2.1 Introduction:

Commercially available poly (ethylene oxide)-poly (propylene oxide) -poly (ethylene oxide) (PEO-PPO-PEO) triblock copolymers (Pluronic®) self-assemble in aqueous media; the nanosize core- shell micelles are made of hydrophobic PPO core surrounded by heavily hydrated PEO chains. The micellar and phase behavior of Pluronic® type copolymeric surfactants has been extensively reviewed [1, 2]. These copolymers have attracted much attention in recent years for their use as vehicles in drug delivery systems [3, 4].

Polymeric micelles can act as nanocontainer to solubilize and release hydrophobic drugs. These nanosized structures exist with variable well-defined geometry where insoluble/sparingly soluble bioactive molecules can be incorporated (solubilized) at different sites in micelles. Solubilization has many potential applications of amphiphilic block copolymers such as in synthesis of nanoparticles, fabrication of mesoporous materials, in chemical extractions for environmental remediation, for the separation of biotechnological products etc [5-7]. Solubilization and interaction of some hydrophobic drugs viz. propofol [8], pilocarpine [9], ibuprofen [10], paeonol [11], hydrochlorothiazide [12], nimesulide [13], octaethylporphine, meso-tetrapheny l porphine and camptothecin [14] in EO-PO copolymer micelles has been examined by few researchers. The solubility of drug in micelle was found dependant on copolymer composition. For potentially useful solubilized drug formulation, it is necessary to adjust the copolymer structure and properties such as hydrophilic-lipophilic balance, efficiency and site of solubilization in micelles thus providing attractive nanotechnologies to enhance the water solubility and stability of poorly water soluble and unstable drugs.

Antioxidants protect cells against the oxidative damage of phospholipids bi-layer caused by free radicals; latter are produced as chain reaction when food is metabolized, or by environmental exposures. Antioxidants molecules terminate these chain reactions by removing free radical intermediates, inhibit other oxidation reactions and are widely used in food, cosmetics, fuel and rubber industries to prevent oxidative degradation. They are also used in many industrial processes, such as plastic and oil processing where, high temperature and pressure cause oxidative damage to products. The ability of phenolic compounds to quench free radicals arises because of their acidity (ability to donate
protons) and their delocalized π-electrons of benzene rings (ability to transfer electrons while remaining relatively stable). These are particularly interesting because as recently as a few years ago, they were believed only to be important for flavor [15].

The interactions of antioxidants with emulsifier have a strong influence on the partitioning of antioxidants [16]. Richards et al [17] observed that oxidative stability of salmon oil-in-water emulsions remained unaffected on solubilization of phenolic antioxidants in nonionic Brij micelles. Vora and Boroujerdi [18] examined enhanced solubility of phenolic antioxidants in cyclodextrins due to the formation of inclusion complexes. Heins et al [19, 20] have studied solubilization of antioxidant with surfactant micelles. Using ESR and NMR, these authors concluded on the location of antioxidants in micelle dependant on their hydrophobicity. The locations of antioxidants are essential to control the antioxidant activity at interfaces present in a wide range of foods, cosmetics, pharmaceuticals (emulsions and carrier systems) and of biological membranes. The location of quercetin in the Triton X-100 investigated by Liu and Guo [21, 22] showed quercetin - micelle binding was spontaneous; the antioxidant when solubilized in spherical micelles of Triton X-100 existed as dimer and as monomer in rod-like micelles. These authors [23] also showed that interaction of quercetin with ionic surfactants undergoes micellar transition. Singh and Marangoni [24] used calorimetric analysis to evaluate the effect of quercetin on the binding with sodium salt of polystyrene sulfonate/ sodium dodecyl sulfate complex. The solubilization of antioxidants in microheterogeneous media results in different interactions compared to homogeneous environment. The activity of antioxidants can vary strongly depending on the micelle size, shape and their location in micelles [25, 26]. Thus, the location and properties of antioxidant in micellar environment can be of crucial importance for its activity. However, no report is found for solubilisation of antioxidant in Pluronics® micelles.

In the present study, the solubilization of four antioxidants was investigated in micellar solutions of Pluronic® P104. The effects of temperature and salt on the solubilization of antioxidant were also investigated. The antioxidants used were p-hydroxy benzoic acid, syringic acid, sinapic acid and quercetin. The solubilization and interaction of these structurally similar phenolic compounds in Pluronic® P104 micelles was examined by UV-Vis spectrophotometry. Changes in micelle size with solubilized
antioxidants and their location in micelles were studied by dynamic light scattering and 2D NMR, respectively.

2.2 Experimental:

2.2.1 Materials:

Pluronic P104 (scheme I) was received as gift from BASF Corp. Parsippany, NJ, USA and used without further purification. Its characteristics are as follows.

![Pluronic P104 Structure](image)

<table>
<thead>
<tr>
<th>Pluronic®</th>
<th>mol wt.</th>
<th>% EO</th>
<th>CMC (%) at 30 °C</th>
<th>CMT(°C) for 1%</th>
<th>CP (°C) of 1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>P104</td>
<td>3540</td>
<td>40</td>
<td>0.04</td>
<td>23.5</td>
<td>81</td>
</tr>
</tbody>
</table>

**Scheme I. Properties of the Pluronic® P104 used in this study.**

The antioxidants used (Scheme II) were from Sigma-Aldrich. All these were used without any further purification. Solutions for solubilisation and pH adjustment were prepared in triply deionised water.
2.2.2 Methods:

2.2.2.1 Cloud Point (CP):

Cloud points of 1% P104 in water and in NaCl solutions with and without antioxidant were determined by gently heating solution in thin 20 ml glass tube immersed in a beaker containing water well stirred with a magnetic bar while being heated. Temperature of the first appearance of turbidity was taken as the cloud point. The obtained results were reproducible up to ±0.5°C.

2.2.2.2 Viscosity:

The viscosities of solutions were measured by Ubbelohde suspended level capillary viscometer kept in a thermostat at 30°C with temperature stability ± 0.1°C. The flow time of the solvent system and the solutions were measured with a calibrated stopwatch.

Scheme II. Structures of antioxidants
2.2.2.3 2D NMR:

The 2D NMR experiments (\(^1\)H, gradient NOESY with and without solvent suppression) were performed on a Bruker AVANCE-II 400 MHz spectrometer at StFX University. The mixing times and the delay times for the NOESY experiments were estimated from the spin-lattice relaxation times (T\(_1\) values) of the surfactant determined in separate experiments. In all cases, an acquisition delay of » 3 x T\(_1\) and a mixing time of » 1 x T\(_1\) were used to obtain the NOESY spectra. For all acquisitions, 256 transients of either 2 or 4 scans over 512 complex data points were acquired. All experiments were done in phase sensitive mode, with and without the saturation of the water resonance at ~ 4.70 ppm. The data were zero-filled twice in dimension 1 and multiplied by a squared sine function in both dimensions before 2DFT.

2.2.2.4. Dynamic light scattering (DLS)

The dynamic light scattering (DLS) experiments were carried out at a fixed scattering angle of 90° on solutions using Zeta sizer Nano-ZS 4800 (Malvern Instruments, UK) equipped He-Ne laser operating at a wavelength of 633 nm. The average diffusion coefficients and hence the hydrodynamic size was obtained by the method of cumulants. Each measurement was repeated at least three-four times.

2.2.2.5. Solubilization studies

The antioxidant-containing samples were prepared by dissolving appropriate amount of P104 in aqueous solution with gentle agitation. Saturated antioxidant-loaded solutions were prepared by mixing excess powdered antioxidant with 2% P104 solution and stirring at constant temperature (30, 35, 40°C) at 200 rpm for 48 hours. The solutions were filtered (Millipore, 0.45 μm) to remove unsolubilized drug. Aqueous solubilities were determined for each antioxidant using the standard analytical shake-flask method, with constant-temperature (30°C) jacketed glass cells. In a solubilization experiment, the filtered solution was diluted 10 to 100 times with methanol, the amount of water after dilution being low enough to allow direct use of the calibration plot. Solubilization studies were carried out on Shimadzu (UV-2450) UV-vis double beam spectrophotometers with matched pair of stoppered fused silica cells of 1 cm optical path length. The wavelengths used for analysis were 254, 270, 322 and 371 nm for p-hydroxy
benzoic acid, syringic acid, sinapic acid and quercetin respectively. Calibration with dilute solutions of the drugs ranging from 0.01 - 0.1 mM dissolved in methanol gave satisfactory Beer-Lambert plot with $R^2=0.9991$.

2.3 Results and Discussion:

2.3.1 Solubilization of antioxidants in micelles:

The solubilization of four antioxidants viz p-hydroxy benzoic acid, syringic acid, sinapic acid and quercetin was examined spectrophotometrically. The aqueous solubility was observed to be 0.6, 0.26, 0.02 and 0.00026% around $30^\circ C$ for p-hydroxy benzoic acid, syringic acid, sinapic acid and quercetin, respectively.

A linear increase in solubility of the antioxidant with increase in P104 concentration (Fig. 2.1) can simply be attributed to interaction between antioxidant and copolymer molecules and increased number of micelles at higher concentration [27-29].

Figure 2.1 Solubilization of antioxidants in P104 (○) p-hydroxy benzoic acid, (●) syringic acid, (▲) sinapic acid, (■) quercetin at $37^\circ C$.

PHBA, being most polar antioxidant among all these, is likely to be solubilized in palisade layer of P104 micelle and reduces the hydrogen bonding of PEO unit to water molecules, similar to “salting out” effect of salt [30]. It can be assumed that the central core of triblock copolymer micelles contains substantial quantity of water. It was
anticipated that solubilization initially takes place through a replacement process in which water is displaced from the micellar core. This process is driven by hydrophobic interactions of the solubilize in the aqueous phase. For more hydrophobic antioxidant like quercetin, the free energy of the transfer process increases since it becomes more difficult to displace water from the core and thus leads to decrease in solubility [31].

Fig. 2.2 (as a representative) shows the solubility of sinapic acid at three different temperatures (30, 35, 40 °C). The apparent solubility of antioxidant in water is slightly increased with an increase in temperature, but dramatically increases in aqueous copolymer solutions. In general, with increase in temperature, dehydration of PEO shell occurs and PPO becomes more hydrophobic [32], and micelle may even grow at higher temperature in the presence of antioxidant.

![Figure 2.2](image)

**Figure 2.2 Effect of temperature on solubilization of sinapic acid.**

As it is well known that addition of salt can have pronounced effect on micellization of pluronics, the solubilization of antioxidant in 2% P104 was therefore studied in presence of varying concentration of NaCl at 30°C. Fig 2.3 reveals that for the copolymer, the amount of the solubilized antioxidant is increased linearly as the ionic strength of the solution is increased and effect is more pronounced for syringic acid.
As a general trend, it appears that the presence of added inorganic salts depresses the cloud point by “salting-out” effect (this point will be highlighted in next section). The ions from the salt get hydrated in an aqueous solution and act like a solvent pump to dehydrate the PEO chains [33, 34]. The addition of salt increases micelle hydrodynamic size ($D_h$) which is responsible for the enhanced solubilization of antioxidant.

### 2.3.2 Cloud Point:

The cloud points of 2% P104 solutions with varying antioxidant concentration are shown in Fig. 2.4. A progressive decrease in cloud point was observed with progressive addition of antioxidants.
Antioxidants, other than PHBA, get incorporated into the PPO region of micelles causing swelling and retardation in interfacial curvature and are therefore likely to decrease cloud point. Whereas in case of PHBA, a progressive increase in PHBA concentration (> 0.65%) bring the CP below room temperature and shows phase separation. This property is of interest in terms of separation of organic molecules by cloud point extraction. This offers the possibilities of Pluronics being used to separate PHBA and its derivatives from effluents through cloud point extraction.

2.3.3 Size of P104 micelles in water and salt solution: Empty and antioxidant loaded:

The DLS measurements on 2% solutions of P104 in water at 23, 30 and 35°C show that copolymer exists as micelles; at 23°C unimer peak (~5 nm) is also seen. The size distribution plots (Fig. 2.5a) indicate that at higher temperature unimer peak disappears due to predominance of micelles. The size initially decreases due to dehydration of PEO shell but at high temperature, an increase is noticed due to micelle growth at temperature close to CP. The copolymer micelles were with low polydispersity.

Figure 2.4 Clod point for 2% P104 as a function of antioxidant concentration (□) p-hydroxy benzoic acid, (■) syringic acid, (●) sinapic acid (▼) quercetin.
with an apparent hydrodynamic diameter about 20 nm at 30°C. Such a behavior for other Pluronic has been observed before [35].

Figure 2.5 Intensity vs. size plot for 2% P104 at different temperatures (a) and in different salt concentration at 23°C (b).

Fig 2.5b shows distribution plots for P104 at different NaCl concentration. The presence of salt diminishes the unimer peak and a progressive addition of salt first decreases the size and later increases. An initial slight decrease with increase in salt concentration can be attributed to tightening of PEO shell and later the size considerably increases due to the quenching of water molecule from the core and micellar growth/transition [36, 37]. The trend in hydrodynamic diameter ($D_h$) is plotted as a function of salt concentration in Fig. 2.6.
The change in size of micelles with solubilized antioxidants was also monitored and results are listed in Table 2.1. It is evident from Table 2.1 that the micelle diameter is increased with addition of antioxidant and polydispersity is greatly decreased. These results reveal that additions of antioxidants decrease the solvation of PPO block of the copolymer and consequently increase in hydrodynamic diameter and decrease in polydispersity [38].

Table 2.1 Micelle size after antioxidant solubilisation in water/ salt solution at 23°C.

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>H₂O</th>
<th>1M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dₜ, (nm)</td>
<td>PDI</td>
</tr>
<tr>
<td>Nil</td>
<td>5, 16</td>
<td>0.37</td>
</tr>
<tr>
<td>p-Hydroxy benzoic acid</td>
<td>16.8</td>
<td>0.134</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>18.5</td>
<td>0.208</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>17.8</td>
<td>0.203</td>
</tr>
<tr>
<td>Quercetin</td>
<td>19.0</td>
<td>0.192</td>
</tr>
</tbody>
</table>

Figure 2.6 Hydrodynamic diameter (Dₜ) of 2%P104 as a function of salt concentration.
2.3.4 Viscosity behaviour:

Performed carefully, viscosity measurement can be a very reliable method to study morphological changes of self-assembled polymers in solution. As one of the antioxidants PHBA shows greater solubility in P104, we studied the viscosity behavior of copolymer solution with PHBA concentration. Fig. 2.7 reveals the viscosity determined for P104-PHBA solutions. The PHBA concentration ranged from 0.1% to 0.65% in 2% P104 micellar solution. The apparent viscosity of micellar solutions was constant up to a PHBA concentration of 0.4% and dramatically increased above that. Initially, no change in viscosity up to 0.4% concentration of PHBA shows solubility in water and after that it starts to solublize in micelle leads to increase in viscosity which pointed towards the possibility of sphere to rod transition [38]. PHBA seems to be solubilized in the palisade layer of the P104 micelle due to the -OH and -COOH polar group which reduce the hydrogen bonding of PEO unit to water and consequently viscosity increases [39, 30]. This shows that polar groups (-OH, -COOH) are responsible for a stronger solubilization due to their ability to enter into interactions like H-bonds in an environment of highly ordered water structure [40]. At higher concentration of PHBA, PEO chains become less soluble in water which flattens the micelle interface curvature (by shrinking in their volume) between the core and the shell regions, resulting in the observed morphological changes [41]. Mata et al. [42] have shown similar increase in viscosity of P65 solutions in presence of phenol and accounted it for micellar growth.
2.3.5 Thermodynamics of solubilization:

The drug-micelle interaction can be better explained by determining its magnitude through the elucidation of micelle-water partition coefficient (P) and thermodynamic parameter (ΔG_s^o) of solubilization.

\[ P = \frac{C_m}{C_w} \]

where \( C_m \) and \( C_w \) are the concentrations of drugs in the micelles (based upon total polymer mass) and in the water, respectively.

From the thermodynamic point of view, the solubilization can be considered as a normal partitioning of the drug between micelle and aqueous phases and the ΔG_s^o can be represented by the following expression:

\[ ΔG_s^o = -RT\ln P \]

ΔG_s^o in water for sinapic acid was calculated using above equation. Figs. 2.8 and 2.9 also show the effect of temperature on the P and ΔG_s^o for sinapic acid solubilized in varying pluronic® concentration.

Figure 2.7 Viscosity of 2% P104 solutions with solubilized p-hydroxy benzoic acid.
Figure 2.8 Effect of copolymer concentration on partition coefficient for solublized sinapic acid at different temperature.

Figure 2.9 The Gibbs energy change for sinapic acid solubilized in P104.

The negative value of $\Delta G_s^0$ indicates that the migration of the antioxidant molecules in the monomer state to the air-water interface is a spontaneous process favored by their hydrophobicity. However, more negative $\Delta G_s^0$ for the copolymer with
increasing concentration (1 to 5%) indicates more favored solubilization. A significant increase could be seen for partition coefficient with increase in temperature (Fig. 2.8) and corresponding decrease for $\Delta G_s^\circ$ can be seen in Fig. 2.9. The values of free energy change are also in agreement with the results obtained.

2.3.6 Location of antioxidants in P104 micelle:

Two dimensional-nuclear overhauser enhancement spectroscopy (2D-NOESY) is an established non-invasive method to gain significant information on the intra- and intermolecular proximity of proton in a number of systems [43, 44]. In case of micellar solubilization, the existence of the cross-peaks provides vital information regarding the locus of solubilization in micelles. Hence, we used NOESY to obtain the location and the resulting chemical microenvironment of the antioxidants in micellar solutions of P104. The location of an antioxidant in the colloidal environment can be of crucial importance for its activity, as there is a strong correlation between antioxidant activity and its surrounding environment.
Figure 2.10 NOESY spectra for antioxidants in P104.
The NOESY contour plots for the all systems are presented in Figure 2.10. For PHBA-P104 system (Fig. 2.10a), cross-peaks are observed between the benzene protons (δ≈7.3ppm) of the PHBA and both the PPO (δ≈1ppm) and PEO (δ≈3.5ppm) protons of P104. The NOESY evidence presented here indicates that PHBA is distributed uniformly in the micelle and is interacting with both PPO and PEO blocks; this information can be easily translated to the existence of PHBA in palisade layer of the P104 micelles where it is not in close proximity to the core of the micelle. This is in complete agreement with viscosity behavior where relative viscosity increases with PHBA concentration. This in turn would mean a greater ability of PHBA to diffuse freely in micelles.

The NOESY spectra for syringic acid and sinapic acid are given in Fig. 2.10b and c. From the spectrum, I observed strong correlations between the A-B proton of the syringic acid (δ≈3.8 ppm) and the PPO-methyl protons of P104 (δ≈1ppm). Syringic acid is located in the micelle with its -OH and -OCH₃ groups in intimate contact with hydrophobic PPO chain of P104. For sinapic acid-Pluronic® systems significant cross peaks are observed in the NOESY spectra, a clear indication of the proximity of certain protons of the antioxidant and the Pluronic® surfactant. A strong correlation between the PPO-methyl protons of P104 (δ≈1ppm) and the C-D-E protons of sinapic acid (δ≈6.7, δ≈6.44, δ≈7.54 respectively) can be interpreted in terms of close proximity of these protons. As well, a strong correlation between the PEO-methylene proton of the P104 (δ≈3.5 ppm) and the B protons of sinapic acid (δ≈3.8 ppm) is observed. These results indicate that the sinapic acid is orienting itself in the micellar interior with the OH groups interacting with the hydrophilic PEO chain and more hydrophobic parts of the antioxidant reside in the micelle palisade layer, while COOH group of the antioxidant appears to orient towards the core of the micelle.

In the case of the quercetin-P104 system (Fig. 2.10d), the NOESY spectra indicates that the quercetin is solubilized in intimate contact with the core of micelle. For sinapic acid(Fig. 2.10c), the NOESY spectra indicate that it resides in the micelle in such a way that one of the part containing the ethylene carbon and -COOH group are directed towards the core-shell region due to their hydrophobic nature and remaining part interacts with the PEO chain. In contrast, the -COOH group of syringic acid (Fig. 2.10b) interacts with the PEO chain and the -OH group and methoxy group interact with the PPO chain.
The microenvironment of solubilized antioxidants into the P104 micelles is reliably determined from NOESY and results are summarized in Fig. 2.11.

**Figure 2.11** *Schematic representation for location of different antioxidants in Pluronic® micelle.*

**2.4 Conclusions:**

The solubilization of four antioxidants in P104 aggregates at different temperatures, copolymer concentrations, with or without added salt has been investigated by UV-Vis spectrophotometry. In each case significantly enhanced solubility of antioxidant with increasing copolymer/salt concentration or temperature was observed.

The NOESY spectra for antioxidants-P104 system indicate that the locus of solubilization of these typical solubilizates can be reliably determined. In the case of the
quercetin-P104 system, the NOESY spectrum indicates that quercetin is solubilized with intimate contact with the core of micelle. Sinapic acid, resides in micelle in such a way that one of the part containing ethylene carbon and -COOH group are directed towards core-shell region due to hydrophobic nature and remaining part interacts with PEO chains. In contrast, -COOH group of syringic acid interacts with PEO chains and the hydroxyl and methoxy groups interact with PPO chains. For PHBA, the NOESY spectrum indicates a rather even distribution in the micellar palisade region. However, strong effects of micelles transition were found for PHBA-P104 systems. This was attributed to the solubilization of PHBA in the palisade layer of P104 micelle. Consequently, the ratio of PEO chains to micellar surface area increased after the micellar transition, which means a tighter micellar packing. These effects were accompanied by an increase in viscosity and decrease in CP as a function of PHBA concentration. Finally, significant quantitative information on the solubilization sites of antioxidants that is crucial to control the antioxidant activity present in pharmaceuticals and of biological membranes is derived from 2D NOESY experiments. These experiments give a detailed picture for the location of the antioxidant in the P104 micelles, which in turn gives an indication of where their solubilization site could be in a membrane type system.

References
Chapter 2  Interaction and solubilization...


