

Development and Characterization of Chitosan-Pluronic Polymeric Nanoparticles for the Breast Cancer Treatment

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Abstract - The chitosan-pluronic nanoparticles have been prepared by an ionic gelation (IG) method. Particle size analysis, Scanning electron microscopy (SEM), Zeta potential measurements, Fourier transform infrared spectroscopy (FT-IR) and differential scanning calorimetry (DSC) were used for nanoparticles characterization. The optimized tamoxifen loaded nanoparticles had a spherical shape with positive charge and mean diameter 150 to 300 nm. The FT-IR and DSC studies found that the drug was dispersed in amorphous form due to its potent interaction with nanoparticles matrix. The maximum encapsulation efficiency was obtained at 8mg/ml tamoxifen. The tamoxifen loaded chitosan-pluronic nanoparticles had good blood compatibility. All study results suggest that the nanoparticles could be used as an effective drug delivery carrier for the breast cancer treatment.

Keywords - Chitosan, ionic gelation, nanoparticles, tamoxifen.

I. INTRODUCTION

Cancer is one of the leading causes of death in many developed countries. The most common treatments include surgery, radiotherapy, immunotherapy and chemotherapy [1]. Chemotherapy in addition to surgery has proven useful in a number of different cancer types including breast cancer, pancreatic cancer, ovarian cancer, testicular cancer and certain lung cancers. So the chemotherapeutic drugs in a chemotherapy regime are an attractive strategy of effective anticancer treatment. Research efforts to improve chemotherapy over the past 25 years have led to an improvement in patient survival. However, the efficacy of the therapy and the possible side effects vary among different agents. Some drugs may have excellent efficacy, but also serious side effects affecting the quality of life [2].

Nanoparticles are receiving considerable attention for the delivery of therapeutic drugs. Nanoencapsulation

of drugs involves forming drug loaded nanoparticles with diameters ranging from 1 to 1000 nm [3]. Nanocarriers act as a vehicle and improve cellular uptake and distribution inside the tumor regions [4].

Recent research efforts have been directed towards developing safe and efficient chitosan based particulate drug delivery systems. Chitosan is a polysaccharide, similar in structure to cellulose. It is composed of 2-amino-2-deoxy- β -D glucan composed with glycosidic linkages. Compared to many other natural polymers, chitosan has a positive charge and is mucoadhesive. Therefore, it is used extensively in drug delivery applications [5]. Chitosan is obtained from the deacetylation of chitin, a naturally occurring and abundantly available biocompatible polysaccharide. Chitosan is insoluble in acidic solution (PH<6.4) as a result of the protonation of the amino groups on the D-glucosamine residues. Because of its advantageous properties including biodegradability, biocompatibility, anti-bacteria and non toxicity, chitosan can be used in the fields of food processing, pharmaceuticals, cosmetics, biomaterials and agriculture [6].

Many methods have been developed to prepare chitosan nanoparticles including emulsion, spray drying, emulsion-droplet coalescence technique and ionic gelation. Ionic gelation is mild and rapid procedure with the counter-ion sodium TPP [7]. These nanoparticles show excellent capacity for drug entrapment and absorption by several routes. The positive charge of chitosan caused by primary amino group shows its mucoadhesive properties. So these systems have great utility in oral absorption of anticancer drugs [8].

Pluronic is a triblock copolymer, composed of poly (ethylene oxide) (PEO) and poly (propylene oxide) (PPO) with a PEO-PPO-PEO structure. It has good stabilizing property and capability to increase the solubility of drugs. Pluronic spontaneously forms micelles with the diameter approximately 30-50 nm at

concentrations equal to above the critical micelle concentration. This micellar structure is used to encapsulate hydrophilic and hydrophobic drugs [9]-[12].

Tamoxifen (Tmx) has been used since many years to treat breast cancer in women and men. It is majorly used to treat patients with early stage breast cancer, as well as those with metastatic breast cancer. As the treatment for metastatic breast cancer, the drug slows or stops the growth of cancer cells that are present in the body [13].

Tamoxifen binds to estrogen receptors on tumors and other tissue targets, producing a nuclear complex that decreases DNA synthesis and inhibits estrogen effects. Tamoxifen causes cells to remain in the G0 and G1 phases of the cell cycle. Because it prevents (pre) cancerous cells from dividing but does not cause cell death, tamoxifen is cytostatic rather than cytotoxic. Different approaches were performed to improve its delivery to the tumor regions. Different formulations such as liposomes, nanotubes, dendrimers, polymeric nanoparticles and drug conjugates were designed for advanced tamoxifen delivery [14].

The drug carrying combination of chitosan and pluronic deliver good combinations by advancing the controlled release profile using pluronic with protection and transfection-enhancing effects using chitosan.

In the present study, we prepared biodegradable chitosan-pluronic nanoparticles, tamoxifen loaded chitosan-pluronic nanoparticles by an ionic gelation technique. Such nanoparticles evaluated for their hemocompatibility studies.

II. MATERIALS AND METHODS

A. Materials

Tamoxifen, Chitosan (low molecular weight), PF, Sodium tripolyphosphate (TPP), 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Triton X-100, glacial acetic acid were all purchased from Sigma-Aldrich chemicals Private Ltd. (Bangalore, India). All other reagents were of analytical grade and obtained from Merck (Mumbai, India).

B. Methods

i. Preparation of nanoparticles

Chitosan nanoparticles were prepared by ionic gelation method [15]. Nanoparticles were obtained up on mixing 0.03% of (w/v) TPP solution with 0.1% (w/v) chitosan solution using magnetic stirrer at room temperature for 1 hour. The suspension assigned as nanoparticles. Chitosan-Pluronic combinations were obtained by adding TPP solution to chitosan aqueous solution containing 1% (w/v) concentration of pluronic.

Tamoxifen loaded chitosan-pluronic nanoparticles were performed by addition of tamoxifen in methanol (8mg/ml) to the chitosan solution and followed by the TPP solution. The nanoparticles were centrifuged (Eppendorf 5804 R, Germany) at 16000 x g in a 10 µl glycerol bed for 30 minutes. The supernatants were discarded, and the pellet resuspended with 5% sucrose solution. This suspension was freeze-dried (Operon, Korea) and stored at 4°C until use.

ii. Characterization of chitosan nanoparticles

a) Particle size analysis

The diameters of nanoparticles are determined by dynamic light scattering using Particle sizer (Beckman Coulter, Delsa Nano C). The dynamic light scattering is done with a wavelength of 633 nm at 25°C with an angle detection of 90°C.

b) Zeta potential analysis

The zeta potential of nanoparticles was determined by laser doppler electrophoresis using Zetasizer (Malvern instruments, UK).

c) Fourier transform infrared spectroscopy (FT-IR)

Fourier transform infrared (FT-IR) spectra were analyzed using a spectrometer (Nicolet 5700, Thermo Electron Corporation) at 4 cm⁻¹ resolution. The lyophilized nanoparticles were mixed with potassium bromide to analyze the chemical reactions between the drug and nanoparticle conjugate.

d) Differential scanning calorimetry (DSC)

The thermal behavior of the particles was analyzed by differential scanning calorimetry (Mettler Toledo, DSC). An approximate amount of samples (4-8mg) were weighed and scanned in a temp range of 0 to 250°C with a heating rate of 15°C/min per cycle. Inert atmosphere was maintained by purging nitrogen at a rate of 360 cm³/minute.

e) Scanning electron microscopy (SEM)

The morphology of nanoparticles was observed using a scanning electron microscope (EVO, Carl Zeiss, Germany). The freeze-dried samples were dried on an aluminum disk at room temperature, and coated with gold using a Cressington sputter coater.

f) Evaluation of drug encapsulation efficiency (EE)

The encapsulation efficiency (EE) was measured by measuring the amount of remaining drug collected after centrifugation. The tamoxifen present in the medium was determined spectrophotometrically by reading absorbance at 249 nm (Biospectrophotometer,

Eppendorf, Germany). The EE of the nanoparticles were calculated by the following equation [16];

$$EE(\%) = \frac{\text{Total amount of Tamoxifen} - \text{Free Tamoxifen}}{\text{Total amount of Tamoxifen}} \times 100 \quad (1)$$

g) *Drug release studies*

Drug release studies were carried out by dialysis method. Four milligrams of nanoparticles were dispersed in phosphate-buffered saline (PBS; pH = 7.4) as a release medium in a dialysis membrane sac (12 kDa; Sigma Aldrich) after tying at both ends. The dialysis sac was placed in PBS (150ml) containing jar and incubated at 37°C in continuous shaking water bath at 90 rpm. For each sample 1 ml of released medium was withdrawn at predetermined time intervals (0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 36, and 48 h) and replaced by the same medium at the same condition. The samples were analyzed for drug by ultraviolet spectrophotometer at 249 nm.

h) *Blood compatibility studies*

Five milliliters of blood sample was obtained for two healthy men (20-30 years old) and added with EDTA. The contents mixed properly and centrifuged at 1500 x g for 10 minutes. The collected Red blood cells (RBCs) were washed thrice with phosphate buffer solution (PBS) for 7 minutes at 1000 x g. The washed RBC's were resuspended in PBS and diluted to prepare erythrocyte stock solution. The lyophilized samples were redispersed and sonicated in PBS to give 0.2 % suspensions. The nanoparticles suspension was added to erythrocyte stock solution. The mixtures were incubated at 37°C in continuous shaking water bath for 1 hour. After the centrifugation at 1000 x g for 5 minutes, the supernatant read at 540 nm. The saline solution alone was used as negative control (0 % lysis) and 0.1 % triton in PBS used as positive control (100 % lysis) [17]. The amount of release of hemoglobin was monitored in spectrophotometer (Biospectrophotometer, Eppendorf, Germany) at 540 nm. Percent hemolysis was calculated using the formula;

$$\text{Hemolysis} (\%) = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the positive control}} \times 100 \quad (2)$$

i) *Statistical analysis*

The results were shown as mean \pm standard deviation. Statistical significance was done using one way-analysis of variance with a P-value < 0.05 as the minimal level.

III. RESULTS AND DISCUSSION

A. *Mean diameter, Size distribution and zeta potential of nanoparticles*

The nanoparticles with sizes below 300 nm were obtained by ionic gelation method by addition of TPP to chitosan in acetic acid under gentle magnetic stirring at room temperature. Chitosan and TPP can form nanoparticles in specific concentrations. Smaller size nanoparticles show valuable characteristics such as minimum toxicity, long circulation in blood and advanced drug delivery [18].

Table I Effect of sodium tripolyphosphate (TPP) concentration on mean diameter and zeta potential value

Chitosan concentration (% w/v)	Mean diameter (nm)	Polydispersity index	Zeta potential (mv)
0.2	150 \pm 2.27	0.263 \pm 0.019	37.1 \pm 0.27
0.4	238 \pm 5.23	0.357 \pm 0.033	39.6 \pm 0.53
0.6	255 \pm 2.61	0.309 \pm 0.054	43.3 \pm 1.74
0.8	330 \pm 4.18	0.381 \pm 0.032	47.4 \pm 1.38
1	456 \pm 2.84	0.429 \pm 0.058	48.7 \pm 0.61

Note: Chitosan = 0.1% w/v.

Table I shows the effect of TPP concentration on particle size. It was analyzed that TPP with higher concentration significantly increase the size of the particles. This is due to the increase in the amount of anionic groups in the preparation medium, which causes more electrostatic interaction with the positive amino sites on the chitosan, which reduce the positive surface charge and increase the nanoparticles size [19]. The zeta potential values show the stability of the nanoparticles through electrostatic repulsion [20]. The higher TPP concentrations lead to form aggregated solutions.

Table II Effect of chitosan concentration on mean diameter and zeta potential

TPP concentration (% w/v)	Mean diameter (nm)	Polydispersity index	Zeta potential (mv)
0.01	75 \pm 5.34	0.264 \pm 0.284	43.4 \pm 0.64
0.02	108 \pm 3.21	0.206 \pm 0.296	30.72 \pm 1.33
0.03	168 \pm 6.67	0.190 \pm 0.032	26.33 \pm 0.62
0.04	481 \pm 8.32	0.221 \pm 0.023	20.17 \pm 0.37
0.05	684 \pm 4.37	0.244 \pm 0.059	18.41 \pm 0.28

Note: TPP = 0.03% w/v.

Table II explains the size of the chitosan nanoparticles increases while increasing its

concentrations. The large chitosan nanoparticles show high zeta potential value due to effective ionic interaction between TPP and chitosan.

The pluronic incorporated nanoparticles are prepared for controlled release studies [21]. Addition of PF helps for the size reduction and rigid gel formation. The rigid gel helps to reduce the water uptake to the nanoparticles. The increased PF concentrations show no significant change in the zeta potential value (Table III). So it can be concluded that PF can easily incorporated inside the nanoparticles matrix without changing their surface charge. This is due to the formation of packed multimolecular aggregates that are trapped inside the nanoparticles matrix.

Table III Effect of PF concentration on mean diameter and zeta potential value

PF (% w/w)	Mean diameter (nm)	Polydispersity index	Zeta potential (mv)
10	181±1.14	0.168±0.049	26.35±0.26
20	169±2.17	0.182±0.024	24.18±1.47
30	108±3.63	0.151±0.006	23.85±0.43

Note: Chitosan = 0.1% w/v, TPP = 0.03% w/v.

Tamoxifen loaded nanoparticles were prepared up on addition of tamoxifen dissolved in methanol to the chitosan-pluronic solution followed by the TPP solution. To analyze the tamoxifen concentration on particle size, various concentration of tamoxifen in methanol were applied. Table IV shows increased tamoxifen increases the particle size and does not affect their zeta potential value.

Table IV Characterization of chitosan nanoparticles by varying tamoxifen (TMX) concentration

TMX (mg/ml)	Mean diameter (nm)	Polydispersity index	Zeta potential (mv)	EE (%)
2	186±3.16	0.159±0.028	27.63±0.57	52.34±0.76
4	203±4.64	0.228±0.034	26.52±0.63	51.18±0.11
6	218±5.17	0.207±0.061	26.14±0.51	50.66±1.55
8	244±2.83	0.186±0.045	25.41±1.78	61.05±0.58
10	287±3.42	0.214±0.057	24.89±1.46	57.47±1.53

Note: Chitosan = 0.1% w/v, TPP = 0.03% w/v.

The tamoxifen loaded chitosan-pluronic nanoparticles size changes while increasing the

tamoxifen concentration (Table V). Tamoxifen is a low molecular weight anticancer drug. So the diameter of the tamoxifen loaded nanoparticles increases up to its maximum capacity. Tamoxifen concentration did not affect the zeta potential of the prepared nanoparticles.

Table V Effect of tamoxifen (TMX) concentration on chitosan-pluronic nanoparticles

TMX (mg/ml)	Mean diameter (nm)	Polydispersity index	Zeta potential (mv)
2	164±1.17	0.179±0.024	27.31±0.72
4	173±2.39	0.204±0.016	25.47±1.13
6	144±3.46	0.216±0.023	26.82±0.64
8	186±6.38	0.193±0.037	25.76±1.93
10	179±4.52	0.127±0.018	25.87±0.85

Note: Chitosan = 0.1% w/v, TPP = 0.03% w/v, PF=20% w/w.

B. Nanoparticles encapsulation efficiency

The effect of different concentrations of tamoxifen on the encapsulation efficiency (EE) of chitosan nanoparticles was determined. The maximum encapsulation efficiency was achieved at 8 mg/ml of tamoxifen concentration (Table IV).

Table VI Encapsulation efficiency of chitosan-pluronic nanoparticles with different PF concentrations

PF (% w/w)	EE (%)
10	58.34±0.56
20	52.28±0.84
30	47.15±1.63

Note: Tamoxifen (TMX) = 8mg/ml, Chitosan =0.1% w/v, TPP =0.03% w/v.

To analyze the effect pluronic incorporation on the EE, 8mg/ml of tamoxifen was added to different PF concentrations (10%, 20%, 30% w/w) in chitosan solution. The addition of more PF concentration reduces the encapsulation efficiency of drug loaded nanoparticles (Table VI).

C. Morphology of nanoparticles

SEM images (Fig.1) of chitosan and chitosan-pluronic nanoparticles loaded with tamoxifen, show that particles are spherical in size. The size of the nanoparticle is increased after loading the drug tamoxifen.

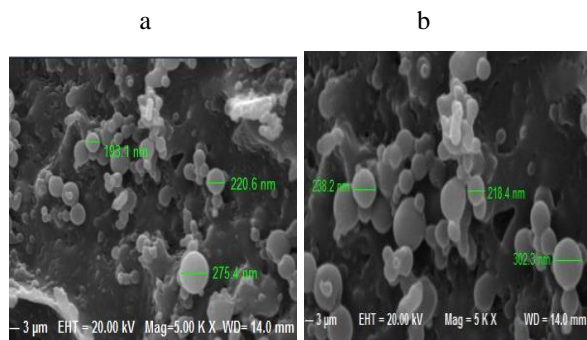


Fig.1 : SEM micrographs of (a) Tamoxifen loaded chitosan nanoparticles (b) Tamoxifen loaded chitosan-pluronic nanoparticles.

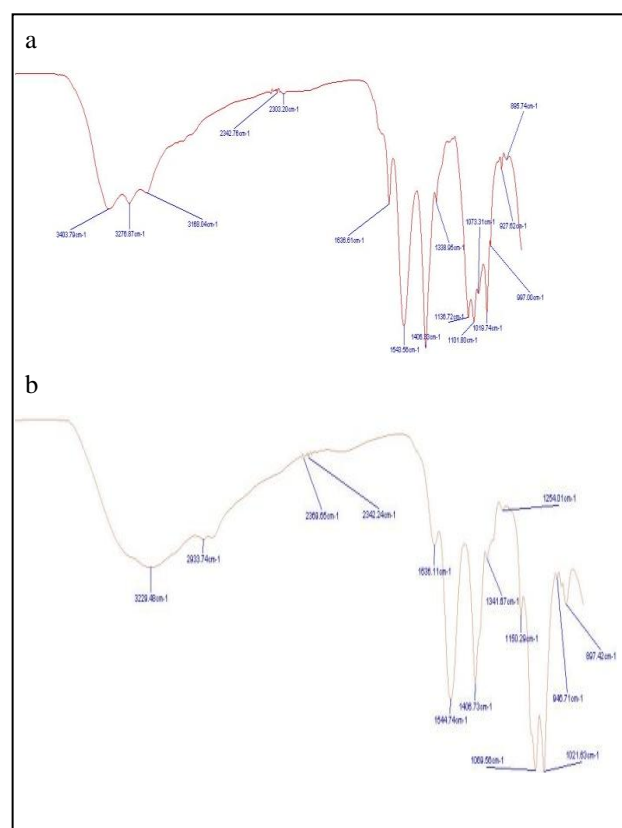
D. Fourier transform infrared spectroscopy (FT-IR) analysis

FT-IR spectroscopy was used to find out the nature of interaction between tamoxifen, chitosan or TPP. The various physiochemical interactions between tamoxifen, chitosan, pluronic and TPP can alter the absorption peaks or broaden the absorption peaks. Nanoparticles are formed in the medium, leads to positive charge for the chitosan due to electrostatic interaction between the primary amino group in the chitosan and TPP. The negative charges in the tamoxifen show affinity towards the chitosan. So the electrostatic interaction and chemical reaction occurs between the tamoxifen and chitosan.

In the spectra of chitosan in Figure 2C, the 1649.13 cm^{-1} represents the primary amino group present in the chitosan, while the stretching bands at 1026.99 cm^{-1} , 1062.52 cm^{-1} , and 3329.03 cm^{-1} are due to the C-O, C-H and hydroxyl group present in the chitosan, respectively. The peak at 2874.88 cm^{-1} represents the stretching band of methylene in chitosan structure. For blank chitosan nanoparticles (Figure 2B), the amino band is shifted to 1636.11 cm^{-1} which indicates the ionic interaction between TPP and NH_2 of chitosan. The broad band is maximum at 3229.48 cm^{-1} represents hydrogenic bonds between the hydroxyl groups in chitosan with TPP. These interaction leads to a decrease in chitosan solubility and nanoparticles formation. In the case of tamoxifen (Figure 2D), there is a characteristic peak at 1732.16 cm^{-1} which shows alkene -C=C- stretching in the tamoxifen molecular structure. A peak at 3423.38 cm^{-1} is represent alcohol O-H stretching in this area. The vibrational peak at 1477.04 cm^{-1} is due to the C=C ring stretching in the tamoxifen. When the tamoxifen was encapsulated in chitosan nanoparticles a small vibration band 1636.61 cm^{-1} appeared instead of 1649.13 cm^{-1} band. Therefore a strong interaction between tamoxifen and free amino groups of the chitosan has occurred. The solvation of the chitosan is related to the protonation of

free amino groups and break down of strong intra and intermolecular hydrogen bonding. The shift in the peak of tamoxifen loaded chitosan nanoparticles indicates the interaction of chitosan with tamoxifen. For the PF (Figure 3B), a strong stretching band at 2879.37 cm^{-1} represents the stretching vibrational band of methylene group. As in blank chitosan-pluronic nanoparticles (Figure 3A), this peak remains same and confirms the incorporation of PF in chitosan-pluronic nanoparticles. In the tamoxifen loaded chitosan-pluronic nanoparticles (Figure 3C), the C=C stretching band remains and confirms the encapsulation of tamoxifen in the nanoparticles matrix.

In the tamoxifen loaded chitosan-pluronic nanoparticles, the primary amino peak of chitosan at 1543.16 cm^{-1} in blank nanoparticles was shifted to 1466.06 cm^{-1} . The same peak existed in the tamoxifen loaded chitosan nanoparticles (Figure 2A). This proves the interaction between the chitosan and the drug. The H-bonding established through NH_2 linkage makes the molecule behave as a resonating structure owing to unshared electron pairs that are less available for protonation. The nitro groups withdraw electrons, making them less accessible at the secondary amide and primary amide groups during the interaction. This indicates the possible interaction of chitosan nanoparticles with tamoxifen.



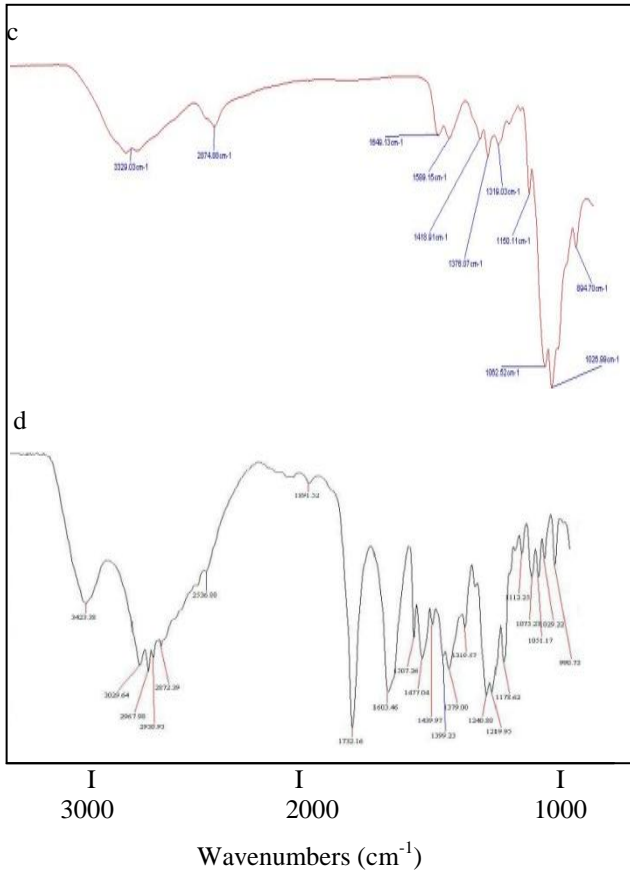


Fig.2 : FTIR spectra of (a) Tamoxifen loaded chitosan nanoparticles (b) Blank chitosan nanoparticles (c) Chitosan polymer alone (d) Tamoxifen.

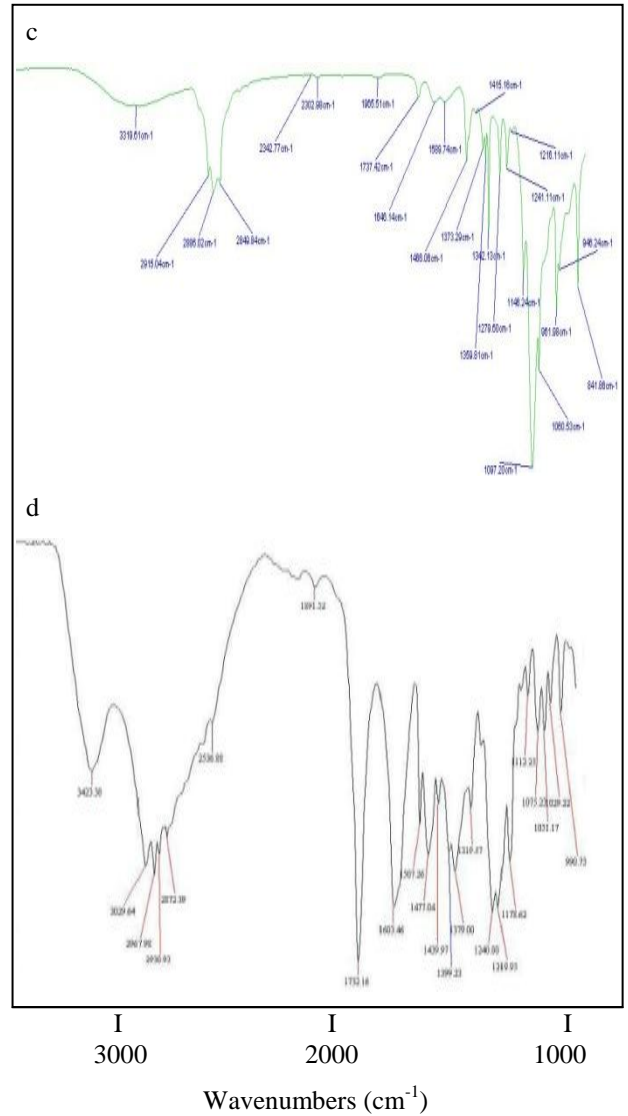
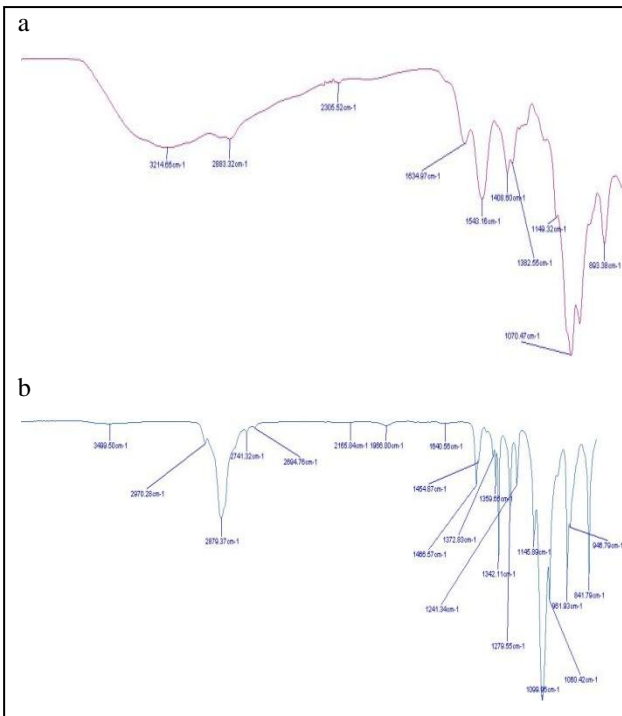


Fig.3. FTIR spectra of (a) Blank chitosan-pluronic nanoparticles (b) PF (c) Tamoxifen loaded chitosan-pluronic nanoparticles (d) Tamoxifen.



E. Differential scanning calorimetry (DSC)

DSC studies confirm the physical status of drug in the nanoparticles. As shown in Figure 4E, the melting point of tamoxifen is observed with a sharp peak at 90°C to 110°C. In blank chitosan nanoparticles (Figure 4B) the endothermic peak observed at 60°C to 80°C. The same peak observed in the blank chitosan-pluronic nanoparticles (Figure 4D) at 60°C to 80°C. For the PF (Figure 4C) the peak is observed at 60°C represents the melting point of a surfactant. The tamoxifen loaded chitosan nanoparticles (Figure 4F) and tamoxifen loaded chitosan-pluronic nanoparticles (Figure 4G) shows similar peaks at 60°C to 80°C. This indicates the incorporation of drug in the nanoparticles matrix.

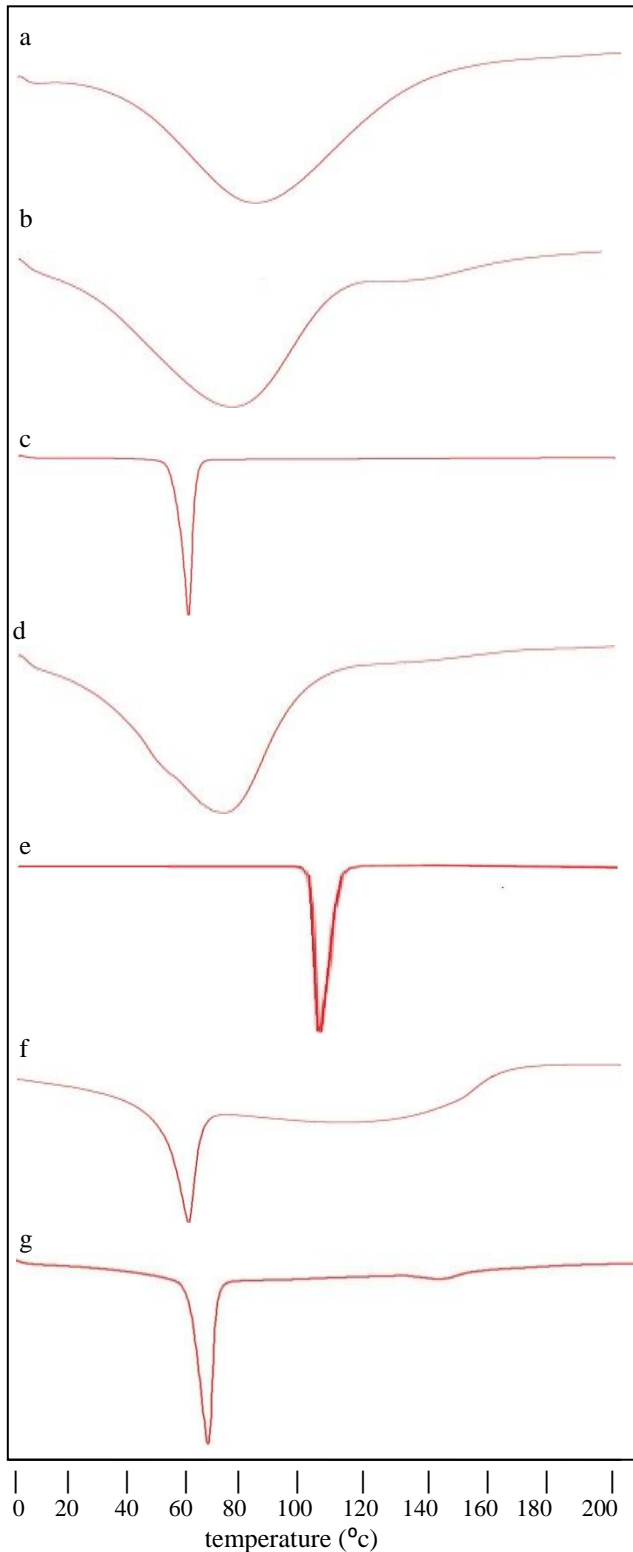


Fig.4. DSC thermographs of (a) Chitosan alone (b) Blank chitosan nanoparticles (c) PF (d) Blank chitosan-pluronic nanoparticles (e) Tamoxifen (f) Tamoxifen loaded chitosan nanoparticles (g) Tamoxifen loaded chitosan-pluronic nanoparticles.

F. *In vitro* drug release

The drug release studies were carried out PH 7.4 and the tamoxifen release from the tamoxifen loaded chitosan nanoparticles, tamoxifen loaded chitosan-pluronic nanoparticles was compared with the free tamoxifen as a reference standard (Figure 5). The percentage of release of tamoxifen from tamoxifen loaded chitosan nanoparticles and tamoxifen loaded chitosan-pluronic nanoparticles was much slower when compared to the free tamoxifen release in six hours. The release of tamoxifen from tamoxifen loaded chitosan nanoparticles and tamoxifen loaded chitosan pluronic nanoparticles was initial burst of 36% and 30% from the dialysis bag respectively. In the first 12 hour 36.85% of drug release was occurred in the tamoxifen loaded chitosan nanoparticles. The tamoxifen loaded chitosan-pluronic nanoparticles shows 30.67% of release at first 12 hour. The release was continued up to 54 hours. The dissolved drug rapidly diffuses in to the release medium near the surface of the nanoparticles and shows a rapid burst release. The incorporation of PF shows lower release rate for chitosan-pluronic nanoparticles when compared to chitosan nanoparticles. The polymer aggregates in to micelles at body temperature. The micellar nature prolongs the release rate of tamoxifen from the nanoparticles. This kind of rapid release followed by slow and sustained release of tamoxifen indicates biphasic release pattern of tamoxifen loaded chitosan-pluronic nanoparticles. The tamoxifen in tamoxifen loaded chitosan-pluronic nanoparticles can be released slowly at a specific concentration for a long time both in vivo and in vitro, which is very important for the clinical application [22].

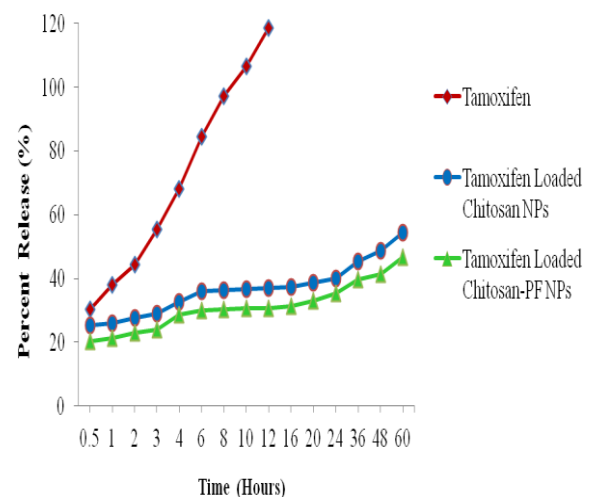


Fig.5. In vitro release of tamoxifen from tamoxifen loaded chitosan nanoparticles and tamoxifen loaded chitosan-pluronic nanoparticles at 37°C.

G. Blood Compatibility

Hemolysis study was conducted to analyze the action of nanoparticles with the human red blood cells (RBC). The samples were taken in four different concentrations 100 μ g, 75 μ g, 50 μ g and 25 μ g respectively (Figure 6). The assay was conducted to analyze the percent of lysis with respect to various concentrations. The lysis was observed at 0.84% to 3.33% for tamoxifen loaded chitosan nanoparticles and 0.96% to 4.07% for tamoxifen loaded chitosan-pluronic nanoparticles. Incorporation of PF did not show any significant change in the hemolysis [23]. But the hemolysis was still higher in the drug tamoxifen, showing 1.56% to 11.55%. From the data, it can be concluded that at tamoxifen loaded chitosan pluronic nanoparticles shows less hemolysis than the tamoxifen drug. The 5% of hemolysis may not cause much adverse effect to the system and may be accepted as within permissible limit [24]. According to the studies the hemolysis caused by chitosan is less than 15% considered as not hemolytic and that is used for the drug delivery purposes [25].

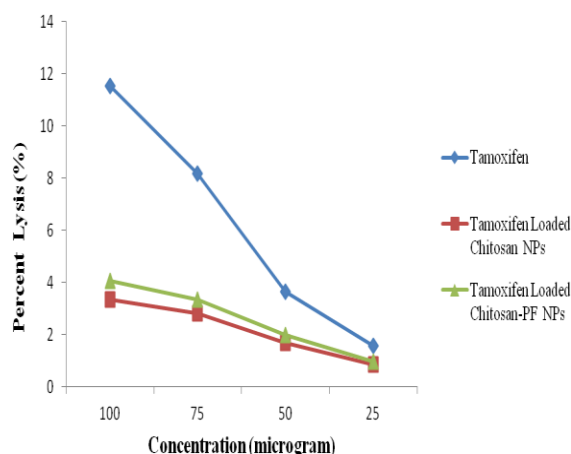


Fig.6. Hemolysis in the presence of tamoxifen, tamoxifen loaded chitosan nanoparticles and tamoxifen loaded chitosan-pluronic nanoparticles at different concentrations.

IV. CONCLUSION

The tamoxifen loaded chitosan polymeric nanoparticles were prepared using an IG method. The particle size of less than 300nm showed high encapsulation efficiency. The tamoxifen loaded nanoparticles showed less hemolytic activity. So it can be used for many pharmacological applications. The tamoxifen loaded nanoparticles could serve as a useful form of targeted delivery system towards breast cancer.

The further studies should be performed to increase the nanoparticles efficiency.

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