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Silk fibroin mediated delivery of liposomal emodin to breast cancer cells

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Abstract

The efficacy of a drug is dependent on its mode of delivery and its potency at the tumor site. In this study, the drug delivery and efficacy of silk fibroin coated liposomes (SF-ELP), encapsulating a receptor tyrosine kinase inhibitor, emodin, on Her2/*neu* over-expressing breast cancer cells, was investigated. This study demonstrates that SF-ELP was more efficacious in suppressing the growth of Her2/*neu* over-expressing breast cancer cells MDA-MB-453 and BT-474 as compared to uncoated emodin loaded liposomes (ELP). Reduced levels of phosphorylated Her2/*neu* correlated with growth inhibition observed in the MDA-MB-453 cells, treated with both ELP and SF-ELP. ELP treatment of MDA-MB-453 breast cancer cells resulted in inhibition of the PI3K pathway whereas SF-ELP treatment inhibited both the PI3K and MAPK pathways, which contributed to the enhanced growth inhibitory effects of Her2/*neu* over-expressing breast cancer cells. Coating of ELP with silk fibroin did not alter the target specificity of emodin, on the other hand the emodin efficacy was enhanced. Higher uptake of emodin delivered by SF-ELP lead to increased cell death as compared to emodin delivery via ELP. Silk fibroin coating around the liposome imparts an extra layer that emodin has to extravasate in order to release from the encapsulating liposome. This increases retention of the drug in the cell for a longer time and protects emodin from quick release and metabolism. Longer intracellular retention may lead to the longer availability of emodin for down-modulation of various Her2/*neu* pathways. This study demonstrates that silk fibroin coating enhanced emodin delivery in Her2/*neu* over-expressing breast cancer cells thereby increasing the overall efficacy of the drug.

Keywords: Drug delivery; Liposome coating; Polymer; Breast cancer; Receptor tyrosine kinase

1. Introduction

One of the major problems facing cancer chemotherapy is achieving specificity of drug action. The key is administration of the required therapeutic concentration of the drug at the desired tumor site for the desired period of time without causing undesirable side effects on other organs after systemic administration (Allen and Cullis, 2004). In the last two decades, many studies have focused on development of drug delivery systems to achieve controlled release and mechanisms to enable drug targeting to specific tissue (tumor) sites (Gregoriadis, 1988, 1995; Lasic, 1998; Yoshioka et al., 1995). Solid tumors such as breast cancer have historically provided many challenges to systemic therapy (Cianfrocca and Gradishar, 2005; Johnston, 2005). Barriers to drug penetration in solid tumors include heterogeneous vascular supply and high interstitial pressures within tumor tissue, particularly in necrotic zones (Grantab et al., 2006; Tannock et al., 2002; Tong et al., 2004). Delivery systems can even exacerbate these problems due to the slow diffusion of macromolecular agents through tumor tissue (Tannock et al., 2002).

Targeted drug delivery to solid tumors, therefore, is desirable in order to achieve optimum therapeutic outcomes. Macromolecular agents with highly restricted volumes of distribution and the capacity for greatly prolonged circulation

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will preferentially extravasate from these abnormal vessels and accumulate in tumor tissue (Shenoy et al., 2005; Tsuno et al., 2005). The leading examples of such 'passively targeted' agents include long term circulating liposomal drugs (Fenske and Cullis, 2005; Kshirsagar et al., 2005; Ramsay et al., 2005).

Intravenously administered liposomes and other colloidal particles, however, tend to be quickly removed by phagocytic cells existing primarily in the liver and spleen as a result of their recognition as foreign bodies by the host's immune system (Papahadjopoulos and Gabizon, 1987; Poste and Kirsh, 1982; Senior, 1987). Many attempts, such as surface modifications, have been investigated in order to decrease the adsorption of blood components and consequently increase the blood circulation time. For example, surface modifications of liposomes with several biological materials including proteins, polysaccharides, and glycolipids were found to improve the circulation time by decreasing the uptake of liposomes in the reticuloendothelial system (RES) (Lasic, 1998; Woodle, 1993; Woodle et al., 1992). Based on these findings, many efforts were undertaken to identify alternative materials for coating of liposomes, and further success was obtained using stealth liposomes. Sterically stabilized liposomes (SSL) were a major breakthrough in prolonging circulation time and achieving improved tumor targeting (Gabizon and Martin, 1997; Poznansky and Juliano, 1984). It has been demonstrated that SSL can accumulate in tumor tissue due to the effect of enhanced permeability and retention (EPR) (Xiong et al., 2005). However, anticancer drug accumulation in tumor tissue via SSL seems to be prerequisite, but far from sufficient criteria to guarantee a therapeutic improvement. For a variety of chemotherapeutic agents with an intracellular site of action, efficient intracellular uptake by the tumor cells determines their relative anti-tumor activity (Xiong et al., 2005). While the use of Polyethyleneglycol (PEG) enables the liposome's ability to accumulate in tumor tissue, it creates a steric barrier that could cause a reduction in liposomal interaction with target cells, leading to low uptake of entrapped drugs via cell endocytosis or membrane fusion (Keil et al., 2005). In particular, when the liposomes are composed of rigid lipids and the entrapped drugs cannot be released easily from them, the intracellular delivery by diffusion is often limited (Bhadra et al., 2002; Dan, 2002; Hashizaki et al., 2005; Laverman et al., 2001).

Arginine-glycine-aspartate modified liposomes (RGD liposomes) have been demonstrated to improve binding ability and targeting efficiency of chemotherapeutic drugs into tumor cells (Xiong et al., 2005). It is well accepted that the polymer coating formed on the liposomal surface effectively protects the liposomes from interacting with the plasma proteins in the blood, leading to a reduction in RES uptake and prolonged circulation time in blood which translates into better uptake and thus effects of the drug (Senior et al., 1991).

Biologically derived polymer coating is an alternative method of modifying the surfaces of liposomes (Gobin et al., 2006). Biologically degradable polymers are of first consideration to use as coating material for *in vivo* administration of drugs. The ideal polymeric coating material for liposomes should be biocompatible and degradable. The degradation product should be non-toxic and should not create an inflammatory response. In addition, the coating or the coating process should not interfere with or alter the activity of the drug within the liposome. Natural polymers are degradable as they undergo enzymatically promoted digestion. Examples of such natural polymers include collagen, albumin, cellulose, chitosan, gelatin, elastin, and silk.

In a previous study, we demonstrated that the silk fibroin coated 1,2-dimyristol-*sn*-glycero-3-phosphotidylcholine (DMPC) liposomes loaded with emodin (SF-ELP) localizes drug delivery, increases residence time, and specificity for cell recognition versus uncoated emodin loaded liposomes (ELP) (Gobin et al., 2006). *Bombyx mori* silk fibroin is a natural fibrous protein polymer that provides a medium to cell adhesion and has characteristically unique mechanical properties (Ha et al., 2006, 2005a,b). The coating of the liposome with the silk fibroin changed the lamella properties by organizing a tightly packed lamella versus a loose lamella found in the uncoated liposome. These properties possibly slowed the release rate of emodin from the liposome. Silk-fibroin (SF) modified liposomal structure (SF-ELP) decreased emodin release rates by changing the release kinetics from a combination of swelling accompanied with diffusion to a purely diffusional process probably due to steric hindrance of the large silk fibroin protein (Gobin et al., 2006). Despite the changes in structure of the liposome upon silk fibroin coating, the overall liposomal diameter did not vary significantly (ELP = 232.2 ± 28.4 nm, SF-ELP = 316.6 ± 43.0 nm).

In this study we examined and compared the efficacy, adhesive targeting, and drug specificity of emodin delivered via SF-ELP versus ELP, against breast cancer cells that over-express the Her2/*neu* proto-oncogene. The Her2/*neu* oncogene (also called erbB-2) and its encoded gene product p185 belong to the epidermal growth factor receptor tyrosine kinases (Hung and Lau, 1999) (Fig. 1). Her2/*neu* acts as ligand-less signaling molecule for three other receptors that bind directly to a large number of stromal-derived growth factors (Hung and Lau, 1999). Her2/*neu* signaling pathways play critical roles in normal and neoplastic cell growth, differentiation, malignant transformation, and resistance to chemotherapeutic agents (Hung and Lau, 1999; Kurebayashi, 2001). Over-expression of Her2/*neu* has been observed in ~30% of breast cancer cells and is associated with resistance to hormone therapy, a higher rate of disease recurrence, and in general a poorer prognosis (Kurebayashi, 2001). Current therapies targeted against Her2/*neu* include trastuzumab (Herceptin; Genentech, South San Francisco, CA), a monoclonal IgG1 class humanized murine antibody that binds to the extracellular domain of the Her2/*neu* protein. However, there are risks of cardiomyopathy and hypersensitivity reactions associated with the use of trastuzumab and a need for alternate therapies remains (McKeage and Perry, 2002). Therapeutic agents against Her2/*neu* have been intensively sought over the past decade (Wang et al., 2001).

Emodin (3-methyl-1,6,8-trihydroxyanthran-quinone), a selective receptor tyrosine kinase inhibitor, is a natural product that is a promising agent for tumor patients that develop drug resistance to other traditional remedies (Caldon et al., 2006; Jayasuriya et al., 1992; Majsterek et al., 2005; Wakeling, 2005). Emodin has shown impressive activity in mouse models of Her2/*neu* over-expressing breast cancer, while sensitizing the

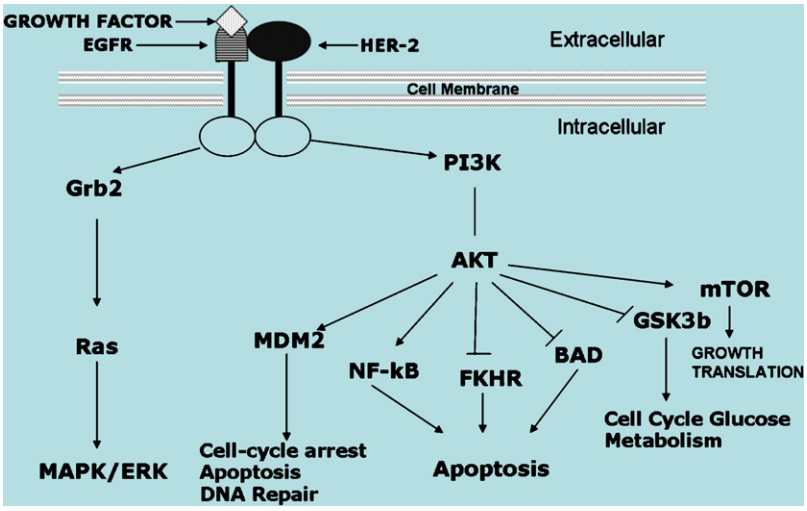

Fig. 1. Schematic representation of some of the possible signaling pathways activated via Her2/*neu* signaling.

cells to other anti-tumor therapies such as cisplatin, doxorubicin, etoposide, and paclitaxel (Zhang et al., 1995, 1998, 1999). However, emodin is highly hydrophobic and requires dissolution in organic solvents like dimethyl sulfoxide (DMSO), which make its use as a therapeutic agent problematic. Thus, in this study the improvement of emodin delivery and efficacy due to silk fibroin coated liposomes was evaluated.

2. Materials and methods

2.1. Emodin loaded liposomes

Stock solutions of emodin (98% HPLC grade, China Xuzhou International Corporation, Xuzhou, Jiangsu, China) at 1 mg/mL, Tween-20 at 10% (v/v), 1,2-dimyristol-*sn*-glycero-3-phosphocholine (DMPC) at 5 mg/mL were prepared in *tert*-butanol. Tween-20 solution, at 5% of the 10%, was added to the liposomal solution. Emodin solution was mixed with the Tween-20-liposomal solution to result in emodin-loaded liposomes (ELP) and frozen at −80 °C overnight. Frozen ELP were lyophilized and stored at −20 °C until used.

2.2. Preparation of silk fibroin coated emodin loaded liposomes

Aqueous silk fibroin (SF) was prepared as described previously (Gobin et al., 2005, 2006; Mathur et al., 1997). Empty liposomes as well as liposomes loaded with 2.86 mg of emodin were added to 1 mL of aqueous SF (1%, w/v). The solution was mixed for 10 min with gentle agitation. The mixture was then frozen at −80 °C and lyophilized. To crystallize the SF coated liposomes, methanol was added and mixed for 10 min. After centrifugation, the methanol was removed and allowed to evaporate in the hood until dry. Samples (SF-ELP) were stored at −20 °C until used.

Incorporation efficiency of emodin in liposomes was determined using HPLC. Following preparation of emodin-loaded liposomes, ELP, supernatant was separated from the liposomal pellet using ultracentrifugation (12,000 rpm, RT, 10 min). The supernatant was collected in another tube. The liposomal pellet was re-suspended with cold saline followed by another supernatant separation in order to eliminate residues of free, non-loaded emodin. ELP was dissolved using a detergent (1% SDS) to release the encapsulated emodin from the liposome. This suspension was frozen at −20 °C until the HPLC measurements. An aliquot (60 μL) was removed from the dissolved ELP suspension and 20 μL were injected twice per sample in to the HPLC (Waters 600S Controller, 474 fluorescence detector, 417 autosampler, Empower software) (Waters United States, Milford, MA). Analytical column, Alltech Allsphere ODS-1, 5 μ, 250 × 4.6 and Guard column, Waters C18 was used with a 25:75 0.1% acetic acid and 5:95 acetonitrile:methanol, mobile phase. Absorbance of the release emodin was measured at an excitation wavelength of 410 nm and an emission wavelength of 510 nm. The drug content was calculated using a calibration curve generated for emodin previously. Encapsulation efficiency was calculated by using the formula below:

$$\%\ \text{encapsulation efficiency (\%IE)} = \frac{\text{liposomal bound drug}}{\text{total initial drug added}} \times 100$$

2.3. Cell lines and culture

MCF-7, BT-474, and MDA-MB-453 breast cancer cells were obtained from the American Type Cell Culture (Manassas, VA). All cell lines were grown in DMEM/F12 supplemented with 5% fetal bovine serum (FBS).

2.4. Growth inhibition of breast cancer cells following exposure to ELP and SELP

MTS assay (Promega, Madison, WI) was performed to assess survival rates of cells after exposure to either ELP or SF-ELP. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) is

reduced by cells into a formazan product, which is detectable by absorbance and is directly proportional to the number of living cells in culture. MCF-7, breast cancer cells (1000 cells/well), BT-474 and MDA-MB-453, breast cancer cells (2000 cells/well) were seeded into 96 well plates in 5% FBS supplemented DMEM and incubated overnight. Medium was aspirated and 200 μL of emodin (ranging in dose from 1 to 100 μM) loaded in coated or uncoated liposomes, were added for 96 h. The liposomes were removed and the cells resuspended in 100 μL fresh media. MTS reagent (20 μL) was added per well. The formazan product was allowed to develop for 1 h and the absorbance was read at 490 nm on a MRX plate reader (Dynex Technologies, Chantilly, VA). Absorbance of ELP and SF-ELP treated cells was compared to untreated cells and plotted as % viability over the concentration range ($N = 3$).

2.5. Western blot analysis

For Western blotting, cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris base pH 8.0, 1 mM EDTA, 0.5% sodium deoxycholate, 1% NP40, 0.1% sodium dodecyl sulfate, 1 mM PMSF, 1 mM Na_3VO_4, and 1 mM NaF). Cultured cell lysates were prepared by washing cells in PBS followed by incubation in lysis buffer for 25 min at 4 °C. Cells were centrifuged at 13,000 rpm for 10 min at 4 °C, following incubation with the lysis buffer. Protein concentration was determined using a modified Lowry's method (BioRad Inc., Hercules, CA) and separated using 7.5% SDS-PAGE. Samples were transferred to a nitrocellulose membrane by wet transfer (28 V, overnight). Following blocking in 5% milk-TBST for 1 h at room temperature, the membranes with the transferred protein were incubated with appropriate antibody overnight at 4 °C, in 2.5% TBS-T. Detection was performed using an appropriate secondary antibody conjugated to horseradish peroxidase and developed using enhanced chemiluminescence detection kit (Pierce Biotechnology, Rockford, IL). Membranes were also probed for β-actin to confirm equal loading of protein. Anti-phospho-tyrosine antibody, anti-Her2/*neu* antibody, phospho-AKT, AKT, phospho-ERKI/2, and ERK2 were all purchased from Cell Signaling (Danvers, MA).

2.6. Cellular uptake of emodin by flow cytometry analysis

MDA-MB-453 (5×10^5/well) breast cancer cells were seeded into six-well chamber slides (Fisher Scientific, Pittsburg, PA, USA) and incubated overnight at 37 °C. The next day medium was aspirated and fresh media (1 mL/well) was added. ELP or SF-ELP dissolved in normal saline (100 μM/well) were added to the wells. The cells were collected 15 min following drug treatment and analyzed by fluorescence activated cell sorting (FACS) or flow cytometry. For the FACS analysis, the excitation wavelength was at 410 nm and emission wavelength at 510 nm.

2.7. Statistical analysis

All data were compared using one-way analysis of variance (ANOVA) with a Tukey–Kramer post-test. Values are reported as means ± standard error of mean. p-Values less than 0.05 were considered significant (GraphPad InStat 3.06, GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Repression of Her2/neu over-expressing human breast cancer cell line by SF-ELP

The cytotoxicity of emodin delivered to breast cancer cells by ELP and SF-ELP was compared by assessing emodin inhibition of Her2/*neu*. The growth rate of breast cancer cells exposed to ELP and SF-ELP was examined since activated Her2/*neu* is known to stimulate cell growth and emodin blocks its activation by inhibiting phosphorylation (Hung and Lau, 1999; Jayasuriya et al., 1992). MDA-MB-453 and BT-474 breast cancer cells that over-express activated Her2/*neu* were compared to low Her2/*neu* expressing cells, MCF-7.

Delivery of emodin from ELP and SF-ELP inhibited growth of BT-474 cells as the dose increased from 1 to 100 μM (Fig. 2A). While there was a significant difference in growth inhibition between ELP and SF-ELP at 60–100 μM concentrations, there was no significant difference in growth inhibition as the concentration increased from 60 to 100 μM for ELP and SF-ELP. Growth inhibition was significantly higher with SE-ELP delivered emodin at 1, 10, 60, 80, and 100 μM concentrations as compared to ELP. There was no dose dependant effect due to the administration of empty liposomes. A maximum of 60% growth inhibition was achieved with SF-ELP delivery and ∼50% achieved with ELP delivery at 100 μM concentration.

Dose dependant growth inhibition was more drastic in MDA-MB-453 breast cancer cells (Fig. 2B). SF-ELP delivered emodin had significantly higher growth inhibition than ELP delivered emodin, especially at higher doses of 40–100 μM. A ∼70% growth inhibition at 100 μM of ELP was similar to the ∼75% growth inhibition observed at just 20 μM of SF-ELP. There was a slight effect of liposome on the growth of breast cancer cells as seen by increase in dose dependant growth inhibition by empty liposomes. At doses of 20–60 μM there was no significant difference between empty liposomes and ELP, whereas a significant increase in growth inhibition with SF-ELP was observed at all doses except for 10 μM as compared to the empty liposomes. Hence, in the two cell lines over-expressing Her2/*neu*, delivery of emodin via SF-ELP was observed to exert a greater effect on growth inhibition than delivery via ELP at similar concentration of drug.

In the low Her2/*neu* expressing cell line, MCF-7 (Fig. 2C), there is a slight growth inhibition as the concentration of ELP was increased from 1 to 10 μM, whereas there was no significant change in growth inhibition as the concentration was increased from 10 to 100 μM. In the case of SF-ELP, there is significant growth inhibition observed between 1 μM and the concentrations of 40–100 μM, whereas there was no change in growth inhibition as the concentration was increased from 10 to 100 μM. The maximum growth inhibi-

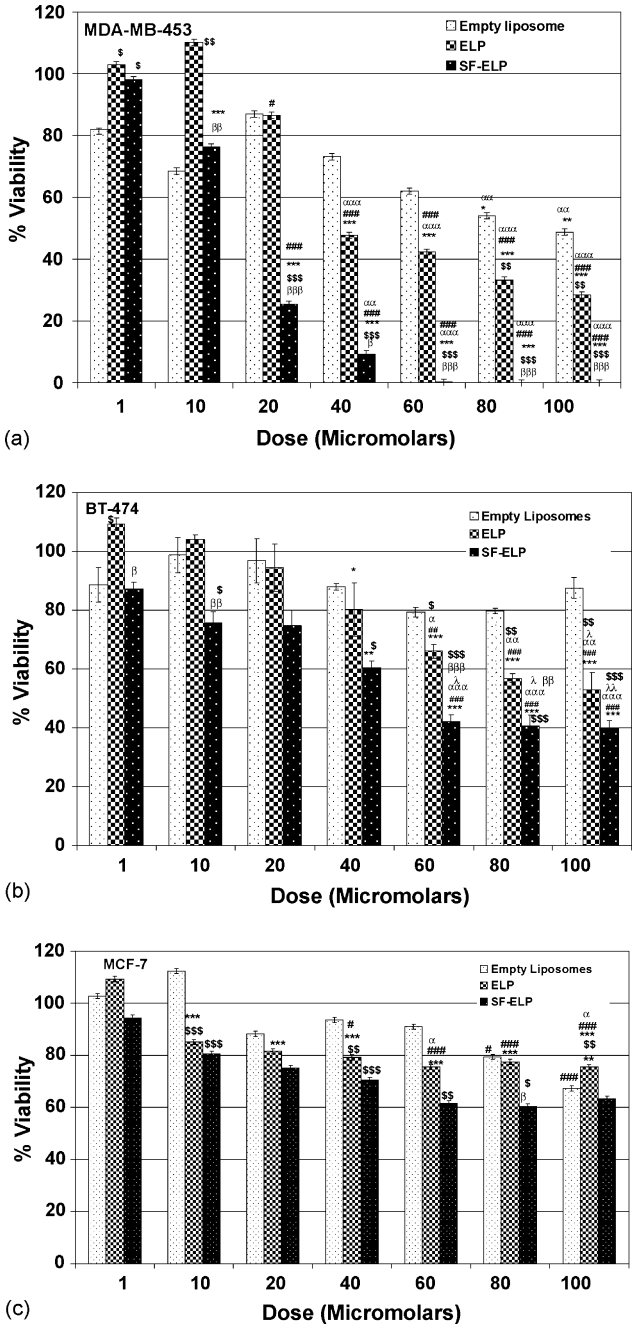

Fig. 2. Cell viability of human breast cancer cells over-expressing Her2/*neu*, (A) BT-474 and (B) MDA-MB-453, and low-expressing Her2/*neu*, (C) MCF-7 was determined using MTS assay and compared after exposure to empty liposomes, emodin-loaded liposomes (ELP) or silk-fibroin coated emodin-loaded liposomes (SF-ELP) at 1–100 μM concentrations. Data are presented as mean ± S.E.M., $N = 3$. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ as compared to empty liposomes. $^{\beta}p < 0.05$, $^{\beta\beta}p < 0.01$, $^{\beta\beta\beta}p < 0.001$ as compared to liposomal emodin, ELP. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared to 1 μM within each treatment group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ as compared to 10 μM within each treatment group. $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$ as compared to 20 μM within each treatment group. $^{\lambda}p < 0.05$, $^{\lambda\lambda}p < 0.01$ as compared 40 μM within each treatment group.

tion observed for SF-ELP in MCF-7 was ~40%, at emodin concentrations greater than or equal to 60 μM. No significant effect on the growth was observed with the empty liposomes.

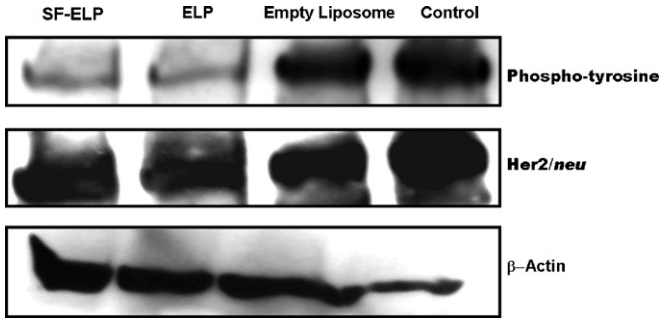

Fig. 3. Unaltered efficacy of emodin was observed following delivery from silk fibroin coated liposomes shown by inhibition of Her2/*neu* phosphorylation. Down-modulation of tyrosine phosphorylation was observed in MDA-MB-453 breast cancer cells over expressing Her2/*neu* following 96 h treatment with ELP and SF-ELP (40 μM each).

3.2. SF-ELP and ELP block the tyrosine phosphorylation of Her2/neu protein in human breast cancer cells

In order to investigate whether the observed differences in growth inhibition, in MCF-7 and MDA-MB-453 breast cancer cells, are a consequence of differences in the extent of inhibition of the activity of Her2/*neu*, the tyrosine phosphorylation status of Her2/*neu* following treatment with ELP and SF-ELP was assessed. Inhibition of tyrosine phosphorylation of a high molecular weight protein (~185 kDa) in MDA-MB-453 cells treated with both ELP as well as SF-ELP was observed. This high molecular weight protein was identified to be Her2/*neu* upon blotting with a Her2/*neu* specific antibody (Fig. 3). The silk-fibroin coating or the coating process in itself preserved the activity of emodin similar to uncoated liposomes. Phosphorylated Her2/*neu* was undetectable, under the blotting conditions used, in MCF-7 cells since these cells exhibit low expression of Her2/*neu* (data not shown).

Since the extent of inhibition of tyrosine kinase activity of Her2/*neu* was similar in MDA-MB-453, and did not account for the observed differences in growth inhibition following treatment with ELP and SF-ELP, the kinetics of the repression of phospho-tyrosine activity was compared. The effects of SF-ELP on the inhibition of tyrosine phosphorylation were evident at the 48 h time point following treatment, as compared to ELP at the 72 h time point (Fig. 4A and B). Delivery of emodin via SF-ELP is effective in inhibiting the tyrosine phosphorylation faster than ELP delivery. Quantification of band intensities of the kinetics data in Fig. 4 showed that there is relatively no change in phospho-tyrosine expression from 72 to 96 h for ELP delivered emodin. At the 72 h time point, maximum down modulation for phospho-tyrosine was measured when treated with SF-ELP. Despite that, at 96 h SF-ELP down-modulation of phospho-tyrosine was significantly greater as compared to ELP.

3.3. SF-ELP inhibits both the PI3K as well as the MAPK pathways, while ELP preferentially inhibits the PI3K pathway only

Activation of Her2/*neu* leads to breast cancer cell proliferation presumably by inducing activation of the

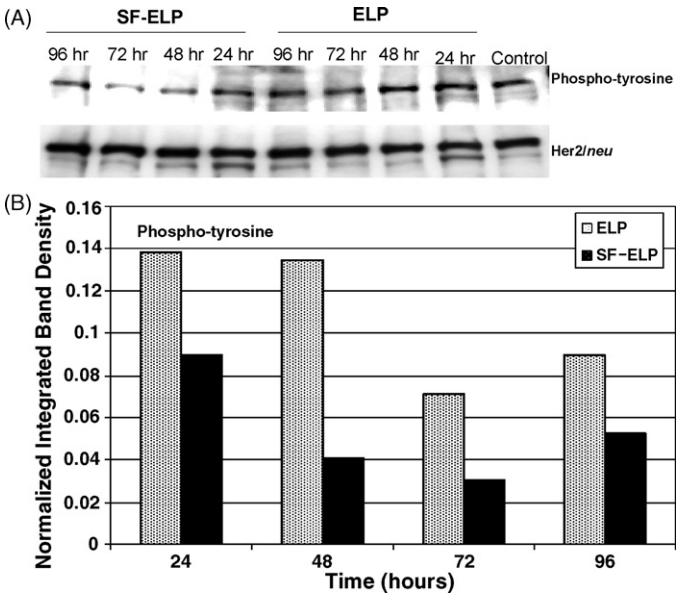

Fig. 4. Comparison of kinetics of inhibition of tyrosine phosphorylation of Her2/*neu* in MDA-MB-453 breast cancer cells treated with SF-ELP and ELP. (A) Expression of phosphorylated Her2/*neu* was detected using Western blotting from total cell lysates from MDA-MB-453 breast cancer cells either treated with ELP or SF-ELP for 24–96 h. Total Her2/*neu* expression was determined and used as a loading control. SF-ELP and ELP were administered at 40 μM concentration. (B) Band intensities were measured for phospho-tyrosine for 24–96 h and normalized to total Her2/*neu* expression.

phosphotidylinositol-3 kinase-AKT pathway and the MAPK pathway (Fig. 1) (Bacus et al., 2002; Pianetti et al., 2001). In order to examine whether ELP or SF-ELP treatment could inhibit activation of the MAPK and PI3K/AKT pathways, MDA-MB-453 cells were treated with 40 μM of either ELP or SF-ELP for 96 h. Phospho-AKT (active form of AKT), total AKT, phospho-MAPK (active form of MAPK), and MAPK were analyzed by Western blotting using specific antibodies.

The results demonstrated a significant decrease in phospho-AKT without any effect on the total AKT expression following ELP or SF-ELP treatment. In addition, SF-ELP was able to

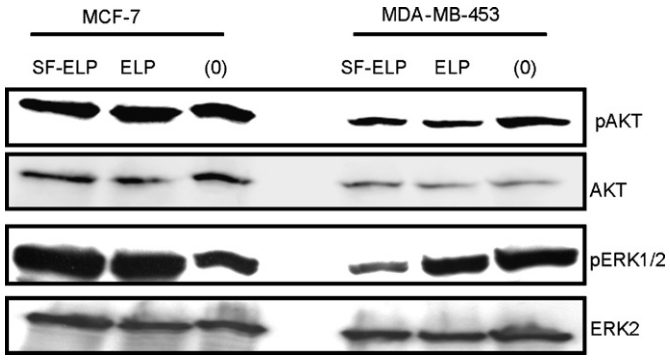

Fig. 5. SF-ELP treatment of MDA-MB-453 breast cancer cells results in selective inhibition of both PI3K and the MAPK/ERK1/2 pathways in Her2/*neu* over-expressing cells. Cell lysates from MDA-MB-453 breast cancer cells treated with 40 μM of either ELP or SF-ELP for 96 h were analyzed by Western blotting for the expression of activated AKT and ERK1/2. Total AKT as well as total ERK2 served as loading controls.

inhibit phospho-MAPK, without reducing total MAPK. ELP treatment, on the other hand, did not inhibit phospho-MAPK or affect total MAPK, at the dose tested (Fig. 5).

3.4. Cellular uptake of emodin by flow cytometry

In order to determine the mechanism for the inhibition of growth measured in MDA-MB-453 cells, emodin uptake in SF-ELP and ELP treated cells was assessed using flow cytometry (Fig. 6). Flow cytometric data indicated that there were two populations of cells post-treatment with ELP and SF-ELP, live and dead. There were more dead cells in SF-ELP treated population of cells and the overall mean fluorescent intensity was higher than ELP treated cells. Mean fluorescent intensity (MFI) for 10,000 events for MDA-MB-453 treated with ELP (MFI: 835; %CV: 128) and SF-ELP (MFI: 1139; %CV: 102) showed that the cellular emodin level for SF-ELP treated MDA-MB-453 cells was higher than that observed for ELP treated cells. Untreated cells served as controls (MFI: 3; %CV: 56).

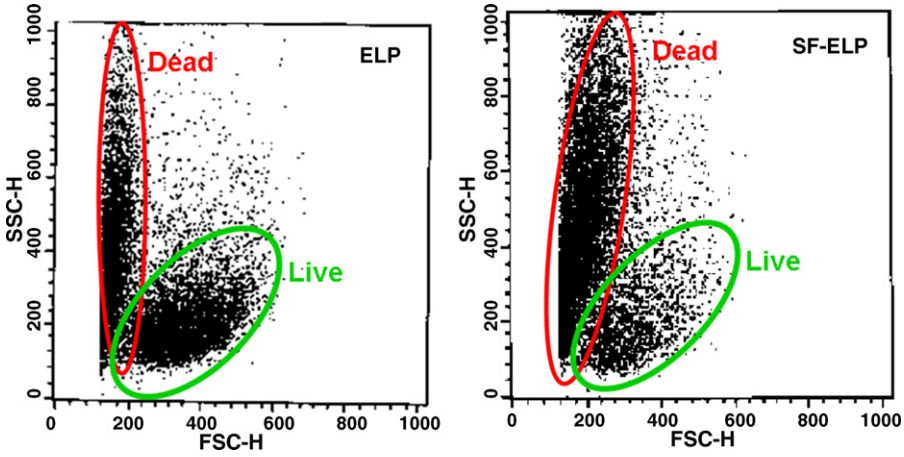

Fig. 6. Representative flow cytometric measurements of the fluorescent intensity of emodin in MDA-MB-453 cells treated with ELP or SF-ELP at equivalent drug concentrations (100 μM). There are two populations of cells detected, live and dead. There are more dead cells in SF-ELP treated cells and the mean fluorescent intensity is higher than ELP treated cells.

4. Discussion

In this study, the delivery of emodin via SF-ELP inhibited the growth of Her2/*neu* over-expressing breast cancer cells to a greater extent than delivery via ELP at the same concentration. Furthermore, we observed that SF-ELP was able to mediate inhibition of Her2/*neu* phosphorylation to the same extent as ELP thereby, indicating that the silk-fibroin coating itself or the process involved in coating of ELP did not alter the target selectivity or the activity of emodin. While ELP and SF-ELP both mediated inhibition of phosphorylation of Her2/*neu* in MDA-MB-453 cells via the PI3K/AKT pathway, SF-ELP mediated inhibition of Her2/*neu* phosphorylation was accompanied by inhibition of the MAPK pathway also. In addition, as compared to ELP, SF-ELP demonstrated a better uptake/retention of emodin in MDA-MB-453 breast cancer cells. Thus, our data demonstrated that the silk-fibroin coating of ELP leads to better delivery of emodin and improves efficacy without altering the target selectivity.

Delivery of emodin via the uncoated and coated liposome was ineffective in inhibiting the growth of MCF-7 breast cancer cells that lack the phosphorylated Her2/*neu* target for emodin. However, uncoated as well as silk-fibroin coated emodin loaded liposomes effectively inhibited the growth of Her2/*neu* over expressing breast cancer cells. Inhibition SF-ELP mediated a greater growth inhibition as compared to ELP in the breast cancer cell lines over expressing activated Her2/*neu* receptor tyrosine kinase.

Both SF-ELP as well as ELP decreased RTK activity to the same extent thus indicating that the coating itself or the process of coating did not alter the targeting ability or the activity of emodin, on the other hand the effectiveness of emodin towards the breast cancer cells was enhanced using SF-ELP delivery. Despite the fact that the phosphorylated Her2/*neu* is inhibited similarly by ELP and SF-ELP, there was increased growth inhibition for SF-ELP treated cells versus ELP treated cells. The difference in the growth inhibition is reflected by examining the pathways affected by Her2/*neu* blocking. Phosphorylated Her2/*neu* signals via the PI3K as well as the MAPK pathways (Siegel et al., 1999) and activation of PI3K as well as the ERK1/2 pathways by RTKs result in the uncontrolled proliferation of cells (Caldon et al., 2006; Majsterek et al., 2005; Wakeling, 2005). The inhibition of PI3K and MAPK pathways by SF-ELP may have contributed to the greater growth inhibition observed using the SF-ELP delivery method of emodin in MDA-MB-453.

We examined whether SF-ELP mediated inhibition of the two oncogenic pathways (PI3K as well as MAPK) in the MDA-MB-453 breast cancer cells, was due to better uptake of SF-ELP to MDA-MB-453 breast cancer cells. The uptake of a similar dose of SF-ELP was found to be greater, for MDA-MB-453 breast cancer cells, as compared to ELP. Not only was the uptake higher for SF-ELP treated cells, the cells that received emodin via SF-ELP also had a higher proportion of their population as dead cells versus the ones treated with ELP. These results indicate that silk-fibroin is contributing to better uptake of the drug-encapsulated liposome leading to higher drug efficacy. The silk fibroin coating possibly renders changes in the liposome that allows for better absorption of the drug by the cells. Therefore, the increased efficacy observed as growth inhibition of Her2/*neu* over-expressing breast cancer cells may also be attributed to more drug entering the cell due to the silk fibroin coating and knocking down more than one pathway.

The dose–response growth inhibition data suggests that increasing the drug dosage above 40 μM did not result in the same level of growth inhibition as witnessed with emodin being delivered with SF-ELP at lower doses. Forty micromolars of SF-ELP had more growth inhibition as compared to 100 μM of ELP. If uptake of emodin is the only factor contributing to the greater efficacy of the drug, then higher dosage of ELP should show better efficacy than a lower dose of SF-ELP. In contrast, the lower dose of SF-ELP had better efficacy than higher dose of ELP.

Additionally, uptake may be increased by increasing exposure time to ELP. Kinetics data indicates that there is relatively no change in phospho-tyrosine expression from 72 to 96 h for ELP delivered emodin. Phospho-tyrosine expression is still significantly less for SF-ELP as compared to ELP even at 96 h, alluding to a more complex mechanism than just increased drug uptake.

We have previously studied the uptake of emodin via ELP and SF-ELP delivery in keloid fibroblasts (Gobin et al., 2006), where a mechanism for higher uptake was discussed. The first barrier that a small drug or a nanoparticle has to overcome as it approaches a cell is the entry into the cell. Nanoparticle interaction with a cell is dominated by the Brownian motion of the particle. The adhesiveness or attraction of the silk fibroin to the outer muco-polysaccharide layer increases the probability of the liposomal nanoparticle attaching to a cell surface as it comes in contact with a cell, thereby overcoming the shortcomings of the Brownian motion of the nanoparticle. Once the particle gets into the cell, the silk fibroin coating acts as a barrier or protective mechanism for emodin release. While the total amount of the loaded emodin in ELP and SF-ELP was the same at each dose, the delivery of emodin from SF-ELP may have been longer lasting due to the protective coating of the silk fibroin.

Our previous study (Gobin et al., 2006) showed that release kinetics from the coated and uncoated particles are very different. While the mechanism for release from ELP was dependant on swelling of the liposome and then quick diffusion, the release mechanism from SF-ELP was purely slow diffusion of emodin from SF-ELP. Of course, once the particle gets into the cell, the metabolism of the particle and the drug in it are also factors in terms of the time it takes to utilize the drug delivered to the cell. The silk fibroin probably protects this quick metabolism of the released drug and increases availability of emodin for a longer time. Therefore, what appears to be better uptake may be an indication of better retention and availability of usable emodin over time in the case of SF-ELP delivery.

The greater growth inhibition of breast cancer cells by SF-ELP delivered emodin can also be explained by the multiple cell pathways that are affected by greater emodin availability. It is well established in literature that the Her2/*neu* proto-oncogene encodes a growth factor receptor that is over-expressed in 20–30% of metastatic breast cancers. This over expression is

associated with decreased survival and decreased relapse-free periods. The aggressiveness of the Her2/*neu* expressing breast cancer cells is attributed to the Her2/*neu*-mediated activation of the mitogenic signaling pathway (MAPK) and the survival signaling pathway (AKT) (Reese and Slamon, 1997). Inhibition of the tyrosine phosporylation of Her2/*neu* results in elimination of mitogenic signaling through MEK and p44/42 MAPK pathway and survival signaling through the Akt pathway. Emodin has been shown to inhibit the tyrosine phosphorylation of Her2/*neu* receptor (Zhang and Hung, 1996). In this study, cells treated with emodin delivered via SF-ELP demonstrated better uptake/retention of emodin versus cells treated with ELP. Higher amount of emodin within the SF-ELP treated cells translated into inhibition of both the mitogenic (MAPK) as well as the survival (PI3K) pathways in these cells as compared to only one pathway (PI3K) in cells treated with ELP. The inhibition of both pathways by SF-ELP translated into greater growth inhibition as compared to ELP treated cells.

Further, activation of Her2/*neu* involves autophosporylation of five tyrosine residues located in the c-terminal of the protein (Akiyama et al., 1991; Hazan et al., 1990; Saga et al., 1991; Segatto et al., 1990). These autophosphorylated tyrosine residues act as docking sites for proteins containing a structural motif, called the Src-homology 2 domain (SH2) (Pawson and Gish, 1992). It has been demonstrated in many tyrosine kinase molecules that the amino acid sequence surrounding each phospho-tyrosine residue determines the identity of the SH2-containing protein that will dock at that site (Songyang et al., 1993) and hence the downstream pathway that shall be activated. Based on this, it can be speculated that SF-ELP delivers enough emodin to inhibit the phosphorylation of perhaps all or more tyrosine residues of Her2/*neu* versus inhibition of phosphorylation of some residues by ELP. Hence, in the case of SF-ELP one may be preventing docking of SH2 molecules responsible for the activation of the MAPK and the PI3K pathways, whereas for ELP one is inhibiting the docking of SH2 molecules responsible for the activation of PI3K pathway alone. In addition, if the docking of SH2 molecules is an inter-dependant or a sequential loading process, then the longer retention or availability of emodin delivered via SF-ELP may play a role in knocking down of the additional pathways and result in greater growth inhibition.

In conclusion, silk fibroin mediated delivery of liposomal emodin showed higher efficacy in breast cancer cells. While the targeting is associated with the specificity of emodin for Her2/*neu* over-expressing cells, silk fibroin coatings of the liposomes provide an enhanced delivery system to these cells by increasing uptake/retention of emodin. The growth inhibition is a function of dual pathway (PI3K and MAPK) inhibition via the SF-ELP delivery system. Down modulation of more than one pathway due to longer availability of emodin is more likely the mechanism for growth inhibition.

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