	60.50	90.60	0.080	138.0	Spherical micelles
PCFP (1)		R_v=262.0 Å t_v= 37.1 Å			Unilamellar vesicles
PCFP (2)	56.70	–	–	–	Spherical micelles
PCFP (3)	59.80	106.70	0.10	133.0	Spherical micelles

"The mixed F127/P123 micelles showed better results than the individual F127 and P123 as they shifted the PC vesicles into spherical micelles with an even smaller core size of Rc = 56.7 Å at total 2 %w/v concentrations. Generally, Pluronics affect the vesicle structure of the PC and lead to the formation of mixed micelles of PC and Pluronics. In Figure 5.2, we observed the increase in the scattering intensity upon the addition of F127, P123, and a mixed F127/P123 system in the 1 %w/v PC solutions. It showed the increase in anisotropy of the aggregates present in the system. The PC shows stabilized openings in the bilayer structure [Figure 5.2(c)]. It is noteworthy that the high concentration of the Pluronic micelles in the PC vesicle samples shows the vesicle-to-spherical transition. Results indicate that mixed F127/P123 spherical micelles with the inclusion of PC have very good solubilizing ability [47].**"**

The change in the viscosity as a role of shear rate for blank PC and other mixed PC/Pluronic micellar solutions are presented in Figure 5.3. The viscosity of PC reduces

significantly with an increase in the concentration of F127, P123, and mixed F127/P123 micellar solutions. The order of decrease in the viscosity of micellar solutions is as follows: PCFP>PCP>PCF. It was clearly found that the viscosities of mixed micellar solutions with different compositions of Pluronics show a non-newtonian fluid and shear-dependent behavior with shear thinning. The viscosity reaches towards a plateau at low shear rates, clearly seen in the plot for all the studied solutions. This gives us to evaluate the zero shear viscosity (η_0) of the mixed systems by extrapolating the viscosity data (Figure 5.4). The η_0 for different mixed PC/Pluronic micellar solutions is tabulated in Table 5.4. Though the viscosity at low shear rates approaches a plateau (Figure 5.3), at intermediate shear rates, the behavior is same like a power-law fluid. Such an observation is also evident in the shear stress versus shear rate plot of the blank PC and other mixed PC/Pluronic micellar systems (Figure 5.5).

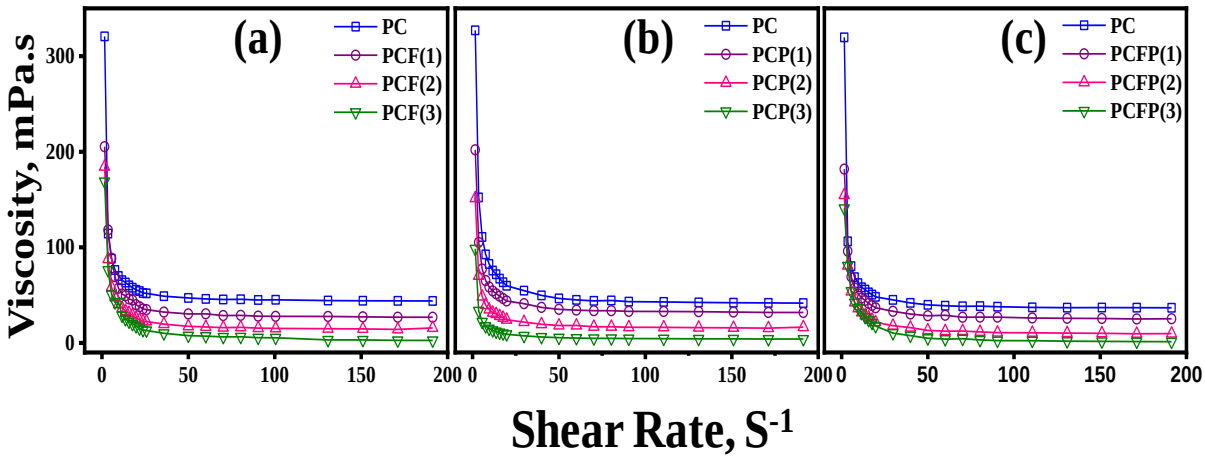


Figure 5.3: Variation of the shear viscosity as a function of shear rate for mixed (a) PCF, (b) PCP, and (c) PCFP micellar solutions (at 30±0.5°C).

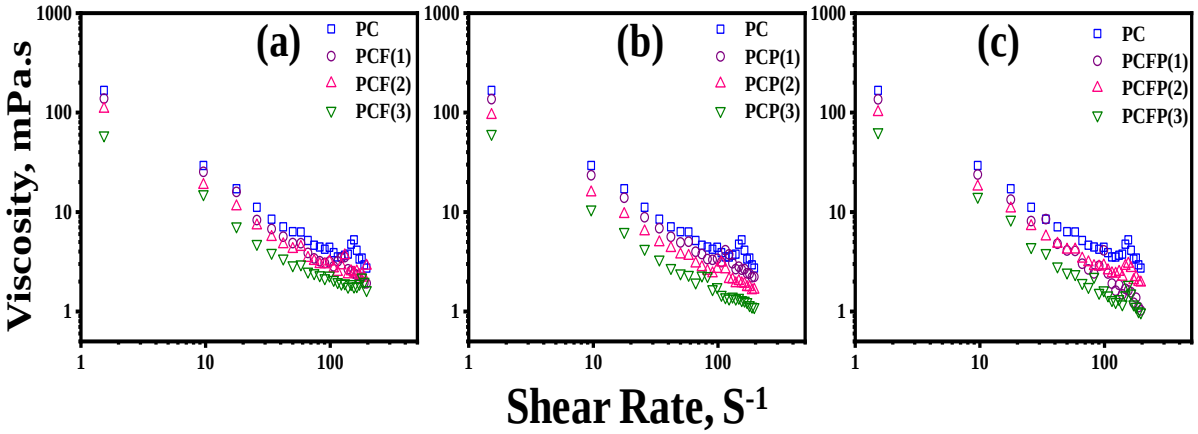


Figure 5.4: Variation of the shear viscosity as a function of shear rate for (a) PC and PCF (b) PCP and (c) PCFP micellar solutions at 30°±0.5 °C for estimate the zero shear viscosity (η_0) of the mixtures by extrapolating the viscosity data.

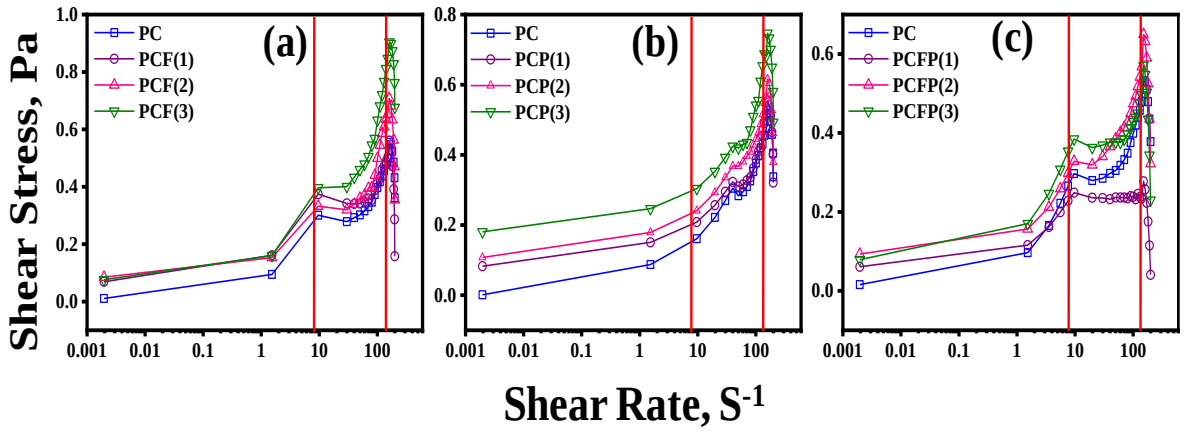


Figure 5.5: Variation of the shear stress (τ) as a function of shear rate (γ) for mixed (a) PCF, (b) PCP, and (c) PCFP micellar solutions (*at 30±0.5°C*).[Fitting parameter was done using the selected range of data shown in-between the red-vertical lines].

It was clearly showed that the shear stress rises with an increase in shear rate, approaches a maximum, and then decreases with further rises in shear rate. In the close region to the stress maximum, the shear stress can be acted by a power-law equation.

$\tau = K\gamma^n$, where τ =shear stress, K = flow consistency index, γ = shear rate, and n = flow behavior index.

Table 5.4: Rheological power law fitting parameters of mixed PC/Pluronic micellar systems (*at 30±0.5°C*).

Mixed PC/Pluronic system	η_0	K	n	R^2
PC	269.81	248.0	0.43	0.9943
PCF (1)	247.17	211.6	0.46	0.9889
PCF (2)	246.37	201.6	0.49	0.9814
PCF (3)	239.77	95.82	0.52	0.9908
PCP (1)	236.23	105.2	0.48	0.9923
PCP (2)	230.45	100.3	0.50	0.9902
PCP (3)	228.18	71.71	0.51	0.9986
PCFP (1)	226.26	95.82	0.52	0.9943
PCFP (2)	207.04	85.62	0.55	0.9988
PCFP (3)	190.57	89.53	0.53	0.9968

A newtonian fluid means n=1. However, the grater the deviation of *n* from 1, the more non-newtonianbehavior of the fluid. Hence, n>1 is for a dilatant fluid, while n<1 for a pseudoplastic fluid. In the regime below the critical shear rate, equation 1 was applied to fit the shear stress vs. shear rate data (Figure 5.5). Table 5.4 summarized the values of n, K, and R^2(statistical correlation coefficient) produced from fitting.

The *n values* found less than 1 for all the mixed PC/Pluronic micellar solutions, which indicating that the micellar systems are pseudoplastic in nature. Moreover, the value of *n* increases with increasing Pluronic concentration, suggesting a deprivation in the pseudoplastic behavior, which indicates the morphological transition of large PC vesicles to spherical micelles. The value of the K also decreases with an increase in the polymer concentration. All the rheological results suggest that the inclusion of Pluronics to the PC significantly changes the flow behavior, possibly due to the penetration of Pluronic molecules into the lamellar vesicle layer. Such changes in the rheological behavior also confirmed the micellar transitions that could be seen in the SANS measurements [48].

Figure 5.6 shows representative TEM micrographs of the blank PC and mixed PCFP micellar solutions. Here, the PC blank and PC to Pluronics different ratios were explored. Before introducing Pluronics, the vesicles had a membrane thickness of 3.81 nm, for which the bilayer structure is visible, typical of phospholipid vesicles, as shown in Figure 5.5(a**)**. On the other hand, after the addition of mixed Pluronic F127/P123 micelles with increasing

concentration, two major morphological shapes are observed, as shown in Figure 5.6(b-d). In Figure 5.6(b), the main occupant is constituted of vesicles with a membrane thickness of 3.71 nm. The thickness value is slightly below that of pure PC vesicles. The unilamellar vesicles shown in PCFP(1) now transform into small-sized mixed spherical micelles in mixed PCFP(2) and PCFP(3) micellar systems, as shown in Figures 5.6(c) and 5.6(d**)**. Moreover, the sizes of aggregates change between microscopic techniques and scattering techniques like DLS, which have been well documented [49].

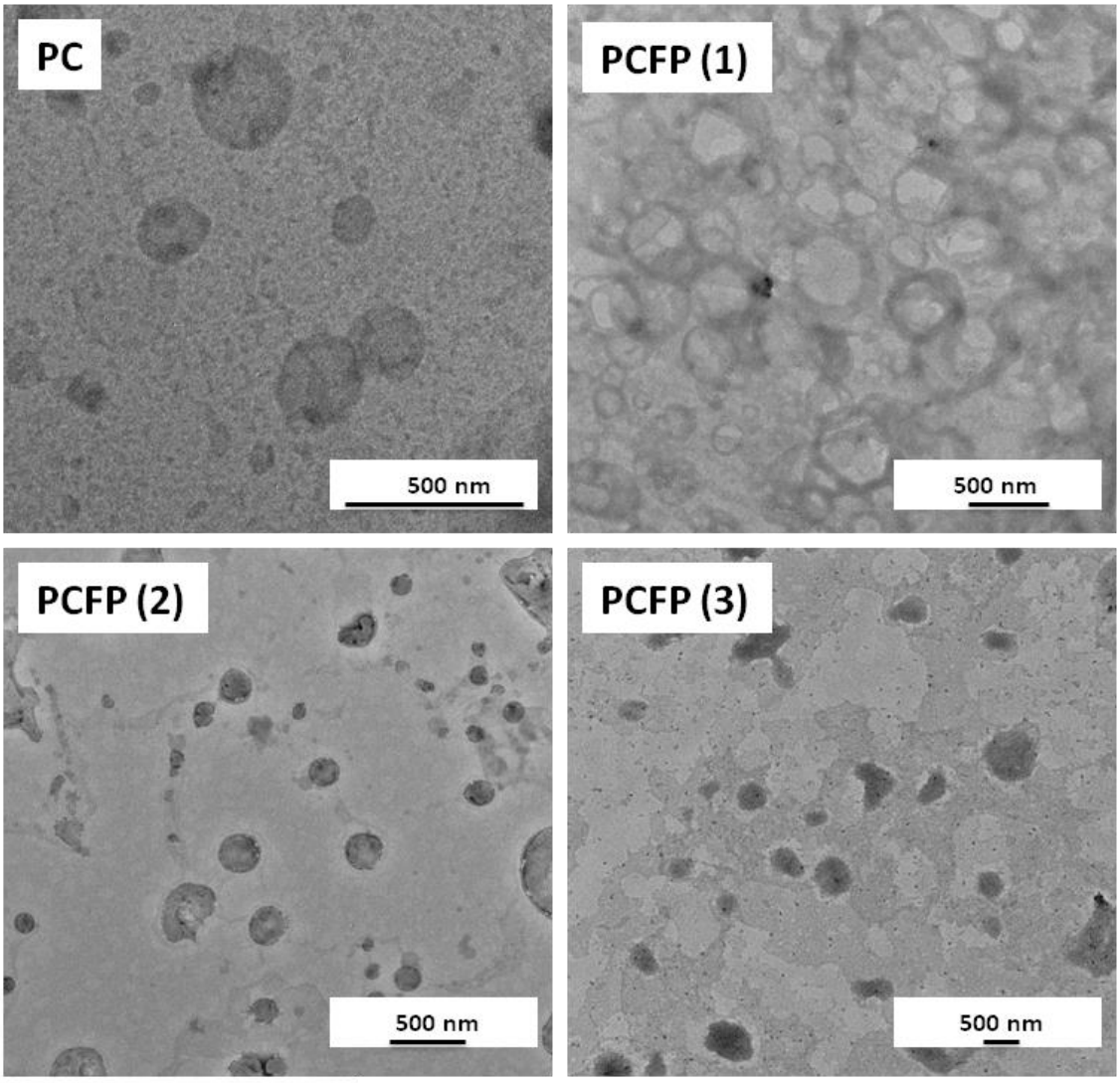


Figure 5.6: TEM results of (a) PC, (b) PCFP(1), (c) PCFP(2), and (d) PCFP(3) micellar systems.

The interaction between PC and Pluronics depends typically on the HLB, which depends on the length of hydrophilic and hydrophobic blocks ratio. The addition of Pluronic might have interfered with PC molecules, causing perturbations in the surface of the lipid bilayer, which is reflected in the changes in the order and relaxation parameters of the bilayer. It decreases the bending rigidity and transitions from a unilamellar vesicle to the relatively small mixed vesicle phase. The presence of mixed micelles could explain the high Pluronic concentration; spherical micelles were observed rather than vesicle morphology [50]. TEM confirmed the spherical morphology of the mixed PCFP micelles.

5.3.2. Solubility of CUR in mixed PC/Pluronic micellar solutions

The mixed Pluronic micelles are significantly modified in the presence of PC in water, which has a substantial impact on their solubilization properties. If you add PC to a mixed micelle to make it more hydrophobic, you can lower the CMC and CMT, making it easier for them to change shape in the water.

Figure 5.7 shows the solubility of CUR in mixed micellar solutions of PCF, PCP, and PCFP at RT (30±0.5°C). Here, blank and mixed F127/P123 micellar solution concentrations increase with a fixed concentration of PC for solubilization of CUR. The results showed that as the concentration of Pluronics increased, the CUR solubility increased. The order of increasing the solubility is as follows: PCP > PCFP > PCF.

Due to the hydrophobic-hydrophobic interaction created by P123, a high PPO block dissolves more CUR. On the contrary, the highly hydrophilic nature of F127 showed less solubility of the drug. The mixed F127/P123 micelles show intermediate results in the solubility of CUR. All drug formulations of PCF and PCP showed precipitation of CUR crystals after storage for 15 days at RT. Pluronic micelles were reported to have certain disadvantages, like their particle size, low stability, and the possible reversion to phase separation.It was reported that the hydrophilic long PEO shell of the micelles formed by F127 and P123 had a protective effect on the micelle dispersion. Therefore, we focused on mixed F127/P123 micelles by incorporating PC for the bioavailability of CUR. Thus, the incorporation of PC would increase the thermodynamic stability of the micelles due to the tight hydrophobic interactions with the hydrophobic PPO blocks of the Pluronics [51]. However, there was no precipitation observed in mixed PCFP(2) micellar solutions (the composition of PC: F127: P123 was 1%w/v, 1%w/v, and 1%w/v in the dispersion) for more

than 2 months. Hence, we have performed *in-vitro* biological investigations of CUR using the mixed PCFP(2) micellar system for better understanding.

The most significant parameters for micellar formulation are to establish the %DL and %EE, which are evidence of the effective loading of drugs into micelles. The %DL and %EE of the mixed PCFP(2) micellar system are 4.68% and 75.29%, respectively measured with UV-Vis analysis. These data indicate the effective encapsulation of insoluble CUR drugs in the mixed micellar system.

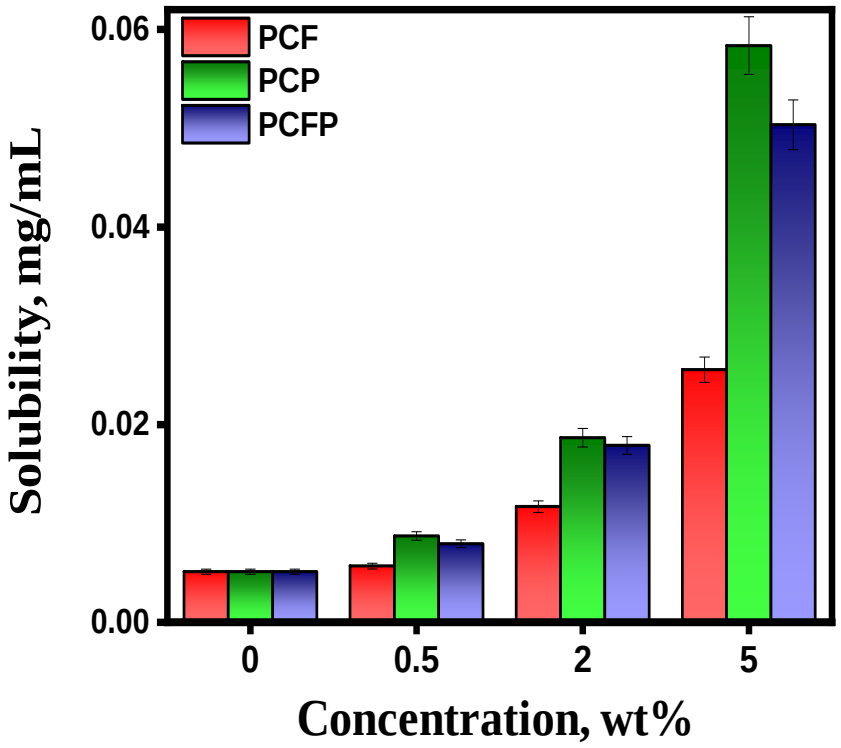


Figure 5.7: The solubility plot of CUR in different concentrations of the mixed PCFP micellar solutions (*at 30 ± 05 °C*).

5.3.3. Location of CUR in the mixed PC/Pluronic micellar system

The location of CUR in the mixed PCFP micelle was determined by using ^{1}H NMR spectroscopy, which is an excellent non-invasive tool [24]. The ^{1}H NMR was used to identify the chemical shifts(δ) of pure CUR, mixed PCFP micelles, and CUR-loaded PCFP micelles. The ^{1}H NMR spectra are presented in Figure 5.8**.** The spectrum of CUR in $CDCl_3$ [Figure 5.8(a)] clearly showed the δ at 7.28 (OH), 6.94–6.96 (Ar-H), 7.59–7.63 (2H, adjacent to the

benzene ring), 3.96 (2H, -OCH_3), and 5.94 (CO-CH_2-CO) ppm. The spectrum of blank mixed PCFP micelle in D_2O [Figure 5.8(b)] showed the δ of CH_3, CH, CH_2 of PPO, and CH_2 of PEO at 0.99, 3.37, 3.39, and 3.54 ppm, respectively. The signals of PC were found to be very weak in the spectrum. It confirmed that the Pluronic F127/P123 micelles are dominant in the mixed PCFP micellar system. In the spectrum of CUR-loaded PCFP micelles in D_2O [Figure 5.8(c)], the peaks of the CUR are hardly observed. Here, three signals of the PPO at 0.98(CH_3), 3.36 (CH), and 3.38 (CH_2)showed the 0.01 ppm upfield shift of PPO in comparison to the blank PCFP micelle spectra. An upfield or downfield shift of copolymer protons probably indicates the location of drug solubilization [52]. These results clearly indicated that CUR was successfully encapsulated into the hydrophobic inner PPO core of mixed PCFP micelles [53].

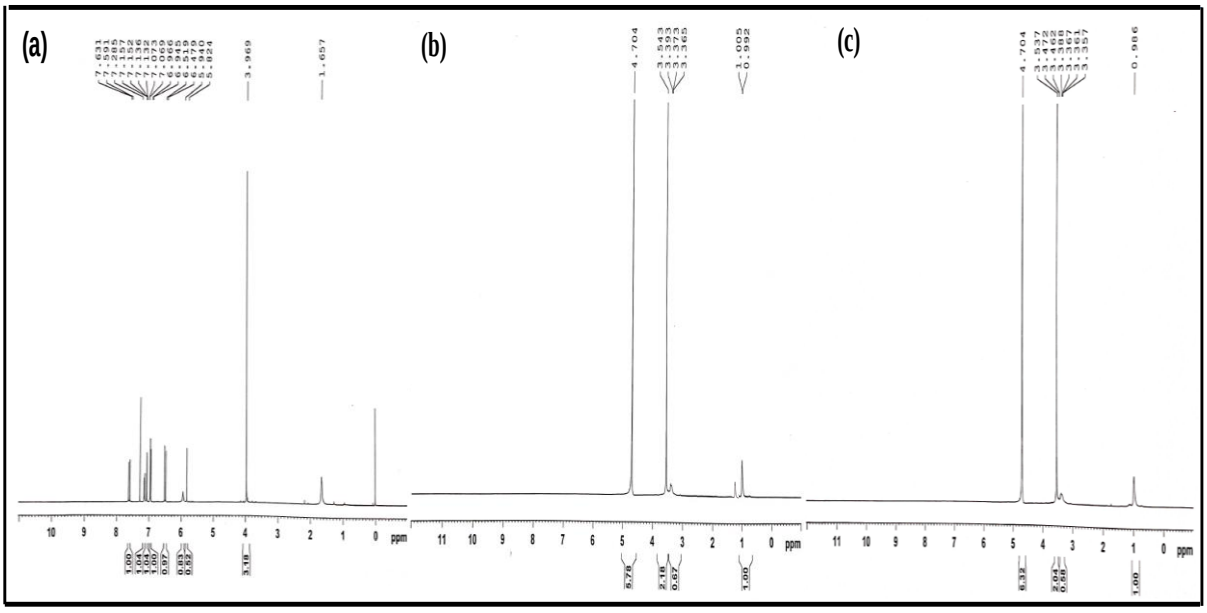


Figure 5.8: ^1H NMR spectra of (a) CUR in $CDCl_3$, (b) blank mixed PCFP micellar system in D_2O and (c) CUR-loaded mixed PCFP micellar system in D_2O.

5.3.4. Biological evaluation

5.3.4.1 In-vitro release study

In order to explore the pharmacological uses of mixed PCFP micellar systems as drug delivery vehicle, the *in-vitro* drug release study was performed. CUR is brilliant yellow and not dissolved in water. So, CUR was solubilized in propylene glycol(PG) solution as a free

CUR solution for the drug release analysis. As shown in Figure 5.9, the most of the CUR was released within 24 h, and this suggests that CUR could facilely diffuse from the dialysis membrane. The release profile of CUR from mixed PCFP micelles were investigated under reservoir-sink conditions at 37±0.5°C. The release of CUR molecules from mixed PCFP micelles followed a time-dependent release profile. Almost 28% of the CUR was released from mixed PCFP micelles at pH 7.4 after 100 hrs. Results indicated the slow release of CUR from mixed PCFP micelles. The slow release of CUR from various drug delivery systems is well observed in the literature [54]. The results show that the shorter inter-micellar distance leads to a more significant number of cross-links between neighboring micelles, leading to higher viscosity and a lower rate of drug release [15]. It is also clearly understood that as CUR was entrapped in the hydrophobic matrix of PC and PPO moieties of Pluronics in the mixed micellar system, it resulted in a slow and more sustained release of the drug.

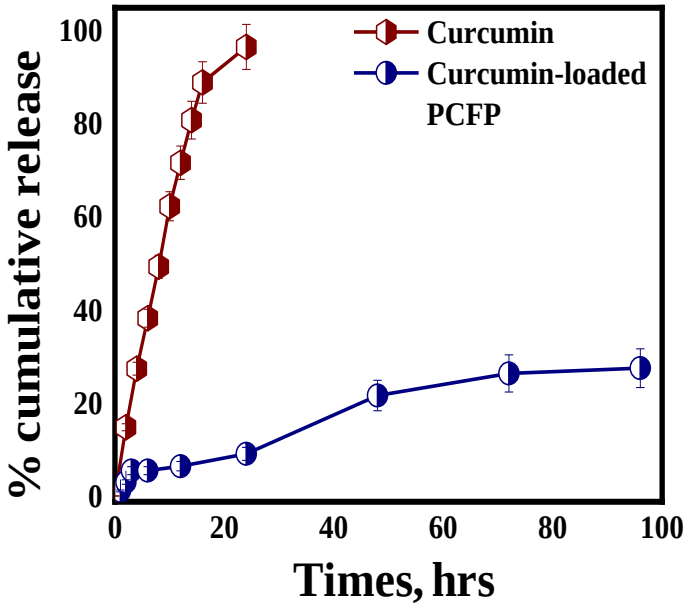


Figure 5.9: *In-vitro* drug release study of CUR and CUR-loaded PCFP system (*at 37 ± 0.5°C*).

5.3.4.2 Antioxidant activity analysis

CUR is a highly effective antioxidant that dissolves in lipids. It is believed to function through both pro- and anti-oxidant actions. Such drugs work by giving electrons to free radicals without changing into electron-scavenging substances themselves. They also participate in mechanisms that repair DNA and maintain the health of the cells.

The antioxidant scavenging activities of free CUR and CUR-loaded mixed PCFP micellar systems were assessed by measuring their capacity to scavenge DPPH free radicals. The results are shown in Figure 5.10. The free CUR showed 30% scavenging activities up to 3.0 µg/mL, while mixed PCFP micellar solution inhibited almost 85% at the same concentration. The IC_{50} (concentration of CUR required to achieve 50% scavenging activity) value of free CUR and the CUR-loaded PCFP micellar system was calculated. The IC_{50} values of CUR and CUR-loaded PCFP were 5.7996 µg/ml, and 1.4631 µg/ml, respectively. The variation in antioxidant scavenging activity could be attributed to the effective of the antioxidant ability of CUR by the mixed micellar system. This is because the mixed PCFP micellar system resulted in smaller particle size and better solubility, which improved the drug's DPPH radical neutralization and antioxidant effect [55]. It was found that CUR-loaded PCFP micelles had more antioxidant power than the free CUR drug.

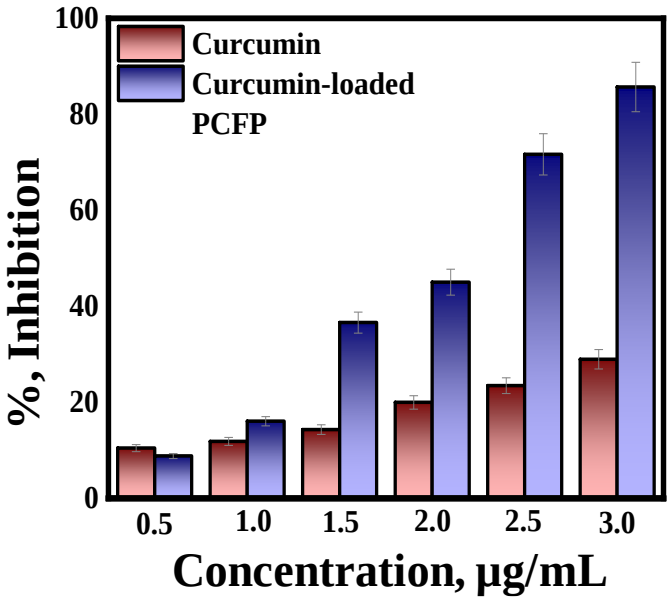


Figure 5.10: % inhibition capacity of DPPH at different concentrations of CUR and CUR-loaded PCFP micellar solutions.

5.3.4.3 Cell proliferation activities

The MTT assay was carried out to evaluate the effect of a mixed PCFP micellar system with and without CUR on the proliferation of MCF-7 cells. The results showed that an MCF-7 cell treated with a CUR-loaded mixed PCFP micellar system for 24 significantly inhibits cell proliferation. Interestingly, the IC_{50} value of CUR-loaded mixed PCFP micelles

was found at 0.32 µg/ml in MCF-7 cells, but no inhibitory concentration of mixed PCFP micellar system was found in the case of blank [Figure 5.11(a)].

However, the blank PCFP micellar system did not show inhibition of cell proliferation of MCF-7 cells as compared to the CUR-loaded mixed PCFP micellar system. The results suggest that the micellar system is safe, biocompatible, and nontoxic. The mixed PCFP micellar system may solubilize the number of poorly water-soluble compounds and act as a safe nano-carrier for drug delivery. These results indicate that an aqueous soluble CUR-loaded mixed PCFP micellar system exhibits significant inhibition in the proliferation of MCF-7 cells.

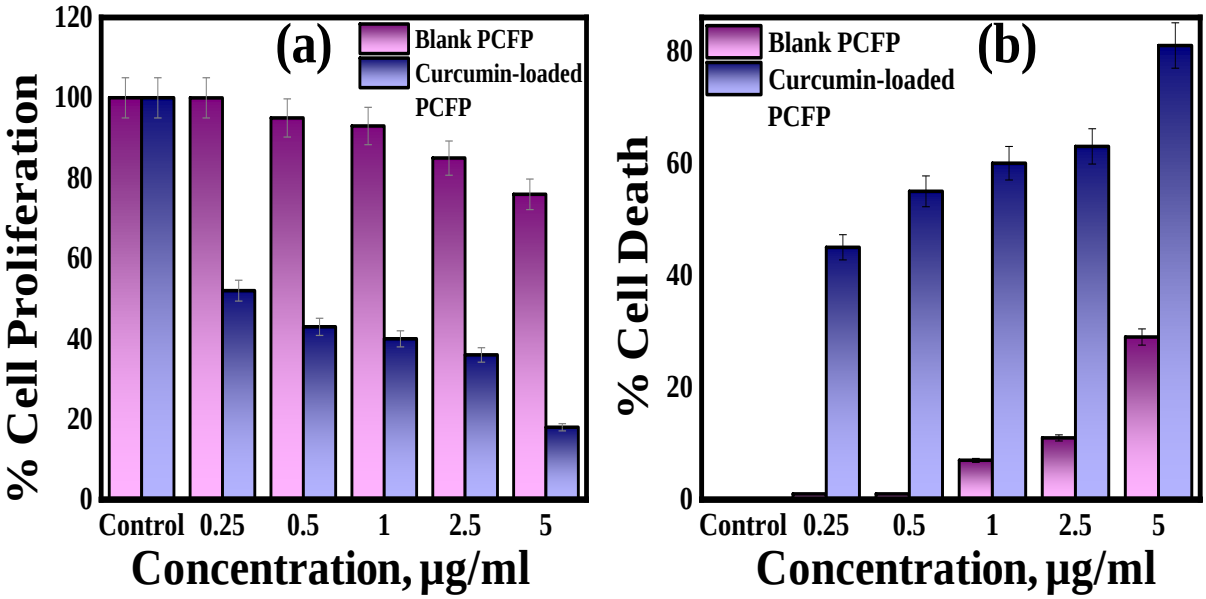


Figure 5.11: (a) % Cell Proliferation of blank PCFP and CUR-loaded mixed PCFP (b) % Cell viability of blank PCFP and CUR-loaded mixed PCFP micellar system.

5.3.4.4 Cell viability assays

Trypan blue is a negatively charged dye that stains only dead cells with the compromised plasma membrane. A Trypan blue exclusion assay was performed to evaluate the cell death potential of a mixed PCFP micellar system. The cells were treated for 24 hrs with a mixed PCFP micellar system with different concentrations (0.25, 0.5, 1, 2.5, and 5 µg/mL) and the percentage of cell death was determined [Figure 5.11(b)]. The cells treated with the mixed PCFP micellar system exhibited substantial cell death in MCF-7. However, no significant cell death was observed in cells treated with a blank mixed PCFP micellar

system as compared to a CUR-loaded mixed PCFP micellar system. Therefore, these results indicate that CUR-loaded mixed PCFP micelles significantly inhibit cell proliferation and induce cell death in MCF-7.

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