Epirubicin hydrochloride loaded poly (butylcyanoacrylate) nanoparticles: Formulation optimization, characterization and *in-vitro* evaluation in human breast cancer cell lines

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ABSTRACT

Metastatic spread is the major cause of breast cancer deaths. The tumor associated lymphatics have long been believed to be the main route for early spread of tumor cells. In the present study polybutylcyanoacrylate (PBCA) nanoparticles containing Epirubicin Hydrochloride (EPI) were synthesized by anionic polymerization in acidic medium in the presence of Dextran 40, Poloxamer 407 and Poloxamine 904. The influence of stabilizer concentration and EPI loading on particle size and entrapment efficiency were studied. The *in-vitro* drug release study was carried out in phosphate buffer saline pH 7.4 at 37°C. The optimum particle size and entrapment efficiency was observed at 0.05% w/v EPI loading, at pH 2.5 and at 1% monomer and surfactant concentration. The *in-vitro* release of EPI from all the EPI-PBCA nanoparticles in phosphate-buffered saline pH 7.4 at 37°C showed biphasic release with an initial burst release followed by sustained release phase of EPI from nanoparticles. *In-vitro* cell studies were carried out in human breast cancer cell lines MCF-7 and MDA-MB-231and the results revealed similar cytotoxicity when compared with free drug in the breast cancer cells evaluated.

Keywords: Nanoparticles, poly (butylcyanoacrylate), Epirubicin Hydrochloride, MCF-7, MDA MB 231

1 INTRODUCTION

Breast cancer is the second leading cause of deaths in women today after lung cancer, the mortality rate of patients with breast cancer around the world is rising steadily over time. Metastatic spread is the major cause of breast cancer deaths. The tumor associated lymphatics have long been believed to be the main route for early spread of tumor cells. Polyalkylcyanoacrylate nanoparticles have gained significant interest in targeting and drug delivery in recent times because of ease of synthesis, biodegradability, ability to alter biodistribution of drugs, lower the drug toxicity and above all it also overcomes multidrug resistance associated with tumors. In the present study poly (n-butylcyanoacrylate) (PBCA) nanoparticles containing Epirubicin Hydrochloride (EPI) were synthesized by anionic polymerization in acidic medium[1] in the presence of Dextran 40 (DX-40), Poloxamer 407 (P-127) and Poloxamine 904 (T-904).

2 METHODS

2.1 Preparation of EPI loaded poly (butylcyanoacrylate) nanoparticles:

EPI-PBCA nanoparticles synthesis was done by drop wise addition of monomer (1% w/v) into the acidic medium containing EPI and stabilizer/surfactant (DX-40 or P-127or T-904) under controlled stirring at 700 rpm at 37°C. After 2.5hrs of polymerization, the nanoparticle dispersion was neutralized by the addition of phosphate buffer containing NaOH (pH 7.4), and stirred for 1 h to allow complete polymerization. The nanoparticle dispersion was concentrated using ultra filtration through 300kda molecular weight cut off filtration membrane (Microsep, Pall) and washed thrice with 0.2 micron filtered distilled water to remove excess surfactant.

3 CHARACTERIZATION OF NANOPARTICLES

3.1. Particle size, Zeta potential and Surface Morphology:

Particle size and zeta potential analysis was carried using Malvern Zetasizer (NanoZS, Malvern Instruments, and UK) that follows Dynamic light scattering (DLS) principle. Surface morphology and particle size was determined by Transmission electron microscopy (TEM).

3.2. Determination of entrapment efficiency (%):

Entrapment efficiency was determined by dissolving known amount of the lyophilized nanoparticles in Acetonitrile: Water (50:50) and measuring the absorbance at 480nm in a UV spectrophotometer (Shimadzu 1700, Japan) against suitable blank.

3.3. In-vitro drug release:

The *in-vitro* drug release of the EPI loaded nanoparticles was performed in phosphate-buffered saline (PBS) pH 7.4 at 37°C. EPI loaded nanoparticles were suspended in 10 ml of PBS in a screw capped tubes, which were placed in a shaking water bath maintained at 37°C. At specific time, samples were taken out and centrifuged at 15000rpm for 30min. The supernatant was collected and absorbance measured at 480nm on a UV-Visible spectrophotometer (Shimadzu 1700, Japan).

3.4. *In-vitro* cell line studies:

The human breast cancer cell lines MCF-7 (estrogen receptor-positive) and MDA-MB-231 (estrogen receptor-negative) were obtained from NCCS, PUNE. MCF-7 cells were maintained in MEM supplemented with 10% heat inactivated FBS at 37°C in a humidified incubator containing 5% CO_2. MDA-MB-231 human breast cancer cells are maintained in L -15 Medium supplemented with 10% heat inactivated FBS at 37°C in a humidified incubator without CO_2.

3.4.1. *In-vitro* cell cytotoxicity studies:

The cytotoxicity of EPI and EPI-PBCA nanoparticles against MCF 7 and MDA-MB-231 breast cancer cells were determined by using the MTT dye reduction assay[2, 3]. Briefly, known quantities of cell suspension (5000cells/well) in the exponential growth phase are plated in 96-well flat-bottom tissue-culture plates. The cells were incubated at 37°C in an incubator for 24 h. After 24hrs the medium was changed with 200-µl medium containing EPI loaded NPs and EPI solution of different concentrations in triplicate (200 µl each). Control wells were treated with equivalent volumes of drug-free media and blank nanoparticles. After 48 h and 72 h, the medium was replaced with 100µl of fresh medium containing 1mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT, (Sigma Chemical Co.) and incubated for 4 h. After 4hrs the unreduced MTT and medium were discarded and 100µl of DMSO was added to dissolve the formazan crystals. Absorbance measured after shaking the plates to dissolve the formazan crystals at 540 nm using the micro plate reader (Tecan). Cell viability was determined using the formula.

$$\text{Cell viability } (\%) = \frac{\text{Abs of test cells}}{\text{Abs of control cells}} * 100$$

where Abs test cells and Abs control cells represent the amount of formazan determined for cells treated with the different formulations and for control cells (non treated), respectively.

3.4.2. Cellular uptake by Fluorescent imaging:

Fluorescence microscopy was performed using an Olympus Fluorescence microscope (Olympus BX51, Japan). 1 X 10^5 cells were seeded on sterile cover slips and allowed to grow. After 24hrs, cells were treated with 5µg/ml EPI and EPI PBCA NPs and incubated for 24hrs. After 24hrs the cells were washed with PBS and the cells were fixed by adding 4% solution of paraformaldehyde in PBS for 10 min. This solution was then removed and 20µL of 0.5µg/mL solution of 4'-6-diamidino-2-phenylindole (DAPI) in PBS was added and observed under microscope.

4 RESULTS AND DISCUSSION

EPI-PBCA nanoparticles were prepared by anionic emulsion polymerization method. Initial studies on the influence of aqueous phase pH on particle size resulted in minimum particle size with low PDI at a pH of 2.5. At higher pH amorphous polymer mass formed due to the rapid polymerization rate, preventing the discrete particle formation. At lower pH, larger particles were observed. This may be due to the extension of polymerization time leading to longer polymer chains[4]. Aqueous phase volume (20ml), stirring speed (700rpm), polymerization temperature (25°C) and polymerization time (2.5h) were fixed to constant. The influence of stabilizer concentration and EPI loading on particle size and entrapment efficiency were studied.

4.1. Particle size, Zeta potential and Surface Morphology:

Particle size and zeta potential of nanoparticles was carried out using Malvern Zetasizer after dilution with 0.2µm filtered double distilled water. A reduction in particles size was observed with increase in concentration of surfactants[5]. The decrease in mean size indicates proper surface coverage and effective stabilization of polymeric particles, preventing agglomeration. Nanoparticles prepared with surfactants T- 904 and P -127 exhibited lowest particle size compared to DX- 40. The resulting difference in particle size distribution may be due to the diverse polymerization mechanisms associated with DX40 and PEO-PPO block copolymers (T- 904 and P -127). For DX 40 the polymerization mechanism is dispersion polymerization and for T- 904 and P -127 it is emulsion polymerization[6]. The mean hydrodynamic diameter decreased with an increasing EPI concentration. This may be due to the basic nature of the drug which enhances the rate of polymerization reaction, leading to decreased mean size (Fig. 1, Fig.2 and Fig.3).

The zeta potential of EPI-PBCA nanoparticles produced in the presence of all surfactants carries a negative charge. The observed zeta potential values are -11.6mv, -7mv and -16mv for DX-40, P-127 and T-904 at 1% concentration.

Transmission Electron Microscopy studies indicated that nanoparticles had spherical shape and confirmed the nanosize (Fig. 4).

4.2. Entrapment efficiency:

The entrapment efficiency of the nanoparticles increased with increase in EPI loading up to 0.05%w/v and a decline afterwards (Fig. 1, Fig.2 and Fig.3). This decline may be due to attaining equilibrium concentration and hence further drug addition resulted in decreased entrapment efficiency. Nanoparticles prepared with DX-40 showed better entrapment efficiency compared to surfactants T-904 and P-127. This might be because of the larger particle size and due to the superior coverage of the porous structure of PBCA nanoparticles[7] which minimizes the drug loss during the preparation process. An increase in surfactant concentration beyond 1% resulted in decreased entrapment efficiency, this can be attributed to the decreased size and increased surface area of the particles at higher surfactant concentrations, which eventually leads to higher drug loss in to the aqueous phase[5].

4.3. *In-vitro* drug release:

The *in-vitro* release of EPI from all the EPI-PBCA nanoparticles in phosphate-buffered saline pH 7.4 at 37°C showed biphasic release, with an initial burst release phase followed by sustained release phase (Fig. 5). The initial burst release might be mainly the result of the larger surface area and desorption of the surface bound EPI and drug diffusion across the thin boundary layer of the nanoparticle matrix. The sustained release phase might be related to the polymer degradation rate[8] and/or due to the stronger interaction between EPI and the PBCA[9] resulting slower diffusion of entrapped drug from the nanoparticles.

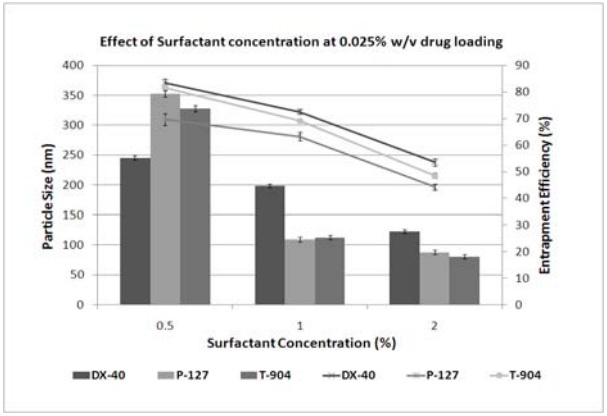

Fig. 1: Effect of surfactant concentration on Particle size (nm) and entrapment efficiency (%) at 0.025% drug loading

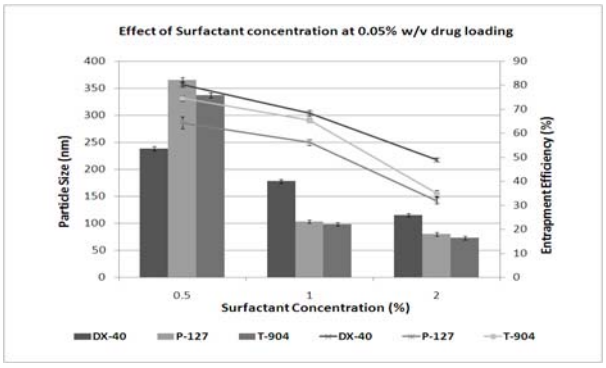

Fig. 2: Effect of surfactant concentration on Particle size (nm) and entrapment efficiency (%) at 0.05% drug loading

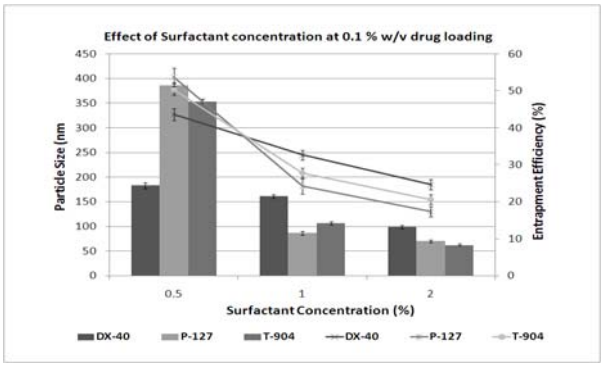

Fig. 3: Effect of surfactant concentration on Particle size (nm) and entrapment efficiency (%) at 0.1% drug loading

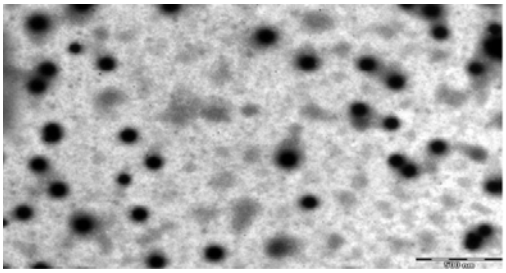

Fig. 4: TEM image of EPI loaded PBCA nanoparticles

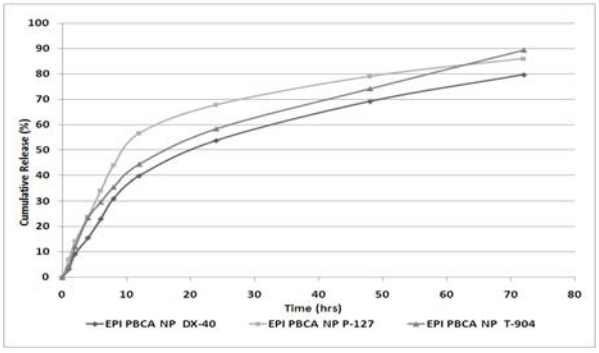

Fig. 5: *In-vitro* drug release of EPI from EPI - PBCA NPs

4.4. In-*vitro* cell cytotoxicity studies:

The cell cytotoxicity studies by MTT reduction assay were conducted to measure the sensitivity of MCF-7 and MDA MB 231 cells to EPI and EPI-PBCA NPs. Data obtained on cell viability showed MCF-7 cells are more sensitive to EPI compared to MDA MB 231. A dose and time dependent cytotoxicity was observed in both the cell lines with not much change in cytotoxicities observed between EPI and EPI PBCA NPs treated cells (Fig.6). Even though the *in - vitro* cytotoxicity results did not showed any superior cytotoxicity compared with the free drug, results conclude that EPI PBCA NPs are at least as effective as the free drug.

4.5. Cellular uptake by Fluorescent imaging:

Fluorescence microscopic images reveal EPI fluorescence inside the cells attributed to the cellular uptake of EPI loaded NPs. Nuclear staining with DAPI and overlay images revealed the nuclear localization of the EPI (Fig.7).
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5 CONCLUSION

Particulate systems, administered interstitially, for lymphatic targeting purposes should drain well from the site of injection and be well retained in the regional lymph nodes[10-12]. The currently investigated polymeric nanoparticles with their small size and negative surface charge satisfy the physicochemical aspects required for preferential uptake of the nanoparticles from the interstitium to the lymph nodes. The prepared PBCA nanoparticles demonstrated higher entrapment efficiency of EPI and showed sustained release of EPI from the particles. Cytotoxicity studies on human breast cancer cell lines revealed comparable results with free drug. Further in vivo studies to prove the targeting ability of the nanoparticles to lymph nodes are required.

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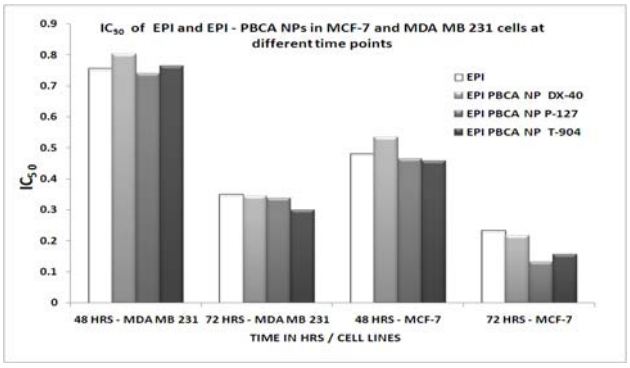

Fig. 6: IC_{50} values of EPI and EPI-PBCA NPs in MCF-7 and MDA MB 231 cells at 48 and 72hrs

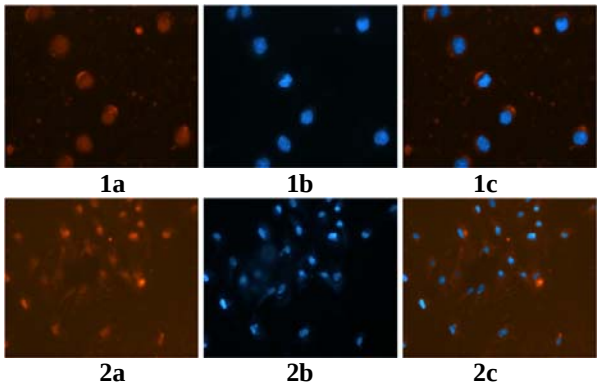

Fig. 7: Fluorescent images of the cellular uptake of EPI delivered by the EPI-PBCA NPs in MCF -7 (1a-1c) and MDA MB 231 cells (2a-2c) (a) EPI Fluorescence detection, (b) DAPI staining, (c) overlay of (a) – (b)