Antibody-Conjugated Nanoparticles of Biodegradable Polymers for Targeted Drug Delivery

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ABSTRACT

Target-specific delivery of diagnostic and/or therapeutic agents has become a main concern in the research of nanomedicine and in the pharmaceutical industry. Formulation of targeting effects can deliver high dose of the formulated diagnostic/therapeutic agents specifically to the diseased cells and leave the healthy cells untouched, which can thus result in much higher diagnostic/therapeutic effects and much less side effects. Fatal diseases such as cancer can thus be cured at their earliest stage. Among various targeting strategies, antibody conjugation may be most promising with high reaction response. In this chapter, we present a proof-of-concept

research to demonstrate how the antibody-conjugated nanoparticles (NPs) of novel biodegradable copolymers can be developed for targeted chemotherapy by using Herceptin (Trastuzumab®), an only United States Food and Drug Administration (FDA) approved antibody drug, as a model ligand and Docetaxel, one of the best antineoplastic drugs, as a model drug. NPs of two component biodegradable copolymers were prepared by a modified solvent extraction/evaporation method with D-α-tocopheryl polyethylene glycol succinate (vitamin E TPGS or simply TPGS) as emulsifier. One component copolymer is poly(lactide)-D-α-tocopheryl polyethylene glycol succinate (PLA-TPGS), which is of desired hydrophobic-lipophilic balance (HLB) for cellular adhesion, and another is carboxyl group-terminated TPGS (TPGS-COOH), which facilitates the conjugation of Herceptin on the nanoparticle surface for targeting. *In vitro* investigation with SK-BR-3 breast cancer cells of HER2 overexpression showed that the Herceptin-conjugated NPs have great advantages versus the nude NPs in cellular uptake and cytotoxicity. The Herceptin conjugated on nanoparticle surface has two functions: one is to target HER2-overexpressing cancer cells and the other is to enhance the cytotoxicity of Docetaxel through synergistic effects. The Herceptin-conjugated, Docetaxel-loaded PLA-TPGS NPs have great potential for targeted chemotherapy to treat HER2-overexpressing cancer.

1. NANOTECHNOLOGY

Nanotechnology is a multidisciplinary field and can be defined as a science and engineering discipline that involves the design, synthesis, characterization and application of materials and devices whose smallest functional organization in at least one dimension is on the nanometer scale.[1] Nanotechnology opens the door to a new generation of techniques and devices for cancer diagnosis, treatment and prevention. For example, intracellular imaging can be achieved with the

aid of nanotechnology, in which multicolor fluorescence imaging is realized by attaching imaging agents such as semiconductor quantum dots (QDs) to selected molecules, or coating the QDs by a layer of amphiphilic molecules. Moreover, nanoparticles with a drug encapsulated in a polymer matrix can realize a controlled and sustained manner for drug delivery. Controlling the localization of nanoparticles to the diseased cells by modification of the nanoparticle surface with targeting moieties is now gaining great interest in the field of nanomedicine. Not only anticancer drugs can be incorporated in the nanoparticles to kill the cancer cells, but diagnostic agents as well, which can be utilized to image the presence of cancer cells. The later will be powerful to detect the development of cancer at very early stage. This, in turn, promises better and more effective treatment for cancer diseases than ever before.

2. NANOPARTICLES

Recently, biodegradable nanoparticles have attracted great interest due to their advantages over conventional therapeutic strategies. Types of such nanoparticles include polymeric micelles, liposomes and polymer-based nanoparticles. Nanoparticles of biodegradable polymers can be used as therapeutics containing small-molecule drugs, peptides, proteins, as well as nucleic acids. Compared to conventional therapeutic strategies, nanoparticles can improve the solubility of poorly soluble drugs, increase the drug half-life as well as the specificity to the target sites e.g. tumors. Most nanoparticles preferentially accumulate within tumors via the enhanced permeability and retention (EPR) effect.[2] Thus, polymeric nanoparticles allow for enhancing the intracellular drug concentration in cancer cells whereas avoiding toxicity in normal cells, resulting in potent therapeutic effects.

There are a variety of nanoparticle systems being developed for cancer diagnosis and therapeutics. The material properties of each nanoparticle system have been developed to enhance delivery to the tumor. For example, hydrophilic surfaces can be used to provide the nanoparticles with stealth properties for longer circulation times and positively charged surfaces can enhance endocytosis. Nanoparticles can be tailor-made to achieve both controlled drug release and disease-specific localization by altering the polymer characteristics and surface chemistry.[3-5]

2.1. Nanoparticle properties and emulsifiers

Physicochemical properties of nanoparticles would determine *in vitro* and *in vivo* performances of the nanoparticles. These properties include particle size and size distribution, surface morphology, surface chemistry, surface charge, surface adhesion/erosion, drug diffusivity, drug encapsulation efficiency, drug stability, drug release kinetics, etc. Several factors influencing an optimal design of nanoparticles include the polymer type as well as the molecular weight, the copolymer blend ratio, the type of organic solvent, the emulsifier/stabilizer, the oil-to-water phase ratio, the mechanical strength of mixing, temperature, pH, etc. Among these, the polymer type, *i.e.* its molecular structure and its molecular weight, and the copolymer blend ratio are the major factors in determining the degradation rate of the nanoparticles and thus the *in vitro* and *in vivo* release of the encapsulated drug.

Emulsifiers, the macromolecules which are added in the emulsification process for nanoparticles preparation, play an important role in stabilizing the colloidal suspension during nanoparticle fabrication. The amphiphilic emulsifier molecules are supposed to stay at the oil-water interface to decrease the interfacial tension, *i.e.* the nanoparticle surface energy per unit area, to facilitate the nanoparticle formation.[6] Poly(vinyl alcohol) (PVA) has been preferentially

chosen as emulsifier in nanoparticles fabrication due to its excellent stabilizing ability to avoid particles aggregation during post-preparative steps (e.g. freeze-drying and purifying), high yield of dry particles powder and ease to be redispersed in solution after lyophilized.[7] It has also been found, however, that PVA may be adsorbed or tightly associated with the surface layer and thus can not be completely removed from the surface of nanoparticles.[8-10] Therefore, PVA should not be desired to be used as emulsifier in preparation of nanoparticle formulation for *i.v.* administration although its safety has been revised.[11] Instead, phospholipids such as Dipalmitoyl phosphatidylcholine (DPPC) and TPGS were found to be more effective emulsifiers in nanoparticles fabrication.[12,13] Phospholipids with short and saturated chains showed higher efficiency in emulsion of polymeric micro/nanoparticles of better physical/chemical properties. There is no significant difference in morphology between TPGS- and PVA-emulsified PLGA nanoparticles. However, TPGS has been found to be able to improve the drug encapsulation efficiency up to 100% compared with PVA-emulsified nanoparticles, which was accounted as only 59%. Moreover, the amount of TPGS needed in the fabrication process was only 0.015% (w/w), which was far less than 1% for PVA needed in similar process.[14,15]

2.2. Nanoparticle fabrication

Nanoparticles can be fabricated by various methods such as polymerization or dispersion of the performed polymers, solvent extraction/evaporation method, salting-out method, dialysis method, supercritical fluid spray technique and nanoprecipitation method. Solvent extraction/evaporation is used in current research due to its acceptable drug loading efficiency, ease of processing and good reproducibility. In this review we highlighted the most useful methods of nanoparticle fabrication: solvent extraction/evaporation, dialysis, and nanoprecipitation methods.

2.2.1. Solvent extraction/evaporation method

Solvent extraction/evaporation method is the most widely used technique for nanoparticles fabrication. In this technique, a selected polymer is firstly dissolved in an organic solvent such as dichloromethane, chloroform and ethyl acetate. The hydrophobic drug is then dissolved in the polymer solution. The formed solution is dispersed in an aqueous phase with or without surfactant/stabilizer such as PVA, gelatin, poloxamer 188, DPPC, TPGS, etc. The mixture is emulsified by either high-speed homogenization or high voltage sonicator leading to the formation of an oil-in-water emulsion. After a stable emulsion is formed, the organic solvent is evaporated under increased temperature, reduced pressure or continuously stirring at room temperature. Further processes including multiple centrifugation/washing and lyophilization will finally result in dried nanoparticles.[12,13] The pharmaceutical characteristics of the nanoparticles can be influenced by many factors of fabrication process such as the polymer concentration in the solvent, the ratio of organic to aqueous phase, the type and concentration of emulsifiers, the drug loading ratio, the strength of mixing energy in emulsifying and evaporation, and post treatment of nanoparticles including centrifugation, washing, lyophilization, sterilization, pH condition as well as temperature.[15]

2.2.2. Dialysis method

Dialysis is another self-assembling method for nanoparticles fabrication, in which nanoparticles can be fabricated with or without surfactant/additive/stabilizer. The drug and polymer are dissolved in a water-miscible organic solvent. The solution is then transferred into a cellulose membrane bag, which is then immersed in a container filled with water for one or two days. The water is exchanged at certain interval to maintain the osmotic pressure, which removes the solvent and unloaded drug from the membrane bag. The nanoparticle dispersion is then

centrifuged to further eliminate the unloaded drug. In dialysis, the molecular weight cut-off (MWCO) of the membrane is one of the additional controlling factors which may influence the suitability of dialysis for some polymers.[16] In addition, the porosity of the membrane controls the rate at which the organic phase is dialyzed, thus affecting the size of nanoparticles. The main limitations of the dialysis method are time-consuming and large volume of tank is required. Therefore, large scale operation may not be feasible.

2.2.3. Nanoprecipitation method

Nanoprecipitation is a nanoparticle synthesis process involving solvent displacement followed by interfacial deposition of pre-formed polymer. This technique was developed by Fessi *et al*.[17] The oily phase commonly used is water-soluble organic solvents such as acetone, acetonitrile and dimethylformamide. The polymer and drug are dissolved in the organic phase. When the organic phase is mixed dropwise with the aqueous phase under gentle magnetic stirring, an emulsification is formed spontaneously. The solvent is evaporated overnight with gently stirring. The particles are collected by filtration to remove the aggregation. The filtrate is then centrifuged and lyophilized.

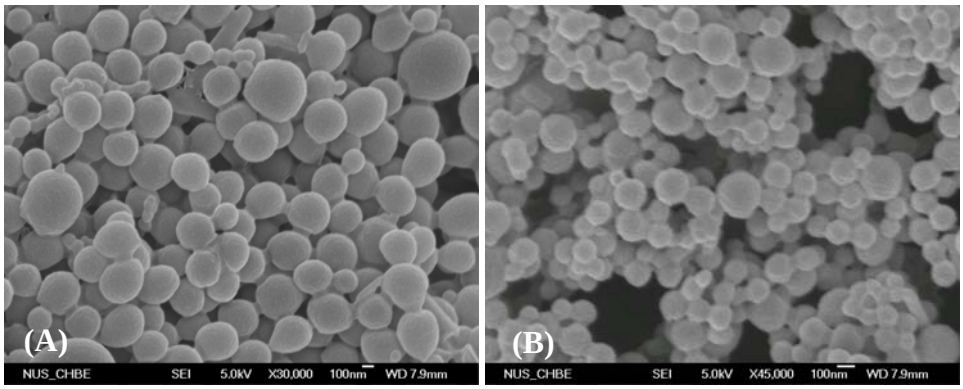

Figure 1. Field Emission Scanning Electron Microscope (FESEM) images of PLGA nanoparticles fabricated by (A) the solvent extraction/evaporation method; and (B) the nanoprecipitation method.

2.3. Nanoparticles for targeted drug delivery

Nanoparticle-based drug delivery systems have considerable potential for treatment of various diseases, especially attractive for difficult-to-treat diseases like cancer.

Targeted therapy is defined as a type of treatment that uses drugs or other substances to identify and attack specific target cells e.g. cancer cells (http://www.cancer.gov). Targeted therapy may be more effective and have fewer side effects than general treatments without targeting effects. Current cancer therapy usually involves intrusive processes to allow chemotherapy to shrink any cancer present or surgery to remove tumor if possible. For the majority of patients with advanced stage of cancer, the treatment is limited to chemotherapy and/or radiotherapy. The purpose of chemotherapy and radiotherapy is to kill the cancer cells as these cells are more susceptible to the actions of these drugs and therapies as they grow at a much faster rate than healthy cells. The effectiveness of the treatment is directly related to the treatment's ability to target and to kill the cancer cells while affecting as few healthy cells as possible. Moreover, the degree of change in the patient's quality of life and eventual life expectancy is directly related to the targeting ability of the treatment.[18,19]

Though there have been several decades of research and development, targeted delivery has not yet fulfilled the initial promise in the treatment of cancer. The targeted delivery of anticancer drugs to solid tumors is a complicated issue because of the impediments to drug delivery.[20,21] The major impediments to drug delivery arise from tumor heterogeneity. Cancer cells typically occupy less than half of the total tumor volume, among which approximately 1~10% is contributed by tumor vasculature, and the rest is occupied by a collagen-rich interstitium. The heterogeneous distribution of blood vessels, antigen and receptor expression in tumors is also a problem in affinity-targeted delivery of drugs to solid tumors.[22,23]

The development of a wide spectrum of nanoscale technologies has tremendous potential to make an important contribution in cancer imaging, diagnosis, treatment and prevention.[24,25] These technological innovations have the potential to turn molecular discoveries arising from genomics and proteomics into widespread benefit for patients. These devices include, but are not limited to, functionalized carbon nanotubes, nanomachines, magnifiers, self-assembling polymeric nanoconstructs, nanomembranes, and nano-sized silicon chips for drug, protein, nucleic acid, or peptide delivery and biosensors and laboratory diagnostics. In terms of biodegradable polymeric nanoparticles, targeting ability can be achieved by functionalizing the surface of drug-loaded nanoparticles with targeting ligands which can then recognize and bind to certain receptors overexpressed on the membrane of the cancer cells.

Targeted cancer therapies interfere with cancer cell growth and division in different ways and at various points during the development, growth, and spread of cancer. Many of these therapies focus on proteins that are involved in the signaling process. Targeted molecular therapies target tumor antigens to alter the signaling either by monoclonal antibodies (mAb) or by small molecule drugs that interfere with these target proteins. By blocking the signals which tell cancer cells to grow and divide uncontrollably, the approaches can help to stop the growth and division of cancer cells. This new type of approaches has been termed as targeted therapy, the goal of which is to provide molecular levels-based agents that are more specific for cancer cells.[26] Targeted therapies are also often useful in combination with cytotoxic chemotherapy or radiotherapy to produce additive or synergistic anticancer activity because their toxicity profiles often do not overlap with traditional cytotoxic chemotherapy. Thus, targeted therapies represent a new and promising approach to cancer therapy leading to beneficial clinical effects. There are

multiple types of targeted therapies available, including monoclonal antibodies, tyrosine kinases inhibitors, and antisense inhibitors of growth factor receptors.

2.3.1. Passive targeting

Targeted delivery can be achieved in two manners, namely, passive and active. Passive targeting makes use of the tumor microenvironment which is characterized by a leaky tumor vasculature and a dysfunctional lymphatic drainage system. Most polymeric nanoparticles display the EPR effect, which is a consequence of the increased vasculature permeability and decreased lymphatic function of tumors. It occurs when nanoparticles extravasate out of the tumor microvasculature, leading to an accumulation of drugs in the tumor interstitium.[27] Thus passive targeting is achieved by incorporating the therapeutic agent into a macromolecule or nanoparticle that passively reaches the target organ through the EPR effect.

Passive targeting also involves the use of other innate characteristics of the nanoparticles which can induce targeting to the tumor, such as surface charge. For example, cationic liposomes are found to exhibit a tendency to bind through electrostatic interactions to negatively charged phospholipid headgroups preferentially expressed on tumor endothelial cells.[28,29] Catheters can be alternatively used to infuse nanoparticles to the target organ or tissues. For example, localized delivery of drug-loaded nanoparticles to sites of vascular restenosis may be helpful for providing sustained drug release at specific sites on the arterial wall.[30]

For passive targeting to be successful, the nanoparticles with chemotherapeutic agents encapsulated have to circulate in the blood for extended time so that there will be multiple possibilities for the nanoparticles to pass by the target sites. Nanoparticles normally have short circulation half-lives due to natural defense mechanisms of the body to eliminate them after opsonization by the mononuclear phagocytic system (MPS).[31] Therefore, the particle surfaces

need to be modified to be invisible to opsonization. A number of studies applied various surface modification approaches to classical nanocarriers to increase their circulation half-lives for effective passive targeting or sustained drug effect. These approaches include incorporation of linear dextrans, sialic acid-containing gangliosides, and lipid derivatives of hydrophilic polymers such as PEG to provide steric stabilization around the liposomes for protection from the MPS uptake.[32,33] For example, polyethylene glycol (PEG), a hydrophilic polymer, is commonly used to lengthen the circulation time. This is because PEG has desirable attributes such as low degree of immunogenicity and antigenicity, chemical inertness of the polymer backbone, and availability of the terminal primary hydroxyl groups for derivatization.[34]

Passive targeting results in high drug concentration in tumors and reduced drug toxicity to the normal tissues. However, the biggest limitation of passive targeting lies in its inability to deliver a sufficiently high level of drug concentration to the tumor site.[35] As this technology lacks tumor specificity and has less ability to control the release of the entrapped agents, the focus currently has gradually shifted from passive to active targeting nanoparticles.

2.3.2. Active targeting

In comparison with passive targeting exploiting the characteristic features of tumor biology that allow nanocarriers to accumulate in the tumor by the EPR effect, active targeting is achieved by delivering drug-encapsulated nanoparticles to uniquely identified sites while having minimal side effects.[36] There are still several limitations in passive targeting approaches although they form the basis of clinical therapy. Certain tumors do not exhibit the EPR effect and the permeability of vessels may not be the same through a single tumor.[22] It is difficult to control the passive targeting process due to the inefficient diffusion of some drugs as well as the random nature of the approach. Thus multiple-drug resistance (MDR) might occur, which is a situation

where chemotherapy treatments fail patients owing to the resistance of cancer cells to one or multiple different drugs. MDR happens when transporter proteins that expel drugs from cells are overexpressed on the surface of cancer cells. This drug resistance at its worst, when the toxic side effects are still there in full force, will kill healthy cells in the body but have no harmful effects towards the cancer cells.[37]

Active targeting attempts to take advantage of overexpressed tumor associated antigen or receptors to selectively target the drug to the tumor. In general, active targeting is achieved through the administration of nanoparticles with cell-specific ligands conjugated on their surface. These ligands can recognize and then bind to specific receptors that are uniquely expressed on cancer cells. In the case of local drug delivery, the cytotoxic drug encapsulated in the nanoparticles can be delivered directly to cancer cells while minimizing harmful toxicity to healthy cells adjacent to the target tissue.[38]

3. ANTIBODY-CONJUGATED NANOPARTICLES FOR TARGETING

3.1. Functionalization of nanoparticles for targeting

As described above, tyrosine kinases inhibitors, monoclonal antibodies and antisense inhibitors of growth factor receptors can function as a cell-specific homing device.

3.1.1. Tyrosine kinases inhibitors

Protein tyrosine kinases (PTKs) are enzymes that catalyze the phosphorylation of tyrosine residues and are especially important targets as they play an important role in the modulation of growth factor signaling and oncogenic transformation of cells.[39] There are two main classes of PTKs: receptor PTKs and non-receptor PTKs. Receptor tyrosine kinases are multi-domain proteins. Several approaches to target PTKs have been developed and classification of such

inhibitors is based on the mode of action. It has been found that activation of protein phosphorylation-related pathway in tumors can occur through overexpression of this protein as compared to normal cells.[40] Therefore, the targeted therapeutics ascribes to therapeutic agents that are as close to be mono-specific as possible to avoid the harmful side effects, which sometimes arise with traditional cancer therapies. For this reason, small molecule inhibitors of protein kinases have emerged as essential for studying targeted therapy and these drugs are also called signal-transduction inhibitors.[41]

Among the tyrosine kinases, the epidermal growth factor receptor (EGFR) family is the most widely investigated. EGFR is the cell-surface receptor for members of the EGFR family of extracellular protein ligands. The EGFR is a member of the ErbB family of receptors, a subfamily of four closely related receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4). Mutations affecting EGFR expression or activity could result in cancer. EGFR exists on the cell surface and is activated by binding of its specific ligands, including epidermal growth factor and transforming growth factor α (TGFα). Overexpression of the EGFR has been associated with a poor prognosis in a variety of solid tumors and thus represents an attractive therapeutic target.[42]

3.1.2. Monoclonal antibody

Monoclonal antibodies (mAbs) were first described by Kohler & Milstein in 1975, who shared the Nobel Prize in Physiology or Medicine in 1984 for the discovery. Monoclonal antibodies target specific molecules and are used as passive immunotherapy to treat various diseases, including certain types of cancer. These mAbs selectively target tumor tissues and have been safely administered in cancer patients. It was in the 1980s that the development of blocking mAbs to EGFR as a cancer therapy was proposed by Mendelsohm for the first time.[43] Since then,

various antibodies have become valuable therapeutic agents for targeting of extracellular proteins in various diseases, including cancer, autoimmunity and cardiovascular disorders.

Development of mAbs of human origin has proven to be a hard task. The first generation of humanized mAbs was simply chimeric antibodies, in which the variable regions of murine mAbs were linked to the constant region of a human IgG.[44] As expected, these humanized molecules should lack the immunogenicity of a murine mAb and interact more efficiently with the human immune system.[45] The second generation of humanized mAbs was the reshaped antibodies, in which the antigen-binding loops of the murine mAbs were built into a human IgG. The main problem was related to the specificity. It is usually not enough to maintain the original binding specificity by combining the complementarity-determining regions (CDRs) from the murine mAb with the frame-work of a human IgG, since several specific residues in the original murine framework contribute to maintain the CDR conformations. Molecular modeling techniques allowed this problem to be resolved, and thus reshaped antibodies have successfully been produced and used for clinical purposes.[46]

The differences between mAbs and small molecule drugs lie in several pharmacological properties. Antibodies are administered intravenously and act only on the receptors expressed on the cell surface.[47] Small molecule tyrosine kinases inhibitors are orally available small, membrane-permeable synthetic compounds that block or compete with adenosine triphosphate (ATP) binding, thus inhibiting the intracellular, downstream signaling cascade stimulated by a receptor or several receptors. The half-life of many tyrosine kinase inhibitors, such as gefitinib, is approximately 24-48 h, whereas the half-life of monoclonal antibodies such as bevacizumab is about 3-4 weeks. mAbs cannot cross the blood-brain barrier efficiently due to their large size, whereas current evidence suggests that small molecules drugs can successfully cross the blood-

brain barrier.[48] Small molecules are generally less specific than therapeutic monoclonal antibodies, therefore a higher risk of toxicity potentially come along with small molecule drugs.[49]

3.2. Trastuzumab-conjugated nanoparticles

3.2.1. HER2 targeted therapy

The EGFR family is thought to play a primary role in the control of epithelial cell proliferation and mutations affecting EGFR expression or activities that could result in cancer. The human epidermal growth factor receptor 2 (HER2) is a member of the EGFR family, which is a receptor tyrosine-specific protein kinase family consisting of four semi-homologous receptors, EGFR (ErbB1), HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). These receptors can interact with several ligands and generate intracellular signals as homodimer or heterodimer pairs.[50] HER2 is a cell membrane surface-bound receptor tyrosine kinase and is normally involved in the signal transduction pathways leading to cell growth, survival, and differentiation in a complex manner. HER2 levels correlate strongly with the pathogenesis and prognosis of breast cancer. The level of HER2 in human cancer cells with gene amplification is much higher than that in normal adult tissues, which potentially reduces the toxicity of HER2-targeting drugs. It is well known that HER2 is overexpressed in 25–30% of invasive breast cancers but in normal tissues its expression is at a much lower level.[51] HER2 overexpression is found in both the primary tumor and metastatic sites, indicating that anti-HER2 therapy may be effective in all disease sites.[52] Thus, HER2 is of great interest as an important therapeutic target in breast cancer.

3.2.2. Assessment of HER2 status

The HER2 status of a tumor is the critical determinant of response to Trastuzumab as it provides prognostic information. Thus, accurate assessment of HER2 expression levels is

essential for identifying breast cancer patients who will benefit from Trastuzumab.[53] There are several methods to test the HER2 levels and testing can be done at the same time as initial breast cancer surgery, or samples of cancer cells from previous biopsies or surgery may be used. Two most widely used methods of measuring HER2 levels in the clinical setting are immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). Both methods have been approved by FDA for selecting patients for Trastuzumab-based therapy.

IHC is widely used as it entails staining paraffin-embedded tissue with a HER2-specific antibody. When using commercially available kits such as HercepTest (Dako, Carpinteria, CA) and Pathway HER2 (Ventana, Tucson, AZ), staining is graded semiquantitatively on a scale from 0 (no detectable HER2) to 3+ (high HER2 expression) on the basis of comparison with cell lines of known HER2 receptor density. Tumors with a staining score of 3+ are the most responsive to Trastuzumab.[54] The disadvantages of IHC include the subjective interpretation and semiquantitative nature of results. Currently available IHC kits provide control slides against which samples are compared. Such standardization is essential to assuring accurate assessment of HER2 status.

FISH detects HER2 gene amplification and is more specific and sensitive than IHC.[55] Importantly, FISH offers quantitative results, possibly eliminating subjectivity and variability among different laboratories. Furthermore, FISH predicts prognosis and response to Trastuzumab more accurately than IHC does, as the subset of patients whose tumors overexpress HER2 in the absence of gene amplification are less likely to respond to Trastuzumab-based therapy. In general, a concordance rate of approximately 80% can be achieved by IHC and FISH.[56]

3.2.3. Trastuzumab (Herceptin®)

Trastuzumab (Herceptin®) is a humanized monoclonal antibody that can specifically bind to the membrane region of HER2/neu with a high affinity and inhibit signal transduction as well as cell proliferation, which offers an excellent strategy for drug targeting due to the easy accessibility of HER2. Trastuzumab was the first HER2-targeted therapy approved by FDA for the treatment of metastatic breast cancer (MBC), either in the first-line setting in combination with Paclitaxel, or as monotherapy for patients who had received at least one prior chemotherapy regimen.[57]

3.2.4. Mechanism of action of Trastuzumab

Trastuzumab consists of two antigen-specific sites binding to the HER2 receptor, which prevent the activation of its intracellular tyrosine kinase. The mechanism of action of Trastuzumab has not been fully characterized and appears to be complex. Trastuzumab exerts its antitumor therapeutic effects against tumor cells with HER2 overexpressed by several possible mechanisms.

Herceptin reduces signaling from pathways that are activated by HER2, and thus promotes cell cycle arrest and apoptosis. These pathways include the PI3 kinase (PI3K) and MAP kinase (MAPK) cascades. Research shows that HER2 remain the same level after Trastuzumab-based treatment, therefore it is still unclear whether Trastuzumab down-regulates HER2.[58] Trastuzumab therapy also increases membrane localization and activity of PTEN. Nagata *et al* demonstrated that the interaction between HER2 and the Src tyrosine kinase is disrupted in response to Trastuzumab treatment, leading to inactivation of Src with subsequent activation of the PI3K inhibitor PTEN, the protein product of the phosphatase and tensin homolog deleted on chromosome 10 gene.[59] Thus, Herceptin activates the PTEN phosphatase, resulting in rapid Akt dephosphorylation and inhibits cell proliferation.

Trastuzumab possesses not only targeting ability but also therapeutic effect compared to many targeted agents. The cytotoxic property enables Trastuzumab to block proliferation and to promote cell death, which may be related in part to induction of an immune response. Trastuzumab activated an antibody-dependent cellular cytotoxicity (ADCC) response in multiple breast cancer cell lines.[60,61] Natural killer (NK) cells, expressing the Fc gamma receptor, are a principal immune cell type involved in ADCC. NK-mediated cell lysis is activated after Trastuzumab binds to the Fc gamma receptor. Trastuzumab recruits immune effective cells that are responsible for antibody-dependent cytotoxicity, which has been put forward in preclinical models. Results showed that mice bearing BT474 HER-2-overexpressing xenografts demonstrated a tumor regression rate of 96% when treated with Herceptin.[62] However, mice lacking the Fc receptor lost much of the protective effect of Herceptin, with only 29% tumor growth inhibition observed. Therefore, NK cells and ADCC are important contributors to the cytotoxic activity of Herceptin. However, it should be noted that patients with advanced metastatic breast cancer are immuno-suppressed and may not be the optimal population to study. Additional studies are needed to better understand the importance of ADCC in mediating the response to Herceptin.

Overexpression of HER2 in human cancer cells is associated with increased angiogenesis.[63] Trastuzumab might also play a role as an antiangiogenic agent, as it has been shown to induce normalization and regression of the vasculature in a human breast tumor model with HER2 positive.[64] Trastuzumab suppresses angiogenesis by both induction of antiangiogenic factors and repression of proangiogenic factors. Expression of multiple proangiogenic factors was reduced, while expression of antiangiogenic factors was increased in tumors with therapy of Trastuzumab compared with control-treated tumors *in vivo*.[65] Moreover, the combined use of Trastuzumab and

Paclitaxel more effectively inhibited HER2-mediated angiogenesis than either treatment alone, which reflects more pronounced antitumor effects.[64] Trastuzumab may also prevent ligation of HER2 with its ligand, by which apoptosis may be induced.[66]

3.2.5. Clinical efficacy of Trastuzumab

The therapeutic efficacy and tolerability of Trastuzumab have been investigated in various studies. The initial clinical trials investigating the safety of Trastuzumab were performed on women with metastatic breast tumors overexpressing HER2. Results from Phase I and II trials in patients demonstrated that Herceptin has an acceptable tolerability profile and promising clinical efficacy in patients.[67] A response rate of 15-35% was reported for Trastuzumab monotherapy in MBC, showing that Herceptin has significant biostatic activity as a single agent.[57] Cobleigh *et al* reported a study in which the benefit of Herceptin as a single agent for MBC was evaluated.[68] In the study, two hundred and twenty-two women were enrolled, of whom all were extensively pretreated and a quarter had received more than two prior therapies and two-thirds had received Paclitaxel. Two hundred and thirteen patients received Herceptin and at 11 months, the overall response rate was found to be 15% by an independent response evaluation committee. The median response duration was 8.4 months with an estimated median survival of 13 months. Treatment was well tolerated, with only two patients discontinuing therapy due to toxicity. In addition, the side effects attributable to Herceptin include fever, chills, pain, asthenia, nausea, vomiting and cardiac dysfunction were minimal.

Herceptin has been approved for the first-line use in combination with chemotherapy. It has been found that Herceptin helps to increase the clinical benefit such as response rate, time to disease progression as well as overall survival of first-line chemotherapies such as Paclitaxel, Docetaxel, doxorubicin and cisplatin in patients with HER2 overexpressed MBC.[54,69,70] Several

studies have examined different Trastuzumab-taxane combinations. For example, higher response rates have been reported for Docetaxel, which yielded a 73% response rate in three phase II trials.[71] An increased response rate of 50-80% was reported when Trastuzumab was combined with single-agent cytotoxic chemotherapy in first line MBC.[72] Several studies have evaluated the role of Herceptin as preoperative therapy in patients with early-stage breast cancer. The greatest effect *in vitro* and *in vivo* was seen with the combination of Paclitaxel and Herceptin.[73] The approved combination therapy indication for Trastuzumab is with 3-weekly Paclitaxel and after that both weekly Paclitaxel and Docetaxel regimens are widely used. A phase II study of preoperative Herceptin in combination with Paclitaxel was reported by Burstein *et al* and a pathologic complete response rate of 18% was found.[74] Seidman *et al* reported that women with MBC of HER2 positive or negative response differently to Herceptin.[75] In the study, patients were treated with standard-dose Trastuzumab plus weekly Paclitaxel (90 mg/m^2). As expected, toxic effects were typical of single-agent Paclitaxel, and cardiac function was preserved for at least a year. The response rate was 81% in patients who were HER2-positive by IHC evaluation and 43% in those who were HER2 negative.

The success with Herceptin in treatment of metastatic breast cancer in combination with chemotherapy has inspired intensive studies on developing effective targeted drug delivery systems. Combination of Trastuzumab and chemotherapeutic agents such as Paclitaxel and Docetaxel for the treatment of HER2 overexpressed cancer has been suggested as a promising means of targeted chemotherapy.

3.3. Trastuzumab-conjugated nanoparticles

Nanoparticles of biodegradable polymers as a drug delivery system have aroused continuous interest in recent years. Drug-loaded nanoparticles have considerable potential to provide an

ideal solution for the major problems encountered in chemotherapy. It has been established that nanoparticles can become concentrated preferentially to tumors by virtue of the EPR effect of the vasculature. Once accumulated at the target site, biodegradable polymeric nanoparticles can act as a local drug deposit and provide a source for a continuous supply of encapsulated therapeutic agent. The advantages of nanoparticles-based drug delivery systems include the sustained and controlled drug release, improved bioavailability, reduced systemic side effects and high capability to cross various physiological barriers.[29,76] Nevertheless, the low selectivity of NPs towards the cancer cells hinders the advantages of the nanoparticle formulation for efficient chemotherapy as the therapeutic agents possess high toxicity also to the healthy cells. Therefore, it is necessary to develop effective therapies with specific effect for the cancer cells.

Effective drug targeting can be realized by the functionalization of nanoparticles with small molecule ligands such as folate and thiamine, peptides such as Arginine-Glycine-Aspartic acid (RGD), sugar residues such as galactose, antibodies and antibody fragments such as anti-HER2, nucleic acid aptamers such as anti-PSMA aptamer, as well as proteins such as transferrin.[76-83] Among various ligands, Trastuzuamb, the monoclonal antibody directed against HER2, is of great interest because it arises synergistic therapeutic effects with chemotherapy in addition to its targeting ability.[84]

Synergistic antitumor effects were found when Trastuzumab is administered in combination with chemotherapeutic agents such as Paclitaxel, Docetaxel and doxorubicin.[74,85] Two different targeting approaches have been reported by Nobs *et al* for immunotargeting with Trastuzumab conjugated to nanoparticles[86]. One is a direct method using Trastuzumab-labeled NPs and the other is a pretargeting method using the avidin-biotin technology. Specific Trastuzumab-labeled NPs binding to tumor cells produced a mean 10-fold or higher signal increase compared to

control. The two-step method was evaluated *in vitro* by incubating SKOV-3 cells first with biotinylated mAbs followed by NPs. The relative fluorescence associated to the specific binding of NPs produced a 6-fold increase in flow cytometry signal compared to nonspecific binding, which suggested that Trastuzumab-functionalized NPs might be a useful drug carrier for tumor targeting.

Steinhauser *et al* reported a time-dependent cell uptake study, in which a significant enrichment of Trastuzumab-modified nanoparticles in 64.23% of HER2-positive SK-BR-3 cells was achieved compared to 3.59% of the nude NPs in the case of for 30 min incubation. However, after incubation of 24 h the nonspecific uptake of PEGylated nanoparticles without Trastuzumab increased dramatically (39.62%) compared to Trastuzumab-modified nanoparticles (51.22%).[87] Research on doxorubicin-loaded, Trastuzumab-modified human serum albumin nanoparticles was reported by Anhorn *et al*, which was the first demonstration of doxorubicin-loaded nanoparticles with a specific Trastuzumab-based targeting of HER2-overexpressing breast cancer cells.[88] It was also reported that there was specific targeting with Trastuzumab-functionalized doxorubicin-loaded nanoparticles with a cellular binding of 73.80%, whereas doxorubicin-loaded nanoparticles without Trastuzumab had a marginal cellular binding of 5.57%. By such a targeting system with Trastuzumab as targeting ligands binding the HER2 receptor, higher drug levels in tumor tissue was achieved. Lee *et al* employed cationic P(MDS-co-CES) micelles to delivery Paclitaxel and Herceptin simultaneously, which is convenient to adjust the dosage of Paclitaxel and Herceptin by simply altering the initial loading.[89] Their results showed that cytotoxicity of Paclitaxel was increased with co-delivery of Herceptin, and the degree of increment in cytotoxicity depends on the level of HER2 expression. All these results indicate that the Trastuzumab-functionalized nanoparticles can significantly promote targeted delivery of the

drug to the corresponding cancer cells and therefore greatly enhance its therapeutic effects and also reduced its side effects.

4. TRASTUZUMAB-CONJUGATED NANOPARTICLES OF PLA-TPGS/TPGS-COOH COPOLYMER BLEND

In this section, we report a strategy developed in our earlier research how to prepare nanoparticles of biodegradable novel copolymers for targeted chemotherapy, by which the targeting effect can be controlled. The NPs formulation consisted of a blend of two-component copolymers. One is poly(lactide)-D-α-tocopheryl polyethylene glycol succinate (PLA-TPGS), which is of desired hydrophobic-lipophilic balance (HLB) and can thus result in high drug encapsulation efficiency and high cellular adhesion/adsorption. Another is carboxyl group-terminated TPGS (TPGS-COOH), which plays the role as linker molecules when appearing on the NP surface. This combination provides a simple technique for conjugation of the molecular probes on the NP surface. Moreover, the targeting effect can be quantitatively controlled by adjusting the copolymer blend ratio. Docetaxel was used as a prototype drug, which is a hydrophobic anticancer drug and has excellent therapeutic effects against a wide spectrum of cancers such as breast cancer, non-small-cell lung cancer, ovarian cancer and head and neck cancer.[90] Docetaxel is semi-synthetic analogue of Paclitaxel, but more effective as an inhibitor of microtubule depolymerization due to its ability to alter tubulin processing within the cells. Moreover, there is a synergistic antitumor effect when Trastuzumab is combined with Docetaxel.[85]

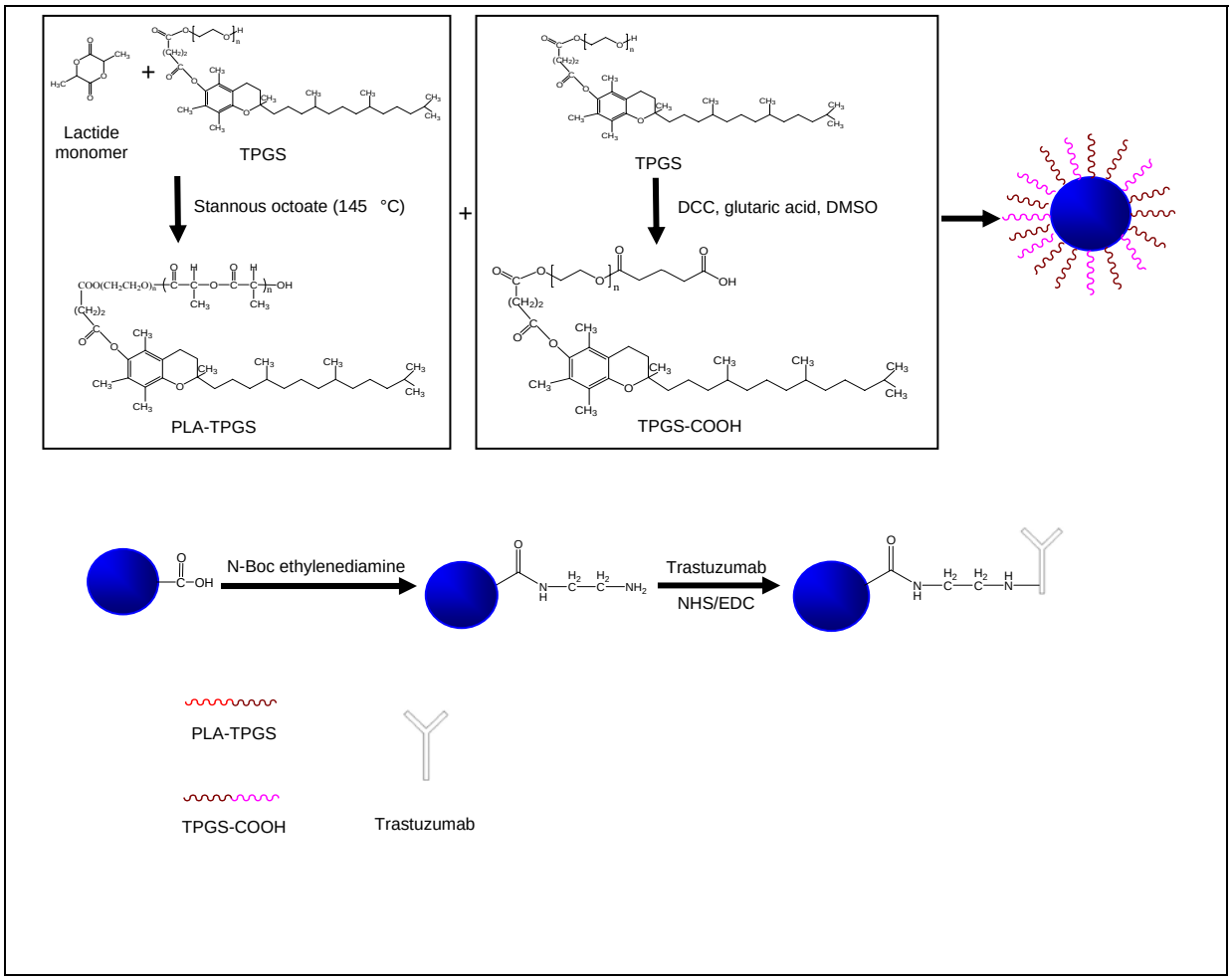

Figure 2. Schematic illustration for preparation of the Trastuzumab-functionalized, Docetaxel-loaded PLA-TPGS/TPGS-COOH nanoparticles.

Docetaxel-loaded nanoparticles of PLA-TPGS and TPGS-COOH blend were prepared by a modified solvent extraction/evaporation method. Four levels of the mass ratio between PLA-TPGS and TPGS-COOH were used in this research, which are 1:0, 9:1, 4:1 and 2:1, for which the weight amounts of TPGS-COOH in the blend are thus 0%, 10%, 20% and 33.3%. In brief, 10 mg Docetaxel and 100 mg of the PLA-TPGS/TPGS-COOH blend at various weight ratios were dissolved in 8 ml DCM. The formed solution was poured into 120 ml aqueous phase containing 0.03% (w/v) TPGS as emulsifier under gentle stirring, and sonicated at 25 W output for 120 s.

The emulsion was evaporated overnight and then centrifuged at 10,500 rpm for 20 min. The pellet was resuspended in water and freeze-dried for 2 days. Hereinafter, the Docetaxel-loaded PLA-TPGS/TPGS-COOH NPs of 0%, 10%, 20% and 33.3% TPGS-COOH were termed NP0, NP10, NP20 and NP33, respectively and these NPs with no Trastuzumab conjugated are called nude NPs in this article.

Before Trastuzumab conjugation, the Docetaxel-loaded PLA-TPGS/TPGS-COOH NPs were first activated according to a procedure similar to that described by Wuang *et al*,[91] where polypyrrole nanoparticles were synthesized to formulate iron oxides (IOs) for targeted MRI imaging. In brief, 10 mg of the PLA-TPGS/TPGS-COOH NPs were dispersed in 4 ml DI water followed by adding 20 mg of 1-ethyl-3-(3-dimethylamino)-propyl cabodiimide (EDC) and 8 mg of N-hydroxysuccinimide (NHS). One milligram of N-Boc ethylenediamine was added to introduce amine groups to the NPs. The pH of the reaction mixture was adjusted to 8 with triethylamine. After 4 h of reaction at room temperature, the mixture was centrifuged, washed with DI water and dried under reduced pressure. Then the product was treated with trifluoroacetic acid (TFA) at 0 °C for 30 min to remove N-Boc protection. After evaporation of TFA, the product was wash with DI water and dried. Trastuzumab was then conjugated to the NPs by using carbodiimide chemistry. The NPs were dispersed in 2 ml of 0.1 M PBS and placed in an ultrasonic bath for 30 min. Then 10 mg of NHS and 50 mg of EDC were added. After that, 500 μl of Trastuzumab (10 mg/ml) was added for reaction and triethylamine was used to adjust the pH of the reaction mixture to 8. After 4 h reaction at room temperature, the resulting product was centrifuged, washed with DI water and dried under reduced pressure. Hereinafter, the Docetaxel-loaded, Trastuzumab-conjugated NPs of 10%, 20% and 33.3% TPGS-COOH were

termed NP10-HER, NP20-HER and NP33-HER, respectively. The detailed scheme for preparation of nanoparticles is shown in Figure 2.

The coumarin-6 loaded PLA-TPGS/TPGS-COOH NPs with/without Trastuzumab conjugation were prepared in the same way as for the docetaxel-loaded NPs except that the drug was replaced by 0.05% (w/v) coumarin-6. The coumarin-6 loaded NPs were used for the *in vitro* cellular uptake experiment.

5. CHARACTERIZATION OF F TRASTUZUMAB-CONJUGATED NANOPARTICLES

Size and size distribution

Particle size, polydispersity, zeta-potential and drug encapsulation efficiency of the Docetaxel-loaded PLA-TPGS/TPGS-COOH NPs of 0, 10, 20 and 33.3% TPGS-COOH with or without Trastuzumab surface modification are listed in Table 1, from which it can be found that the NPs of all formulations in this study were in a size of around 300 nm in diameter with polydispersity of around 0.15. Surface modification of the NPs by Trastuzumab slightly increased the particle size and polydispersity, which, however, seem not affected by the amount of Trastuzumab on the NP surface. In our point of view, the NPs of around 200 nm in diameter would be appropriate to be used as drug carrier to achieve high cellular uptake through a mechanism called endocytosis, in which the individual nanoparticle would first adhere to the cell surface and then bend a small piece of the lipid bilayer membrane. The nanoparticles would then be enveloped and engulfed into the cytoplasm. In this process, the surface energy of the NPs is sacrificed to provide the bending energy. Too small NPs would not have enough surface energy to complete the cell membrane bending process. Moreover, too small NPs would result in too small drug encapsulation efficiency and cause too fast drug release. We found that the placebo

nanoparticles have similar size and size distribution with those of their corresponding drug-loaded nanoparticles. Other physicochemical properties such as surface morphology, surface chemistry and surface charge were also not effected by the drug loading since the XPS investigation would show in the Surface Chemistry section below that no drug molecules appear on the nanoparticle surface due to their high hydrophobicity.

Surface morphology

The surface morphology of the Docetaxel-loaded PLA-TPGS/TPGS-COOH NPs was investigated by a field emission scanning electron microscopy system (FESEM, JEOL JSM-6700F, Japan). The samples were prepared by dripping a drop of the NP suspension onto the copper tape placed on the surface of the sample stub and dried overnight. The stub was coated with a platinum layer by the Auto Fine Platinum Coater (JEOL, Tokyo, Japan) for 40 s before measuring.

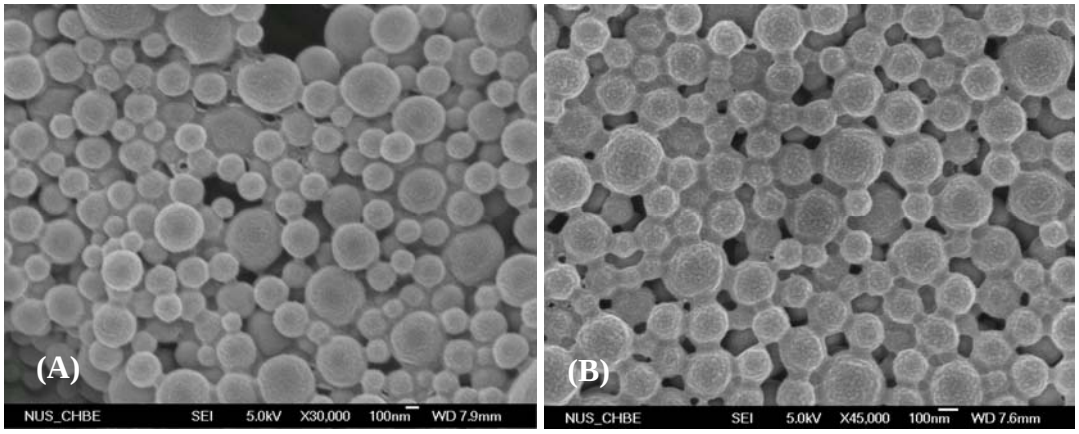

Figure 3. FESEM images of Docetaxel-loaded nanoparticles of PLA-TPGS (67%) and TPGS-COOH (33%) copolymer blend: (A) NP33 and (B) NP33-HER.

Figure 3 shows surface morphology of the Docetaxel-loaded PLA-TPGS/TPGS-COOH NPs and the images were studied by FESEM. The Docetaxel-loaded NPs without Trastuzumab-

modification (Figure 2(A)) were found spherical in shape with smooth surface within the FESEM resolution level. The image of NPs with Trastuzumab-conjugation was found of more blurry surface and there seemed to be adhesion between the NPs compared with NP20, which was probably caused by the Trastuzumab on the nanoparticle surface.

Surface charge

Zeta potential is an important factor to determine the stability of the NPs in dispersion. Surface charge also plays important role in the interaction between the cell membrane and the NPs. Low absolute value of the zeta-potential implicates colloidal instability, which could lead to aggregation of the NPs in dispersion. High absolute value of the zeta potential indicates high surface charge of the NPs, which leads to strong repellent interactions among the NPs in dispersion and thus high stability. It can be seen from Table 1, the zeta potential of Docetaxel-loaded PLA-TPGS/TPGS-COOH NPs without Trastuzumab modification was found to be around -40 mV, indicating the nanoparticle dispersion is stable. The absolute values of zeta potential for Trastuzumab-functionalized NPs are lower than those without Trastuzumab on the surface and the more the Trastuzumab on the surface, the lower the absolute values would be resulted. This is because of the positive charge of Trastuzumab.

Nanoparticles	Size (nm)	Polydispersity	Zeta-Potential (mV)	EE (%)
NP0	301.8±9.5	0.143±0.02	-42.73±3.88	54.7±4.73
NP10	294.7±9.2	0.151±0.03	-41.68±4.12	55.8±4.62
NP10-HER	308.5±9.0	0.202±0.03	-32.25±2.95	54.5±6.25
NP20	286.2±10.2	0.156±0.03	-40.33±4.01	57.4±4.19
NP20-HER	305.7±11.8	0.175±0.05	-25.08±1.15	55.7±5.75
NP33	292.5±9.8	0.121±0.03	-41.50±3.28	48.2±4.03
NP33-HER	311.2±9.4	0.155±0.04	-23.94±2.09	47.5±4.58

Table 1. Characteristics of Docetaxel-loaded, Trastuzumab-functionalized nanoparticles of PLA-TPGS and TPGS-COOH copolymer blend at various component ratios.

Drug encapsulation efficiency

The drug encapsulation efficiency (EE) of the Docetaxel-loaded PLA-TPGS/TPGS-COOH NPs is also included in Table 1, from which it can be found that (1) the EE data have no significant difference among all types of NPs and (2) the slight difference in EE among all types of NPs is caused by the component ratio between PLA-TPGS and TPGS-COOH. For example, the EE of NP20, which contains 20% TPGS-COOH, is the highest among all the NPs. Either lower or higher content of TPGS-COOH in the polymeric matrix caused lower EE. Surface functionalization should increase the EE in general since it would decrease the drug diffusion from the NPs into the aqueous phase in the preparation process. However, drug diffusion from the NPs should also depend in the porosity of the NP matrix. The porosity of the polymeric

matrix would be high if too much TPGS-COOH was included, which would speed the drug diffusion from the NPs into the aqueous phase in the preparation process.

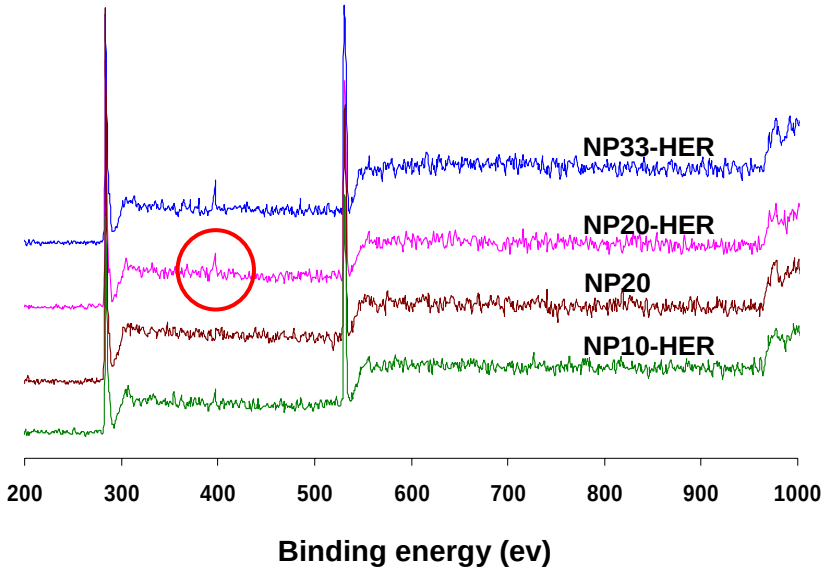

Figure 4. The X-ray photoelectron spectroscopy (XPS) wide scan spectra of the Docetaxel-loaded PLA-TPGS/TPGS-COOH nanoparticles.

Surface chemistry

X-ray photoelectron spectroscopy (XPS) was applied to study the surface chemistry of the Docetaxel-loaded PLA-TPGS/TPGS-COOH NPs with or without surface modification. Figure 4 shows the XPS wide scan spectrum of the Trastuzumab-functionalized, Docetaxel-loaded NPs of various component copolymer blend ratios. From their chemical structure, it can be noticed that PLA-TPGS and TPGS-COOH do not contain any nitrogen atoms, while Docetaxel and Trastuzumab contains nitrogen atoms. Therefore, nitrogen could be used as a "marker" to detect the presence of Docetaxel and Trastuzumab on the NPs surface. It can be seen from the third spectrum from the top (for NP20) in Figure 4, there is no N 1s signal at the 397.6 eV bond

energy position, which means there is no Docetaxel molecules on the NP surface. This agrees with our earlier research on Paclitaxel-loaded polymeric nanoparticles prepared with either phospholipids, or PVA, or TPGS as emulsifier. From the second spectrum from the top (for NP20-HER), however, the nitrogen peak at the 397.6 eV bond energy position (as shown in the red circle in the Figure 4) must come from Trastuzumab, which confirms the successful conjugation of Trastuzumab on the NP surface. It can be found from a close comparison of the XPS spectra among NP10-HER, NP20-HER and NP33-HER that there was an increase tendency in the percentage of the N 1s region as the more TPGS-COOH is included in the polymeric matrix, the more Trastuzumab could be conjugated on the NP surface.

In vitro Docetaxel-release kinetics

Figure 5 shows the 30-day *in vitro* release profiles of Docetaxel from the drug-loaded PLA-TPGS/TPGS-COOH NPs with/without Trastuzumab-conjugation. Docetaxel was released from the NPs in a pH 7.4 PBS buffer at 37 °C. From Figure 5, it can be found that Docetaxel was released in a biphasic style with an initial burst and subsequent accumulative release. It is obvious that the drug release of NPs with Trastuzumab-decoration is significantly faster than that without Trastuzumab-conjugation, which means the modification of NP surface significantly speed up the drug release. This can be explained by effects of the surface modification on the surface morphology, which makes the NPs blurry and thus increases the surface area, which increases the speed at which the drug is released from the NPs.

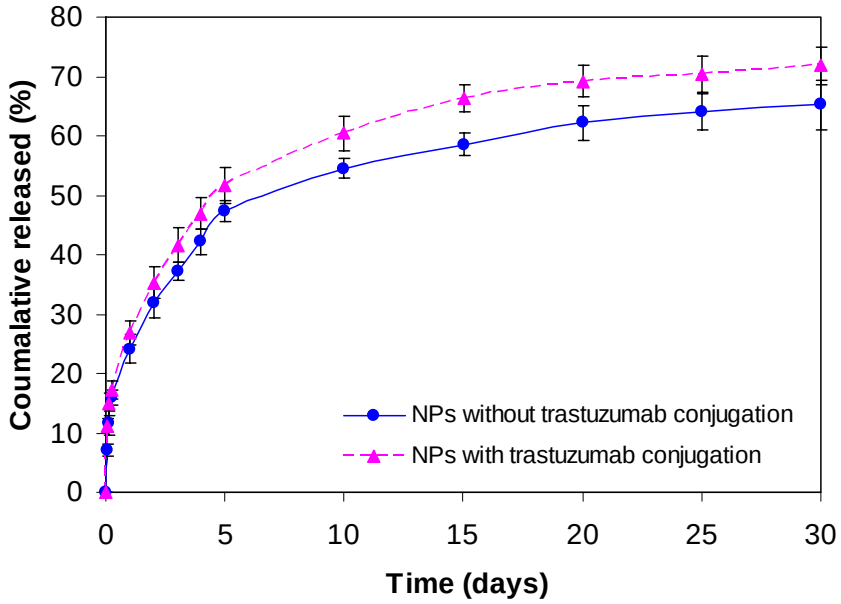

Figure 5. ***In vitro*** **drug-release profiles of Docetaxel-loaded nanoparticles of PLA-TPGS (67%) and TPGS-COOH (33%) copolymer blend within 30 days.**

6. *IN VITRO* EVALUATION OF TRASTUZUMAB-CONJUGATED NANOPARTICLES

6.1. *In Vitro* Cellular uptake

The MCF-7 breast adenocarcinoma cells have a moderate level of HER2 overexpression on their surface and the SK-BR-3 breast adenocarcinoma cells are found of high overexpression of HER2. The targeting effect of the Trastuzumab-functionalized nanoparticles can thus be evaluated by employing these two cell lines to investigate the cellular uptake of coumarin-6-loaded nanoparticles. Figure 6 shows the cellular uptake efficiency of coumarin-6-loaded NP20 and NP20-HER by (A) SK-BR-3 breast cancer cells and (B) MCF-7 breast cancer cells at the same 125 μg/ml NP concentration for 0.5, 1.0, 2.0, 4.0 h, respectively. It can be seen from Figure 6 that NP20-HER demonstrated much higher cellular uptake efficiency for both of MCF-7 and SK-BR-3 cells than the NP20. For example, it can be found from Figure 6(A) that the NP20-

HER achieved 1.36-, 1.33-, 1.31-, and 1.28-fold higher cellular uptake efficiency than NP20 after 0.5, 1.0, 2, 4 h incubation with SK-BR-3 cells, respectively. It can be found from Figure 7(B), instead, that the NP20-HER showed 1.21-, 1.17-, 1.15-, and 1.17-fold higher cellular uptake efficiency than NP20 after 0.5, 1.0, 2, 4 h incubation with MCF-7 cells, respectively. NP20-HER thus showed 1.12-, 1.34-, 1.14-, and 1.09-fold higher targeting effect for SK-BR-3 cancer cells (of high HER 2 overexpression) than for MCF-7 breast cancer cells (of moderate HER2 overexpression) after 0.5, 1.0, 2, 4 h cell culture, respectively.

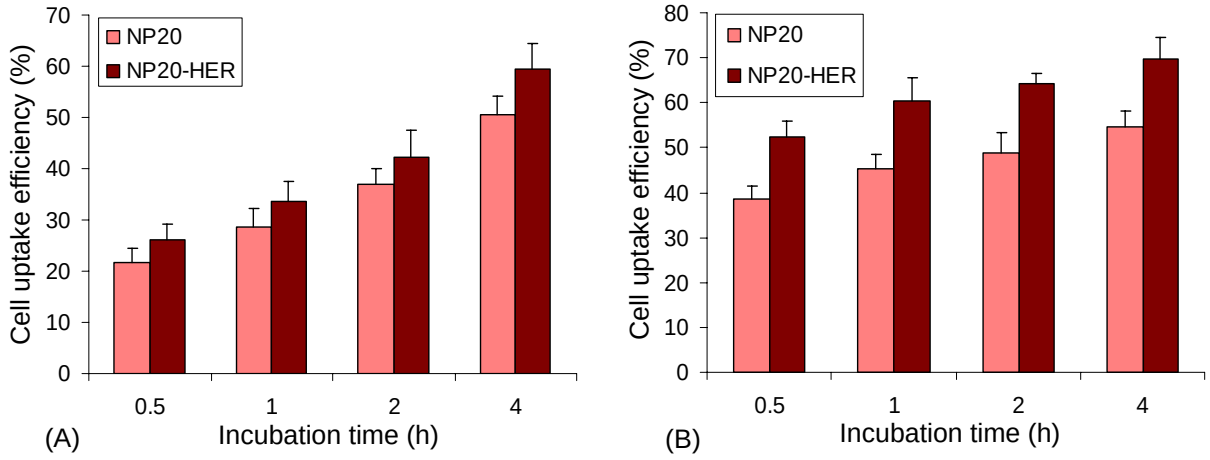

Figure 6. Cellular uptake efficiency of coumarin-6-loaded nanoparticles after 0.5, 1, 2, 4 h incubation at 125 μg/ml nanoparticles concentration by (A) MCF-7 breast cancer cells and (B) SK-BR-3 breast cancer cells. Each point represents mean ±SD (n=6, $p<0.05$).

It can be read from the first group in Fig. 6(B) that the cellular uptake efficiency for a 30-min-incubation of naked nanoparticles and trastuzumab-modified nanoparticles are 38.6% and 52.5% for SK-BR-3 cells, respectively. This demonstrates that in such a short incubation period, the uptake of trastuzumab-modified nanoparticles was enhanced in comparison with the cellular uptake of the naked nanoparticles, which is in accordance with literature. Moreover, it is

straightforward from both of Figure 6A and Figure 6B that the cellular uptake depends on the incubation time and the longer the incubation time, the higher cellular uptake efficiency would be achieved. Similar trends were also found in our earlier work, where the Trastuzumab is physically attached on the PLGA-MMT NPs for targeted delivery of Paclitaxel.

6.2. *In Vitro* Cytotoxicity

Figure 7(A) and (B) show the *in vitro* viability of (A) MCF-7 breast cancer cells (of moderate HER2 overexpression) and (B) SK-BR-3 breast cancer cells (of high HER2 overexpression) treated with placebo nanoparticles with (Blank-NP20-HER) and without (Blank-NP20) Trastuzumab conjugation, Taxotere®, and Docetaxel-loaded PLA-TPGS/TPGS-COOH nanoparticles of 20% TPGS-COOH with (NP20-HER) and without (NP20) at 25 μg/ml Docetaxel concentration after 24, 48, 72 h treatment, respectively (n=6). The results are shown in the form of cell viability. The sum of viability and mortality is always 100%. The reason we chose 25 μg/ml concentration of Docetaxel is that the IC50 of the MCF-7 breast cancer cells has been found to be 13.2 μM, *i.e.* 10.7 μg/ml (Molecular weight of Docetaxel is 808). From Figure 7, the following conclusions can be obtained.

The viability for both cell lines was decreased with increase of the incubation time, which means the longer the culture time, the higher the cytotoxicity. This is straightforward.

From the first column of each group for Blank-NP20, it can be found from Fig 8(A) for MCF-7 cells that the viability for placebo PLA-TPGS/TPGS-COOH NPs without Trastuzumab-functionalized was 96.1±2.6%, 95.5±3.8%, 94.8±5.2% after 24, 48, 72 h incubation, respectively. In comparison, similar results can be found from Figure 8B, *i.e.* the viability of SK-BR-3 cells for placebo PLA-TPGS/TPGS-COOH NPs without Trastuzumab-functionalized was 98.4±4.0%,

95.5±5.7%, 95.7±4.2% after 24, 48, 72 h incubation, respectively. This means that the cytotoxicity of the placebo PLA-TPGS/TPGS-COOH NPs have high biocompatibility with fractional cytotoxicity.

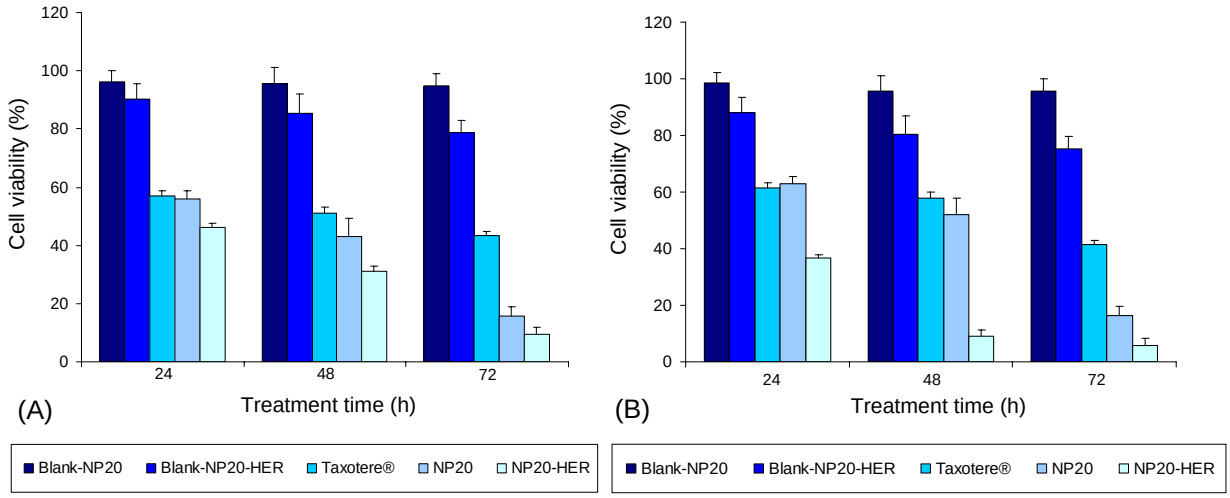

Figure 7. *In vitro* viability of (A) MCF-7 breast cancer cells and (B) SK-BR-3 breast cancer cells treated with placebo nanoparticles (blank-NP20 and blank-NP20-HER), Taxotere® and Docetaxel-loaded nanoparticles (NP20 and NP20-HER) at 25ug/ml Docetaxel concentration after 24 (left group), 48 (middle group), 72 h treatment (right group), respectively (n=6).

The second column of each group in Figure 7(A) and (B) stands for the cell viability of placebo PLA-TPGS/TPGS-COOH NPs with Trastuzumab surface modification (Blank-NP20-HER). In Figure 7A, the viability of MCF-7 cells is 90.3±3.3%, 85.3±4.8%, 78.7±5.2% after 24 h, 48 h, and 72 h cell culture, respectively. In Figure 8B, however, the viability of SK-BR-3 cells is slightly lower to be 88.2±5.1%, 80.3±6.6%, 75.3±4.3% for 24 h, 48 h and 72 h, respectively. The results clearly show the targeting effects of the Trastuzumab-functionalized NPs for the breast cells of high HER2 overexpression.

The third column of every groups in Figure 7 shows the viability of the two breast cancer cells after 24 h, 48 h, 72 h treatment with Taxotere®), which is 56.9±2.6%, 51.3±4.3%, 43.2±3.6% for MCF-7 cells and 61.3±1.9%, 57.8±2.1%, 41.4±1.4% for SK-BR-3 cells, respectively. This means that Taxotere® may be more effective for MCF-7 breast cancer cells than for SK-BR-3 breast cancer cells in short time (24 h and 48 h), and the effect may become inverse in long time (72 h). In comparison, the *in vitro* viability shown in the fourth column of each group in Figure 7(A) and (B) is 55.8±3.1%, 43.2±3.0%, 15.6±3.4% for MCF-7 cells and 62.7±2.8%, 51.9±6.1%, 16.4±3.2% for SK-BR-3 cells after 24 h, 48 h, 72 h treatment, respectively. This means that evaluated by cellular mortality, the NP formulation has comparable *in vitro* therapeutic effect (1.025-fold for MCF-7 cells and 0.964-fold for SK-BR-3 cells) with Taxotere® after 24 h cell culture However, the NP formulation of Docetaxel would be 1.166-, 1.486-fold for MCF-7 cells and 1.140-, 1.427-fold for SK-BR-3 cells more effective than Taxotere® after 48 h, 72 h treatment, respectively. This may be attributed to the sustainable drug release manner of the NP formulation.

The fifth column at the right of every group in Figure (A) and (B) shows the viability of the MCF-7 and the SK-BR-2 breast cancer cell lines after 24 h, 48 h, 72 h treatment with NP20-HER, which is 46.3±2.7%, 31.0±3.2%, 9.4±3.1% for MCF-7 cells and 36.7±1.3%, 9.2±2.0%, 5.9±2.5% for SK-BR-3 cells, respectively. It can thus be concluded that judged by cellular mortality, the Trastuzumab-functionalized NP formulation can be 1.215-, 1.215-, 1.073-fold for MCF-7 cells and 1.697-, 1.886-, 1.126-fold more effective for SK-BR-3 cells than the NP formulation with no Trastuzumab functionalization after 24 h, 48 h, 72 h treatment, respectively. The targeting effects of Trastuzumab functionalization thus showed 39.7%, 55.2%,

4.9% higher cancer cell mortality for SK-BR-3 cells of high HER2 overexpression than for MCF-7 cells of moderate HER2 over expression after 24 h, 48 h, 72 h treatment, respectively.

In combination of the advantages of the PLA-TPGS/TPGS-COOH NP formulation and the Trastuzumab functionalization, the Trastuzumab-functionalized NP formulation can be 1.245-, 1.417-, 1.594-fold for MCF-7 cells and 1.635-, 2.150-, 1.606-fold more effective for SK-BR-3 cells than Taxotere® after 24 h, 48 h, 72 h treatment, respectively.

7. SUMMARY

Nanoparticle formulation has great advantages over the conventional drug formulations, resulting in a sustained and controlled manner for drug release thus with increased the therapeutic effect and reduced side effects. Size and surface modifications are the two key factors determining the performance of biodegradable polymeric NPs for cancer therapy. Targeted chemotherapy can be realized by functionalization of the NPs surface by targeting ligands such as monoclonal antibodies. A copolymer strategy can be further employed to synthesize nanoparticles of two component copolymers: one component copolymer has ideal HLB for high drug encapsulation efficiency, high cellular adhesion and internalization, and another copolymer facilitates the conjugation of the ligand molecules on the NPs surface. The targeting effects can thus be quantitatively controlled by adjusting the blend ratio between the two copolymers. Development of multifunctional NPs are also feasible by co-encapsulation of the imaging, therapeutic, and reporting agents in the polymeric nanoparticles with their surface functionalized by targeting ligands. In this chapter, we provided a preliminary, proof-of-concept investigation for a targeted drug delivery system of molecular probe-conjugated nanoparticles of a blend of two biodegradable copolymers, PLA-TPGS and TPGS-COOH. It was found that

judged by *in vitro* cellular mortality, the Trastuzumab-functionalized PLA-TPGS/TPGS-COOH NP formulation of Docetaxel can be 1.215-, 1.215-, 1.073-fold for MCF-7 cells and 1.697-, 1.886-, 1.126-fold more effective for SK-BR-3 cells than the PLA-TPGS/TPGS-COOH NP formulation with no Trastuzumab functionalization after 24 h, 48 h, 72 h treatment, respectively. The targeting effects of Trastuzumab functionalization thus showed 39.7%, 55.2%, 4.9% higher cancer cell mortality for SK-BR-3 cells of high HER2 overexpression than for MCF-7 cells of moderate HER2 over expression after 24 h, 48 h, 72 h treatment, respectively. The strategy greatly simplified the existing nanoparticle technologies for targeted drug delivery.

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