CHAPTER 7

FABRICATION OF CHITOSAN – FMPC SCHIFF BASE NANOPARTICLES AND EVALUATION OF THEIR ANTIOXIDANT AND ANTICANCER PROPERTIES

7.1 INTRODUCTION

Researchers are in search to form chitosan micro and nano systems for wide range of biomedical application (Bodnar et al. 2005). However, majority of the attempts were made to prepare chitosan nanoparticles (Liu et al. 2008). Nano sizing of chitosan and its derivatives were found to have high saturation solubility and dissolution rate due to large surface area and small diffusion layer thickness compared to larger particles (Kesisoglu et al. 2007). Hence, polysaccharide nanoparticles with ultra-tiny volume can penetrate through the cells and tissue gap easily and enhance the availability of the drug at the target site (Jung et al. 2000).

Chitosan derivatives will form nanoparticles by ionic gelation method. Recently, N-(2-hydroxyl) propyl-3-trimethylammonium chloride (Xu et al. 2003), N-trimethyl chitosan (Amidi et al. 2006) and glucomannan-chitosan (Alonso-Sande et al. 2006) nanoparticles were fabricated for the delivery of proteins by ionic gelation method. Among wide variety of crosslinked chitosan nanoparticles, vanillin crosslinked chitosan nanoparticles have received considerable attention because vanillin is regarded as a safe material (Lirdprapamongkol et al. 2005) for its potential antioxidant (Kamat et al. 2000), antibacterial (Sangsuwan et al. 2008) and anti-inflammatory activity (Murakami et al. 2007).
Owing to its vast therapeutic applications, there has been a great need to overcome the short shelf-life of vanillin (Kayaci & Uyar 2012). This can be achieved by crosslinking vanillin with chitosan to form chitosan particles with prolonged functionality (Uhrich et al. 1999). It has recently been reported, that vanillin cross linked chitosan microspheres was explored as bioactive microcarrier of drugs (Zou et al. 2015) such as the control release of resveratrol (Peng et al. 2010). In addition, chitosan vanillin nanoparticles were fabricated, that can act as a promising vehicle for the delivery of 5-flurouracil against HT-29 cells (Li et al. 2016). Kwon et al., developed a novel polymeric prodrug of vanillin termed poly (vanilin oxalate) nanoparticles that have great potential as novel antioxidant therapeutics and drug delivery systems (Kwon et al. 2013). The objective of the present study is to develop a novel, facile route to fabricate C-FMPC-Nps, which would act as a potential biomaterial in therapeutics. The incorporated FMPC will undergo hydrolysis at the site of carbonate linkages producing hydroxyl groups (Groot & Koert 2007). This would act as a radical scavenger at the targeted site making the C-FMPC-Nps a better antioxidant and anticancer agent.

7.2 PREPARATION OF SCHIFF BASE C-FMPC-NPS

7.2.1 Preparation of Chitosan Nanoparticles

Initially, with 1g of chitosan prepared in 100 mL of 1% aqueous acetic acid, 0.1g of TPP in 100 ml of water was gently dripped and kept at room temperature for 10 min to get an opalescent suspension. The obtained suspension was subjected to centrifugation at 10,000 rpm for 30 min at 4°C. The formed nanoparticles were again re-suspended in distilled water, sonicated, centrifuged and freeze dried.

7.2.2 Fabrication of C-FMPC-Nps

100 mg of chitosan nanoparticles dispersed in methanol was taken in a round bottom flask. Excess amount of FMPC was added to the same and refluxed for 48 h at 60°C with continuous stirring. FMPC crosslinked chitosan
nanoparticles were collected, after washing the particles with methanol several times by centrifugation at 10,000 rpm for 20 minutes. The C-FMPC-Nps were finally dried in a vacuum oven at 40 °C for 12 h.

7.3 CHEMISTRY OF C-FMPC-NPS FORMATION

In Scheme 7.1, the condensation reaction of aldehyde functionality of vanillin with the free amine groups on chitosan resulted in the formation of Schiff base chitosan, that exhibits the presence of azomethine (-CH=N-) linkage in C-FMPC (Jin et al. 2009).

Scheme 7.1 Schematic structure of newly synthesized Schiff base C-FMPC-Nps
7.3.1 Elemental Analysis of C-FMPC-Nps

The successful formation of Schiff base was confirmed by elemental analysis (Table 7.1). From the results it could be inferred that the C-FMPC-Nps showed an increase in nitrogen percentage but a decrease in carbon and hydrogen percentage compared to chitosan (Kocak et al. 2012).

This behaviour would lead to a conclusion, that FMPC has been successfully incorporated onto chitosan backbone. In addition, the decrease in the C/N ratio (Laus & Favere 2011) is an indicative of the successful crosslinking of chitosan with the pendent FMPC to form Schiff base as specified in Scheme 7.1.

Table 7. 1 Elemental analysis of C-FMPC-Nps

<table>
<thead>
<tr>
<th>Sample</th>
<th>Element %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Chitosan</td>
<td>40.43</td>
</tr>
<tr>
<td>C-FMPC-Nps</td>
<td>33.63</td>
</tr>
</tbody>
</table>

7.4 CHARACTERIZATION STUDIES OF C-FMPC-NPS

7.4.1 FTIR Spectrum of C-FMPC-Nps

In the spectrum of C-FMPC-Nps (Figure 7.1(b)), the peak at 1668 cm\(^{-1}\) corresponds to free aldehyde group of vanillin (Figure 7.1(a)) was found to be absent and a new peak appeared at 1646 cm\(^{-1}\) indicates the formation of expected Schiff base (Peng et al. 2010).
Figure 7.1 FTIR Spectra of (a) chitosan and (b) C-FMPC-Nps

Table 7.2 FTIR absorptions of C-FMPC-Nps

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>Characteristic Absorption(s)(cm(^{-1}))</th>
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<tbody>
<tr>
<td>-CH=N- stretching</td>
<td>1646</td>
</tr>
<tr>
<td>C=C aromatic stretching</td>
<td>1600</td>
</tr>
<tr>
<td>-CH(_3)-sym angular deformation</td>
<td>1374</td>
</tr>
<tr>
<td>ester C=O asymmetric stretching</td>
<td>1254</td>
</tr>
<tr>
<td>-C-O-C- anti symmetric stretching</td>
<td>1152</td>
</tr>
<tr>
<td>-C-O-C- symmetric stretching</td>
<td>887</td>
</tr>
</tbody>
</table>
Furthermore, the absence of peak at 1559 cm\(^{-1}\), in C-FMPC-Nps corresponds to the amide II band of chitosan was a clear evidence for the covalent attachment of FMPC with chitosan (Jin et al. 2009). In addition, the identification of peaks at 1600 cm\(^{-1}\) and 1152 cm\(^{-1}\) (Table 7.2) is due to the presence of C=C aromatic stretching vibration of vanillin and C-O-C anti symmetric bridging vibration of chitosan confirms the successful incorporation of FMPC with chitosan (Santos et al. 2005).

7.4.2 (CP/MAS) \(^{13}\)C-NMR of C-FMPC-Nps

In the (CP-MAS) \(^{13}\)C-NMR spectrum of C-FMPC-Nps (Figure 7.2), the peak at 195 ppm (Krishnapriya & Kandaswamy 2010) resulted from aldehyde carbon disappeared and a new signal at 167 ppm observed was attributed to the formation of schiff base. The product was obtained from the condensation reaction between chitosan and FMPC (Amarasekara & Razzaq 2012). The chemical shift values obtained from the \(^{13}\)C-CP MAS of C-FMPC-Nps is represented in Table 7.3 as given below.

**Table 7.3 (CP-MAS) \(^{13}\)C-NMR of C-FMPC-Nps**

<table>
<thead>
<tr>
<th>Type of carbon</th>
<th>Chemical shift ((\delta) ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-(C=O)-O / (C(_1))</td>
<td>171</td>
</tr>
<tr>
<td>-HC=N- / (C(_8))</td>
<td>167</td>
</tr>
<tr>
<td>O-C=C- and CH(_3)O-C-Ar / (C(_a) and C(_b))</td>
<td>148</td>
</tr>
<tr>
<td>–N=CH-C-(Ar) / (Cd)</td>
<td>126</td>
</tr>
<tr>
<td>-C=CH-CH-(Ar) / (C(_6) and C(_7))</td>
<td>116</td>
</tr>
<tr>
<td>CH(_3)O-C=CH-C-(Ar) / (C1 and C(_c))</td>
<td>103</td>
</tr>
<tr>
<td>(C4)</td>
<td>82</td>
</tr>
<tr>
<td>(C5 and C3)</td>
<td>74</td>
</tr>
<tr>
<td>CH(_3)O-(Ar) / (C(_h), C6 and C2)</td>
<td>56</td>
</tr>
<tr>
<td>CH(_3)CO- of chitosan</td>
<td>22</td>
</tr>
</tbody>
</table>
7.4.3 XRD of C-FMPC-Nps

Figure 7.3 indicates the XRD profile of pristine chitosan, vanillin and C-FMPC-Nps respectively. The high degree of crystalline morphology of chitosan (Figure 7.3(a)) is indicated by the presence of sharp diffraction peak at $2\theta = 20.3^\circ$ and a weak diffraction peak at $2\theta = 11.6^\circ$ (Kumar et al. 2009). The presence of a sharp diffraction peak of vanillin at $2\theta = 13.3^\circ$
(Figure 7.3(b)), appeared as semi crystalline peak at 2θ = 13.7° in C-FMPC-Nps (Figure 7.3(c)) (Stroescu et al. 2015). Herein, for C-FMPC-Nps the observed peak at 2θ = 13.7° with weak reflection exhibits the existence of interdigited hydrogen bond between chitosan and vanillin unit present in FMPC (Marin et al. 2013). In addition, C-FMPC-Nps exhibits a broad peak at 2θ= 20.6° with decrease in magnitude that is an indicative of molecular miscibility between the chitosan, TPP and FMPC. Thus, the successful formation of C-FMPC-NPs has been confirmed by the XRD patterns.

Figure 7.3 XRD of (a) chitosan, (b) vanillin and (c) C-FMPC-Nps

7.4.4 TGA of C-FMPC-Nps

The thermal stability of chitosan, vanillin and prepared C-FMPC-Nps studied from TGA results are indicated in Figure 7.4. For chitosan, vanillin and C-FMPC-Nps, the initial weight loss at around 50-150 °C is due
to the moisture vaporization and bound water of hydration. As already stated in the previous literature, chitosan shows a $T_{\text{max}}$ at 298 °C, (Figure 7.4(a)) was an indicative of disruption of strong inter and intra molecular hydrogen bonds, due to the depolymerisation and thermal decomposition of the acetylated and deacetylated units of polymeric chitosan (Singh et al. 2014). For vanillin (Figure 7.4(b)) $T_{\text{max}}$ value has been observed at 230 °C corresponds to the degradation or thermal evaporation of vanillin (Kayaci & Uyar 2011).

It is evidenced from Figure 7.4(c), for C-FMPC-Nps ($T_{\text{max}} = 269$ °C) the thermal stability, was comparatively higher than vanillin. This could be due to the incorporation of stable carbonate backbone present in FMPC moiety. But rather the decrease in the stability of C-FMPC-Nps compared to chitosan was because of the introduction of the FMPC moiety in chitosan. FMPC decreases the number of amine groups on chitosan by obstructing the chain packing resulting in the decreased stability of C-FMPC-Nps (Tirkistani 1998).

Figure 7.4 TGA of (a) chitosan, (b) vanillin and (c) C-FMPC-Nps
7.4.5 HRTEM observation for C-FMPC-Nps

The visualization of the nanoparticles as performed by HRTEM analysis indicates the size and morphology of the formed C-FMPC-Nps (Figure 7.5). TEM image revealed that the size of the nanoparticles were around 25 to 30 nm in diameter with smooth surface and spherical morphology. The electrostatic repulsion, hydrophobic interaction and hydrogen bonding exists between different functionalities present in C-FMPC-Nps would result in the transparent colloidal aggregates of the formed nanoparticles that could be clearly observed from TEM analysis (Tree-Udom et al. 2011).

Figure 7.5 HRTEM image of C-FMPC-Nps

7.5 BIOLOGICAL ASSAY

7.5.1 Biocompatibility Study

Biocompatibility of the C-FMPC-Nps was carried out by MTT assay using VERO cell lines to evaluate the suitability of the fabricated...
nanoparticles for biomedical applications. Cell viability of VERO cells with different concentrations of pure C-FMPC-Nps (0.2-1.0 mg/mL) along with control (100%) was assessed by MTT assay. Here, the actively respiring cells convert the water soluble yellow tetrazolium MTT to an insoluble purple formazan which gives rise to extracellular deposits of needle-shaped crystals by exocytosis (Liu et al. 1997).

These crystals were dissolved in DMSO. The resulting purple solution was spectrophotometrically measured. The effect of the nanoparticles on the cell proliferation was expressed as (%) cell viability (Zhang et al. 2011).

From the results (Figure 7.6) it could be inferred that the C-FMPC-Nps at 0.2 mg/mL dilution after 72 h showed remarkable biocompatibility to VERO cell lines with a maximum cell viability of 99%. As the concentration of the C-FMPC-Nps increased from (0.2-1.0 mg/mL), the cell viability decreases indicating the dose dependent cytotoxicity of the nanoparticles (Rejinold et al. 2011). The significant increase in the cell viability of C-FMPC-Nps at all given dilutions with increasing time interval (12 h to 72 h) suggested the positive time dependence property, which was a neat indication of the cell adherence and proliferation over the C-FMPC-Nps (Thangaraju et al. 2012).

The phase-contrast images of the viable cells in VERO cell lines seeded on control and C-FMPC-Nps after 72 h of incubation were shown in Figure 7.7. The results suggested that the C-FMPC-Nps has low cytotoxicity effects towards normal cell lines. Hence, the nanoparticles were found to be biocompatible with the living cells and act as a potential material for biomedical application.
Figure 7.6  Cell viability test performed on vero cell lines after cultivating with different concentrations of C-FMPC-NPs. Each value represents the mean ± SD (n=3)

Figure 7.7 MTT assay of (a) Control and (b) C-FMPC-Nps
7.5.2  *In-vitro* Antioxidant Assay

7.5.2.1  DPPH free radical scavenging activity

DPPH is a relatively stable nitrogen centred free radical. It has the ability to react with electron/hydrogen atom donating reducing agents and converting it into a nonradical 1,1-diphenyl-2-picryl hydrazine (yellow) diamagnetic molecule which can be measured spectrophotometrically (Patel Rajesh & Patel Natvar 2011).

7.5.2.2  DPPH scavenging activity of FMPC

The $IC_{50}$ values for the newly synthesized FMPC and the standard ascorbic acid for scavenging DPPH radical were found to be 72.7% and 90.29% respectively. FMPC was found to be almost equally potent as compared to ascorbic acid in DPPH assay. The functional groups present on the targeted compound are highly responsible for the free radical scavenging activity. It could be understood, that the antioxidant activity of the FMPC might be attributed to the electron donating nature of the substituent -OCH$_3$ (Jagtap & Pardeshi 2014). The presence of -OCH$_3$ on FMPC increases both the electron density and electron mobility in the core structure (Zhang et al. 2011) and stabilizes the generated radical during oxidation (Mohana & kumar 2013) thus, exhibiting predominant DPPH radical scavenging activity.

7.5.2.3  DPPH scavenging activity of C-FMPC-Nps

Figure 7.8 represents the antioxidant activity of the C-FMPC-Np. The $IC_{50}$ values of the chitosan nanoparticles and C-FMPC-Nps were found to be 1.859 and 0.9739 mg/mL respectively. The antioxidant activity of C-FMPC-Nps indicates that the nanoparticles act as a potent antioxidant in scavenging DPPH free radicals.
The antioxidant property is far higher than the synthesized FMPC. The reason behind this high activity might be due to the synergistic effect of both FMPC and chitosan (Lopez-Matta et al. 2015). Notably, the electron donating -OCH$_3$ present in vanillin moiety, the hydroxyl groups present at C3 and C6 position of chitosan and the presence of (-C=N-) linkage enhances the radical scavenging activity of the C-FMPC-Nps. Wherein, the high electron density over nitrogen present in the Schiff base (-C=N-), overlaps with the neighbouring (-O') radical electrons formed at the C3 carbon of chitosan would stabilize the free radical formed (Zai-Qun et al. 2007). Further, the increased electron fluidity of the prepared nanoparticles upon formation of the Schiff base (Zhang et al. 2011) would result in the enhanced antioxidant property of the Schiff base C-FMPC-Nps (Guo-quing et al. 2011).

Figure 7.8  DPPH radical scavenging property (a) chitosan and (b) C-FMPC-Nps. Each value represents the mean ± SD (n=3)
7.5.3 *In-vitro* Anticancer Activity

7.5.3.1 Inhibition of FMPC on MCF-7 cell line

As the antioxidant result of FMPC is encouraging, further screening of FMPC for anticancer activity against MCF-7 cell line was undertaken. The *in-vitro* cytotoxic activity accessed for different concentrations (1.4-2.9 µg/mL) is graphically represented in Figure 7.9. The IC$\text{50}$ value was determined to be 218 µg/mL.

From the result it could be inferred that the electron donating -OCH$_3$ group is highly responsible for the positive effect against stress related H$_2$O$_2$ scavenging anticancer activity of the synthesized compound (Naik et al. 2011). The synthesized FMPC with carbonate ester backbone structure has high response towards H$_2$O$_2$.

Thus under the cancer induced acidic environment the carbonate back bone might be cleaved (Park et al. 2010) to release vanillin, which suppress the generation of ROS, such as H$_2$O$_2$ and exerts, excellent activity against oxidative stress (Kim et al. 2011). Thus FMPC exhibits dose dependent cytostatic/cytolysis behaviour (Ho et al. 2009) towards MCF-7 cell line.

The high levels of H$_2$O$_2$ found in cancer cell line induce the hydrolytic cleavage of carbonate ester bonds present in FMPC (Chen et al. 2014). Thus, the release of vanillin from FMPC would result in the anticancer activity of the synthesized FMPC.
7.5.3.2 Inhibition of C-FMPC-Nps on MCF-7 cell line

The percentage inhibition of C-FMPC-Nps (0.9-3.0 μg/mL) on MCF-7 cancer cell line is indicated in Figure 7.10. Here, the activity of the nanoparticles was compared with chitosan nanoparticles. The inhibitory effect of C-FMPC-Nps was higher with IC$_{50}$ of 35.39 μg/mL compared to chitosan nanoparticles with IC$_{50}$ 69.06 μg/mL. The increase in the anticancer activity of C-FMPC-Nps arises from the Schiff base formation between vanillin and chitosan. Here, the reactive oxygen species generated at the targeted site were quenched by the localized electron dense nitrogen atom present in the schiff base. In addition to the aforementioned property, the hydrolytic cleavage of the carbonate backbone (Gillies & Frechet 2003) and the C=N linkage (Souza & Topp 2004) would release vanillin and chitosan from C-FMPC-Nps (Scheme 7.2).
Scheme 7.2 Hydrolytic cleavage of C-FMPC-Nps in acidic environment

Figure 7.10 Anticancer effects (a) chitosan and (b) C-FMPC-Nps. Each value represents the mean ± SD (n=3)
The synchronous effect of both chitosan and vanillin moieties released at the targeted carcinogenic site would enhance the anticancer activity thus curtailing the growth of cancer cells (Figure 7.11). The inhibitory effect led us to speculate, that there is a steady and linear increase in the cytotoxic effect with increase in concentration of the prepared nanoparticles (Reddy et al. 2015).

Figure 7.11  Images of (a) Control and (b) C-FMPC-Nps treated MCF-7 cells

7.6 SUMMARY

In conclusion, the prepared C-FMPC-Nps were characterized by various spectral studies. The synthesized FMPC and prepared C-FMPC-Nps were subjected to in-vitro antioxidant and anticancer bioassay. The prepared nanoparticles were found to be more effective than FMPC in the screening of anticancer activity against MCF-7 cell line. Both FMPC and C-FMPC-Nps exhibits a dose dependent cytotoxicity towards the MCF-7 cell line. The structure revealed that in addition to -OCH₃ moiety the -CH=N- imine moiety plays a vital role for the enhanced activity of the C-FMPC-Nps to fight against the ROS. Hence it has been concluded that the present work can open new vistas in chitosan schiff base chemistry and further research is needed to fine-tune the structure of FMPC for advanced biological studies.