Self-assemblies of mixed PEO-PPO-PEO triblock copolymer-Phosphatidylcholine (PC) systems for Curcumin drugs

7.1: Introduction

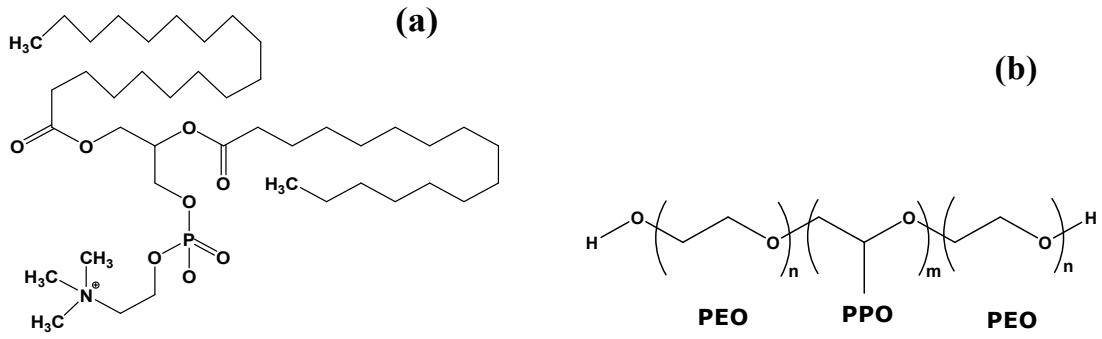
In the pharmaceutical industry, various biocompatible materials are used to improve the aqueous solubility, stability, and better bioavailability of hydrophobic drugs. One type of biocompatible material is Pluronic® block copolymers, which are amphiphilic triblock copolymer (polyethylene oxide-polypropylene oxide-polyethylene oxide, PEO-PPO-PEO) and FDA-approved system for pharma applications [1,2]. Pluronic block copolymers exhibit nonionic surfactant properties and widely studied for a number of biomedical applications [3-5]. The Pluronic molecules dissolve and exist as unimers in dilute aqueous solution at below cmt and cmc. They forms micelles at above cmt as well as cmc, having a hydrophobic core surrounded by a hydrophilic shell. The hydrophobic core works as a compartment for the encapsulation of hydrophobic drugs, while the hydrophilic shell works as a stabilizer for the hydrophobic core. The shape and size of the Pluronic micelles are depending on concentration, block lengths, and temperature [6,7].

Due to high stability and enhanced solubilization capacity reported by researchers, the mixed micelles composed of two or more kinds of Pluronic polymers found better systems for an investigation nowadays [5,8]. The mixed micelles of Pluronic F127 and Pluronic P123 have been widely investigated in therapeutic application due to the kinetic stabilization effect of long PEO chains of hydrophilic F127 blended with thermodynamical stability through hydrophobic P123 with long PPO chains [8-12].

These mixed micelles have lower cmc values and hence high dilution of concentrations also remain above the cmc of mixed systems [7]. Rapoport et al. reported that the small concentration of vegetable oil when added into Pluronic micellar solutions, it decreases the degradation of micelles upon dilution without compromising the drug loading capacity of oil-stabilized micelles [13].

The ability of mixed micelle formulation to keep the drug in solubilized form even after the dilution in GI tract is the key factor for bioavailability of drugs rather than the solubility of drugs in the formulation itself. Looking to this concern, it seems necessary to develop new hydrophobic thermodynamically stabilized Pluronic mixed micellar systems which could resist the precipitation upon dilution through the incorporation of biocompatible additive like L-α-phosphatidylcholine in Pluronic micelle structure [14]. L-α-

phosphatidylcholine (PC) is prepared from natural phospholipids and hence their use is free of issues of any cytotoxicity. The PC shows high stability, narrow size distribution, and good skin penetration ability. PC vesicular systems have attracted much attention because of their capability to solubilize both hydrophilic molecules in their inner aqueous phase and hydrophobic molecules in their lipid bilayer. Being biocompatible and biodegradable systems, these vesicles have been widely used as carriers for many drugs in the medical field. The interaction of PC vesicles with Pluronic block copolymers has particular importance in drug delivery. By controlling the interactions between the Pluronic and PC, these drug delivery systems can be better designed for better therapeutic outcomes. Majority of literature reported the interaction of PC vesicles with Pluronic polymers at low concentration and produced rather very diverging findings [15-20]. While some literature reported negative effects, such as a high rate of encapsulated material leakage and a shorter blood circulation time [21,22]. It was also found that Pluronic not only provides good stealth stabilization to vesicles but also enhances the micromechanical properties of the bilayers [23]. The purpose of the present work is, therefore, to focus on the self-assembly based on Pluronic block copolymers and L-α-Phosphatidylcholine(PC) vesicles (shown in Scheme.7.1).



Scheme .7.1: Molecular structures of a) L-α-Phosphatidylcholine(PC) and b) Pluronic block copolymers

Curcumin, a traditional medicinal ingredient called as Indian solid gold, is used to provide frontline pharmacotherapy for a variety of diseases [24, 25]. Advanced researchers reflect that curcumin possesses a broad range of medicinal properties, including anti-inflammatory, anticancer, antioxidant, and chemopreventive activities[26]. As we seen in

earlier studies, the main issue with curcumin is the low oral bioavailability (1%), which hurdle in achieving the concentration of curcumin within therapeutic ranges [27–29].

In view of the advantages of mixed micelles of Pluronic F127 and Pluronic P123 for pharmaceutical applications, it is critically important to investigate the fundamental understanding of how these Pluronic mixed micelles interact with biocompatible useful compound lecithin (L-α- phosphatidylcholine). The objective of the study is to investigate the mixed micellar solutions of PC-Pluronic F127/P123 using complementary tools like DLS, SANS and TEM. The evolution of the structure from vesicle aggregates to spherical micelles by the addition of Pluronic polymers is evident from SANS and TEM studies. The solubilization of curcumin in mixed PC-Pluronic F127/P123 solutions are evaluated. The in-vitro release and stability of curcumin from these mixed PC- Pluronic F127/P123 solution was evaluated. At a physiological environment, the antioxidant activity of curcumin drug and curcumin loaded mixed PC and Pluronic F127/P123 micelles were observed for its better therapeutic efficacy.

7.2: Experimental Section

7.2.1: Materials

PEO-PPO-PEO triblock copolymers, Pluronic F127, and Pluronic P123 were obtained from Sigma-Aldrich, Banglore, India. L-α Phosphatidylcholine (PC) and curcumin were purchased from Sigma-Aldrich, USA. The 2,2′-diphenyl-1- picrylhydrazyl (DPPH) was received from Merck, India. All the chemicals were used as received. Samples were prepared in deionized water. All the solvents are of Anala R grade and used with proper purifications.

7.2.2: Preparation of mixed PC-Pluronic solutions

The stock solutions of 10 wt% concentration of Pluronic F127 and Pluronic P123 were prepared in HPLC grade water at 4 °C temperature and then equilibrated at room temperature (30 °C) for more than 3 hrs. The mixed PluronicF127/P123 solutions with different-different concentrations were prepared using respective stock solutions and kept at 30°C for 2 hrs before for use. In the required number of 25 mL RBF, the fixed amount of phosphatidylcholine(PC) was dissolved in chloroform flask and kept overnight. The solvent was then removed thoroughly under the vacuum. The dry lipid film formed in the bottom of the flask was resuspended with above prepared mixed PluronicF127/P123 solutions. These samples were sonicated and vortexed several times until all the PC molecules were well dispersed. The amount of PC and Pluronics along with the designated name are presented in Table.7.1.

7.2.3: Preparation of curcumin-loaded mixed PC-Pluronic solutions

Curcumin was used as a model hydrophobic drug to estimate the solubility and release behavior of PC-Pluronic solutions. The fixed amount of curcumin was added to the PC and Pluronic solutions. Curcumin-loaded PC- Pluronic solutions were prepared as discussed above. The free curcumin molecules were separated by centrifugation (4 min), followed by filtration through a 0.8 μm filter membrane, and a yellow color dispersion of curcumin loaded PC- Pluronic solutions were obtained.

Table .7.1: Composition of PC and Pluronic F127/P123 in weight percentage studied in the present work.

PC, (wt%)	Pluronic , (wt%)		% Composition	Code name
	F127	P123	$X_{PC} : X_{pluronic}$	
1.0	0.0	--	1.0 : 0.0	PC
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)
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)
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)

7.2.4: Characterizations of mixed PC-Pluronic solutions

7.2.4.1: Dynamic light scattering (DLS))

The mean diameter (D) of the mixed PC-Pluronic F127/P123 solutions (PCF, PCP & PCFP) were determined through DLS analysis using a Zetasizer Nano-SZ 100 (Horiba) instrument. The scattering angle was kept at fixed 90°. All the samples were extruded and filtered through a polycarbonate membrane of 0.2 μm pore size. All measurements were done at room temperature (30°C). The experiments are repeated at least 5 times to take an average value of mean diameter for each sample.

7.2.4.2: Small-angle neutron scattering (SANS)

SANS was performed for evaluation of sizes and shapes of mixed PC-Pluronic F127/P123 solutions in D_2O at 30°C. These experiments were carried out on the SANS instrument operating at Dhruva Reactor, BARC, Mumbai, India. The mean incident neutron beam wavelength (λ) was 5.2 Å with a resolution ($\Delta\lambda/\lambda$) of about 10%. The scattered neutrons were searched in an angular range of 0.5–15° through a linear position sensitive detector. The scattering vectors 'Q' in the range of 0.015-0.3 Å−1 of scattered neutrons were observed. The measured SANS data were corrected and presented on an absolute scale with standard protocols.

7.2.4.3: Transmission electron microscopy (TEM)

TEM investigations of morphological changes in mixed PC-Pluronic F127/P123 solutions were performed with a Transmission electron microscope (JEOL, Japan, JEM-2100, 120kV). The sample solution drop was put on the carbon-coated copper grid (200 mesh) followed by drying for a few minutes (at RT). The drop of freshly prepared uranyl acetate solution as negative staining was placed on the grid having the dried sample. The grid was again dried at room temperature for analysis.

7.2.5: Phase-solubility of curcumin in mixed PC-Pluronic solutions

The amount of the curcumin in the various mixed PC-Pluronic F127/P123 solutions was determined using the UV–Visible spectroscopy (Shimadzu, UV-2450, Japan, double beam mode 200-800 nm). The solubility of the curcumin drug is evaluated by measuring its absorbance at the wavelength of 425 nm using the calibration curve (reported in Chapter.2). All the measurements were made in triplicate.

7.2.6: In vitro release study

In-Vitro release of curcumin drug from representative mixed PC-Pluronic F127/P123 solution (designated as PCFP(2)) was investigated by diffusion method using a dialysis membrane. The release medium was simulated in PBS with pH 7.4. The PCPF (2) solution was weighed and filled in the dialysis tube well prepared using the membrane. The tube was suspended in a 1000 mL glass beaker containing 500 mL of freshly prepared release medium. The experiment was carried out at a constant temperature in a shaking water bath adjusted to 37 ± 0.2 °C. The aliquot (4 mL) was withdrawn from the release medium at a pre-determined time interval. The release medium was replaced with an equal volume of the freshly prepared release medium every time. The aliquots were diluted as required and the amount of curcumin released was determined by measuring the absorbance at λ=425 nm using UV-Vis spectrophotometer (Shimadzu, UV-2450, double beam). All the experiments were measured in triplicate. The experiment was completely performed in dark. The release profile of curcumin was obtained by plotting the % cumulative amount of drug released from each formulation against time

7.2.7: Stability study

The drug stability of representative mixed PC-Pluronic F127/P123 solution, PCFP(2), was also performed. The PCFP(2) sample was well packed in a glass bottle sealed by aluminum foil and stored at room temperature in a desiccator over anhydrous Calcium chloride salt for 15 days. The stability of the PCFP(2) sample was evaluated for change in drug retention at each time interval with measuring the drug content using UV-Visible spectroscopy technique.

7.2.8: Antioxidant activity

The antioxidant activities of curcumin drug and representative mixed PC and Pluronic F127/P123 solutions, PCFP(2) with the different-different amount 0.05, 0.1, 0.4, 0.8, 1.0, 2.0, and 3.0 µg/mL were checked by measuring their ability to scavenge the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) stable radicals. The sample solutions were vigorously mixed and kept for half an hour in the dark at room temperature for incubation. The color intensity of the sample changed by the scavenging of free radicals from DPPH was measured by a spectrophotometer at a wavelength of 517 nm. The scavenging capacity of the tested samples was calculated by comparison of sample color with the control. The experiments were carried out in triplicates. The percentage of inhibition was calculated using the following formula:

$$\% \text{ inhibition} = (A_0 - A_1)/A_0 \times 100$$

Where A_0= control and A_1= sample present

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

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

7.3: Results and discussion

7.3.1: Aggregation behavior of mixed PC-Pluronic solutions

Fig.7.1(a) shows the mean diameters(D) of various PCF,PCP & PCFP solutions. The DLS stacks i.e. intensity profile of each solution was also presented in Fig.7.1(b). Here, the concentrations of Pluronic F127, Pluronic P123, and its mixture were taken at above their respective cmc values reported in the literature [30]. The large bilayer vesicles of PC with the diameter of 250.2 nm was found in the absence of Pluronics. The sizes of PC vesicles are comparable with reported values [31-33].

In the Fig.7.1(a), the mean diameter of PC vesicles has been decreased with added Pluronic F127, Pluronic P123, and mixed Pluronic F127/P123 solutions to total 5 wt% concentration regime. It might be attributed to solubilization of the vesicle bilayer with the formation of mixed micelles of Pluronics as previously reported [34]. A similar trend for a decrease in vesicle size was also reported for the addition of many nonionic surfactants [35-37].

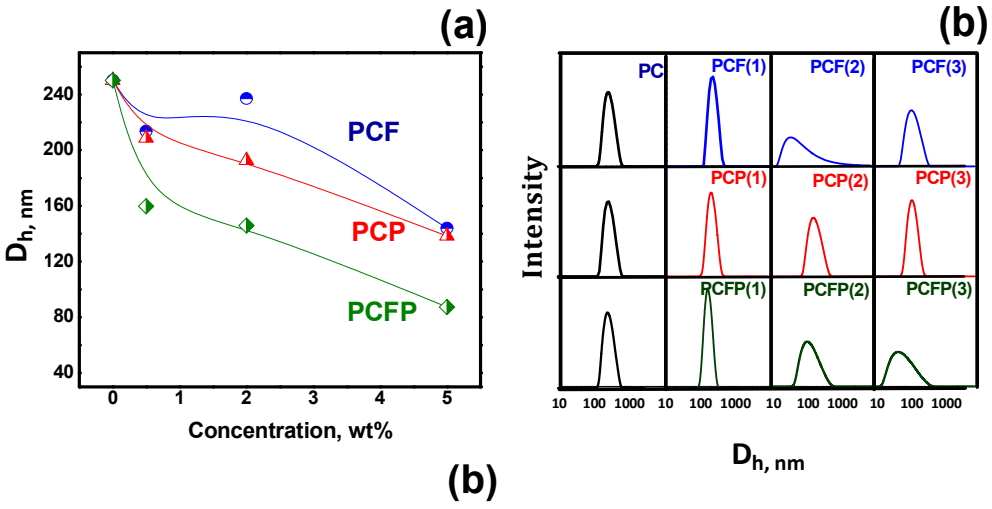


Fig.7.1: (a)Plot of mean diameter(D) versus concentration of mixed PCF, PCP and PCFP solutions and (b) D_hLS stacks of mixed PCF, PCP and PCFP solutions.

Table 7.2 also denotes that PC vesicles with Pluronic F127 i.e. PCF solutions had high size values compared to those containing Pluronic P123(PCP solutions). This might be because of Pluronic F127 has a large molecular weight and high percentage of PEO compared to Pluronic P123 (12600 $g.mol^{-1}$ versus 5750 $g.mol^{-1}$). All vesicles had PDI values

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 (Fig.7.2(b)). Results of PCFP solutions found lower diameter values in comparison to PCF and PCP solutions. It was known that the mixed micelles of Pluronic F127/P123 are thermodynamically stable as Pluronic P123 tighten hydrophobic interactions and kinetically stable as the long hydrophilic chain of Pluronic F127[38]. Hence, these mixed micellar solutions are more effective in the transformation of large PC vesicles into spherical micelles.

Table.7.2: Mean diameter (D) and PDI index values of mixed PC-Pluronic solutions at 30°C.

System	D, nm	PDI
PC	250.2	0.442
PCF (1)	213.6	0.334
PCF (2)	237.1	0.531
PCF (3)	144	0.439
PCP (1)	208.7	0.403
PCP (2)	192.7	0.732
PCP (3)	138.3	0.360
PCFP (1)	159.5	0.588
PCFP (2)	145.9	0.792
PCFP (3)	87.4	0.256

In order to understand an association mechanism of the formation of mixed PC-Pluronic F127/P123 solutions, we performed the SANS measurements. SANS is an important measurement for characterizing soft-condensed matter and has been widely applied to examine Pluronic micelles and its interaction with PC vesicles.

Fig.7.2 depicts the experimentally fitted SANS curves of 1 wt% PC, PCFs, PCPs, and PCFPs solutions in D_2O at 30 °C. Various morphological parameters like mean core radius (*Rc*), hard sphere radius (*Rhs*), the volume fraction of micelles (ϕ), aggregation numbers (N_{agg}) are listed in Table.7.3.

Table .7.3: SANS parameters of mixed PC-Pluronic solutions at 30°C.

System	Core radius R_c (Å)	Hard sphere radius R_{hs} (Å)	Volume fraction ϕ	Aggregation number N_{agg}	Micellar structure
PC	Size of vesicle = 210 Å Vesicle thickness, *t*= 38.1 Å				Vesicles
PCF (1)	Size of Vesicle =280 Å Vesicle thickness, *t*= 34.0 Å				Unilamellar vesicles
PCF (2)	Size of Vesicle =285 Å Vesicle thickness, *t*= 35.6 Å				Unilamellar vesicles
PCF (3)	56.1	85.9	0.13	110	Spherical micelles
PCP (1)	Size of Vesicle =285 Å Vesicle thickness, *t*= 39.1 Å				Unilamellar vesicles
PCP (2)	58.3	–	–	–	Spherical micelles
PCP (3)	60.5	90.6	0.08	138	Spherical micelles
PCFP (1)	Size of Vesicle =262 Å Vesicle thickness, *t*= 37.1 Å				Unilamellar vesicles
PCFP (2)	56.7	–	–	–	Spherical micelles
PCFP (3)	59.8	106.7	0.10	133	Spherical micelles

It was noticed that the addition of Pluronic micelles very much influenced the structure of PC vesicles. The addition of Pluronic F127 leads to a decrease in the thickness of PC vesicles from usual. At higher concentration, PCF(3) has shown the change into the spherical micelles with Rc of 56.1 nm. Other Pluronic P123, initially slightly increases the PC vesicle size due to its high adsorption at surfaces and then changed to spherical micelles at 2 wt% concentration (PCP(2)). High hydrophobic Pluronic P123 more effectively converted large vesicles into spherical micelles at lower concentrations. The Pluronic F127/P123 micelles showed better results than individual as shifted the PC vesicles into spherical micelles with an even lower core size of Rc=56.7 nm at total 2 wt% concentrations. Generally, Pluronic solutions affect the vesicle structure of the PC and simply led to the formation of mixed micelles of PC with Pluronics.

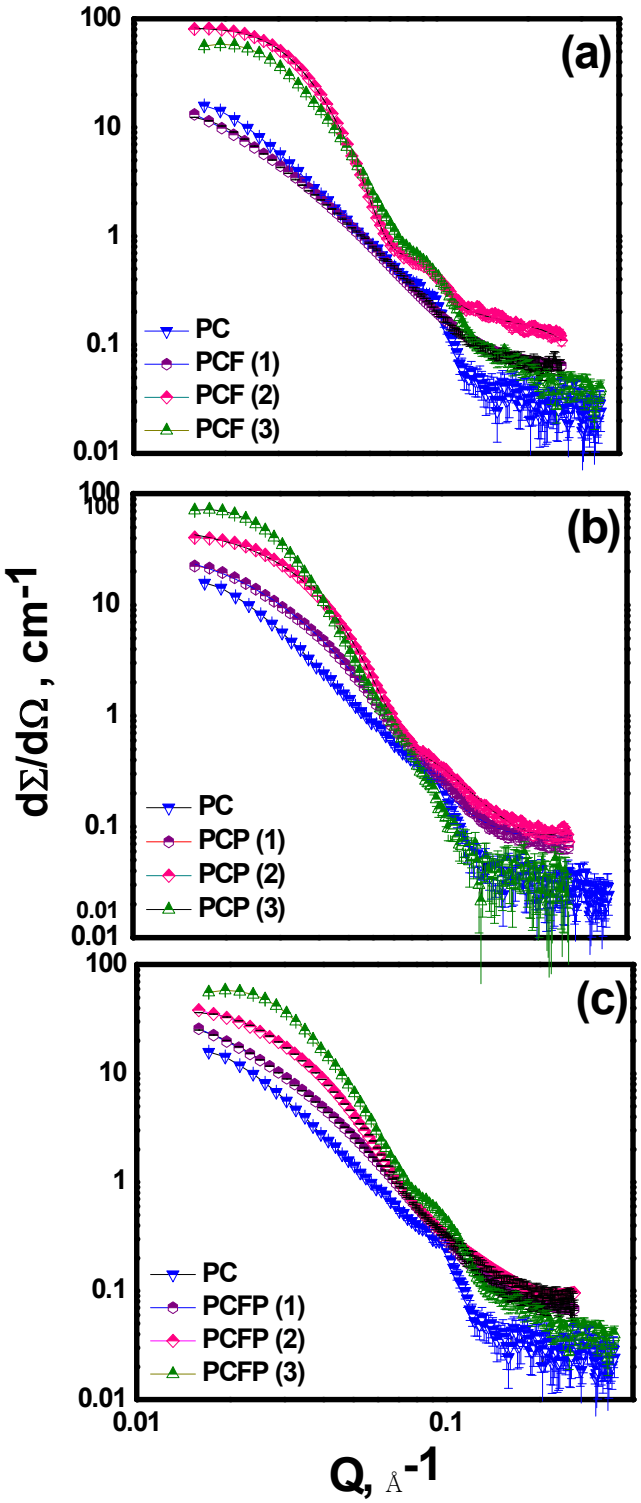


Fig.7.2. Experimental scattering curves for (a) 1 wt% PC and PCFs,(b) 1 wt% PC and PCPs, and (c) 1 wt% PC and PCFPs solutions in D_2O at 30 °C.

In Fig. 7.2, we observed the increase in the scattering intensity upon addition of Pluronic F127, Pluronic P123 and mixed Pluronic F127/P123 in the 1 wt% PC solutions. It showed the increase in anisotropy of the aggregates present in the system. The PC shows stabilized openings in the bilayer structure (Fig.2c). It is noteworthy that the high concentration of the Pluronics micelles in the PC vesicles samples showing the vesicle-to-spherical transition. Results clearly indicate the solubilizing ability of mixed Pluronic F127/P123 spherical micelles is very high as it involves the PC [39].

Fig.7.3 shows representative TEM micrographs of the 1 wt% PC and mixed varied PCFP solutions. Here, the PC blank, PC to Pluronics different ratio was explored. Before introducing Pluronic, the vesicles looked perfectly large spherical. On the other hand, after the addition of Pluronic incorporation, large sized spherical vesicles showed predominant small-sized mixed spherical micelles.

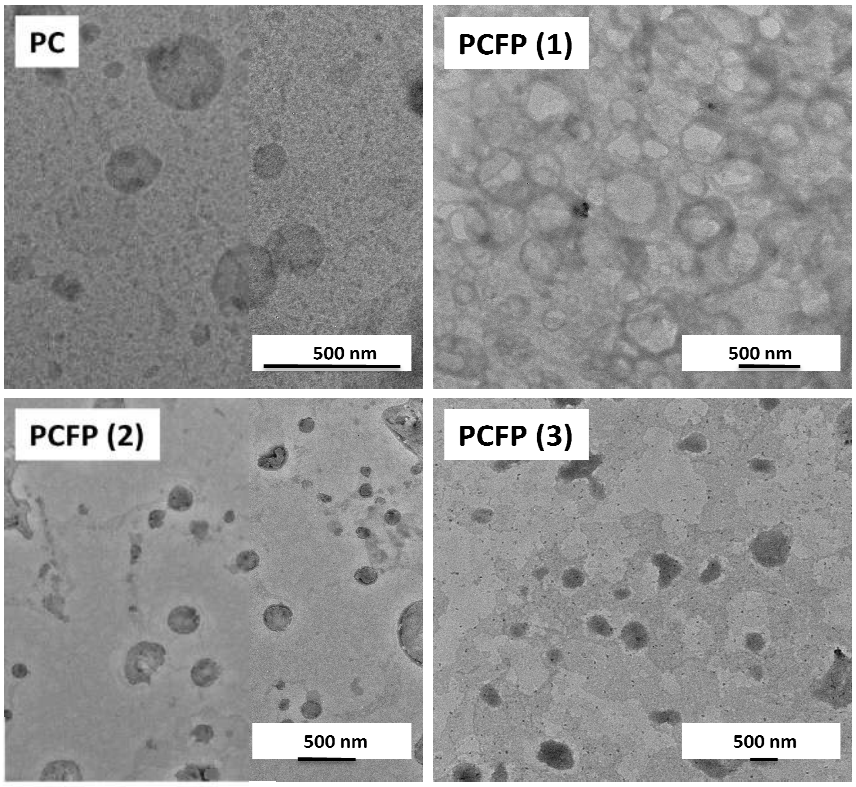


Fig.7.3: TEM images of (a) PC, (b) PCPF (1), (c) PCPF (2), and (d) PCPF (3)

The interaction between PC and Pluronics normally depends on the HLB, which in turn 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 of the order and relaxation parameters of the bilayer. It decreases the bending rigidity and inducing a transition from unilamellar vesicle to the quite small mixed vesicle phase. At high Pluronics concentration might be due to the presence of mixed micelles, the spherical micelles were shown rather than vesicles morphology [40].

The change in morphologies of surfactants has been well discussed with the concept of critical packing parameter (CPP). When surfactants aggregate with each other, they tend to form monolayers that have curvature allowing the most efficient packing of the molecules [41]. According to CPP, spherical micelles are formed when CPP < 1/3, wormlike micelles at 1/3< CPP< 1/2, vesicles when 1/2 < CPP <1, and lamellar or bilayer structure when CPP is 1. Basalious et al [14] have been also reported that mixed micelles of PluronicF127/P123 show morphologies micelles in dilute aqueous solution and consequently have the CPP near 1/3. While PC has two alkyl chains and showed higher CPP (close to unity) and tends to form vesicle structures.

Here PCFP(1) to PCF(3), the ratio of mixed PluronicF127/P123 micelles increases with a fixed amount of PC which leads to transforming the CPP from 1 to nearly 1/3. such spherical morphologies of the PCFP micelles as confirmed by TEM.

7.3.2: Solubilization of curcumin in mixed PC-Pluronic solutions

In order to deliver curcumin to targeted organs of the body, its hydrophobic nature requires to be modified. The mixed micelles of Pluronics® as drug carriers have been already reported for improving the therapeutic efficacy of drugs [28,42,43]. The mixed micelles of Pluronics in water are modified significantly in the presence of additives that have a strong impact on their solubilization characteristics. Increasing the hydrophobic character of these Pluronics, for example through the incorporation of lecithin (PC) in the spherical structure of mixed micelle, favors micelle formation by reducing more the cmc, cmt, and the transition to different morphology in their aqueous micellar solutions.

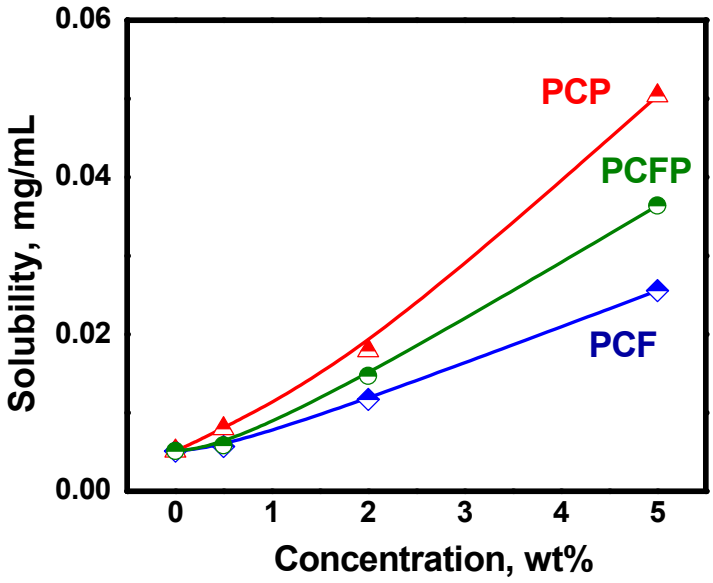


Fig.7.4: The solubility of curcumin in various concentration the PCF, PCP, and PCFP solutions at 30 °C.

Fig.7.4 shows the solubility of curcumin drug in micellar solutions of PCF, PCP, and PCPF at 30°C. Here, the concentration of Pluronic and mixed Pluronic F127/P123 increases with a fixed concentration of PC for solubilization of curcumin. The plot is shown in Fig.7.4. clearly, indicate that the solubility of curcumin increases with the concentrations of Pluronics. The order of increasing the solubility is as PCP>PCPF>PCF. In the PCP systems, due to hydrophobic-hydrophobic interaction created by Pluronic P123 with a high length of PPO block dissolves more curcumin. On the contrary, high hydrophilic nature of Pluronic F127 showed the less solubility of the drug. The mixed Pluronic F127/P123 micelles show moderate results in the solubility of curcumin. All drug formulations of PCF and PCP showed precipitation of curcumin crystals after storage for 15 days at room temperature (data not shown). Pluronic micelles were reported to have certain disadvantages like the large particle size, the low stability, and the possible reversion to the phase separation. It was reported that the hydrophilic long PEO shell of the micelles formed by Pluronic F127 and Pluronic P123 had a protective effect on the micelle dispersion. Therefore, we focused on mixed Pluronic F127/P123 micelles through the incorporation of PC for bioavailability of curcumin. Thus, incorporation of PC would increase the thermodynamic stability of the micelles due to the

tight hydrophobic interactions with hydrophobic PPO blocks of the Pluronic polymers [14]. However, there were no precipitation observed in mixed PFPC(2) micelles (the composition of PC:F127: P123 is 1wt%:1wt%:1wt% in the dispersion) for a few weeks. Hence, we have performed other bio investigation of this PFPC(2) formulations for better understanding.

7.3.3: In-vitro release study of curcumin from mixed PC-Pluronic solutions

In order to explore the pharma uses of PFPC(2) formulations as a drug delivery carriers, we have investigated the release profile of curcumin from PFPC(2) micelles under reservoir-sink conditions at 37°C.

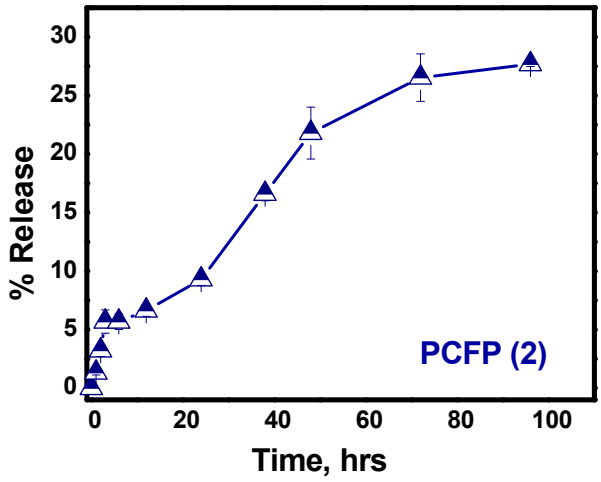


Fig.7.5: In-vitro of selected PCFP (2) PC- mixed micelles at 37°C.

The release of curcumin molecules from mixed PC-Pluronic micelles followed a time-dependent release profile (Fig.7.5). About 28% of the curcumin was released from mixed micelles at pH 7.4 after 100 hrs. Results indicated the slow release of curcumin from mixed PC-Pluronic micelles. The slow release of curcumin from various drug delivery systems is well observed in the literature [44]. The results show that as the shorter inter-micellar distance leads to a larger number of cross-links between neighboring micelles, leading to the higher viscosity and a lower rate of drug release [45]

7.3.4: Stability study

The preliminary storage stability of PCFP(2) was studied for a week at room temperature. Fig.7.6. shows the stability results in the form of drug intake in the PCFP (2) micelles at a time interval. No significant loss in drug retention within the time period was observed. It shows that the PCFP(2) micelles are stable up to the week. These results also proved the compatibility of the curcumin with this mixed PCFP micellar solutions. This mixed system also shows good solubility which observed by Basalious et al [14].

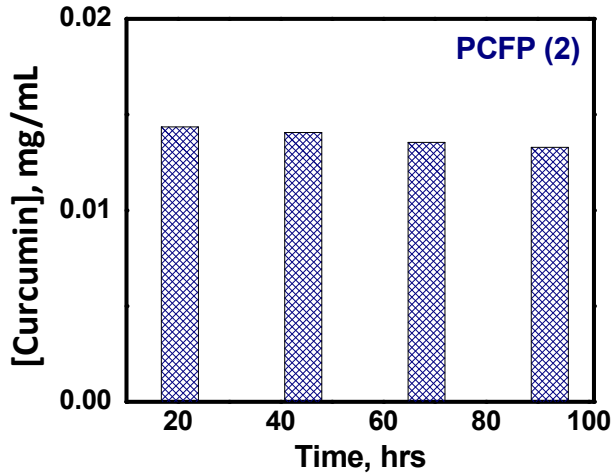


Fig.7.6: The solubility of curcumin versus time plot of mixed PCFP (2) micelles

7.3.5: Antioxidant activity analysis

The antioxidant activities of curcumin drug and curcumin-loaded mixed PC- Pluronic solutions(here representative PFPC(2)) were estimated by measuring their ability to scavenge the DPPH radicals and results are presented in Fig.7.7. The pure curcumin showed 30% inhibition upto 3.0 µg/mL, while mixed PFPC micelles inhibited almost 85% at the same concentration. It was clearly observed that mixed PFPC micelles showed higher antioxidant activity than the pure curcumin drug.

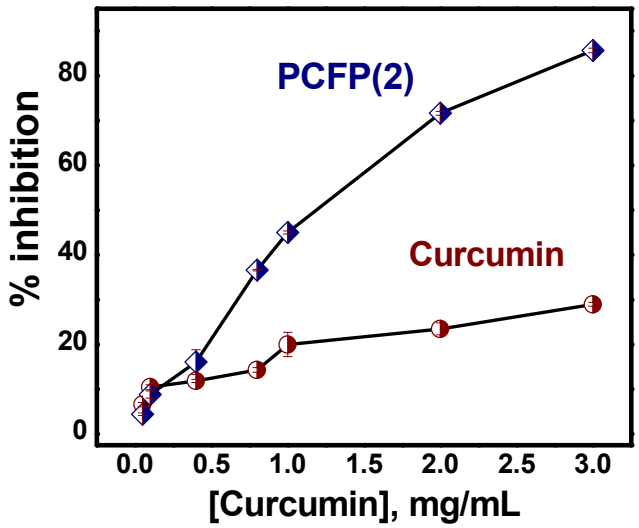


Fig.7.7: Percentage inhibition of DPPH at various concentrations of PCPF (2) micelles and Curcumin.

7.4: References

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