Cancer chemotherapy: Effect of poloxamer modified nanoparticles on cellular function

Grace Lovia Allotey-Babington\(^a\), Henry Nettey\(^b\), Sucheta D’Sa\(^a\), Kimberly Braz Gomes\(^a\), Martin J. D’Souza\(^a\)

\(^a\) School of Pharmacy, Mercer University, Atlanta, GA, United States

\(^b\) School of Pharmacy, University of Ghana, Legon, P.O. Box LG 43, Legon, Ghana

A B S T R A C T

The use of excipients with the ability to synergize the action of active pharmaceutical ingredients is highly recommended. Although a large spectrum of surfactants can be used in the preparation of polymeric nanoparticles, the poloxamer surfactants are currently being explored because they have been shown to preferentially target cancer cells as well as inhibit Multi-Drug Resistant proteins and other drug efflux transporters on the surface of cancer cells. The proper type and concentration of surfactant used plays a major role in the stability of the nanosuspensions. The aim of the study was to assess the effect of three different poloxamer surfactant (Pluronic F-108, pluronic F-127 and kolliphor P-188) and chitosan on the stability, immunogenicity, cytotoxicity of dasatinib nanoparticles, as well as cellular uptake of nanoparticles by various cell types.

A combination of chitosan and the poloxamers enhanced stability of nanoparticles: average size was 190 ± 20 nm just after preparation, changing to 200 ± 40 nm after 28 days of storage. Uptake of dye-loaded nanoparticles into the cells was 1.5 times more than a solution with an equivalent amount of dye. The uptake of nanoparticles into all the cell lines used was highest for P-188 nanoparticles, whilst F-127 nanoparticles were the least taken up. Poloxamers were observed to be non-toxic to the cells, however, stimulation of cell growth was observed in some cases. The properties exhibited by the various poloxamers in this study could guide in the selection of an appropriate poloxamer for the formulation of nanoparticles.

1. Introduction

Nanotechnology for drug delivery has attracted a lot of attention in the past two decades. It has transformed the approach to the design and delivery of medications for the treatment of diseases such as cancer and brain disorders. Among the various types of nanoparticles, polymeric nanoparticles have found excessive use in the field of oncology not just because of its ability to deliver chemotherapeutic agents to cancer cells, but also because they provide the flexibility of modifying the release of the entrapped drug and additionally, allow the attachment of various ligands to the nanoparticle’s surface thereby producing a targeted effect for precision killing of cancer cells and sparing normal cells. The enhanced penetration and retention effect (EPR) observed in the micro-environment of solid tumors promote the accumulation of nano-materials into solid tumors, producing a targeted effect [1]. Maintaining the particle size within the desired range is critical for achieving the necessary effect, making the selection and the amount of the appropriate surfactant one of the important steps in the formulation process.

The nonionic synthetic surfactants - Poloxamers, also known as pluronics were invented in the early 1970’s [19] and have attracted tremendous attention in the field of oncology in the last two decades. Research has demonstrated that poloxamer surfactants interact with multidrug-resistant tumors resulting in increased sensitization of these tumors to various anticancer agents [2,3]. This property is due to their ability to block P-glycoprotein (P-gp) pump activity as well as overcome glutathione/glutathione-S-transferase (GSH/GST) detoxification system [4,5]. These properties in combination with their amphiphilic nature promote their use as stabilizers in the fabrication of polymeric nanoparticles for tumor targeting. Poloxamers are triblock polymers composed of a hydrophobic block sandwiched by two hydrophilic polymer blocks. Their peculiar structure gives them the ability to coat the surface of hydrophobic nanoparticles, leaving the hydrophilic tails to induce stealth properties [6]. Manipulation of the length of the individual polymer blocks has led to the existence of different types of poloxamers with varying properties [22]. In this paper, the effect of three poloxamer types (F-108, F-127 and P-188) and their concentration on the stability of dasatinib nanoparticles were evaluated. Additionally, combination of these poloxamers with chitosan and their effect on some cellular functions were investigated in order to select an appropriate formulation for further studies. Dasatinib is an inhibitor of Src, tyrosine...
kinases, which have been shown to be over-expressed in a number of cancers including triple negative breast cancer. It was chosen as a model drug because has been shown to be effective alone and in combination with other chemotherapy agents [7,8,20].

2. Materials and methods

2.1. Images in this document are presented in color

Pluronic F-108 and Kolliphor P-188 were gifts from BASF Corporation (NJ). Pluronic F-127 was a gift from Dr. Banga, Mercer University, Atlanta. Dasatinib was purchased from LC laboratories (Woburn, MA). Polycaprolactone, Coumarin VI and Tween 80 were purchased from Sigma-Aldrich (St Louis, MO). Dulbecco’s Modified Eagle Medium (DMEM), Dulbecco’s Phosphate Buffer Saline (PBS) and Fetal Bovine Serum (FBS) were purchased from Atlanta Biologicals (Atlanta GA). 1% Penicillin- Streptomycin was purchased from Cellgro (Manassas, VA). MDA-MB-231 & HCC-70 breast cancer cell lines were obtained from Dr. Kenneth Rock at the University of Massachusetts Medical School. MTS cell proliferation assay kit was purchased from Promega Corporation (Madison, WI). Sulfanilamide, 98% was purchased from Acros Organics (NJ). N-(1-Naphthyl)-ethylene-diamine dihydrochloride was purchased from Fisher Scientific Company, (NJ). All other materials used were of analytical grade.

2.2. Preparation and characterization of nanoparticles

Dasatinib nanoparticles were prepared by the nanoprecipitation method using three poloxamer surfactants (pluronic F-108, pluronic F-127, kolliphor P-188) and polycaprolactone (PCL) as the polymer matrix. Four different polymer/surfactant ratios (1:1, 1:2, 2:1, 1:5) were prepared to investigate the effect of poloxamer type and concentration on size distribution of nanoparticles. Dasatinib was dissolved in 5 mL of a 10 mg/mL solution of polycaprolactone in acetone. The mixture obtained was added dropwise to 30 mL of the aqueous surfactant solution on a stirrer in the ratios listed above while stirring. The milky mixture obtained was stirred on a magnetic stir plate for about 6 hours to allow for the evaporation of all the acetone. The same procedure was used to make coumarin-loaded and empty (blank) nanoparticles. Chitosan coated nanoparticles were prepared by adding 1 mL of 0.1% chitosan solution dropwise into the beaker containing the immediately precipitated nanoparticles. The suspension was left stirring to allow the evaporation of the acetone. Nanoparticles were washed twice by centrifugation at 20,000 g for 20 minutes, after which the pellet was lyophilized and stored for subsequent studies.

Nanoparticles were characterized by size and charge using a Malvern Zetasizer Nano-ZS (Malvern Instruments Inc. UK) which utilizes the principle of dynamic light scattering. About 1 mg of drug nanoparticles were weighed and suspended in 10 mL of filtered deionized water. 1 mL of the obtained suspension was put in a cuvette and analyzed. The results reported are average values from triplicate runs of three independent experiments.

2.3. Colloidal stability

To determine physical stability of Dasatinib nanoparticles in suspension, 20 mL of each formulation was stored at two different temperature conditions (25 °C and 40 °C) for four weeks (28 days). The size distribution of the particles was measured weekly during the study period using the Malvern Zetasizer.

2.4. Fourier transform infrared spectra (FT-IR)

FT-IR spectra was recorded for each of the twelve formulations prepared using Shimadzu IRAffinity – IS fourier transform infrared spectrophotometer. Each spectrum was obtained with 50 scans in the range of 600 to 4000cm-1. This was done to determine the effect of surfactant amount on the spectra.

2.5. Drug content analysis and encapsulation efficiency

To determine drug content in the formulation, 10 mg of nanoparticles was weighed and added to 0.2 mL of acetone to dissolve the polycaprolactone polymer matrix. Following dissolution, DMSO was added to solubilize the entrapped dasatinib. The mixture was kept stirring to allow evaporation of the acetone. Following which the mixture was then centrifuged, filtered and diluted for further analysis. The actual drug content of the particles was determined using a reverse phase HPLC method [21]. Experiments were done in triplicates.

After determination of the actual amount of drug in 10 mg of nanoparticles, the theoretical amount of drug supposed to be in 10 mg of nanoparticles was also calculated. The encapsulation efficiency was determined using the formulae below.

\[
\text{Theoretical Loading} = \left( \frac{\text{Theoretical Dasatinib amount}}{\text{Dasatinib + solid content of formulation}} \right) \times 100
\]

\[
\text{Actual loading} = \left( \frac{\text{Experimental Dasatinib amount obtained}}{\text{Dasatinib + solid content of formulation}} \right) \times 100
\]

\[
\text{Encapsulation Efficiency} = \left( \frac{\text{Actual loading}}{\text{Theoretical loading}} \right) \times 100
\]

2.6. Drug release

To determine the drug released from the nanoparticles in each formulation, 10 mg each of the various nanoparticles were weighed into Eppendorf tubes and suspended in 1 mL of phosphate buffered saline (PBS, 20 mM, pH 7.4) supplemented with 0.1% Tween 80 to promote dissolution of the released dasatinib from the nanoparticle into the medium. The Eppendorf tubes were placed on a mechanical shaker with continuous shaking (80 rpm) at 37 °C. At select time intervals, the tubes were centrifuged at 20,000 g for 15 min. 0.5 mL of the supernatant was collected and processed for analysis using a reverse-phase HPLC method as mentioned above in section 2.4. To maintain sink conditions, 0.5 mL of fresh medium (0.1% Tween in PBS) was added to each tube, vortexed and placed back on the shaker each time a sample was taken.

2.7. Cellular uptake of nanoparticle observed by fluorescence microscopy

200 μL of a 10^5/mL suspension of MDA-MB-231 breast cancer cells and Dendritic cells were each seeded on pretreated cover slips placed in petri dishes. The cells were placed in a humidified CO2 incubator overnight to equilibrate, following which the cells were exposed to coumarin VI loaded nanoparticles in media (0.1 mg/mL). Each cell type was exposed to PCL/F-108, PCL/F-127, PCL/P-188 nanoparticles with and without chitosan. After 2 hours of exposure, nanoparticle suspension was gently washed off the surface of the cover slip using cold phosphate buffered saline (PBS). Care was taken not to wash away the cells from the surface of the cover slip. Cells were then introduced to fresh media, coverslip was flipped onto a glass slide and observed under
an Olympus CX41 Fluorescence microscope using a 40X objective. Experiments were done in duplicates.

2.8. Cellular uptake of nanoparticle determined by flow cytometry

Extent of uptake of nanoparticles fabricated with the three poloxamers by various cell types was evaluated by flow cytometry. Coumarin VI, a fluorescent dye was loaded into the nanoparticles and used for the uptake study for easy quantification. Five different cell lines were used, namely: (A) MDA-MB-231 breast cancer cells (B) HCC-70 breast cancer cells (C) B16-F10 melanoma cells (D) WB rat epithelial cells and (E) Dendritic cells (DC.2.4). 5 × 10⁴ cells/well of each cell type were plated in 24 well plates and left to equilibrate in a CO₂ incubator. Cells were then exposed to two batches of nanoparticles (coumarin VI loaded nanoparticles with and without chitosan) in culture media. After 2 hours, cells were trypsinized and washed using cold phosphate buffered saline. Following the third wash, pellet obtained after centrifugation was re-suspended in 0.5 mL of PBS and placed on ice. The mean fluorescence intensity (MFI) of a fixed number of cells was determined by flow cytometry. Three independent wells were analyzed per group.

2.9. Cytotoxicity of blank nanoparticles on different cell types

Cytotoxicity of empty nanoparticles was investigated in-vitro on three different cell types; rat liver epithelial cells (WB), murine kidney cells (MDCK) and triple negative human breast cancer cells (MDA-MB-231). About 5 × 10³ cells/well were plated in a 96-well plate. Cells were treated with various concentrations of empty nanoparticles prepared using the three poloxamers. Percent cell viability was determined after incubating cells with various concentrations of nanoparticles for 24 hours at 37°C. The viability of each cell type was evaluated using MTS assay.

2.10. Effect of PCL-Poloxamer nanoparticles on the release of innate immune marker (nitric oxide) from antigen presenting cells

Nitric oxide is one of the innate immune markers released by antigen presenting cells in response to an exogenous antigen. 10⁵ cells/well of dendritic cells, DC 2.4, were plated in a 24 well plate and allowed to equilibrate overnight. To determine the antigenicity of the poloxamer-stabilized nanoparticles, the plated cells were treated with 0.2 mg/mL drug-free nanoparticles in culture media and then incubated at 37°C. After 16 hours the supernatant was analyzed for nitric oxide content using the Greiss chemical method [9].

2.11. Statistical tests

Statistical analyses were performed using the SPSS software. T-test was used to assess the difference between the formulations prepared. P-value was used to assess the difference between the formulations prepared. P-value was used as p > 0.05 (non-significant differences), p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***) Error bars represent standard deviation of uncertainty.

3. Results

3.1. Physical characteristics

Poloxamers: F-108 and F-127 nanoparticle suspensions produced a bell shaped curve with a narrow size range for polymer/surfactant ratios 1:1 and 1:2. At low concentrations of F-127 (ratio 2:1) a small population of particles with size greater than 1 μm was observed, while at high surfactant concentrations (ratios 1:5) the distribution was bimodal. Notably, a large population of the particles were in the 100 and 1000 nm size range. Unlike the other two surfactants, kolliphor P-188 did not have the characteristic bell shape. Particle size ranged from 1 nm to 10 μm as indicated by the line with the triangle symbols along the size axis (Fig. 1).

![Fig. 1. Effect of poloxamer F-108 (red), F-127 (green) and kolliphor P-188 (blue) on size of nanoparticles at fixed polymer/surfactant ratios. (A) Ratio 1:1, (B) Ratio 1:2, (C) Ratio 2:1 (D) ratio 1:5. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)](image-url)
the Malvern can measure. The size was probably above 6 μm, which is the maximum size of particle size the Malvern can measure.

- The size distribution of nanoparticles increased by > 50% as indicated in Table 2 demanding the need for better stability.

The size of the nanoparticles did not change much after stabilization with chitosan for F-108 and F-127, however, a decrease in particle size was observed for P-188 nanoparticles. It is desirable to maintain small nanoparticle size, since, larger particles are likely to be taken up by the immune cells. For all three sets of nanoparticles prepared with the poloxamers and chitosan, the zeta potential measured gained a positive charge (Table 3). The magnitude of the charge was least in all the formulation prepared with 1:5 polymer/surfactant ratio.

To evaluate the effect of chitosan on the stability of the nanoparticles, the size distribution of nanoparticles coated with chitosan was determined just after production and also after 48 hours of storage at 25 °C. Nanoparticles remained stable during this period as compared with the formulations without chitosan (Table 4). The nano-suspensions were further observed for a period of 28 days under two different temperature conditions (25 °C and 40 °C). The size distribution of the particles was measured weekly during the study period.

3.2. Stability of nanoparticles during storage

Chitosan increased the hydrophilicity on the surface of the nanoparticles leading to the introduction of steric hindrance. Aggregation of particles was immensely controlled (Table 4). The average size of nanoparticles after the 28-day study period was 200 ± 40 nm. The polydispersity index (PDI), however increased from about 0.09 to 0.5 (Fig. 2).
3.3. Fourier transform infrared Spectroscopy (FTIR) of nanoparticles

The presence of Dasatinib in the formulations was confirmed by the presence of its characteristic functional groups. The FTIR spectra of the various formulations were similar (Fig. 3). Poloxamers contain polyethylene oxide and polypropylene oxide in various ratios, thus, the same functional groups. Hence, FTIR spectra could not bring out the differences between the various formulations.

3.4. HPLC data for dasatinib

Dasatinib was quantified using a reverse phase HPLC method. The mobile phase was a combination methanol, acetonile and TEA buffer. Dasatinib eluted at 9.02 min (Fig. 4). Content analysis was conducted after the polymer matrix was dissolved. The amount of drug entrapped in the PCL matrix was influenced by the type of poloxamer used as well as the ratio of polymer to poloxamer, that is, the amount of surfactant used.

3.5. Release studies

The release was conducted from formulations containing PCL/poloxamer ratios 1:1 and 1:2, since they were the most stable. The cumulative release was conducted over a 5-day period into PBS release-media containing 0.01% Tween 80. Dasatinib release from polycaprolactone/poloxamer matrices was relatively slow. From PCL/F-108 nanoparticles, release of drug from the 1:2 ratio was about 11%, while that from the 1:1 ratio was about 7%. Addition of chitosan to the formulation did not affect the drug release (see Fig. 5).

3.6. Cellular uptake of nanoparticles observed by fluorescence microscopy

Fluorescence microscopic images of the cells after internalization revealed that the dye loaded nanoparticles were taken up into the cells (Fig. 6). Results indicate that poloxamers have some effects on cellular function. Effect of poloxamer on cell membrane of MDA-MB-231 breast cancer cells is revealed in Fig. 7.

3.7. Cellular uptake of coumarin-loaded nanoparticles determined by flow cytometry

Flow cytometry analysis of the mean fluorescence intensity of the cells after internalization of coumarin-loaded nanoparticles and coumarin solution revealed that nanoparticles are taken up better than solution. Additionally, marked differences in the amounts uptaken by the cells were observed. It was also established that, the charge of the nanoparticles influenced cellular-uptake in all the cell lines studied (Fig. 8).

3.8. Cytotoxicity of blank PCL/Poloxamer nanoparticles using different cell types

Cytotoxicity of blank nanoparticles was performed to unravel the effect of the poloxamers (excipients) on the various cell types used. Cell lines of three different species (mice, rat and human) were used. The poloxamers were found to be relatively non-cytotoxic (Fig. 9).

3.9. Effect of nanoparticles on the release of innate immune marker: nitric oxide

Nanoparticles stimulated the release of nitric oxide from dendritic cells (immune cells). The amount of nitric oxide released was highest for F-108 nanoparticles, followed by F-127 nanoparticles, with P-188 nanoparticles producing the least nitric oxide.
4. Discussion

The stability of polymeric nanoparticle-suspensions has been one of the difficult puzzles formulation scientists have had to solve. Nanoparticles are thermodynamically unstable due to the enormous surface free energy they possess. This energy is produced by the increase in surface area observed as the particle size decreases [10]. Surfactants play a crucial role in the stabilization of nanoparticles. The dynamic nature of the interfacial film produced by this group of compounds in solution enables them to reduce the surface tension of the nanoparticles, thereby, inducing stability [11]. Thus, the selection of the proper type and concentration of these surface modifiers, are vital in the fabrication of this delivery system [12,13]. Van Eerdenbrugh et al. demonstrated no correlation between physical drug properties...
and nanosuspension stability using 13 different surfactants [14]. Lee et al. found a correlation between the surface energy of particles, the polymer used and the stability process [15]. These contradictory findings make the selection of the most appropriate stabilizer a difficult task. Unfortunately, there is no formula for stabilizer selection, hence, the preparation processes and the stabilizer system differs from drug to drug [16]. The physical characteristics of nanoparticles are dictated by the interactions between the polymer, surfactants as well as the entrapped drug. The first part of this study investigated the ability of three poloxamers (F-108, F-127 and P-188) in stabilizing dasatinib nanoparticles. F-108 is the most hydrophobic among the three poloxamers and P-188 the least, hence much stronger interaction was expected between F-108 and the hydrophobic polymer matrix, polycaprolactone (PCL) used for the formulation. The average size of nanoparticles prepared with F-108 and F-127 were 182 nm and 184 nm, respectively, with a narrow distribution size range indicated by the low polydispersity index (PDI) value of 0.3 obtained. Just after preparation, P-188 nanoparticles separated out into two distinct phases, demonstrating gross instability. The PDI value of the P-188 formulation was very close to the value “1” (0.8). Poloxamer, P-188, being more hydrophilic may have had less interaction with the polycaprolactone. Haley et al. observed that particles gain some degree of stability as their surface becomes more hydrophilic [17]. As mentioned previously, each poloxamer molecule is composed of a hydrophobic units of poly-propylene oxide (PPO) and two hydrophilic block made of poly-ethylene oxide (PEO). It is reported that, the greater the magnitude of the hydrophilicity exhibited on the nanoparticle surface the greater the steric hindrance and the better the stability [11]. Thus, during the design of the particle, if the poloxamer can be oriented to have the two hydrophilic blocks of PEO on the surface of the particle, a more stable product will be achieved. This, notwithstanding, the hydrophobic part of the surfactant should be able to interact adequately with the matrix to keep the entrapped drug within the particle. As mentioned previously, it was observed that poloxamer, P-188-nanoparticles were highly unstable even though it is the most hydrophilic among the three surfactants. It is stipulated here that there was inadequate interaction between itself and the polycaprolactone used as the matrix, hence, the poor nanoparticle formation and entrapment. A good quality nano-formulation is expected to have a bell shaped size distribution curve with a narrow size range. The amount of surfactant used, had an influence on the size distribution. We observed a broadening of the distribution peak as the surfactant was reduced. At low concentrations of surfactants (formulation with ratio; 2:1), F-127 produced a bimodal distribution (Fig. 1). Majority of the produced particles had an average size of about 202.0 nm, while a few of the particles which are represented by the smaller peak had an average size greater than 1 μm. A similar observation was made by Ecevit et al. who reported that, low surfactant concentrations do not prevent aggregation. The increase in size could have been provoked by the dispersed system, in an attempt to counteract the inefficient surfactant at the nanoparticle surface [18]. Nanoparticle formulations prepared using all three surfactants with the ratio 1:5 (high surfactant content) had more than one population of particles, however, in this case, the distributions were skewed towards smaller particle sizes. The population of particles with average size below 100 nm could be micelles, formed unintended, with subsequent entrapment of drug within them (Fig. 1). Poloxamers, similar to other surfactants are able to form micelles at high concentrations. Thus, it can be concluded that very high and very low surfactant concentrations of the surfactants, lead to low drug entrapment of in the nanoparticles. Nanoparticles in suspension, when left to stand for a period of time, are prone to aggregation due to the increase in Gibbs free energy the
particles possess. After 48 hours of preparation, an increase in average particle size was observed in all the suspensions, thus the amount of surfactant used did not prevent aggregation during storage [1]. A 50% increase in average size was observed in the formulation of nanoparticles prepared with poloxamers F-108 and F-127, 48 hours after production. Suspension of P-188 nanoparticles, had completely separated into two distinct phases and no longer fitted the description of a nanosuspension. Stability of nanosuspensions has been shown to be enhanced by inducing steric hindrance to the surface the particles. Stabilizers, such as chitosan have been used to modify the surface of nanoparticles by increasing the hydrophilicity on their surface [11]. Due to the instability observed in the prepared nano-suspension on standing, chitosan was adsorbed unto the surface of the nanoparticles. Following this action, the charge of the nanoparticles became positive with magnitudes of about 13 to 21 units (Table 3), which was expected, however, size was not affected. To evaluate the ability of chitosan to enhance the stability of the nanosuspension, the colloidal stability study was conducted over a 28-days period. Chitosan-stabilized-nanoparticles maintained a reasonable degree of stability during the study period. Although, aggregation could not be totally halted, about 95% of the nanoparticle population had an average size of $\pm 240$ nm. The graphs in Fig. 2, indicate a reduction in the average size of the nanoparticle after day 21. Polycaprolactone has a low melting point ($60^\circ$C), hence the reduction in size could have been due to softening of the hard polymer matrix in the aqueous medium over time, followed by its erosion. Based on the results obtained thus far, formulations with ratios 1:1 and 1:2 showed much promise and hence the stability and release studies were performed on the formulations prepared with these ratios.

The yield of nanoparticles prepared with pluronic F-108 and F-127 was $\geq 80\%$. Kolliphor, P-188 produced nanoparticles with very low yield (Table 5). The particles produced were precipitating out and sticking to the walls of the container. As mentioned before, P-188 was not able to reduce the surface free energy as the nanoparticles were forming, explaining why aggregation was observed just after the nanoparticles were formed, subsequently, phase separation was observed. The amount of dasatinib entrapped in the PCL matrix was adequate for formulations prepared with F-108 and F-127: above 80% for ratios 1:1, 1:2 and 2:1. In formulations with high surfactant concentrations (ratio 1:5), the percentage of dasatinib entrapped reduced by $\geq 10\%$. This observed phenomenon run across formulations with all three surfactants. Since the surfactant concentration in this ratio was above their CMCs, micelles may have been formed, leading to solubilization of the

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**Fig. 6.** Fluorescence microscopic images showing the internalization of coumarin VI loaded nanoparticles prepared with and without chitosan by MDA-MB-231 and Dendritic cells (phagocytic cells), after incubation with equivalent amount of nanoparticles for 2 hours. Coumarin VI loaded nanoparticles (green dots) can be seen inside the cells after 2 hours of exposure to culture media containing nanoparticles.

**Fig. 7.** Fluorescence microscopic images showing the blebbing of the cell membrane of MDA-MB-231 cells after exposure to coumarin VI loaded nanoparticles prepared with and without chitosan.
drug, which could have been lost during the washing step due to their small size. In the preparation of nanoparticles, the optimum amounts of surface modifiers need to be accurately determined and optimized, as too less a concentration will lead to instability of the colloid and too much of it will solubilize the drug leading to low entrapment efficiency.

In cancer therapy, one of the reasons for entrapping chemotherapy agents in a matrix is to protect normal cells from the effect of these drugs until they reach the site of action. The lower the amount of drug released during the period the delivery system travels to the site of action, the lower the adverse effects patients will experience, which will produce better tolerance. Another advantage of delivering chemotherapeutic agents as nanoparticles is because of the ability of this delivery system to produce a passive targeting effect as mentioned above, that is, by harnessing the enhanced permeability and retention (EPR) effect observed in the tumor microenvironment.

Cells have the ability to uptake micro-, nano-particles by phagocytosis and or pinocytosis. This also been demonstrated in Figs. 6 and 7. It is postulated that the nanoparticles that enter the tumor microenvironment are taken up whole into the cells with subsequent release of the entrapped drug after the matrix is broken down by the action of intracellular enzymes. It is worth mentioning that drug entrapment or encapsulation masks the drug structure, thereby, preventing its recognition by the efflux transporters. From the release profile in Fig. 5, a maximum of about 10% of entrapped drug was released after 5 days. The release of dasatinib was relatively similar between PCL/F-108 matrix and PCL/F127 matrix. PCL/F-108 matrix with ratio 1:2 released about 3% more than the same matrix with ratio 1:1. However, after both formulations were stabilized with chitosan, the release profile of F-108 1:1 + chitosan (ch.), and F-108 1:2 + chitosan (ch.) overlapped indicating a normalization of the processes. It was expected that a coat of chitosan on the nanoparticles will slow down the release, however that was not observed (Fig. 5). Although PCL/P-188 matrix entrapped much less drug than the other two matrices, the rate of release was similar among all three PCL/Poloxamer formulations.

Nanoparticles after administration into the blood stream, have been shown to accumulate in the tumor microenvironment following which they stand a greater chance of being taken up into the cells to produce the desired effect. The nanoparticles are said to be trapped within the tumor microenvironment because upon entry into this space from the blood stream, they are unable to flow back into the blood stream and

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**Fig. 8.** Cellular uptake of coumarin loaded nanoparticles by (A) MDA-MB-231 cells, (B) HCC-70 cells, (C) B16 F10, (D) WB rat epithelial cells and (E) DC 2.4 cells by flow cytometry. A histogram comparing the intensities between the various groups. There was a significant difference between the dye solution and all the nanoparticles. A significant difference in uptake was similarly, observed between nanoparticles with and without chitosan. (*p < 0.05, **p < 0.01, ***p < 0.005).
Table 5

Percentage of dasatinib entrapped in nanoparticles stabilized with poloxamer and chitosan. Entrapment efficiency was > 75% for nanoparticles prepared with F-108 and F-127. Kolliphor P-188 entrapped < 70% of Dasatinib in all ratios used. Its yield was similarly low.

<table>
<thead>
<tr>
<th>Polymer/Surfactant Ratio</th>
<th>Poloxamer F-108</th>
<th>Poloxamer F-127</th>
<th>Kolliphor P-188</th>
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<tr>
<td></td>
<td>Yield (%)</td>
<td>Entrapment (%)</td>
<td>Yield (%)</td>
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<tr>
<td>1:1</td>
<td>86.58</td>
<td>88.97</td>
<td>88.68</td>
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<td>1:2</td>
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<tr>
<td>1:5</td>
<td>87.50</td>
<td>66.37</td>
<td>86.18</td>
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Additionally, the lymphatic drainage in solid tumors is usually not well developed. Due to this fact, it is important to use biocompatible and biodegradable materials like PCL in the manufacturing of nanoparticles for tumor targeting.

Five cell lines were used to study the cellular uptake of the formulations by flow cytometry. Three of them were cancer cell lines, MDA-MB-231, HCC-70 (breast cancer cell lines) and B16 F10 (melanoma cell line). A phagocytic cell line DC 2.4 (dendritic cells) was the fourth and rat epithelial liver cells (WB cells) representing normal cells the fifth. The uptake of nanoparticle was 1.5 times greater than solution in all the cell lines. Among the three poloxamers, uptake of P-188 and F-108 nanoparticles were relatively, higher than F-127 nanoparticles. Addition of chitosan to the formulation greatly enhanced cellular uptake. The positive charge inferred on the particle by the chitosan contributed to the increase in uptake. The surface of most cells are negatively charged, hence the particles adhere to the cell surface by electrostatic interaction followed by their subsequent engulfment into the cell, as revealed by the fluorescence microscopic images in Fig. 6. Poloxamers have been reported to have influence on the cell membrane of cancer cells. This phenomenon is clearly depicted in the microscopic images. The presence of the poloxamer led to blebbing of cell membrane of the cancer cells used (Fig. 7). This observation was not as obvious in the phagocytic (DC 2.4) cell line. Additionally, less blebbing was observed in the cells treated with chitosan coated nanoparticles. It is postulated that the chitosan may have masked the hydrophilic wings of the poloxamer on the surface of the nanoparticles.

After more than three decades of research, toxicity of nanoparticles still remains an issue. Mostly, the organs of interest are the kidneys, liver, heart, and brain. In this study we investigated the cytotoxicity of the above mentioned poloxamers on murine kidney, rat liver and human breast cancer cell lines. To determine the effect of the excipients used, cells were treated with empty (blank) nanoparticles and viability determined. After 24 hours of exposure, the viability of all the cell types were greater than 80% indicating that the particles, formulated with the poloxamers were relative non-cytotoxic (Fig. 9). Cells exposed to nanoparticle concentration of 1000 μg/mL may have suffocated as the particle density was very high. Interestingly, an increase in viability above the control (cells only) was observed in some instances. This observation was pronounced in MDCK kidney cell line and MDA-MB-231 breast cancer cell line. Chitosan coated nanoparticles however, maintained the viability of the cells just as observed in the control groups. The chitosan may have masked the effect of the poloxamers on cell viability. Samith D. Vicente et al. observed increase the viability of neuroblastoma cells cultured with P-188 treated media compared to non-treated cells. Similarly, Sengupta Aritra et al. observed in increase in cell viability after exposing DU145 prostate cells to laser pulses in the presence of nanoparticles and poloxamer F-127 treatment.

The resemblance of nanoparticles to microorganisms make them vulnerable and prone to be attacked by the immune cells. Nitric oxide is released by innate immune cells following recognition and phagocytosis of antigens. Opsonization of nanoparticles by immune cells tends to decrease the concentration of drug intended to reach the target site. Nanoparticles inducing the least immune responses are preferred in cancer therapy. A significant difference in nitric oxide release was observed from dendritic cells after exposure to nanoparticles prepared with the three surfactants. P-188 nanoparticles released the least nitric oxide, thus, it was less antigenic than F-108 and F-127 (Fig. 10). The observed stimulation of the immune cells is postulated to be the result of the hydrophilic part of the poloxamers: poly-ethylene oxide units, which orients to the outer part of the nanoparticle.

5. Conclusion

Stability of the nanoparticles increased with an increase in PEO units of poloxamer surfactant. Addition of chitosan to the formulation, increased the stability of PCL/poloxamer nanoparticles. Uptake of cationic nanoparticles were significantly higher than anionic nanoparticles in all the cell lines studied. Polycaprolactone/poloxamer nanoparticles did not demonstrate cytotoxicity in the cell lines studied. On the contrary, viability of cells in the anionic nanoparticles were slightly higher than that of the control group, while the cationic nanoparticles (chitosan coated) had viabilities similar to the control. Among the three surfactants studied, poloxamer F-127 nanoparticles were more antigenic, followed by poloxamer F-108 and then P-188. Chitosan increased the antigenicity of all the nanoparticles in a similar pattern. Thus, poloxamer and chitosan do affect cellular functions. Chitosan will be a useful excipient to consider in formulations, where immunogenicity need to be enhanced: like vaccines. On the contrary, its use should be limited in formulations where increase in immunogenicity is not desirable. The poloxamer surfactants used in this work were relatively non-cytotoxic, instead, they had the tendency to increase the viability of certain cell types.

Conflicts of interest

We wish to indicate that, there are no known conflicts of interest associated with this publication. All named authors have read and approved of this manuscript and confirm that there has been no significant financial support for this work that could have influenced the results of this project.

References


