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# Development of Biodegradable PLGA Nanoparticles Surface Engineered with Hyaluronic Acid for Targeted Delivery of Paclitaxel to Triple Negative Breast Cancer Cells

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## ABSTRACT

This study aimed at development of poly (lactic-co-glycolic acid ) (PLGA) nanoparticles embedded with paclitaxel and coated with hyaluronic acid (HA-PTX-PLGA) to actively target the drug to a triple negative breast cancer cells. Nanoparticles were successfully fabricated using a modified oil-in-water emulsion method. The effect of various formulations parameters on the physicochemical proprties of the nanoparticles were investigated. SEM imaging confirmed the spherical shape and nano-scale size of the nanoparticles. A sustained drug release profile was obtained and enhanced PTX cytotoxicity was observed when MDA-MB-231 cells were incubated with the HA-PTX-PLGA formulation compared to cells incubated with the non-HA coated nanoparticles. Moreover, HA-PLGA nanoparticles exhibited improved cellular uptake, based on a possible receptor mediated endocytosis due to interaction of HA with CD44 receptors when compared to non-coated PLGA nanoparticles. The non-haemolytic potential of the nanoparticles indicated the suitability of the developed formulation for intravenous administration.

Keywords: PLGA nanoparticles; Hayluronic acid; Paclitaxel; Functionalized nanoaprticles; Degradation studies; cytotoxicity.

## 1. INTRODUCTION

Recently, it has become very clear that the commonly-used methods of cancer therapy aren't always successful, resulting in reduced quality of life for patients as well as poor prognosis [1]. Nanotechnology-based drug-delivery systems have given unprecendented opportunities for cancer treatment. These nano-carriers have shown better and more favourable pharmacokinetics, tumour tissue accumulation via passive targeting (EPR effects – enhanced permeation and retention), and/or active targeting and prolonged systemic circulation time [2]. Nanoparticles (NPs) can be used to deliver paclitaxel (PTX), an extract of the *Taxus brevifolia* tree, directly to cancer cells. PTX has been widely used for chemotherapy of several cancers such as ovarian, colon, bladder, Kaposi's sarcoma, large and small cell lung cancer and breast cancer [3]. The two major issues of intravenous monotherapy with PTX is that- 1. it is a substrate for P-glycoprotein, an efflux pump in cancer cells, therefore it is easily removed by cells 2. it is associated with very serious and life-threatening side effects such as anaemia, super infections, hypersensitivity and myelosuppresion [4]. PLGA is synthetic biodegradable copolymer which has been widely used to formulate NPs that can encapsulate hydrophobic drugs such as paclitaxel [5]. PLGA NPs can passively be taken up by tumours, but cellular uptake may be limited because of lack of targeting. Active targeting, can be achieved by modifying the surface of the PLGA NPs with targeting moieties such as antibodies, nucleotides or peptide ligands. The downfall of using these targeting moieties is the potential to activate an immune response and impaired binding affinity after conjugation [6]. Hyaluronic acid (HA) is desirable as a targeting moiety for PLGA NPs because it is a naturally-ocurring polysaccharide composed of N-acetyl-D-glucosame and D-glucuronic, a substrate that binds specifically to CD44 receptors, which are overexpressed on some tumour types including triple negative breast cancers (TNBC). Advantages of using HA are that it is non-toxic, biocompatible and biodegradable, and non-immunogenic [7]. Furthermore, HA binds specifically to CD44 receptors resulting in the receptor-mediated endocytosis of PLGA NPs [8]. Another advantage of using HA as a targeting moiety for PLGA NPs is its ability to increase circulation time by providing the nanoparticles with necessary characteristics to prevent their uptake by the reticuloendothelial system (RES) [9].

The aim of this study was to improve the chemotherapeutic efficacy of paclitaxel for the treatment of breast cancer by asociating it with HA-PLGA nanopartilces. In the present study PTX-HA-PLGA NPs were synthesised using o/w emulsion technique. The effect of various formulation parameters on the characteristics of the nanoparticles were studied in order to select nanoparticles with dsirable physicochemical proprties. The characteristics of blank or drug-loaded, HA-coated and non-coated PLGA NPs were evaluated for degradation, CD44 receptor-mediated endocytosis, cytotoxicity and cell viability. Coumarin-6 loaded HA-coated and non-coated PLGA NPs were synthesised to evaluate the behaviour of the NPs on cells *in vitro*; to elucidate the targeting and uptake of the various NP formulations by MDA-MB-231 breast cancer cell lines.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Sodium hyaluronate (MW 150KDa – 1.8MDa) was purchased from Lifecore Biomedical (New Zealand). 50:50 PLGA polymer (MW 30,000 – 60,000), Tween 40, cetrimide, polyvinyl alcohol (MW 89,000 – 98,000), and coumarin-6 were purchased from Sigma-Aldrich® (Australia), paclitaxel was purchased from Shaanxi Jintai Biological Engineering Co. Ltd (China), dichloromethane (DCM) was purchased from Schalau Labs (New Zealand). Deionized (DI) water was obtained from Milli-Q system (Millipore, Saint Quentin-en-Yvelines, France).

### 2.2 Methods

#### 2.2.1 Nanoparticle synthesis and characterisation

PLGA nanoparticles were synthesised by o/w emulsion technique. Briefly, 50:50 PLGA copolymer was dissolved in dichloromethane (DCM) with or without PTX (100 mg) or coumarin-6. The solution was added to the aqueous phase composed of 2% w/v PVA and Tween 40, sonicated for 4 minutes, and evaporated at room temperature for 4 hours. Positively-charged nanoparticles were created by the addition of cetrimide to the aqueous phase. Once the DCM had completely evaporated, the surface charge was modified by the addition of negatively-charged sodium hyaluronate. The mean diameter of the NPs and their zeta potential were determined by light scattering using a ZetaSizer NanoZS analyzer (Malvern Instrument, France). The analyses were perfomed in DI water containing 10% v/v of the NPs suspension.

The effect of various formulation parameters on the properties of the nanoparticles were evaluated. The parameters variants were as follows:

1. PVA concentration – 0.1, 0.5 and 2% w/v;
2. Sonication time – 1, 3 and 4 minutes;
3. Polymer concentration – 50, 100 and 150 mg;
4. Polymer type – 50:50 and 75:25 PLGA, and polycaprolactone;
5. Hyaluronic acid concentration – 0.05, 0.1 and 0.5 % w/v

#### 2.2.2 Encapsulation efficiency of NPs

Determination of PTX entrapment efficiency (EE%) was obtained by high performance liquid chromatographic (HPLC) analysis. NPs containing PTX were disrupted in methanol, centrifuged and the supernatant collected and PTX measured. The amount of PTX was calculated using the equation obtained from the calibration curve, and EE calculated according to the following equation:

$$EE\ \% = \frac{Total\ amount\ of\ PTX - Amount\ of\ free\ PTX}{Total\ amount\ of\ PTX}\ x100$$

$$DL\ \% = \frac{Weight\ of\ PTX}{Total\ weight\ of\ PTX\ in\ the\ formulation}\ x100$$

The HPLC method for PTX quantification consisted of a quaternary pump system (Agilent 1260 Infinity Quaternary LC HPLC system) with a reverse phase C18 column (5 µm, 4.6 mm x 150 mm and Cosmosil® column guard 4.6mm x 10 mm). A mobile phase consisting of water (30%) :acetonitrile (60%) :methanol (10%), injected with a volume of 20 µL and a flow rate of 1 mL/min at 35 °C. The UV detector was set to monitor at 230 nm and the HPLC was validated for paclitaxel with $R^2 = 0.9978$.

The encapsulation efficiency of coumarin-6 loaded PLGA NPs was carried out by disrupting the NPs containing coumarin-6 in methanol and acetone. Unloaded coumarin-6 was removed by filtration through a syringe filter (0.45 mm pore size, Millipore, USA). The content of coumarin-6 in the nanoparticles was calculated according to a coumarin-6 calibration curve constructed by spectroflurometry.

**2.2.4 *In vitro* release profile**

The *in vitro* release study was carried out by adding 33 mg lyophilized HA-coated and non-coated PTX-PLGA nanoparticles to PBS containing Tween 40, and placed in a water bath shaker at 37 ± 0.5 °C. Samples of 500 µL were collected at different time intervals and replaced with 500 µL PBS, pH 7.4 with 0.5% w/v Tween 40. The samples were centrifuged at 10,000 RPM for 5 minutes, supernatant collected and drug content measured by the previously-validated HPLC method.

**2.2.5 Nanoparticles degrataion studies:**

**2.2.5.a. Lactic Acid Calibration Curve**

In order to measure the degradation of drug-loaded and drug-free, coated and non-coated PLGA NPs, a method for measuring lactic acid was developed. As PLGA degrades it releases lactic acid, and a standard curve assay was designed to quantify this. Concentrations of lactic acid from 0 – 18ug/mL were made up by dissolving various amounts of lactic acid to test tubes containing deionised water or phosphate-buffered saline solution (PBS) pH 7.4, making a total volume of to 500 µL. To these, 3 mL concentrated $H_2SO_4$ was added and vortexed for 20 seconds. The test tubes were incubated at 95 – 100°C for 10 minutes in a water bath shaker (Grant SubAqua 5 Plus). Next, samples were cooled to below 20 °C using an iced water bath. Followed by an addition of 50 µL of 10 % (w/v) $CuSO_4$-$5H_2O$ in $ddH_2O$ and 100 µL 1.5 % 4-phenylphenol in 95% ethanol, mixed well on a vortex and incubated in a 37 °C water bath for 30 minutes. Samples allowed to cool to room temperature and absorbance was read at 570 nm using a (Libra S32 PC) spectrophotometer.

**2.2.5.b Degradation study**

150 mg of drug-free and drug-loaded, lyophilized NPs were resuspended in dispersing media (20 mL PBS buffer, pH 7.4) and incubated in a shaking water bath at 37 °C, 100 RPM. At determined time periods (Day 1, 5, 10, 15, 18, 20, 22, 24, 26, 28, 30, 35, 40, 45 and 50) samples were collecetd to analyze pH, particle size, zeta potential, lactic acid content and drug content. The samples collected for lactic acid measurement were diluted in a ratio of 1:10 (sample: PBS buffer), where 500 µL was taken for the lactic acid assay, previously validated. The pH was measured using a Sartorius pH meter.

### 2.2.6 Red Blood Cell (RBC) Haemolysis Study

Human blood samples (2.5 mL) were collect into 5 mL EDTA-containing blood tubes by venular puncture. RBCs were collect by centrifugation (3000 rpm, 15 minutes at 4 $^0$C), the plasma removed, and ~100 µL RBC pellet resuspended in 1 mL cold PBS buffer. The RBCs dispersion (0.1 mL) was mixed with HA-coated PLGA NPs and non-coated PLGA NPs suspensions at concentrations of 0.1 µg/mL, 1 µg/mL, 5 µg/mL, 10 µg/mL, and 15 µg/mL NPs in 2 mL PBS buffer. The negative control consisted of RBCs suspended in PBS only and a positive control included PBS with 0.1 % v/v Triton-X100 (to lyse cells). The samples were incubated in a shaking water-bath for 1 hours. The samples were then centrifuged to pellet any intact cells, the supernatant collected and analysed for haemoglobin content by measuring the absorbance at 540 nm by spectrophotometry. The percentage haemolysis was then calculated by subtracting the absorbances of the sample from the negative control and then dividing by the difference in absorbance between the positive and negative controls-

$$\%\ RBC\ haemolysis\ = \frac{ABSsample - ABSnegative\ control}{ABSpositive\ control - ABSnegative\ control} x\ 100$$

### 2.2.7 Cell culture

Human triple negative breast cancer cells (MDA-MB-231; purchased from ATCC) were cultured in RPMI medium (Thermofisher) supplemented with 5 % (v/v) foetal bovine serum and 1 % (v/v) PSG (penicillin, L-glutamine and streptomycin) antibiotics in a humidified incubator at 37° C in 5 % $CO_2$.

### 2.2.8 *In vitro* toxicity of paclitaxel loaded PLGA NPs against MDA-MB-231 cells

#### 2.2.5.1. Cell viability and proliferation studies

On day 1, 2,500 MDA-MB-231 cells in 100 µL RPMI with 5 % v/v FBS were seeded per well into 96-well plates, and, incubated for 24 h at 37° C. On day 2, drug-loaded and drug-free, HA-coated and non-coated PLGA NPs, or also non-encapsulated PTX solution were added to the cells in a two-fold dilution series of PTX from 80 nM to 0.625 nM and cells incubated at at 37° C for 48 h. On day 4, the media was removed from the plates which were then stored at -80° C overnight. The DNA content, as a surrogate measure for cell number, was then determined using a SYBR Green I-based fluorimetric assay as previously described [10]. In brief, the plates were thawed to room temperature then 100 µL SYBR® Green I in lysis buffer (10 mM Tris-HCl ph 8.0, 2,5 mM EDTA, 1 % (v/v) Triton X-100 with 1:4000 (v/v) SYBR Green I (Thermofisher)) added to each well. Plates were incubated overnight at 4° C in the dark and then the fluorescence signal measured at 485 nm/535 nm in spectrophotometer. The effect of the drug, drug-free, drug-loaded, HA-coated and non-coated NPs on the cells were measured by calculating the percentage cell viability relative to the cells receiving no drug. The results were plotted using Microsoft Excel, and the $IC_{50}$ determined.

#### 2.2.5.2 Cellular uptake studies

MDA-MB-231 cells were seeded at 2.5 x $10^5$ cells per plastic well on glass coverslips (35 mm round) and incubated at 37° C incubator containing 5 % $CO_2$. After 12 h the medium was was replaced with 1

mL of fresh medium containing: free coumarin-6, HA-coated and non-coated coumarin-6 loaded NPs, which each contained 25 ng/mL of coumarin-6. The cells were incubated at 37° C for 3 h, washed three times with ice-cold PBS and fixed with 4 % (v/v) formaldehyde solution for 20 minutes. After removing the formaldehyde solution, the cells were further washed with ice-cold PBS and treated with Triton X-100 (0.5 % w/w in DI) for DAPI staining and observed using a confocal laser scanning microscopy (Zeiss LSM 710 Inverted Confocal Microscope).

## 3. RESULTS AND DISCUSSION

### 3.1 Nanoparticle preparation and characterisation

PLGA nanoparticles have received a lot of attention and interest lately due to their superior properties over other polymeric nanoparticles [11]. In the research presented here, we have shown the successful synthesis of PLGA NPs coated with HA and containing PTX (HA-PTX-PLGA). An oil in water (o/w) emulsion technique was used because it is easy to carry out and has produced good quality PLGA nanoparticles as previously reported [11].

We evaluated polyvinyl alcohol (PVA) in the production of our PLGA NPs because it is a surfactant commonly used in the development of polymeric nanoparticles [12]. The impact of 0.1% w/v, 0.5% w/v and 2% w/v PVA on PLGA NPs was investigated in the present study. The results shown in Table 1 reveal that 0.1% w/v was insufficient for the formation of PLGA NP capable of effectively encapsulating PTX, and the formulation precipitated. In contrast, 0.5% w/v and 2% w/v PVA produced nanoparticles of size 225.1±0.43 nm and 207.5±0.35 nm respectively, and the optimal PVA concentration was determined from the polydispersity index (0.429±0.43 and 0.207±0.00) and zeta-potential (-71.2±1.5 and -34.9±0.06 mV respectively). The polydispersity index PDI value indicates the nanoparticle size distribution in a suspension, where 1 is the highest value and 0 is the lowest; the higher the PDI value indicates the distribution of nanoparticles with a variable size range, which culminates in the formation of aggregates indicating poor homogeneity and low stability of the NP suspension [12]. Low PDI (< 0.4) is desirable because it suggests narrow size distribution, which is important to achieve the EPR effect, and plays an important role in tissue accumulation and renal clearance [13,14]. We observed that as the PVA concentration increased, the size and PDI of the nanoparticles decreased, making the formulation more stable.

Another factor that plays an important role in nanotoxicity, distribution and uptake is surface charge of the NPs. This plays a critical role in cellular uptake via a two-step process: 1. NP binding with the cell membrane and 2. Internalization. The surface charge of the nanoparticles (zeta-charge or zeta-potential) is an indication of the stability of the suspension [15]. Although positively-charged nanoparticles have shown better internalization *in vitro* they can easily bind other cells in the body, including endothelial cells, before reaching the target tumour cells, thus it is empirical to keep the nanoparticles as neutral or anionic as possible [16]. Furthermore, positively-charged nanoparticles have shown a dose-dependent haemolytic activity and cytotoxicity against various cell lines *in vitro* whereas negatively-charged ones did not [17]. In addition, other studies have shown that neutral and negatively-charged nanoparticles have increased circulation half-life and have less clearance [20]. Our study also suggests that the zeta-potential of stable PLGA NP formulations were around -30±5mV [18], further confirming the stability of this formulation.

The size of PLGA NPs can be directly affected by sonication time. The results presented here showed that when sonicated at the same power (100%, 70W) and for different durations (1, 3 and 4 minutes), the particle size decreased accordingly. This is because the magnitude or/and exposure time to shear

stress will result in reduced nanoparticle size [26]. The amount of polymer also has an impact on particle size (more polymer = increase in size), drug loading and encapsulation efficiency. As the concentration of polymer increases, the viscosity of the medium increases proportionally and efficiency of stirring decreases leading to an increase in the size of the nanoparticles. Smaller nanoparticles are desired because nanoparticles <300 nm are necessary to achieve the EPR effect and passive targeting [27, 28]. The amount and the type of polymer also have an impact on the nanoparticle size. The present study investigated the effect of 75:25 PLGA and polycaprolactone (PCL) along 50:50 PLGA on the quality of the nanoparticles as well. PCL precipitated during the evaporation stage and 75:25 PLGA formulations produced nanoparticles of >500 nm, which is too big for the aims of this study.

Next, methods to attain the ideal encapsulation efficiency (EE %) using various formulations of PLGA NPs were evaluated. Table 1 shows that the formulation that produced the highest encapsulation efficiency (90.8 ± 1.5) contained 100 mg of 50:50 PLGA and 2 % w/v PVA. Sonication time was also investigated because this is believed to play an important role in EE% by assisting in the formation of the NPs. Our results showed that both EE % and DL % did improve with increased sonication (Table 1).

The concentration of HA was also investigated (Table 1), and this displayed a concentration-dependent impact on the size of the NP, and the PDI value. In order to successfully adhere HA onto the nanoparticle surface, cetrimide (CTAB) was used to neutralise the negative charge. CTAB is a surfactant by nature and this also decreased the nanoparticle size. Upon addition of CTAB to the external phase, the average NP size was 163.4 ± 5.51 nm, and the average zeta potential 35.2 ± 9.20 mV. As HA is a highly negatively-charged proteoglycan, thus upon drop-wise addition to the solution it increases the size of the NPs in a concentration dependent manner, as shown on Table 1. Three concentrations of HA were investigated in the present study- 0.05 % w/v, 0.5 % w/v and 1 % w/v. The, lowest concentration produced NPs that did not exceed 250 nm, were negatively charged and with an encapsulation efficiency and drug loading >84. Whereas the highest HA concentration (0.5%) produced too large particle.

SEM image (Figures 1. A) of the nanoparticles show that they are in a nanometer size scale. The particles appear to be smooth, homogenous and spherical in shape. The NPs have moderate uniformity as expected from the PDI and zeta charge values that were previously acquired. All NPs were discrete entities that did not appear to aggregate after being lyophilized. PTX crystals can be observed on the surface of the PTX-loaded NPs, and this may play a role in the release of the drug.

### 3.2 *IN VITRO* RELEASE STUDY

To evaluate the release of the PTX loaded in PLGA NPs, an *in vitro* release study was performed. The results of this study showed that PTX was released from HA-coated PLGA NPs faster than from non-coated PLGA NPs (Figure 1.B). Approximately 65 % of the cumulative drug release from HA-PLGA NPs occurred in the first 24 hours, whereas for non-coated PLGA NPs the same was achieved after approximately 72 hours (Figure 1.B). This supports the notion that HA creates a porous surface

on the NPs, contributing to the hydrolysis of the PLGA by increasing the water absorption. Our results showed that both formulations had a biphasic release profile, characterised by an initial rapid release during the first 24 hours, followed by a slower and continuous release out to 120 hours. The high initial burst observed in both formulations may be attributed to the release of PTX crystals that were adhered to the surface of the NPs, as seen in Figure 1.A.

### 3.3 NANOPARTICLE DEGRADATION AND STABILITY STUDY

PTX-loaded and non-loaded PLGA nanoparticles were incubated in phosphate buffer at 37 °C for 50 days, and samples were analysed at different time points for lactic acid build up, change in pH, changes in size, PDI and charge, and drug content. The effect of PTX was studied to analyse the role that the drug has on the NPs degradation. We observed that the rate of PLGA NPs degradation was directly related to the concentration of lactic acid in the solution (Figure 2.A). The results showed that the lactic acid concentration began to increase after 10 days and plateaued after 30 days. The concentration of lactic acid release after polymer degradation for both PTX-PLGA and HA-PLGA NPs was also measured (Figure 2.B). It is possible, judging from the graphs, that PTX played a role in the degradation of the nanoparticles, as the concentration of lactic acid from PLGA-only NPs plateaued at ≈85 μg/mL, but the PTX-loaded NPs continued to degrade. Presence of PTX in nanoparticles may have increased the porosity of PLGA nanoparticles, contributing for more water penetration from the aqueous medium [19, 20]. The lactic acid build up observed in HA-coated PTX-loaded NPs followed a similar profile to the non-coated PTX-loaded NPs. However, our data suggests that HA-coated NPs seem to release lactic acid at the same rate as the non-coated NPs, but eventually release more lactic acid suggesting they degrade faster (Figure 2.B). One explanation for this is that HA contributes to the acidity of the environment which aids in the degradation of the polymer.

Next we analysed the pH of PTX-PLGA and HA-PLGA NP suspensions over time. These results showed that the pH was essentially stable at pH 7.4 for at least 20 days and then began to drop sharply from pH 7.4 to pH 3 by Day 30 (Figure 3.A). Explanations for the sudden decrease in pH of the medium may be attributed to an outflux of acidic PLGA oligomers, release of lactic acid or an enhanced proton exchange between polymer matrix and PBS buffer ions.

The changes in NP size were measured over a period of 22 days. The results (data not shown) revealed that the size of both drug-loaded and drug-free nanoparticles increased over time, as the nanoparticle began to degrade (hydrolyse) and/or break down (erode). PLGA degradation is thought to occur due to the autocatalysis of the degradation reaction in the bulk of the polymers [21]. When hydrolysis occurs in the polymer, during the early stages of the degradation process, lower molecular weight polymer chains with carboxylic end groups, namely the degradation product, remains entrapped inside the polymer matrices [22]. The degradation product increases the acidity of the environment due to an increase of terminal acid groups. Consequently, further autocatalysis of the PLGA degradation occurs, hence the fast drop in pH after 15 days. This reflects on the impact of hydrolysis on PTX release (Figure 3.B), as described above previously explained. Moreover when hydrolysis occurs on the NPs surface, the degradation product diffuses out easier and this surface layer contains a relatively large amount of buffer solution which results in the neutralisation of terminal

acid groups formed. Combined, these two effects result in an environment much less acidic. As a result there is significantly less autocatalysis reaction happening, culminating in the plateau of the lactic acid concentration and pH change indicating the complete degradation of the nanoparticles.

A relationship between drug release and the degradation of the NP is apparent when comparing Figures 1.B and 3.B. These show that as the concentration of lactic acid increases (indicating polymer degradation), the concentration of PTX also increases, confirming both drug release and NPs degradation. This type of drug release and degradation profile desired *in vivo* because it confirms that within the system, the polymer biodegrades into lactic acid (and glycolic acid), which can be up taken by the cells and disposed of accordingly, while the drug is released over a sustained period of time.

### 3.4 RBC COMPATIBILITY STUDIES

In the design of NP systems intended for administration via intravenous injection, it is empirical to investigate the haemolytic property of a formulation. The results shown in Figure 4.A, indicate that both formulations, HA-coated and non-coated NPs were able to induce RBC lysis, although the effect was dependent on the concentration of NP tested. At the lowest concentrations, HA-coated PLGA NPs appeared to cause less haemolysis than non-coated NPs consistently at lower concentrations 0.1 – 5mg/mL indicating that HA was able to prevent haemolysis at lower concentrations of nanoparticles, but the opposite was true at the highest concentration tested (Figure 4.A). Next we investigated the potential of both HA-coated and non-coated NPs to cause RBCs aggregation. At the highest concentration (15 mg/mL) the level of RBCs aggregation is not pronounced, as no obvious clumps of cells is observed (Figure 4.B).

### 3.5 CELL VIABILITY STUDIES

It is important to carry out cell viability studies during drug carrier formulation development to determine the safety, potential toxicity and efficacy of the formulation against specific cell lines. In order to determine the potential effects of both drug free and drug loaded NPs on cell viability, HA-coated and non-coated PLGA NPs were tested in an in vitro assay using MDA-MB-231 cells. We then determined the IC50 for the various PTX-containing NP formulations and also for unencapsulated PTX (PTX solution).

The results showed that the IC50 for MDA-MB-231 cells incubated with PTX solution for 72 hours was between ~5.5 and ~5 nM (Figure 5). The cytotoxicity of empty nanoparticles (HA-PLGA and PLGA) was also evaluated by incubation of MDA-MB-231 cells at previously described concentrations. This showed that the blank NPs were not cytotoxic to these tumour cells (Figure 5). Analysis of the cells which were incubated with PTX-PLGA NPs showed a dose-dependent cytotoxicity (Figure 6.A). The IC50 of PTX-PLGA NPs was ~5 nM, which indicated that the NPs successfully delivered the drug load to the nucleus, producing an IC50 similar to that of PTX alone. However, incubation of cells with a dose range of HA-PTX-PLGA NPs showed a decreased IC50. Figure 6.B shows the cell viability results for HA-PTX-PLGA NPs where the IC50 was ~3 nM, which was lower than all other formulations, in addition to PTX solution. This suggests there was greater cellular uptake of HA-PTX-

PLGA NPs, possibly due to the interactions between HA and the CD44 receptors overexpressed by this particular cell line, which increased the intracellular concentration of PTX.

### 3.6 CELLULAR UPTAKE STUDIES & CONFOCAL MICROSCOPY

To investigate the cellular uptake of HA-coated and non-coated PLGA NPs, coumarin-6 loaded NPs were synthesised and the coumarin-6 encapsulation efficiency of these formulations were measured and were found to be (97.50 ± 1.98 for HA-PLGA NPs and 98.34 ± 0.66 for PLGA NPS. In addition to coumarin-6, DAPI, a nucleic acid dye was also utilised in this experiment to stain DNA.

Receptor-mediated endocytosis has been identified as the major uptake mechanism by which cells take up HA-coated NPs through interactions between HA and the cell surface CD44 receptors . As shown in Figures 7 A and B, coumarin-6 was observed to be intracellular following incubation of MDA-MB-231 cells with both formulations of NPs, suggesting these NPs were taken up by the cells via endocytosis. We expected the uptake of HA-coated NPs would be higher than the non-coated NPs, and indeed the fluorescence of HA-coumarin-6 PLGA NPs was slightly stronger than the non-coated NPs (Figure 7 A&B). However both formulations displayed good uptake. Figure 7.B also shows blue fluorescence around the nucleus, and that is not to be mistaken by NPs as DAPI, as it also stains ribonucleic acid (RNA) which is believed to be present in the organelles around the nucleus of those specific cells at the time of imaging.

## 4. CONCLUSION

In conclusion, a stable HA-PTX-PLGA NP formulation was successfully developed and tested *in vitro*, and demonstrated great potential to decrease the $IC_{50}$ of PTX on triple negative breast cancer cells. Future work should include testing of this formulation on further CD44+ and CD44- cancer cell lines (or lines with variable CD44 levels) to confirm the importance of the HA-modified NPs for efficient delivery of drug load into cells. These studies should include also testing of the HA-PTX-PLGA NP formulations on experimental animals to determine their therapeutic efficacy *in vivo.*

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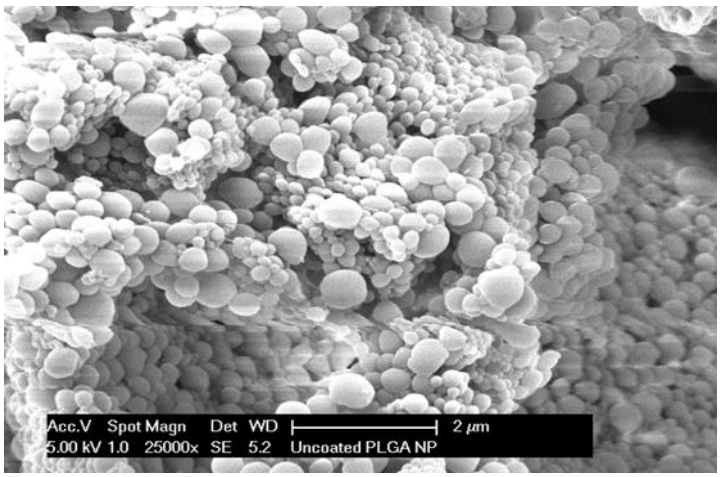



Figure 1. A SEM photomicrograph of PTX-PLGA NPs.

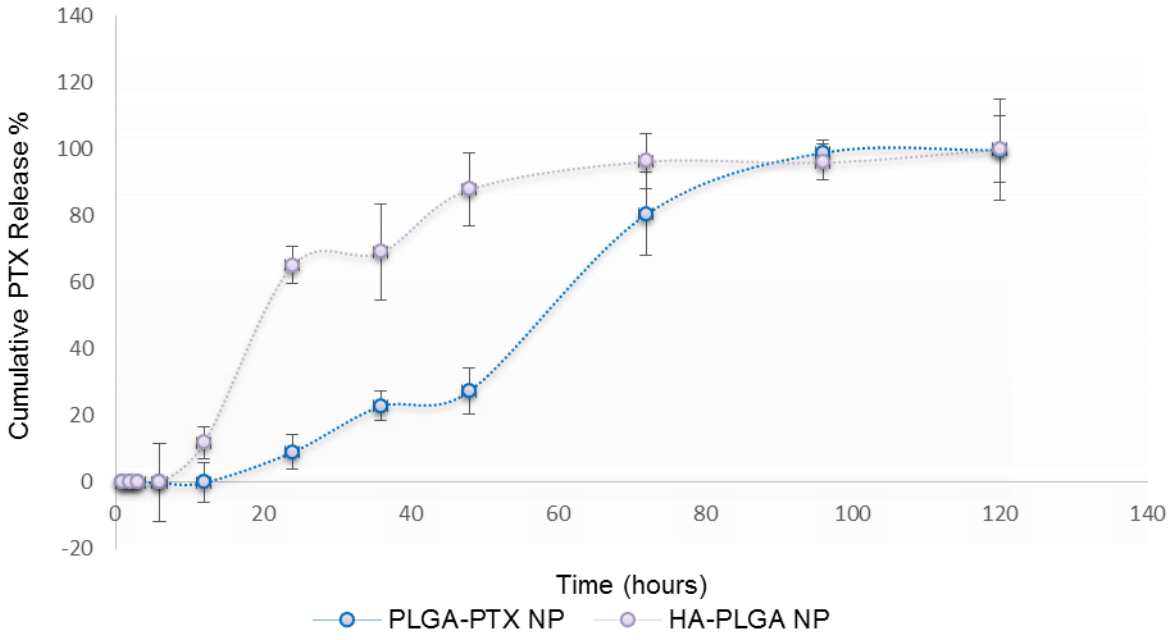
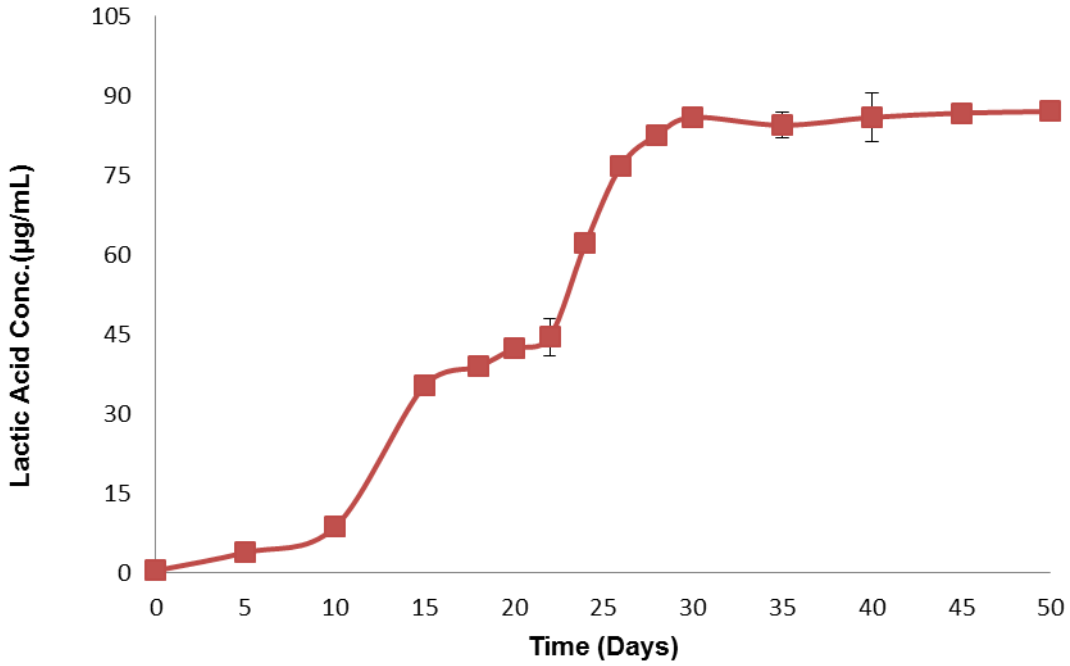



Figure 1.B. Cumulative release of PTX from HA-PLGA and PLGA NPs *in vitro*.

**(A)**




**(B)**

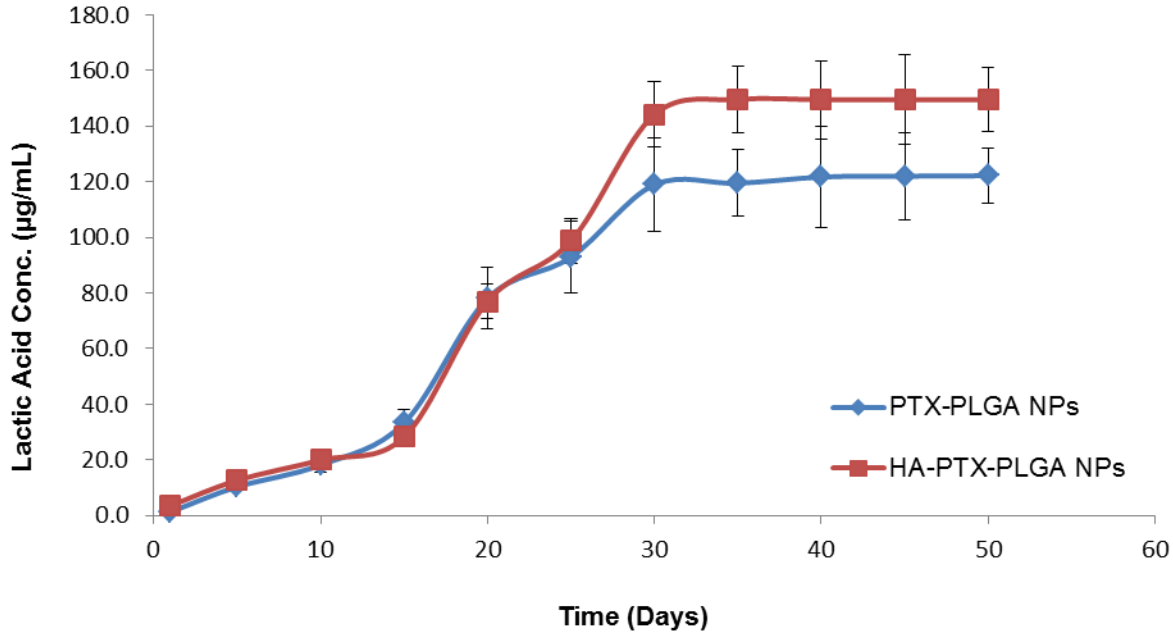



Figure 2. The amount of lactic acid released/produced over time for (A) Drug free PLGA and (B) HA-coated and non-coated PTX-PLGA NPs. The amount of lactic acid in the NP solution was measured over a period of 50 days. Data is represented as mean ± standard deviation (n=3).

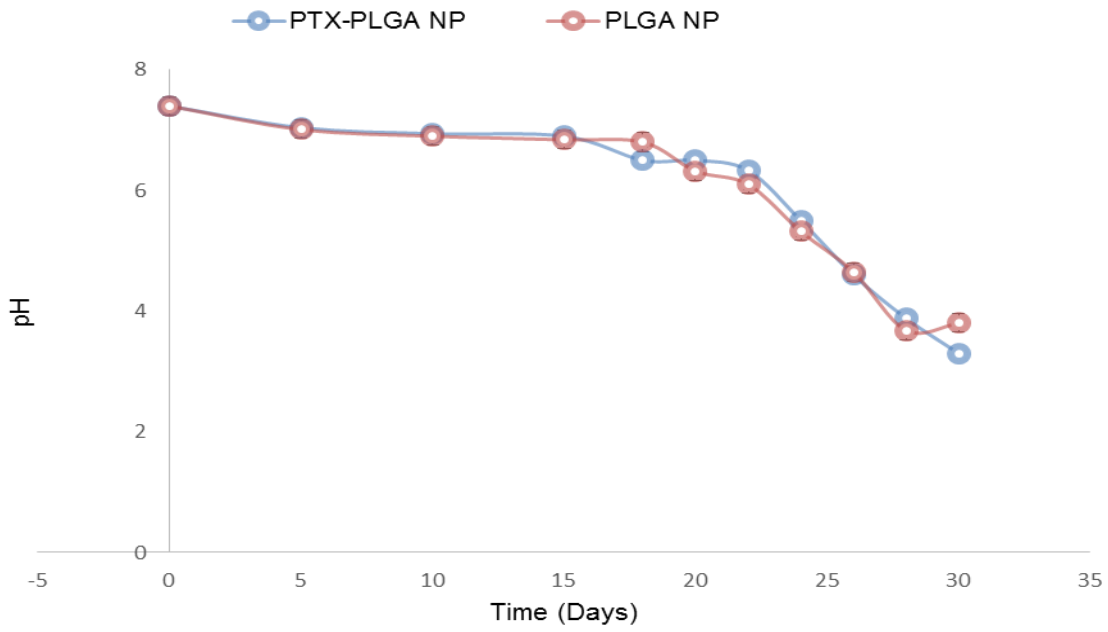



Figure 3.A Measurement of the pH of PTX-free and PTX-loaded NP suspensions over 35 days. Data is represented as mean (n = 3) ± standard deviation.

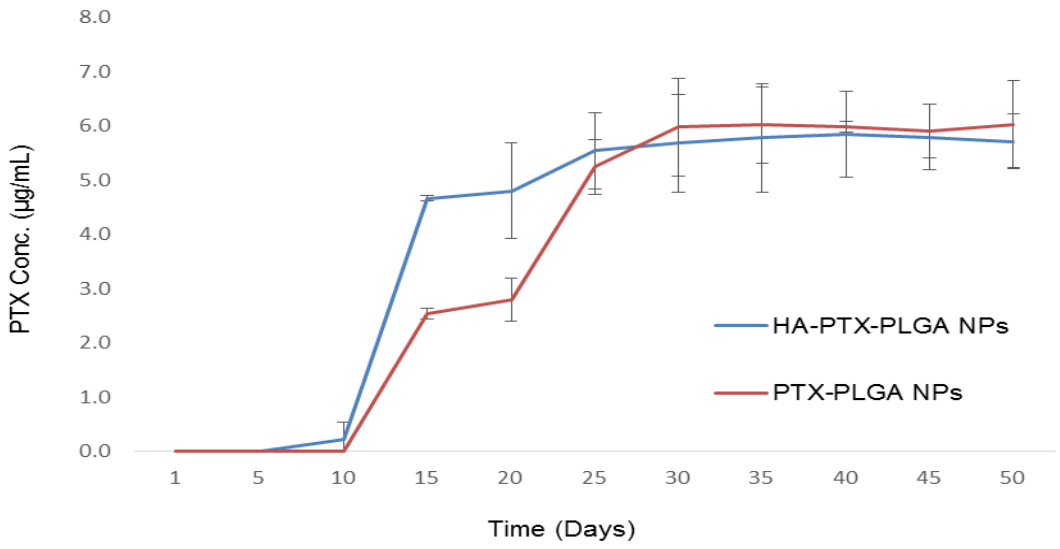
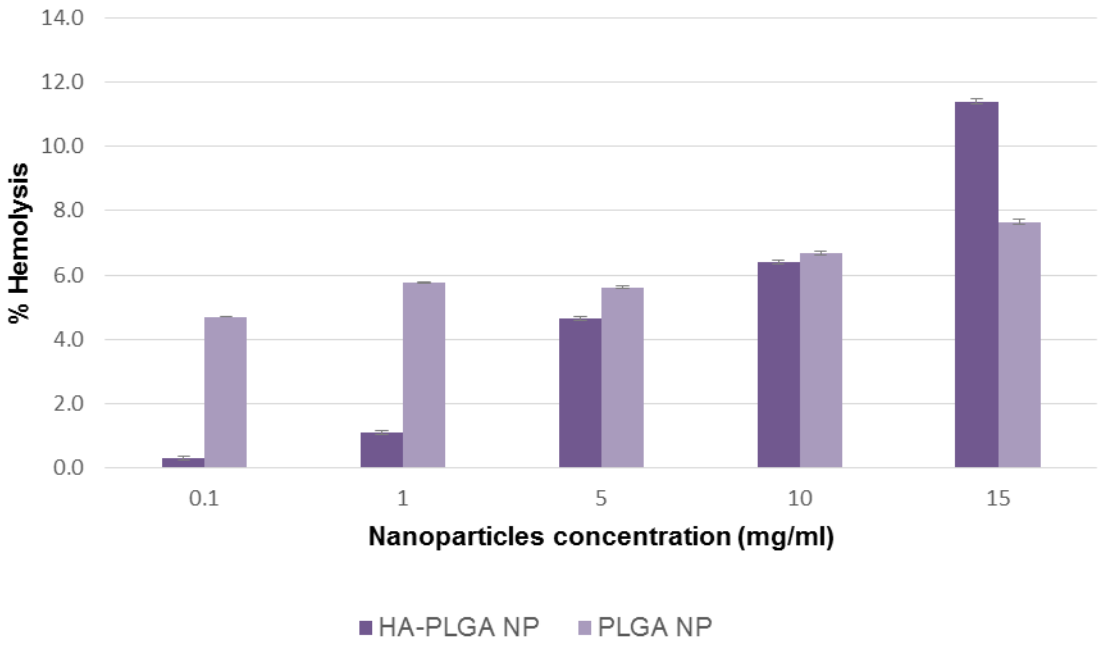



Figure 3. B Concentration of PTX released from HA-coated PLGA and PLGA-only NPs during degradation study.

**(A)**




**(B)**

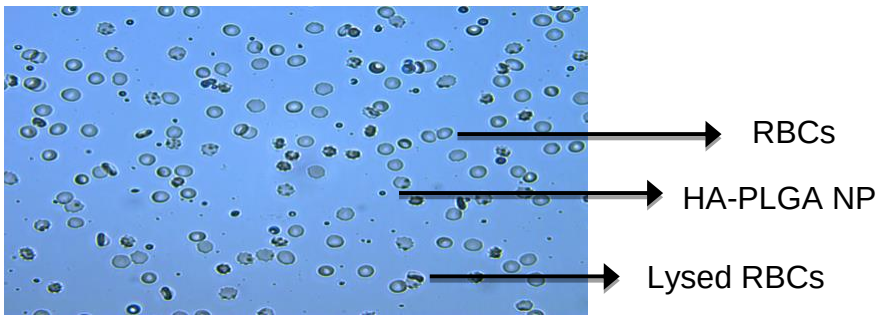
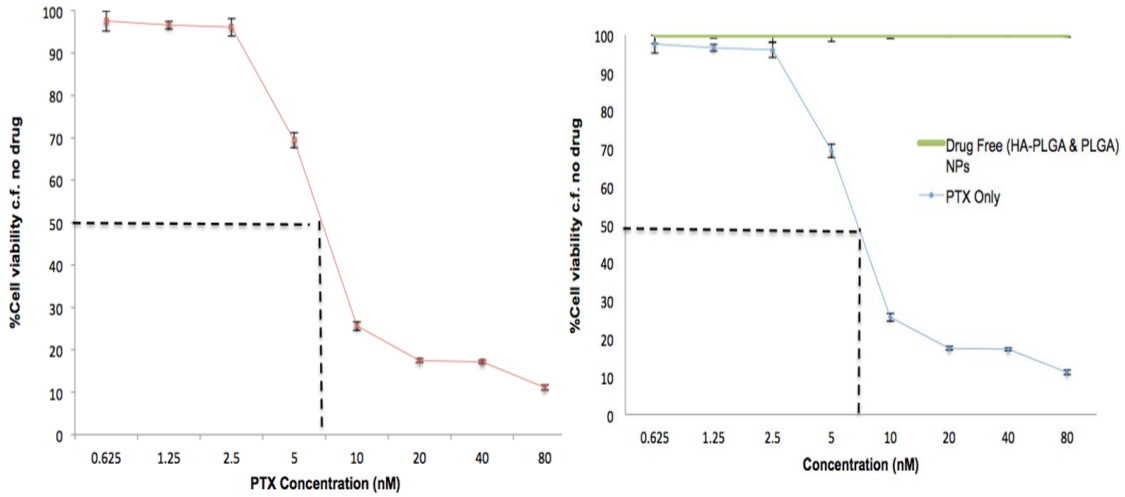
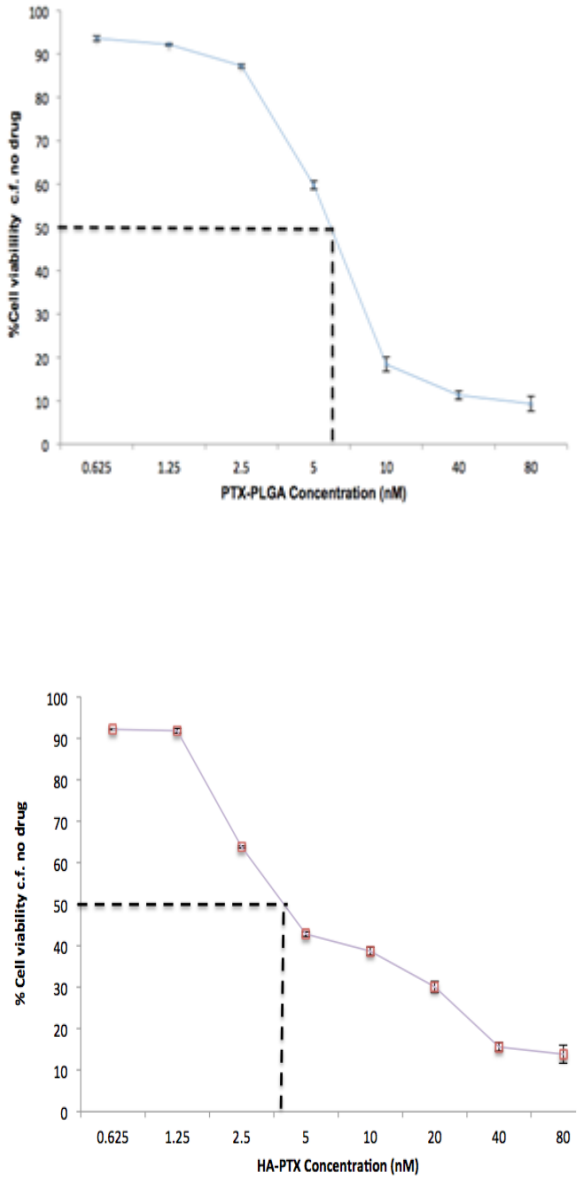



Figure 4. Formulation-blood compatibility studies. (A) Percentage of RBC haemolysis for HA-coated and non-coated PLGA nanoparticles (B) Effect of HA-PLGA NPs (concentration 15mg/mL) on RBCs aggregation




**(A)** **(B)**

Figure 5: (A) $IC_{50}$ of PTX alone after incubation of MDA-MB-231 cells for 72 hours, The $IC_{50}$ value for PTX on the MDA-MB-231 cells is approximately 5 nM (indicated with the dashed line). (B) MDA-MB-231 cells incubated with drug-free HA-PLGA NPs and PLGA NPs or PTX alone, The $IC_{50}$ value for PTX on the MDA-MB-231 cells line is approximately 5-5.5 nM (indicated with the dashed line). The viability of MDA-MB-231 cells was measured, based on DNA content, following incubation with a dose range of PTX for 72 hours. Data was normalised and plotted relative to media-control treated (i.e. no drug) cells and expressed as the mean and the standard error (n = 6).

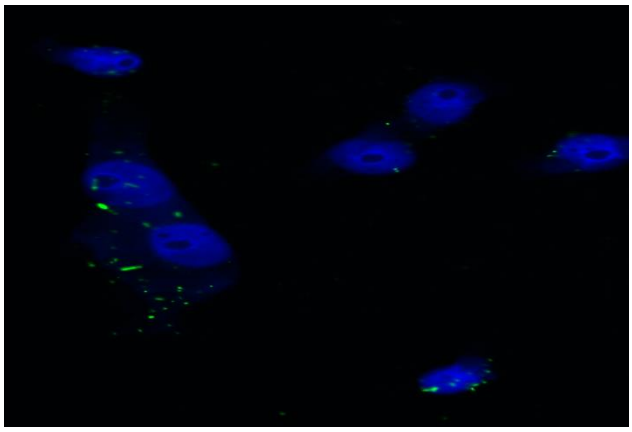



**(A)** **(B)**

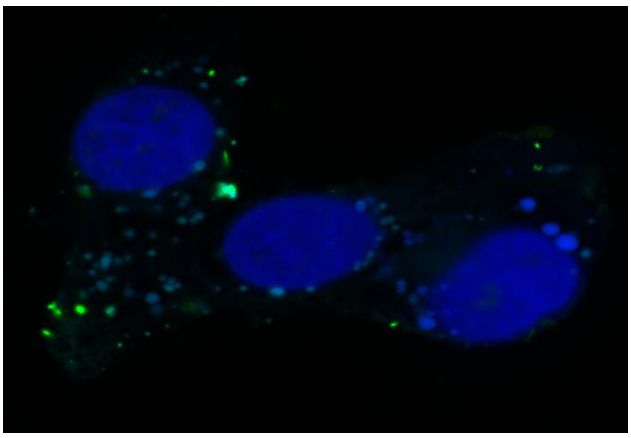
Figure 6: Dose-response curves showing(A) MDA-MB-231 cells incubated with PTX-PLGA NPs (B) MDA-MB-231 cells incubated with HA-PTX-PLGA NPs, The IC50 value for PTX on MDA-MB-231 cell

line is approximately 3 nM (indicated with the dashed line). The viability of MDA-MB-231 cells was measured, based on DNA content, following incubation with a dose range of PTX for 72 hours. Data was normalised and plotted relative to media-control treated (i.e. no drug) cells and expressed as the mean and the standard error (n = 6).

**(A)**



**(B)**



| Parameter | Variable | Size (nm) ± SD | PDI ± SD | Zeta Potential (mV) ± SD | EE % |
|---|---|---|---|---|---|
| **PVA Concentration** | 0.1 % w/v | *PRECIPITATED* | | | |
| | 0.5 % w/v | 225.10 ±0 .43 | 0.429 ± 0.030 | -71.20 ± 1.50 | 78.3 |

Figure 7: A Visualisation of MDA-MB-231 cells incubation with (A) coumarin-6 loaded HA-PLGA NPs (B) coumarin-6 loaded PLGA NPs, coumarin-6 (visible as green) and DAPI (visible as blue).

**Table 1. The effect of formulation parameters on the physicochemical properties of PTX loaded PLGA nanoparticles**

| | | | | | |
|---|---|---|---|---|---|
| | 2 % w/v | 207.50 ± 0.36 | 0.270 ± 0.000 | -34.90 ± 0.06 | 90.8 |
| **Sonication time** | 1 min | 295 ± 7.80 | 0.357 ± 0.040 | -38.90 ± 0.50 | 61.1 |
| | 3 min | 277 ± 1.77 | 0.370 ± 0.020 | -35.30 ± 0.10 | 83.7 |
| | 4 min | 212.30 ± 2.76 | 0.289 ± 0.060 | -29.30 ± 0.49 | 90.8 |
| **Polymer Concentration (50:50 PLGA)** | 50 mg | 209.20 ± 9.92 | 0.326 ± 0.060 | -38.40 ± 3.45 | 33.2 |
| | 100 mg | 252 ± 3.31 | 0.115 ± 0.010 | -31.50 ± 1.85 | 90.8 |
| | 150 mg | 448.30 ± 32.60 | 0.469 ± 0.030 | -58.30 ± 1.27 | 78.3 |
| ***Polymer Type*** | 50:50 PLGA | 206.70 ± 0.36 | 0.270 ± 0.010 | -41.70 ± 0.05 | 90.8 |
| | 75:25 PLGA | 539.90 ± 36.60 | 0.388 ± 0.080 | -23.10 ± 0.15 | 26.7 |
| | PCL | | *PRECIPITATED* | | |

**Table 2. Effect of different concentrations of hyaluronic acid (HA) on the characteristics of PTX loaded PLGA NPs.**

| ***HA Concentration*** | ***Size ± SD*** | ***PDI ± SD*** | ***Zeta Potential ± SD[a]*** | ***Zeta Potential ± SD[b]*** | ***EE %*** | ***DL %*** |
|---|---|---|---|---|---|---|

| | | | | | | |
|---|---|---|---|---|---|---|
| ***0.05 % w/v*** | 246.5 ±5 | 0.250 ± 0.030 | 32.1 ± 6.28 | -37.5 ±0.05 | 89.9 ± 2.15 | 82.4 ± 12.8 |
| ***0.5 % w/v*** | 2143 ± 30 | 0.721 ± 0.221 | 33.7 ± 5.37 | -48.3 ± 1.50 | 84.1 ± 5.25 | 77.1 ± 9.3 |
| ***1 % w/v*** | 1158 ± 53 | 0.715 ± 0.185 | 33.1 ± 2.53 | -37.9 ± 2.10 | 52.6 ± 16.1 | 49.2 ± 3.40 |

[a] **Cetrimide coated NPs**

[b] **Hyaluronic acid coated NPs**