# Targeted Nanoparticle-Polypeptide Bioconjugates for Breast Cancer Therapy

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## ABSTRACT

Novel approaches that allow for a more effective and less toxic chemotherapy for disseminated breast cancer are needed for which the current therapies are largely ineffective. We report the development of controlled release polymer nanoparticles using a poly ($_{D,L}$ lactic)-poly(ethylene glycol) copolymer with a terminal maleimide functional group (PLA-PEG-MAL). These nanoparticles were conjugated to a polypeptide sequence that has preferential binding characteristics for the Her-2 receptor, a well characterized antigen that is over-expressed on the surface of breast cancers. The conjugation of the peptide does not affect the physical properties of the nanoparticles and we did not observe any nanoparticle aggregation. We next demonstrated a differential cytotoxity of these bioconjugates using docetaxel encapsulated targeted nanoparticles. These bioconjugates specifically bound to and were taken up by the Her2 expressing cells resulting in enhanced differential cytotoxity to empty nanoparticle controls. These targeted drug delivery nanoparticles could be used as a powerful therapeutic tool for Her2 expressing cancers.

***Keywords***: Her2, Poly (lactic acid), PLA, targeted nanoparticles, docetaxel

## 1 INTRODUCTION

Nanocarriers have the potential to improve manifold the current cancer chemotherapies by increasing drug efficacy, lowering drug toxicity, and achieving a high drug dose over an extended period of time. Nanocarriers can also improve drug solubility and drug stability, allowing the development of potentially effective new chemical entities that have been stalled during the pre-clinical or clinical development because of suboptimal pharmacokinetic or biochemical properties. Recently the breakthrough potential of cancer nanotechnology is becoming increasingly recognized with several examples of first generation drug delivery nanocarriers approved by the Food and Drug Administration (FDA) for cancer therapy (Abraxane®[1,2], Doxil®[3], DaunoXome®[4]). Abraxane, a 130 nm albumin bound nanoparticle formulation solvent free form of paclitaxel has been recently approved for breast cancer therapy. Abraxane has been shown to allow the delivery of a double dose of paclitaxel compared to Taxol® drug formulation without increasing side effects in breast cancer patients[2]. Cancer therapy using targeted nanoparticles functionalized with ligands that bind specifically to cell membrane antigens is emerging as a powerful approach to increase the therapeutic index of cytotoxic drugs. Therefore, we developed FDA approved based polymeric PLA-PEG long-circulating nanoparticle-polypeptide bioconjugates for targeted delivery and uptake in Her2 positive breast cancer cells. Considering the favorable properties of polypeptides (PP) such as small hydrodynamic size, lack of immunogenicity and ease to scale up synthesis, we became interested to use an anti-Her2 specific polypeptide for targeted docetaxel delivery using biocompatible polymeric nanoparticles.

## 2 MATERIALS AND METHODS

*Polymeric nanoparticles preparation*: Nanoparticles are formed by precipitating the PLA-PEG-MAL copolymer in water. Briefly, the polymer is dissolved in acetonitrile, and then mixed slowly with water. The nanoparticles form instantly upon mixing. The residual acetonitrile in the suspension is evaporated by continuously stirring the suspension at room temperature for 4 hours.

*Nanoparticle characterization*: Size (nm) and surface charge (Zeta-potential) of NPs are evaluated by Quasi-elastic laser light scattering (QELS) using a ZetaPALS dynamic light-scattering detector (15 mW laser, incident beam = 676 nm; Brookhaven Instruments, Holtsville, NY).

*Conjugation and characterization of nanoparticle-polypeptide bioconjugates:* Polymeric nanoparticles are incubated with the polypeptide (anti-Her2 polypeptide, 15kDa) solution and allowed to react for 12 hours under stirring conditions at 4 °C in aqueous buffer (Figure 1). Next, the bioconjugates is purified from free polypeptide using Amicon Ultra centrifuge device with a molecular weight size exclusion of 100 kDa (Millipore). Subsequently, the nanoparticle-polypeptide bioconjugates are characterized using protein gel electrophoresis and Fourier Transform Infrared spectroscopy (FTIR). The purified nanoparticle-polypeptide conjugates are loaded

into NuPAGE 4-12 % Bis-Tris protein gels (Invitrogen) to quantify residual free polypeptide and evaluate the purity of the bioconjugates solution.

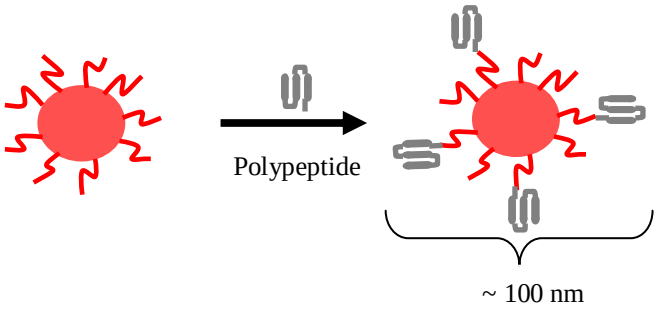


**Figure 1**: Schematic diagram of the synthesis of the PLA-PEG-Polypeptide nanoparticle bioconjugates.

*Binding assays of targeted and non targeted nanoparticles*: SKBR3 and MCF7 cell lines are grown in chamber slides within their respective growth medium to allow 70% confluence in 24 h (i.e., 40,000 cells/cm$^2$). On the actual experiment day, cells are washed with prewarmed PBS and incubated with prewarmed phenol-red–reduced OptiMEM media for 30 minutes, before adding 50 µg of nanoparticles or nanoparticle-polypeptide bioconjugates loaded with rhodamine dye (red). Formulations are incubated with cells for 2 hours at 37°C, washed with PBS three times, fixed with 4% Para formaldehyde, counterstained with Alexa-Fluor Phalloidin (cytoskeleton staining; green) 4',6-diamidino-2-phenylindole (nucleus staining, blue), mounted, and visualized by fluorescent microscopy (DeltaVision system).

*In vitro toxicity study of drug encapsulated into targeted and non-targeted bioconjugates*: SKBR3 and MCF7 are grown in 6-well plates in Modified McCoy's 5a and Ham's 12FK medium respectively (American Type Culture Collection). They are supplemented with 100 units/ml aqueous penicillin G, 100 ug/ml streptomycin, and 10% FBS at concentrations that allow 70% confluence in 24 h (i.e., 40,000 cells per cm$^2$). Defined concentration of the drug encapsulated nanoparticles and nanoparticle-polypeptide bioconjugates (50 ug) are incubated with SKBR3 and MCF7 cell lines in OptiMEM for two hours. Next, cells are washed and fresh media is supplemented. The cells are then allowed to grow for 72 hours and cell viability is assessed colorimetrically with MTT reagents (Invitrogen).

## 3 RESULTS AND DISCUSSION

**Development of polymeric nanoparticle-polypeptide bioconjugates to form stable targeted nanoparticles.** We developed "stealth" nanoparticles using poly ($_{D,L}$ lactic)-poly (ethylene glycol) copolymers to increase their circulation half-life and lower nonspecific interactions targeted nanoparticles. The copolymer is modified with a maleimide (MAL) terminal functional group to specifically react with free thiol modified polypeptide (polypeptide-SH) and form a stable bond necessary for targeted applications. This conjugation approach allows us to efficiently and specifically conjugate the anti-Her2 polypeptide to the polymeric nanoparticles with a proper orientation for specific binding to breast cancer cell membranes antigens. We characterized the formation of the bioconjugates using FTIR spectroscopy. Figure 2a shows the FTIR spectra of the nanoparticles with and without polypeptides. The corresponding peaks of carboxylic acid group (stretching; C=O; 1639 cm$^{-1}$) and amide bond (stretching, N-C=O, 1657 and 1548 cm$^{-1}$) appear for the nanoparticle and nanoparticle-polypeptide bioconjugates, respectively. This result is suggesting that the formation of the stable bond between the polymer and the polypeptide has been successful. To confirm the lack of free polypeptide in the nanoparticle-polypeptide solution, we performed a protein gel electrophoresis assay using the nanoparticle-polypeptide bioconjugates solution. We evaluated the presence of residual polypeptide into the nanoparticle-polypeptide bioconjugates solution using a defined amount of free polypeptide. The results shows a residual amount of polypeptide estimated to be ~ 1% of the initial "feed", hence suggesting the high purity of the nanoparticle-polypeptide bioconjugates. This result supports the actual formation of the nanoparticle-polypeptide bioconjugates.

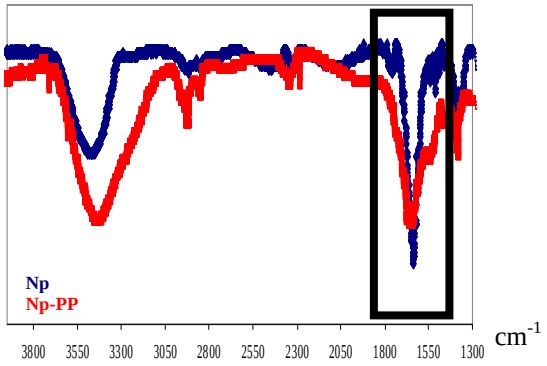


**Figure 2.** FTIR spectra of PLA-PEG-MAL nanoparticles with and without polypeptide.

Next, we formed targeted and non targeted polymeric nanoparticles by nanoprecipitation followed by the conjugation of the anti-Her2 polypeptide on the nanoparticle surface to form nanoparticle-polypeptide bioconjugates. The nanoparticle formulations were characterized for their size, nanoparticle size distribution and surface charge (ζ-potential) using a ZetaPALS dynamic light scattering detector. The nanoparticles generated show an averaged diameter of ~ 115 nm (± 6nm) similarly to the nanoparticle-polypeptide bioconjugates (130 nm; ± 5nm; data not shown). In addition, both targeted and non-targeted nanoparticles showed narrow size distribution (polydispersity index ~ 0.2; data not shown). Therefore, the physico-chemical properties of the polymeric nanoparticles were not affected by the conjugation of the polypeptide ligand.

**In vitro binding and uptake of nanoparticle-polypeptide bioconjugates using targeted breast cancer cells.** We next demonstrated that the binding and uptake of the targeted nanoparticles to Her2 positive breast cancer cells was significantly enhanced compared to non-targeted nanoparticle formulation. In the case the of SKBR3, a well established breast cancer cell line expressing Her2 antigens, we observed the internalization of the targeted nanoparticles within 90 minutes of incubation in contrast to the non targeted formulation (Figure 3). The results suggest that the nanoparticle-polypeptide bioconjugates are internalized via an endocytosis dependent mechanism in SKBR3 cells after binding to the HER2 antigens on the cell membrane. This is consistent with the polypeptide internalization mechanism through specific binding to Her2 antigens. The localization of the nanoparticle-polypeptide bioconjugates using z-axis reconstitution fluorescent image (Figure 3C) is confirming the internalization of the targeted nanoparticles.

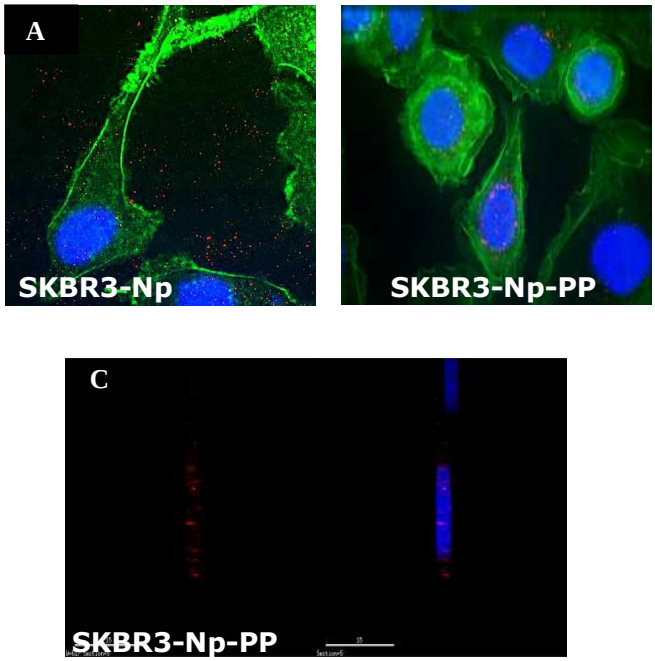


**Figure 3.** Fluorescent microscopy picture of nanoparticles with and without polypeptide incubated for 90 minutes on HER2 positive breast cancer cells (SKBR3). **a)** Nanoparticles (Np), **b)** Nanoparticle-polypeptide bioconjugates (Np-PP), and **c)** Image showing the cross section of a single cell through the mid z-axis point with and without nucleus staining.

**In vitro cellular cytotoxicity of nanoparticles with and without polypeptide using SKBR3 cells.**
In this study, we examined the differential cytotoxicity of encapsulated docetaxel in targeted and non targeted formulation using Her2 positive SKBR3 cells. We incubated the nanoparticle formulations including controls nanoparticles without docetaxel with SKBR3 cells for 2 hours to allow specific interaction and subsequently incubated the cells in growth media for a total of 72 hours prior to the measurements of cell toxicity using colorimetric assays (MTS). The data suggests that the nanoparticle-polypeptide bioconjugates did not show a significant cytotoxicity compared to non-targeted nanoparticles. This is probably due to the non specific toxicity of the docetaxel released from the nanoparticles during the incubation period locally in the media. However, nanoparticle formulations without drug encapsulated did not affect the cell viability, confirming the lack of cytotoxicity of the nanoparticle formulations without docetaxel.

In summary, we have developed controlled release polymeric nanoparticle-polypeptide bioconjugates that can specifically bind to Her2 antigens and internalize into Her2 positive breast cancer cells. This drug delivery system might become a potent technology to deliver chemotherapeutic drugs such as docetaxel to Her2 positive breast cancer cells. Additional in vitro formulation optimization is required to improve the cytotoxicity efficiency of the system and in vivo studies are needed to validate the therapeutic value of the Her2 targeted nanoparticle-bioconjugates.

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