Journal of Young Pharmacists, 2024; 16(4):714-724.
https://www.jyoungpharm.org

Original Article

EGFR-mTOR siRNA Loaded Immumonanoparticles for Targeted Chemotherapy of Triple Negative Breast Cancer

Krishnan Shalini[1,2], Rajavel Varatharajan[3,*], Hamid Shahrul[2,*], Palanimuthu Vasanth Raj[4], Venugopal Vijayan[5]

[1]Biochemistry Unit, Faculty of Medicine, AIMST University, Semeling, Bedong, Kedah Darul Aman, MALAYSIA.
[2]Department of Biomedical Science, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Penang, MALAYSIA.
[3]Faculty of Pharmacy, Pharmacology, Toxicology and Basic Health Sciences Unit AIMST University, Semeling, Bedong, Kedah Darul Aman, MALAYSIA.
[4]School of Pharmacy, Medical Biology Centre, Queen's University of Belfast, Lisburn Road, Belfast, Northern Ireland, UNITED KINGDOM.
[5]School of Pharmacy, Sri Balaji Vidyapeeth University, Pillayarkuppam, Puducherry, INDIA.

ABSTRACT

Background: Triple-Negative Breast Cancer (TNBC) presents a significant clinical challenge due to its aggressive nature and limited therapeutic options. A promising treatment strategy involves targeting Epidermal Growth Factor Receptor (EGFR) signaling and PI3K/Akt/mTOR pathways that are hyperactivated in many breast carcinomas. In our study, we aimed to develop a drug delivery system utilizing Paclitaxel (PTX) bio-conjugated with mammalian Target of Rapamycin (mTOR) small interfering RNA (siRNA) nanoparticles, which were further conjugated with EGFR to specifically target TNBC cells. **Materials and Methods:** The nanoparticles were synthesized using a precipitation method followed by solvent evaporation. They were characterized for size, shape, entrapment efficiency, and drug release kinetics. Additionally, we assessed their cytotoxicity, migration assay, and siRNA binding efficiency using gel retardation. **Results:** The results showed that the EGFR-mTOR siRNA-loaded immuno nanoparticles had a mean size of 186.6 nm with a zeta potential of 46.57±12.3 mV and an entrapment efficiency of 76.3±3.6%. Gel retardation assays confirmed the integrity of mTOR post-conjugation with mTOR immuno nanoparticles. The anticancer activity of mTOR immuno nanoparticles was evaluated using MDA-MB231 and MDA-MB468 TNBC cell lines, demonstrating significant cytotoxicity. The antibody-conjugated immuno nanoparticles showed enhanced targeting specificity and efficacy against TNBC cells. **Conclusion:** In summary, mTOR immuno nanoparticles exhibit potential as a targeted therapeutic strategy for TNBC, providing enhanced effectiveness while reducing the adverse effects associated with conventional chemotherapy. This novel drug delivery system's potential to improve therapeutic efficacy and mitigate systemic toxicity underscores its translational relevance in cancer treatment.

Keywords: Epidermal Growth Factor Receptor, Immunonanoparticles, Nanoparticles, Paclitaxel, Triple-Negative Breast Cancer.

Correspondence:
Dr. Shahrul Bariyah Sahul Hamid
Associate Proffesor, Department of Biomedical Science, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Kepala Batas, Penang, MALAYSIA.
Email: shahrulbariyah@usm.my

Dr. Rajavel Varatharajan
Associate Proffesor, Faculty of Pharmacy, Pharmacology, Toxicology and Basic Health Sciences Unit, AIMST University, Semeling-08100, Bedong, Kedah Darul Aman, MALAYSIA.
Email: varadharajeen@gmail.com

Received: 06-08-2024;
Revised: 20-08-2024;
Accepted: 03-09-2024.

INTRODUCTION

Cancer is characterized by the unregulated growth of abnormal cells, leading to the formation of malignant tumours. The primary cause of illness and mortality in most cancer patients is the spread of tumour cells to nearby tissues and distant organs.[1] Triple-Negative Breast Cancer (TNBC) is a formidable subtype of breast cancer characterized by the absence of three distinct receptors: the Estrogen Receptor (ER), the Progesterone Receptor (PR), and the Human Epidermal growth factor Receptor 2 (HER2). These receptors play vital roles in the growth and division of breast cells. Consequently, breast cancer exhibits resistance to hormone treatment and HER2-targeted medications, both of which have demonstrated efficacy in treating other subtypes of breast cancer.

TNBC comprises around 10-15% of the total incidence of breast cancer. According to projections, the breast cancer is anticipated to emerge as the most prevalent form of cancer among women in the United States in the year 2020. Additionally, it is expected to rank as the second leading cause of mortality, following lung cancer. Cancer has emerged as a significant concern in the field of public health, mostly attributable to its heightened incidence and fatality rates. Multiple approaches can be employed in the management of cancer, encompassing surgical interventions, radiation therapy, chemotherapy, and targeted therapy.[2]

OPEN ACCESS

Manuscript TECHNOMEDIA

DOI: 10.5530/jyp.2024.16.91

Publishing Partner : Manuscript Technomedia. [www.mstechnomedia.com]

The principal aim of chemotherapy is to either impede the development of malignant cells or elicit direct cytotoxic effects on them. Although chemotherapy is widely recognized as a systemic approach to treating cancer, there are many alternate non-systemic techniques available for its administration.[3] As a result; the administration of chemotherapy may lead to the emergence of undesirable consequences, such as the formation of oral ulcers, instances of emesis, and the manifestation of alopecia. Various drug delivery techniques have been developed to address the difficulties associated with chemotherapy.[4]

Paclitaxel (PTX) is a diterpenoid chemical and plant alkaloid that is naturally obtained from the bark of the Pacific yew tree. The compound demonstrates the characteristics of a white crystalline powder, possessing a chemical formula of $C_{47}H_{51}NO_{14}$ and a molecular weight of 853.9 g/mol.[5] PTX is a pharmaceutical compound employed for its anti-cancer characteristics. It is specifically designed to selectively interact with microtubules, which have a vital function in the construction of spindle fibers during cellular mitosis. Microtubules play a vital role in the preservation of cellular architecture, as well as in the facilitation of cellular movement and transportation throughout the cell. PTX serves as a stabilizing agent for microtubules, successfully inhibiting their depolymerization process and, hence slowing cellular division. Although the intravenous injection of PTX does not facilitate its passage through the blood-brain barrier, it has enhanced drug transport characteristics in local administration when compared to other chemotherapeutic agents.[6] The hydrophobic characteristic of PTX poses a difficulty when considering its systemic administration.

The effectiveness of PTX, a frequently used therapeutic drug for the treatment of TNBC, is impeded by the development of resistance in patients with breast cancer.[7] In addition, it has been observed that the conventional PTX formulation, known as Taxol®, is associated with significant hypersensitivity reactions and neurotoxicity that restrict the dosage.[8] The concurrent administration of an inhibitor targeting the mammalian Target of Rapamycin (mTOR) and PTX had a synergistic impact across multiple breast cancer cell lines, resulting in the most favorable synergistic outcome.

A novel formulation of polymeric nanoparticles was developed with the objective of effectively encapsulating both PTX and mTOR siRNA at an optimal synergistic ratio. The present formulation was specifically developed for the simultaneous delivery of both therapeutic agents to TNBC cells. The aim of this study was to produce nanoparticles containing PTX and mTOR siRNA that have the ability to selectively target the Epidermal Growth Factor Receptor (EGFR) through the binding of EGFR-targeted Panitumumab (PmAb) Fab fragments. The application of fragment antigen-binding (Fab) as targeting agents in nanoparticles can overcome the limitations associated with the use of entire antibody-targeted nanoparticles. Fab fragments demonstrate decreased immunogenicity than complete antibodies.[9,10] The objects in the question have reduced dimension, so facilitating a greater concentration of targeting and enhanced alignment of ligands. As a result, this leads to improved cellular internalization, heightened efficacy, reduced interference with the physicochemical characteristics and performance of the nanoparticles.

MATERIALS AND METHODS

Synthesis of PTX loaded PLGA nanoparticles

The Nanoparticle (NP) was synthesized using the nano-precipitation technique, which has been previously documented.[11,12] In the initial step, a 5 mg quantity of PTX was solubilized in 5 mL of Dichloromethane (DCM). Subsequently, a solution of 100 mg of poly(lactic-co-glycolic) acid (PLGA) was dissolved DCM and subsequently combined with the drug solution. Nanoparticles were generated through the drop-wise injection of a drug-polymer solution into an aqueous phase consisting of 12 mL of deionized water containing 2% Polyvinyl Alcohol (PVA) and 0.25% tween 80. This injection process was facilitated by sonification using an Ultra-Probe Sonicator (QSonica, USA) operating at amplitude of 60 W for duration of 5 min, while maintaining the system in an ice bath. The NP suspension was permitted to undergo uninterrupted stirring on a magnetic stirrer overnight at room temperature, without being covered. This was done to facilitate the complete evaporation of DCM and to promote the emulsification of the nanoparticles. Following that, the nanoparticles were retrieved using centrifugation at a speed of 1000 revolutions per minute (rpm) for duration of 10 min. The nanoparticles underwent two rounds of washing with distilled water to eliminate any residual drug, followed by lyophilization as the final step. The desiccated nanoparticles were preserved at a temperature of -20°C in order to facilitate subsequent experimentation.

Anchoring of EGFR monoclonal antibody (mAb) to the PTX- nanoparticles

The initiation of EGFR mAb conjugation on the surface of PTX nanoparticles was carried out through the thiolation of the mAb, as described previously.[13] The EGFR mAb was immobilized on the surfaces of nanoparticles by a three-step process using the cross-linking agent m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS). Initially, the PTX-NP was subjected to cross-linking with MBS. Specifically, 5 mg of PTX nanoparticles were dispersed in 1 mL of phosphate buffered saline (PBS) (pH 7.2). Subsequently, the PTX nanoparticles were incubated with 1 mL of MBS solution (containing 3.14 mg of MBS dissolved in 1 mL of dimethylsulfoxide (DMSO) for duration of 1 hr at a temperature of 20°C, while maintaining continual agitation. The subsequent procedure involved the thiolation of anti-EGFR monoclonal antibody. The third step was the utilization of 2-iminothiolane

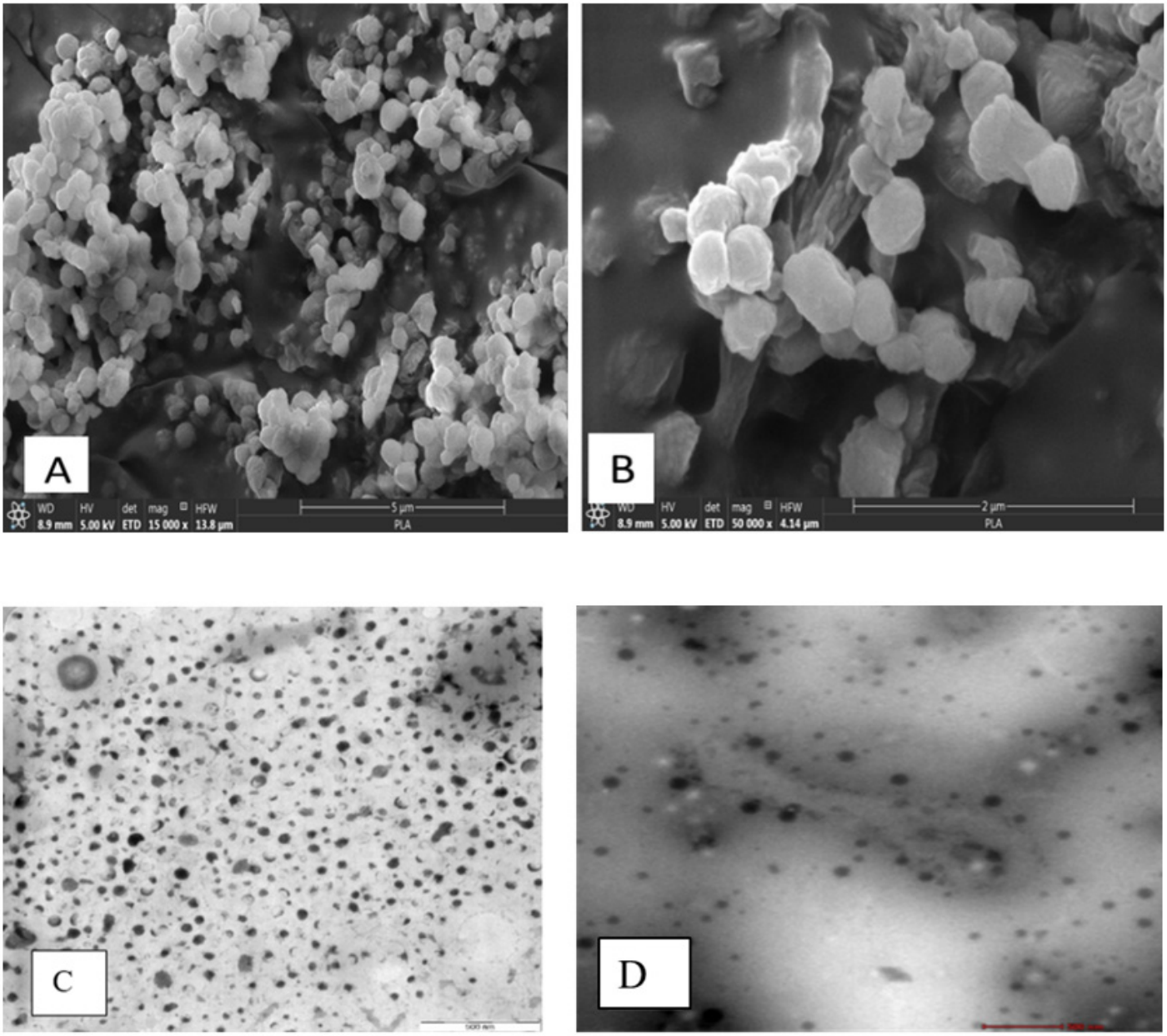

Figure 1: Scanning electron microscope (SEM) and transmission electron microscope (TEM). In SEM (A) PTX NP (B) PTX INP and TEM (C) PTX NP and (D) PTX INP.

(commonly known as Traut's reagent) to perform thiolation of the EGFR monoclonal antibody. A total weight of 5.7 mg of Traut's reagent was dissolved in 5 mL of PBS with a pH of 7.4. Subsequently, 100 µL of a solution containing EGFR monoclonal antibodies at a concentration of 1 mg/mL in PBS with a pH of 8.0 was combined with 4.2 µL of 2-iminothiolane. This mixture was incubated for duration of 3 hr at a temperature of 20°C while being continuously stirred. Subsequently, the thiolated EGFR monoclonal antibody was conjugated onto the surfaces of MBS-activated nanoparticles. The thiolated EGFR was anchored onto the activated surface of the nanoparticles in the last stage by combining the activated solution in equal proportions while subjecting it to magnetic stirring for duration of 3 hr at ambient temperature.

Generation of mTOR siRNA immunonanoparticles (mTOR INP)

The PTX-EGFR NP (10 mg) was mixed in 2 mL of distilled water. In order to immobilize mTOR siRNA onto the surface of immunonanoparticles, a total volume of 500 µL of siRNA solution (10 µg/mL in distilled water) was gradually added to 500 µL of PTX-EGFR NP suspension. The resulting mixture was subjected to sonication for 1 min, followed by incubation at a temperature of 4°C. After the incubation period, the mTOR inhibitor nanoparticles were separated by centrifugation at a speed of 13,000 revolutions per minute for 15 min at a temperature of 4°C. The mTOR inhibitor nanoparticles were washed 3 times with distilled water to eliminate any unbound siRNA and store at -20°C.

Scanning Electron Microscope (SEM) analysis

The samples were examined for their size, shape, and surface morphology using a scanning electron microscope (Hitachi S-4700 FE-SEM, Tokyo, Japan). The preparation of the samples involved the application of a small amount of nanoparticle suspension onto the surface of a silicon wafer that was subsequently subjected to a drying process and gold sputtering.

Transmission Electron Microscope (TEM) analysis

The analysis of nanoparticle morphology was conducted using the transmission electron microscopy technique. The TEM was employed to perform the sizing of the nanoparticle (Eindhoven, The Netherlands). The samples were prepared by negative staining method. Analysis was done on the polymeric nanoparticles encapsulating PTX. A sample of the nanoparticle formulation, measuring 50 µL was placed onto a parafilm surface. The specimens underwent a desiccation procedure on a carbon-coated grid. Subsequently, the samples underwent negative staining by immersion in an aqueous solution of phosphotungstic acid. After the completion of the drying procedure, the specimen underwent microscopic examination at various magnifications, ranging from 10 to 100000-fold, utilizing an accelerating voltage of 100 kV.

Measurement of the nanoparticle size, Poly Dispersity Index (PDI) and zeta potential

The dimensions of nanoparticle were determined using the photon correlation spectroscopy technique on the Zetasizer instrument (Malvern, UK). The nanoparticles, which had undergone freeze drying, were reconstituted by dissolving 10 mg of PTX INP in 5 mL of distilled water. Subsequently, the reconstituted nanoparticles were subjected to analysis using Zetasizer, at 25°C. The measurement of diameter was conducted by analyzing the autocorrelation function of the emitted light intensity originating from nanoparticles. The same method has been implemented for PLGA-PTX and PTX NP. The PDI serves as a quantitative measure for assessing the degree of dispersion uniformity. It was computed to assess the degree of uniformity in dispersion, with values ranging from 0 to 1. A number in close proximity to zero signifies a state of homogenous dispersion, whereas a value of more than 0.7 indicates a high degree of heterogeneity.[13,14]

The zeta potential was measured to assess the surface charges of the nanoparticles. The zeta potential serves as a significant determinant of the stability of the colloidal dispersion. It serves as an indicator of the extent of electrostatic repulsion that exists between adjacent particles within a dispersion, which possess identical charges. The samples were diluted using distilled water and analyzed with Zetasizer at 25°C. The apparatus was utilized with a Helium-Neon (HeNe) laser operating at a wavelength of 632.8 nm.

Cell viability assay

The MDA MB 231 and MDA MB 468 cells were seeded at 2×10^3 cells/mL in a 6-well plate containing Leibovitz's media supplemented with 10% v/v Fetal Bovine Serum (FBS) and 1% w/v penicillin-streptomycin antibiotic combination. After subjecting the cells to a 24 hr at 37°C, they were further grown under both normoxic and hyperoxic conditions (with 5% CO_2) until they reached a confluence of between 70% to 80%. Following this, the medium was substituted with a volume of 1 mL of medium devoid of serum. In this study, PTX, PTX nanoparticles and PTX INPs were employed at a concentration of 100 nmol/L. Following a 6 hr incubation period, the culture medium was substituted with Leibovitz's medium supplemented with 2% serum. Subsequently, the cells were cultivated at 37°C for 24 hr and 48 hr. The cells were collected to measure their viability by Trypan blue exclusion assay[2].

Encapsulating Efficiency (EE), drug release and drug Loading Capacity (LC)

PTX is one of the most efficacious anticancer drugs. Nevertheless, drug is frequently linked to occurrence of unfavorable outcomes like neurotoxicity, myelosuppression, and hypersensitivity responses. Nanotechnology provides a platform achieving sustained release of PTX. This can be a potential strategy for mitigating the adverse effects associated with its administration. For the measurement of drug release, the equilibrium dialysis method was employed.[15] Firstly, the effects of PLGA to PTX ratios on percentage of cumulative drug were studied. Different ratios of PLGA to PTX formulation (5:1, 10:1, 15:1 and 20:1) were studied. Samples were collected from PBS (pH 7.4) at 37°C for 180 hr.

Secondly, further analysis was done to examine the kinetic release of PTX from the nanoparticles at 37°C in PBS (pH 7.4). The dialysis bag contained 1 mL of PTX nanoparticles (equal to 30 ug of the drug), namely PTX, PTX NP, and PTX INP. This suspension was immersed in a 30 mL solution of PBS (7.4 pH) and rotated at 80 rpm at 37°C in a water bath. The sample and solution underwent centrifugation at predetermined time intervals (0, 0.5, 1, 2, 3, 4, 6, 8, 16, 24, 48, 72, 96, 120 and 180 hr). Subsequently, 200 uL of the resultant supernatant was collected to measure the absorbance of PTX using UV-visible spectroscopy at 228 nm. Subsequently, the acquired data was integrated into the calibration curve to determine the concentration of the drug that was delivered. The quantification of PTX produced in each sample was accomplished by employing a calibration curve, and the presented values represent the average of three samples. The results of the *in vitro* drug release experiments were documented and visually represented as a graph displaying the cumulative percentage of drug release over time. The values for EE% and LC% were determined using the following calculation method, with a sample size of three (n=3).

$$\text{Entrapment efficiency (\%)} = \frac{\text{siRNA amount in loading buffer} - \text{siRNA amount in supernatant}}{\text{siRNA amount in loading buffer}} \times 100 \text{------F1}$$

$$\text{Loading efficiency (\%)} = \frac{\text{Total amount of entrapped siRNA}}{\text{Total amount of NPs}} \times 100 \text{---------F2}$$

In vitro cytotoxicity activity

The viability of MDA MB 231 and MDA MB 468 cells was evaluated using the MTT (3-[4, 5-dimethylthiazol2-yl]-2, 5-diphenyltetrazolium bromide) assay. The impact of pure PTX, PTX NP and PTX INPs on cell viability was investigated. The nanoparticles were sterilized by filtration with a 0.2 m Millipore syringe filter prior to *in vitro* analysis. The cells were cultured in Leibovitz's L-15 modified essential medium, which was supplemented with 10% fetal bovine serum, as well as penicillin (100 units/mL) and streptomycin (100 µg/mL). The cells were maintained at 37ºC in a humidified 5% CO_2 incubator. In this experiment, triplicates of 8×10^4 cells per milliliter were seeded in each well of 96-well microtiter plates. The plate was then placed in a 5% CO_2 incubator and cells were grown 24 hr. Following incubation, a dose-response analysis was performed by adding 100 mL solution comprising newly synthesized nanoparticles, of different concentration growth medium. It was subsequently treated for 48 hr.[17] Next, 20 µL of MTT solution (5 mg/mL in PBS) was added into each well and incubated for 4 hr. After the incubation, the solution containing MTT was replaced with 200 µL of DMSO to aid in the breakdown of formazan crystals. The absorbance values obtained were subsequently measured using a microtiter plate reader (Tecan, United Kingdom) at a wavelength of 540 nm, with a reference wavelength of 630 nm. The dose-response curve was plotted based on the absorbance values of the experimental group (cells subjected to treatment) and the control group (cells not subjected to treatment) according to the formula provided.

Gel retardation assay

The stability of mTOR was validated by incubation in FBS. In this analysis 200 µL volume of PTX INPs containing 5 µg of mTOR was subjected to incubation at 37ºC. This incubation was carried out alongside an equal volume of Leibovitz medium that had been supplemented with FBS final concentration of 50% v/v. Each dilution measuring 20 µL was collected at six different time points of 24, 48, 72, 120, 144 and 168 hr. Immediately after incubation, 1 µL of 0.5 M EDTA solution was added to the solution in order to inhibit deterioration. The sample was then separated by 1% agarose gel electrophoresis as described by Gandhi *et al.* (2018).[16] Following the electrophoresis, bands separated were captured with the Biorad multi-image ChemiDoc XRS (California, United States). Subsequently, the acquired images were subjected for analysis with the Biorad Image lab software version 3.0.

RESULTS

In the TNBC therapy, PTX remains the predominant chemotherapeutic drug utilized in the early management. However, the effectiveness of PTX in the treatment of TNBC is impeded by its lack of specificity, limited solubility in water-based environment, high toxicity tendency to lead to ineffective treatment, drug interactions, and unfavorable physicochemical properties that hinder the development of a suitable delivery system.[17] The restricted ability of PTX to pass through the blood-brain barrier (BBB) is a substantial limitation to their potential effectiveness in treating brain malignancies. The BBB is characterized by the presence of endothelial cells that have elevated impermeability as a result of establishment of tight junctions. Additionally, the BBB is characterised by the existence of enzymatic activity and active efflux transport mechanisms. The aforementioned mechanisms combined serve to limit the transportation of molecules just to those that are selectively and indispensably necessary.[18] A wide range of advanced drug delivery technologies, including as nanoparticles and liposomes have undergone thorough investigation and refinement in order to enhance the transport of pharmaceuticals that are typically unable to permeable of BBB. Nanoparticles coated with PLGA and Tween 80 have exhibited the capability to effectively cross the BBB and have been utilized for the delivery of drugs with restricted permeability.[19,20] The phenomenon known as the burst effect is a common challenge observed in controlled release particulate drug carriers, wherein there is an excessive release of drugs from the polymer matrix of particles into the dissolving fluid during a relatively little duration.

Electron Microscopy Analysis

Scanning Electron Microscopy (SEM) was employed to analyze the surface morphology of both PTX NP and PTX INP. The SEM analysis revealed that the PTX NP formulated with PLGA and PVA exhibited a well-defined spherical shape with a consistently smooth surface (Figure 1). The range of particle sizes seen in the distribution was between 200 nm to 10 µm, with a notable abundance of nanoparticles sizes of 200 nm. The nanoparticles exhibited a uniform size distribution of 200 nm and possessed a smooth surface. They exhibited a uniform, sleek, and spherical nature, as evidenced by the surface morphology analyses illustrated in Figure 1.

The particle size and zeta potential of the produced nanoparticles were assessed, and the results are shown in Figure 1 for the SEM and the TEM pictures of PTX NP and PTX INP. The particle size of formulation with PLGA and PTX was found to be 143.53±1.83 nm, with a polydispersity index of 0.43±0.12. The zeta potential measurements exhibited a range of between -27.65 (±11.4) to -46.57 (± 12.3).

The nanosuspension exhibited enhanced homogeneity, as evidenced by the reduced polydispersity index observed with

PTX INP. Zeta potential is the method for the measurement of the electrostatic potential at the electrical double layer surrounding a nanoparticle in solution. This is referred to as the zeta potential. Nanoparticles with a zeta potential between -10 and +10 mV are considered approximately neutral, whereas the values of greater than +30 mV or less than -30 mV are considered as strongly cationic and strongly anionic, respectively.

The zeta potential offers valuable information regarding the physical stability of a formulation, particularly with regards to the surface charge exhibited by the particles. The presence of surface charge is a crucial factor in regulating the stability of the nanoparticle formulation through the promotion of robust electrostatic repulsion among particles. The use of zeta potential analysis makes it feasible to estimate the major constituent that has the most influence on the surface of a particle. In this study, there was substantial increase the surface charge of PLGA nanoparticles, whereby PTX INP had the higher zeta potential than PTX nanoparticles. The reduction in the negative charge can be ascribed to the shielding effect of carboxylic groups by the drug molecules located on the surface of the particles [21]. Data obtained indicated an increase of polymer concentration resulted in a significant rise in EE from 42.3% (PTX NP 5:1) to 83.8±4.7% (PTX NP 20:1) with negligible impact on LC (Table 1). This could be due to the low polymer content, which allows for easy PTX leakage. In addition, a higher PLGA content resulted in a significantly lower burst release. Thus, facilitating gradual drug release for distribution within 180 hr (Figure 5). Moreover, high PLGA concentration results in a higher density of nanoparticles that enable better drug incorporation, delayed release, and slower disintegration.[14]

TNBC growth inhibitory activity by the PTX INP and PTX NP

Chemotherapy elicits substantial adverse consequences, even when utilized in conjunction with other pharmacological drugs. The potential to address the adverse effects by means of dosage reduction is demonstrated through the development of nanodelivery systems. To achieve the desired outcome, the study utilized the nanoprecipitation method to encapsulate EGFR and mTOR siRNA for generating PTX INP formulation. This approach effectively enhanced the therapeutic efficacy of PTX. Findings revealed that the PTX INP exhibited an increased inhibitory effect on both cell lines. For MDA MB 231 the value reduced from 2.5 nM to 1.8 nM with PTX INP than PTX NP (Figure 2A). Similarly for MDA MB 468 the value reduced from 3.8 nM to 2.6 nM (Figure 2B). There was a considerable reduction observed in the IC_{50} values for both PTX INP and PTX NP. The IC_{50} value for PTX NP decreased by a factor of three, while for PTX NP it was lowered by a factor of four. The application of 10

Table 1: Comparison of averaged particle size, polydispersity index, zeta potential, encapsulating efficiency and loading capacity with different formulations of nanoparticles. Each value is represented by mean±SD (*n*=3).

Formulation	Particle size (nm)	PDI	Zeta potential (mV)	EE (%)	LC (%)
PTX NP (20:1)	168.71±1.62	0.37±0.24	-32.51±9.4	83.8±4.7	7.8±0.14
PTX NP (15:1)	174.35± 2.71	0.46± 0.37	-33.76± 6.8	73.5± 3.8	6.93± 0.17
PTX NP (10:1)	188.05± 3.54	0.52± 0.28	-39.62± 4.2	54.7± 4.2	6.42± 0.36
PTX NP (5:1)	231.64± 2.19	0.68± 0.12	-51.49± 7.4	42.3± 3.69	5.03± 0.62
PTX INP	186.57±2.31	0.29±0.09	-46.57±12.3	87.3±3.6	8.9±0.43

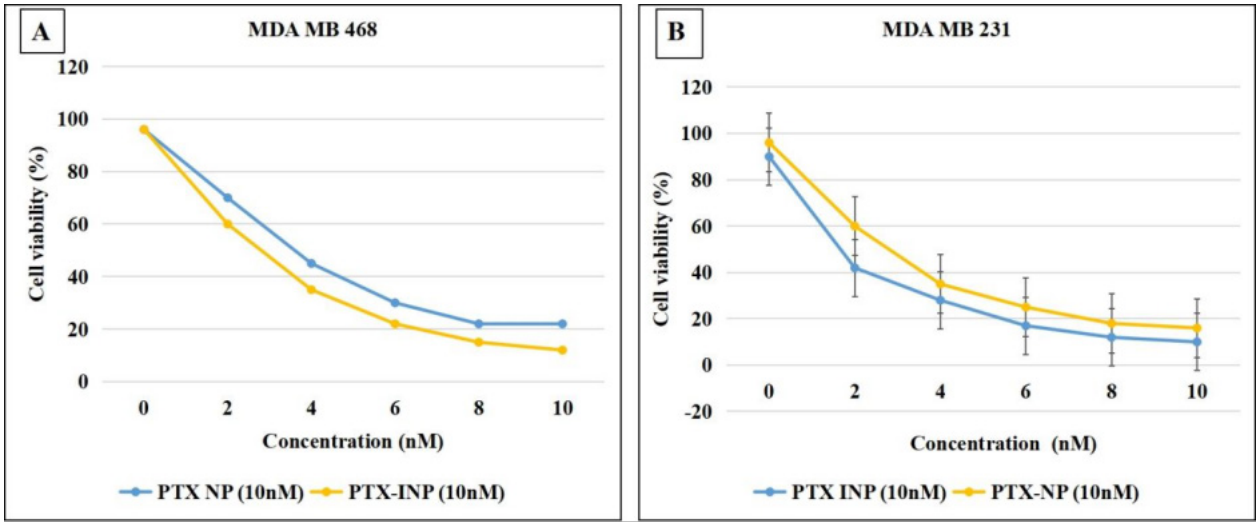

Figure 2: Dose-dependent inhibitory effect of PTX NP and PTX INP (A) MDA MB 231 and (B) MDA MB 468. All experiments were done in triplicates. Data is presented as mean±SD (*n*=3).

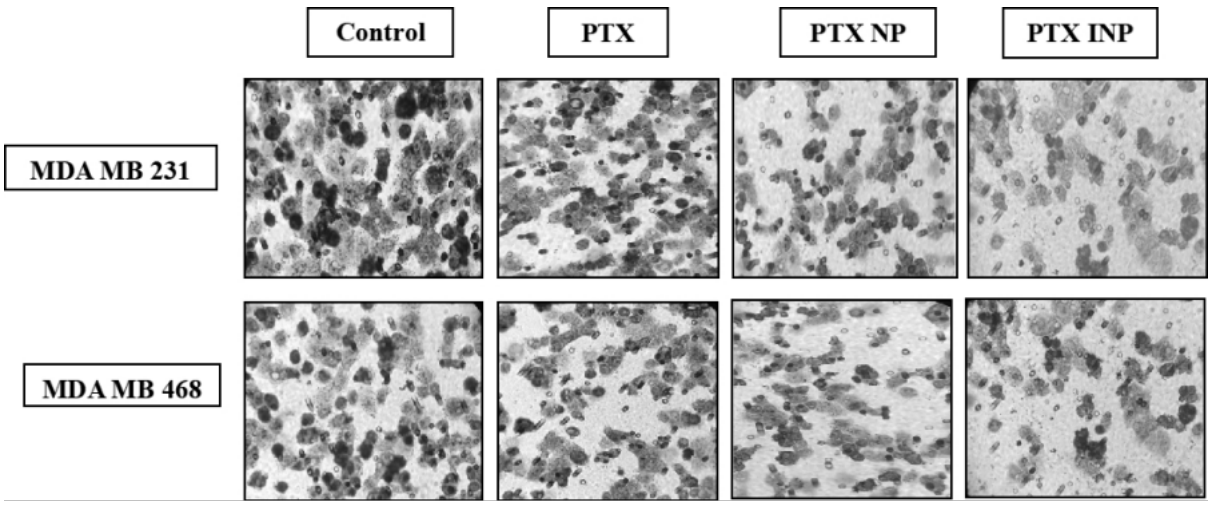

Figure 3: Images of the cell death induced by different formulation. Comparison on toxicity effect in MDA MB 231 and MDA MB 468 with treatment of the PTX, PTX NP and PTX INP after 24 hr suggest PTX INP was the most cytotoxicity. Images were taken at 400X magnifications under a phase contrast microscope.

nM PTX INP and PTX NP led to a further led to a reduction in the IC_{50} of PTX to 0.7 nM for MDA MB 231 cells and 1.2 nM for MDA MB 468 cells, as illustrated in Figure 2A and 2B. The little cytotoxicity of PTX INP was observed. This phenomenon can be attributed to the endocytosis effect. Study suggested that the MDA MB 231 and MDA MB 468 cells exhibited a fast uptake of PTX in the form of PTX INP that contained a higher concentration of PTX compared to free PTX. This feature of the formulation enhanced the cellular uptake of PTX and induced high cytotoxic activity. The potential effectiveness of administering low dosages would address the detrimental effects as reported in the clinical settings. The findings offer supporting evidence of a synergistic therapeutic effect of the PTX INP formulation for further evaluation on TNBC.

Morphological changes induced by the formulation

In addition to the observed decrease in cell viability, as determined using the MTT assay, the combination treatment resulted in significant cell death within the wells after a 24 hrs period, in comparison to cells treated with other formulations (Figure 3). These results gathered from the MTT assay consistently correlated with the cell death analysis. Furthermore, morphological alterations were seen in both cell types after the administration of nanoformulations. By employing tubulin labeling, visualization of the control samples showed that the cells were initially spherical shaped and they transformed into a more fibroblast-like morphology (Figure 3). Nevertheless, the treated cells exhibited a noticeable reduction in the number of cells compared to control cells.

Effect of PLGA to PTX ratio on percentage of Cumulative Drug Release (CDR)

The release curves of PTX exhibited the importance of polymer to drug ratio for drug release studies. There was a clear correlation, indicating that a polymer to drug ratio of 5:1 yielded 92.3±2.1% drug releases by 48 hr. Comparison with the lower ratio of 10:1 and 15:1 ratio indicated that the drug release was lower achieving 84.7±3.5% and 78.2±4.1% respectively (Figure 4). In contrast, high ratio of 20:1 significantly decelerated drug release profile to 52.6±3.4% by 48 hr. These differences can be ascribed to the significant encapsulation efficiency of the drug and the minimal swelling behavior of the polymer in the release medium. These characteristics contribute to a gradual diffusion of drug particles from the polymeric matrices.

The release kinetics of PTX from the nanoparticles exhibited a biphasic profile that was similar to previous studies.[12,22] The biphasic pattern was characterized by an initial rapid release within the 24 hr and followed by a sustained release at a slower rate over 8 days. A notable first burst release can be elucidated by the swift disintegration of polymer and subsequent release associated with the PTX adhered to the surface of the nanoparticles.

The investigation focused on examining the release kinetics of PTX INP at a temperature of 37°C in phosphate-buffered saline (pH 7.4). The objective was to assess the suitability of PTX INP as a carrier for the PTX. The experiments were conducted under submerged settings, with the dialysis medium being regularly replaced to maintain the desired environment. Figure 5 illustrates the *in vitro* release profile of PTX INP with a drug loading of 10%. The findings indicated that around 50% of PTX was released during the first 24 hr period, followed by a consistent release pattern that persisted until the tenth day. The observation that 80% of the PTX was released after a span of 10 days served as evidence for the potential efficacy of nanoparticles as a sustained drug delivery system. Furthermore, it should be noted that PTX INP nanoparticles exhibited long-term stability in liquid settings mimicking physiological blood pH by maintaining their structural integrity for a duration of 10 days.

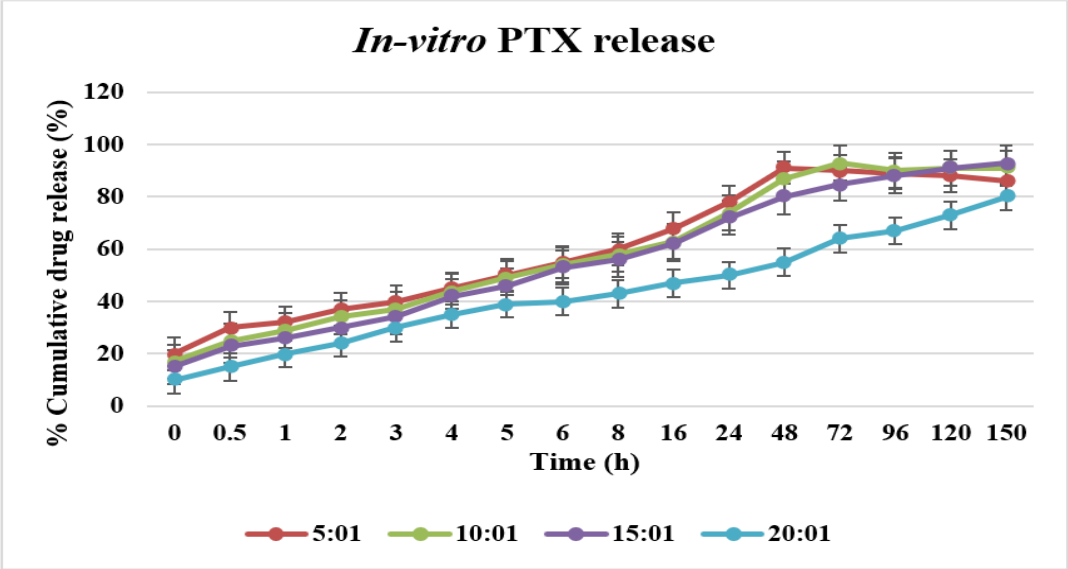

Figure 4: Effect of PLGA to PTX ratio on percentage of cumulative drug release. Different ratios of PLGA: PTX formulations (5:1, 10:1, 15:1 and 20:1) were studied. Samples were collected from phosphate buffer saline (pH 7.4) at 37^{0}C for 180 hr. Values presented are mean±SD of triplicates.

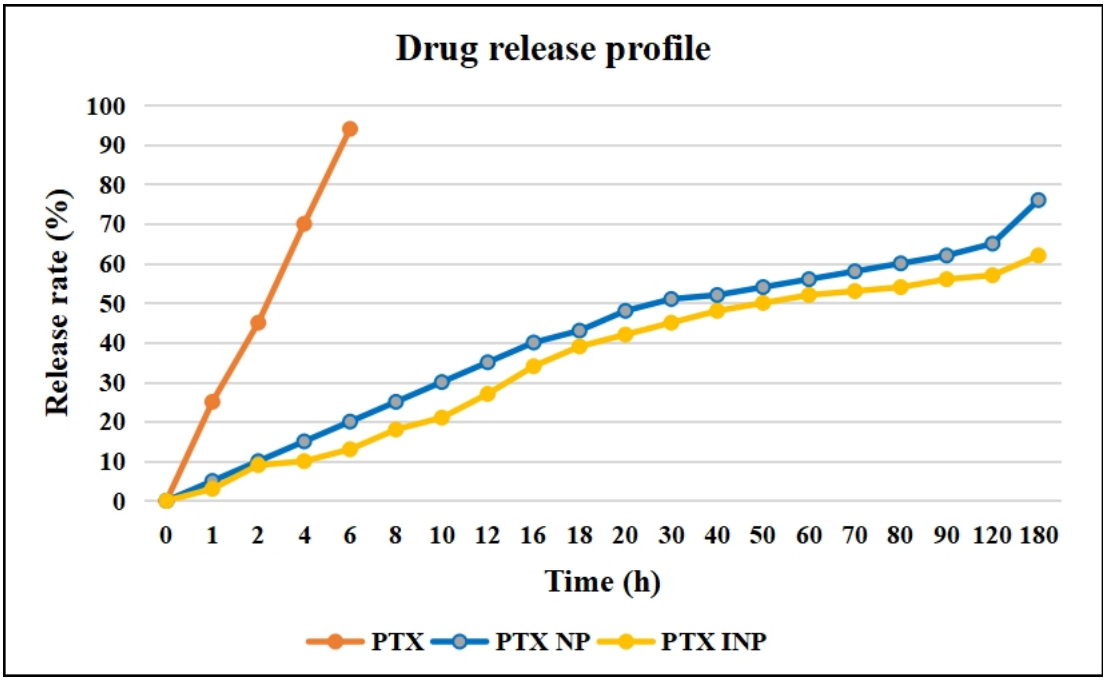

Figure 5: Effect of different nanoparticles formulations on percentage of cumulative drug release. Comparison of release pattern by the (PTX, PTX NP and PTX INP) in phosphate buffer saline at 37^{0}C for 120 hr. Values presented as mean±SD of triplicates.

Gel retardation assay

The gel retardation assay provides evidence of the binding affinity of the PTX NP to form a complex with mTOR siRNA. In this assay, the PTX INP underwent experimentation with different mass ratio (MR), where MR is defined as the ratio of micrograms of NP to micrograms of siRNA. The findings of the study demonstrated that the stability and integrity of PTX INP were maintained during the formulation process. Additionally, the study revealed that mTOR was effectively encapsulated on PTX NP (Figure 6). The results indicated that the binding and complexation of mTOR were not affected by the formulation method, chemical usage, or environmental factors such as temperature variation.

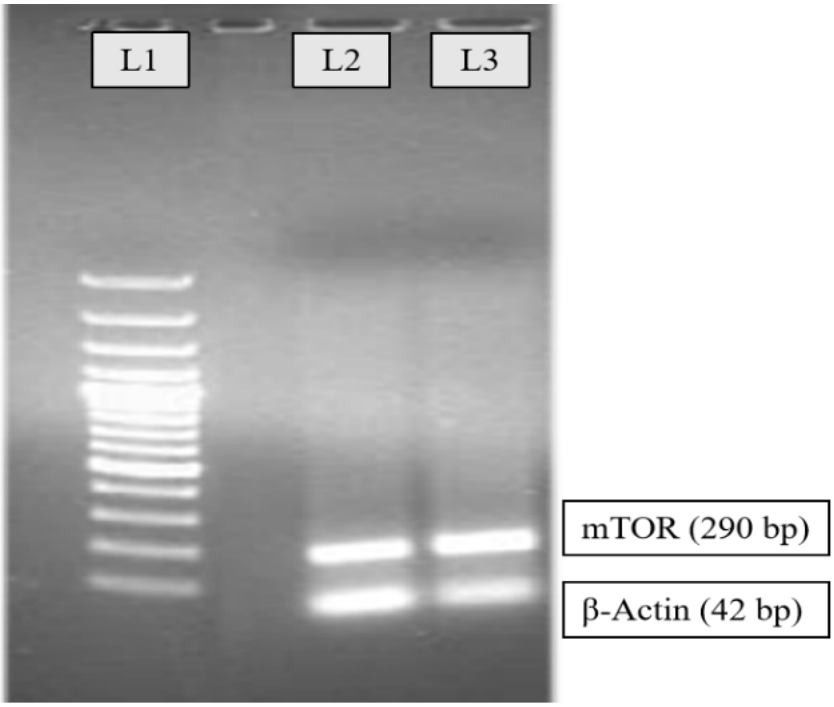

Figure 6: Gel retardation analysis of mTOR for ability to form a complete complex with PTX INP. L1, L2 and L3 indicates ladder, free mTOR and PTX INP respesctively. The β-actin was used as control.

DISCUSSION

PTX is considered to be one of the most efficacious anticancer drugs. In the realm of clinical practice, it has been shown that the medicine is frequently linked to a significant occurrence of unfavorable outcomes, such as neurotoxicity, myelosuppression, and hypersensitivity responses. The proposal of employing PTX INP for the purpose of achieving sustained release of PTX has emerged as a potential strategy for mitigating the adverse effects associated with PTX administration.

The studies showed PTX INP synthesised with PLGA and PVA had a well-defined spherical shape and a consistently smooth surface. This indicates that the formulation of PTX INPs is more homogeneous than the others. The optimised formulation, which ensures a constant dispersion of PTX within the nanoparticles and decreases variability in drug loading efficiency and particle size. Furthermore, with the utilization of zeta potential analysis, it becomes possible to estimate the primary constituent that exerts the greatest influence on the surface of a particle. The presence of surface charge plays an important role in maintaining the stability of the nanoparticle formation by promoting strong electrostatic repulsion among particles.

Nanoparticles exhibiting a high zeta potential are associated with enhanced stability. These nanoparticles demonstrated a reasonable level of homogeneity, exist as distinct entities without aggregation and can be readily dispersed. Taken together, the nanoparticles had a spherical morphology with nano size range. This was further validated by the utilization of TEM and result is provided (Figure 1).

The particle size distribution pattern is a crucial factor in determining the behavior of drug release, the appropriateness of particles for intravenous administration, and their destiny for *in-vivo* injection. The advantage of the small size nanoparticles with 200 nm enables their extravasation. Thus, facilitating their accumulation at tumour sites and impeding their filtration by the spleen. Moreover, the sterilization can be done by employing filter prior testing for *in vivo* and *in vitro* studies. An increase in the PDI of the formulation was seen as the ratio of polymer to drug decreased. This suggests that the formulation of PTX INPs demonstrates better homogeneity that the other formulations. This can be attributed to the optimised formulation, which provides a consistent dispersion of paclitaxel within the nanoparticles and reduces variations drug loading efficiency and in particle size.[23]

Generally, cell membranes are negatively charged, as such zeta potential can affect a nanoparticle's tendency to permeate membranes. Consequently, cationic particles exhibit more toxicity than anionic particles as they are associated with causing disruption of the cell membrane.

It has been reported that an augmentation in the quantity of drug loaded onto the surface of nanoparticles can result in enhanced the rate of drug release. Additionally, it was noticed that nanoparticles exhibiting the highest degree of encapsulation demonstrated a more protracted and regulated pattern of release. A notable disparity was seen in the nanoparticle release kinetics

when comparing the PTX immunonanoparticle formulation was compared to the other formulations.[23]

The assessment of their interaction with the cell membrane *in vivo* is an essential element. The potential for the controlled release of PTX from those nanoparticles, which might potentially be attributed to the process of diffusion, facilitated by the partitioning between the nanoparticle core and the surrounding liquid phase. The study, MDA MB 231 and MDA MB 468 cells absorbed PTX in the form of PTX immunonanoparticle at a faster rate than free PTX. The finding provides significant evidence to support the notion that the simultaneous injection of PTX INPs efficiently suppressed the proliferation of TNBC cell.

The dimensions and electrostatic properties of the PTX immunonanoparticle nanoparticles remained very stable over a period of 6 hr (Figure 6). The complexes displayed a hydro dynamic diameter spanning from 100 nm to 350 nm and featured a surface with a positive charge (Figure 1b). Furthermore, it was observed that the application of modified mTOR led to an improvement in MRs, which can be associated with the decreased in particle size and the simultaneous increase in net charge. The lack of smeared or fragmented bands on the gel suggests that siRNA from the PLGA nanoparticles was intact. This implies that the nanoparticles preserved the encapsulated siRNA's structural integrity and prevented it from being released prematurely into the serum.[24]

CONCLUSION

The study was able to formulate a novel PTX immunonano particle drug delivery method with PLGA and siRNA conjugated with EGFR for controlled release. It demonstrated anti-proliferative activities against MDA MB 231 and MDA MB 468 cells. The nanoparticles were homogeneous and spherical nanoparticles with high stability in physiological condition. Due to their lower toxicity than cationic lipid or polymer vectors, siRNA and antibody-based vectors are attractive gene delivery systems. Polymeric-based gene transfer nanoparticles can rapidly stimulate the innate immune response after local and systemic delivery. These compounds are also more likely to damage cells. Our *in vitro* studies showed that PTX immunonanoparticle was low toxicity nano delivery platform with mTOR and EGFR complexes on it. This study introduced modified amphiphilic PTX immunonanoparticle for breast-targeted mTOR administration in many *in vitro* settings. As with previous cationic delivery systems, these positively charged modified PTX NP engage electrostatically with negatively charged siRNAs. In this study, the self-assembling nanoparticle system bound and complexed with PTX NP-targeted siRNAs to generate nanoscale, positively charged particles. By improving interactions, low particle size and improved surface charge can help to boost cellular absorption.

ACKNOWLEDGEMENT

The authors are grateful for the support and facilities provided by AIMST University and the Oncological and Radiological Sciences Cluster, Advanced Medical and Dental Institute, University of Science and Technology of Malaysia (USM).

FUNDING

This research has been funded by the Fundamental Research Grant Scheme (FRGS) under a grant number of FRGS/1/2017/SKK08/AIMST/02/3 from the Ministry of Education Malaysia.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

BC: Breast cancer; **CDR:** Cumulative drug release; **DCM:** Dichoromethane; **DMSO:** Dimethyl sulphoxide; **EDTA:** Ethylenediaminetetraacetic acid; **EE:** Entrapment efficacy; **EGFR:** Epidermal growth factor receptor; **ER:** Estrogen receptor; **HER2:** Human epidermal growth factor receptor 2; **INP:** Immunonanoparticles; **LC:** Loading capacity; **mTOR:** Mammalian target of rapamycin; **MTT:** 3-[4, 5-dimethylthiazol2-yl]-2, 5-diphenyltetrazolium bromide); **MBS:** m-Maleimidobenzoy l-N-hydroxysuccinimide ester; **NP:** Nanoparticles; **PLGA:** Poly (lactic-co-glycolic acid); **PmAb:** Panitumumab; **PTX:** Paclitaxel; **PVA:** Polyvinyl alcohol; **SEM:** Scanning electron microscope; **TEM:** Transmission electron microscopy.

REFERENCES

1. Haddad R, Alrabadi N, Altaani B, Li T. Paclitaxel drug delivery systems: focus on nanocrystals' surface modifications. Polymer. 2022;14(4):658. doi: 10.3390/polym14040658, PMID 35215570.
2. Gupta U, Sharma S, Khan I, Gothwal A, Sharma AK, Singh Y, *et al.* Enhanced apoptotic and anticancer potential of paclitaxel loaded biodegradable nanoparticles based on chitosan. Int J Biol Macromol. 2017;98:810-9. doi: 10.1016/j.ijbiomac.2017.02.030, PMID 28189791.
3. Anand U, Dey A, Chandel AK, Sanyal R, Mishra A, Pandey DK, *et al.* Cancer chemotherapy and beyond: current status, drug candidates, associated risks and progress in targeted therapeutics. Genes Dis. 2023;10(4):1367-401. doi: 10.1016/j.gendis.2022.02.007, PMID 37397557.
4. Amjad MT, Chidharla A, Kasi A. Cancer chemotherapy. Treasure Island, (FL): StatPearls Publishing; 2024.
5. Sugo K, Ebara M. A simple spectrophotometric evaluation method for the hydrophobic anticancer drug paclitaxel. PeerJ Anal Chem. 2020;2(3):e3. doi: 10.7717/peerj-achem.3.
6. Zhao Y, *et al.* Recent advances in drug delivery systems for targeting brain tumors. Drug Deliv. 2023;30:1-18.
7. Kumar H, Gupta NV, Jain R, Madhunapantula SV, Babu CS, Kesharwani SS, *et al.* A review of biological targets and therapeutic approaches in the management of triple-negative breast cancer. J Adv Res. 2023;54:271-92. doi: 10.1016/j.jare.2023.02.005, PMID 36791960.
8. Da Costa R, Passos GF, Quintão NL, Fernandes ES, Maia JR, Campos MM, *et al.* Taxane-induced neurotoxicity: pathophysiology and therapeutic perspectives. Br J Pharmacol. 2020;177(14):3127-46. doi: 10.1111/bph.15086, PMID 32352155.
9. Chehelgerdi M, Chehelgerdi M, Allela OQ, Pecho RD, Jayasankar N, Rao DP, *et al.* Progressing nanotechnology to improve targeted cancer treatment: overcoming hurdles in its clinical implementation. Mol Cancer. 2023;22(1):169. doi: 10.1186/s12943-023-01865-0, PMID 37814270.
10. Kwak SY, Han HD, Ahn HJ. A T7 autogene-based hybrid mRNA/DNA system for long-term shRNA expression in cytoplasm without inefficient nuclear entry. Sci Rep. 2019;9(1):2993. doi: 10.1038/s41598-019-39407-8, PMID 30816180.

11. Pulingam T, Foroozandeh P, Chuah JA, Sudesh K. Exploring various techniques for the chemical and biological synthesis of polymeric nanoparticles. Nanomaterials (Basel). 2022;12(3):576. doi: 10.3390/nano12030576, PMID 35159921.
12. Hernández-Giottonini KY, Rodríguez-Córdova RJ, Gutiérrez-Valenzuela CA, Peñuñuri-Miranda O, Zavala-Rivera P, Guerrero-Germán P, *et al.* PLGA nanoparticle preparations by emulsification and nanoprecipitation techniques: effects of formulation parameters. RSC Adv. 2020;10(8):4218-31. doi: 10.1039/c9ra10857b, PMID 35495261.
13. Marques AC, Costa PC, Velho S, Amaral MH. Lipid nanoparticles functionalized with antibodies for anticancer drug therapy. Pharmaceutics. 2023;15(1):216. doi: 10.3390/pharmaceutics15010216, PMID 36678845.
14. Gazzi RP, Contri RV, Pohlmann AR, Guterres SS, Frank LA. Pharmaceutical nanocarrier characterization. In: Talevi A, editor. The ADME encyclopedia: A comprehensive guide on biopharmacy and pharmacokinetics; 2022. p. 793-802.
15. Raza F, Zafar H, Khan MW, Ullah A, Khan AU, Baseer A, *et al.* Recent advances in the targeted delivery of paclitaxel nanomedicine for cancer therapy. Mater Adv. 2022;3(5):2268-90. doi: 10.1039/D1MA00961C.
16. Gandhi NS, Godeshala S, Koomoa-Lange DT, Miryala B, Rege K, Chougule MB. Bioreducible poly(amino ethers) based mTOR siRNA delivery for lung cancer. Pharm Res. 2018;35(10):188. doi: 10.1007/s11095-018-2460-z, PMID 30105526.
17. Alves RC, Fernandes RP, Eloy JO, Salgado HR, Chorilli M. Characteristics, properties and analytical methods of paclitaxel: a review. Crit Rev Anal Chem. 2018;48(2):110-8. doi: 10.1080/10408347.2017.1416283, PMID 29239659.
18. Zhang Y, Cui H, Zhang R, Zhang H, Huang W. Nanoparticulation of prodrug into medicines for cancer therapy. Adv Sci (Weinh). 2021;8(18):e2101454. doi: 10.1002/advs.202101454, PMID 34323373.
19. Li QY, Lee JH, Kim HW, Jin GZ. Research models of the nanoparticle-mediated drug delivery across the blood-brain barrier. Tissue Eng Regen Med. 2021;18(6):917-30. doi: 10.1007/s13770-021-00356-x, PMID 34181202.
20. Pandya T, Kulkarni M, Acharya S, Prajapati BG Chapter 11. PLGA mediated drug delivery for Alzheimer's disease. In: Alzheimer's disease and advanced drug delivery strategies; 2024. p. 181-96.
21. Liu Z, Dong X, Liao Y, Fan Y, Cao Y. Effect of carboxyl group position on assembly behavior and structure of hydrocarbon oil-carboxylic acid compound collector on low-rank coal surface: sum-frequency vibration spectroscopy and coarse-grained Molecular Dynamics simulation study. Molecules. 2024;29(5):1034. doi: 10.3390/molecules29051034, PMID 38474546.
22. Aslam A, Berger MR, Ullah I, Hameed A, Masood F. Preparation and evaluation of cytotoxic potential of paclitaxel containing poly-3-hydroxybutyrate-co-3-hydroxyvalarate (PTX/PHBV) nanoparticles. Braz J Biol. 2023;83:e275688. doi: 10.1590/1519-6984.275688, PMID 37970904.
23. Yusuf A, Almotairy AR, Henidi H, Alshehri OY, Aldughaim MS. Nanoparticles as drug delivery systems: a review of the implication of nanoparticles' physicochemical properties on responses in biological systems. Polymer. 2023;15(7):1596. doi: 10.3390/polym15071596, PMID 37050210.
24. Zare M, Pemmada R, Madhavan M, Shailaja A, Ramakrishna S, Kandiyil SP, *et al.* Encapsulation of miRNA and siRNA into nanomaterials for cancer therapeutics. Pharmaceutics. 2022;14(8):1620. doi: 10.3390/pharmaceutics14081620, PMID 36015246.

Cite this article: Shalini K, Varatharajan R, Shahrul H, Vasanth Raj P, Vijayan V. EGFR-mTOR siRNA Loaded Immumonanoparticles for Targeted Chemotherapy of Triple Negative Breast Cancer. J Young Pharm. 2024;16(4):714-24.