

Silk-fibroin-coated liposomes for long-term and targeted drug delivery

Andrea S Gobin¹
 Robyn Rhea²
 Robert A Newman²
 Anshu B Mathur¹

¹University of Texas MD Anderson Cancer Center, Laboratory of Reparative Biology and Bioengineering, Plastic Surgery, Houston, TX, USA; ²Pharmaceutical Development Center, Houston, TX 77054, USA

Abstract: Many barriers to drug delivery into a tumor site require careful consideration when designing a new drug. In this study, the adhesive targeting and drug specificity of modified liposomal vesicles on human-scar-producing cells, keloid fibroblasts, were investigated. Keloids express abundant levels of mucopolysaccharides and receptor tyrosine kinase (RTK). In this report, the structural properties, drug release kinetics, and therapeutic availability of silk-fibroin-coated, emodin-loaded liposomes (SF-ELP), compared with uncoated, emodin-loaded liposomes (ELP), were investigated. SF-ELP had a highly organized lamellae structure, which contributed to 55% of the liposomal diameter. This modified liposomal structure decreased emodin release rates by changing the release kinetics from a swelling and diffusional process to a purely diffusional process, probably due to steric hindrance. SF-ELP also increased adhesion targeting to keloid fibroblasts. Increased retention of SF-ELP is most likely due to the interaction of the fibrous protein coating around the ELP with the pericellular molecules around the cell. SF-ELP also decreased survival rate of keloids that expressed high levels of RTK. These results demonstrated that SF-ELP enhanced emodin delivery by improved diffusion kinetics and specific cell targeting.

Keywords: silk fibroin, liposomes, emodin, keloids, cell targeting

Introduction

Barriers to drug delivery into the tumor site are numerous and require careful consideration when designing a new drug. Drug efficacy depends on its mode of delivery and its potency at the tumor site. An injected drug has to travel via the vasculature path and then diffuse and disperse into interstitial space at effective concentrations. At the site of a solid tumor, the heterogeneous vascular architecture, heterogeneous vascular permeability, high interstitial pressures in the necrotic core, large interstitial distance between the tissue mass and vessels, low convective transport, acidic pH, hypoxia, and lack of lymphatic drainage are all key issues that make drug delivery to tumors a challenge. In addition, due to relative lack of specificity of a drug for tumor cells, administration of high dosage no doubt contributes to the development of drug resistance and production of toxicity in normal tissues (Jain 2001). In this report we will discuss preparation and characterization of a modified drug delivery vehicle with a two-step approach for adhesive targeting and drug specificity against tumor cells.

Liposomes have been used to encapsulate drugs and can be controlled by pH or temperature. Liposomes can encapsulate hydrophilic drugs within their core and hydrophobic or lipophilic drugs in their lipid bilayer. Their size can be varied from nanometer to micron range and they can be size-selected. Although liposomes can reduce drug toxicity, little to no tissue specificity is associated with naked liposomes. Active targeting of liposomes for chemotherapy is less toxic and more effective with

Correspondence: Anshu B Mathur
 University of Texas M.D. Anderson
 Cancer Center, Laboratory of Reparative
 Biology and Bioengineering, Plastic
 Surgery, Unit 602 PO Box 301402,
 Houston, TX 77230 1402, USA
 Tel +1 713 563 7568
 Fax + 1 713 563 0231
 Email amathur@mdanderson.org

increasing in vivo residence time, promoting site-specific delivery with control release rates.

Emodin loaded in liposomes composed of 1,2-dimyristol-sn-glycero-3-phosphocholine (DMPC) was shown to improve efficacy for the treatment of 32D-p210bcr-abl leukemia by Claxton et al (2003), who patented the development, delivery, and formulation. Emodin (3-methyl-1,6,8-trihydroxyanthran-quinone), a relatively selective receptor tyrosine kinase (RTK) inhibitor, naturally occurs and is a promising agent for tumor patients that develop drug resistance to other traditional remedies. In addition, emodin has shown impressive activity with low toxicity in vivo and in preclinical models of Her2 overexpressing breast cancer, while sensitizing the cells to other antitumor therapies such as cisplatin, doxorubicin, etoposide, and paclitaxel (Zhang et al 1995, 1998, 1999).

To localize drug delivery, and increase residence time and specificity for cell recognition of emodin loaded in DMPC liposomes (ELP), we developed an adhesive coating derived from silk fiber. Silk fibroin (SF), a fibrous protein obtained from the larva of the domestic silkworm, *Bombyx mori*, is nonthrombogenic, antiinflammatory, and cell adhesive, making it a highly biocompatible material (Santin et al 1999). *Bombyx mori* SF is composed of two chains—a heavy chain, approximately 325 kDa, and a light chain, approximately 25 kDa—linked by a single disulfide bridge. The heavy chain of SF is composed of crystalline and amorphous domains (Zhou et al 2001; Altman et al 2003). The crystalline domain consists of glycine–alanine repeats interconnected with serine and tyrosine amino acids. The amorphous domain consists of the more bulky amino acids such as aspartic acid. The crystalline domains, which form antiparallel β -sheet secondary structures, are interspersed by the more flexible amorphous regions (Zhou et al 2001; Altman et al 2003). The two chains are bound together by a sericin coating and the removal of this sericin coating, before fibroin processing, removes the thrombogenic and inflammatory responses of SF (Santin et al 1999). Additional properties of SF include its strong affinity to polysaccharides (Roden et al 1985; Falini et al 2003); mechanical properties that include high strength and flexibility (Altman et al 2003); and swelling properties that depend on solution pH (Yeo et al 2003). These dynamic properties of fibroin microstructure make it a unique candidate for controlled and sustained drug or gene delivery. Previously, peptide sequences similar to fibroin have been conjugated with elastin to prepare particles for the controlled delivery of naked DNA (Megeed et al

2004). Silk fibroin microspheres have also been prepared for drug delivery applications (Yeo et al 2003).

In this study we examined the adhesive targeting and drug specificity of modified liposomal vesicles for human-scar-producing cells, keloid fibroblasts. Keloids are chronic dermal wounds resulting from a cutaneous injury caused by surgery or inflammation. They are characterized as raised pathological scars, causing pain and persistent itching, and are considered to be benign dermal tumors (Rockwell et al 1989; Ehrlich et al 1994; Niessen et al 1999). Chronic dermal wounds consist of fibroblasts that overproduce collagen and chondroitin sulfate, have high contractile activity, high levels of secreted cytokines, and are similar to tumors in overexpression of RTK, a transmembrane receptor that binds to growth factors such as fibroblast growth factor (FGF) (Mancini and Quaipe 1962; Berman and Bielely 1995). Assessment of modified liposomes on this cell line will aid in characterization of adhesion targeting and drug specificity.

Methods and materials

Aqueous SF

Raw silk was generously donated by Dr Sam Hudson (North Carolina State University, Raleigh, NC, USA). Sericin coating was removed with 0.25% w/v sodium lauryl sulfate (SDS; Sigma-Aldrich, St Louis, MO, USA) and 0.25% w/v sodium carbonate (Sigma-Aldrich) in boiling water for 1 hour. The degummed fibroin was then washed in boiling water for 1 hour and rinsed again in distilled water to remove remaining sericin and surfactants. Dried SF was then dissolved in calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$; Fisher Scientific, Pittsburgh, PA, USA) methanol solution. To achieve this, calcium nitrate tetrahydrate was dissolved in methanol at 1:4:2 molar ratio ($\text{Ca}:\text{H}_2\text{O}:\text{MeOH}$) at room temperature for 1 hour while stirring. The solution temperature was then raised to 70 °C and SF was added to a final concentration of 10% w/v. The SF was allowed to dissolve for 4 hours with continuous stirring at 70 °C. Aqueous SF was eventually obtained after the dissolution mixture was dialyzed against deionized water for 4 days with a change of water each day (6000–8000 Da MWCO; Fisher Scientific, Pittsburgh, PA, USA). Aqueous SF was then stored at 4 °C until used.

ELP

Emodin was dissolved in tert-butanol at 1 mg/mL and Tween 20 solution was prepared in t-butanol at 10% v/v. DMPC was separately dissolved in tert-butanol at 5 mg/mL and then

5% of the 10% Tween 20 solution was added to this overall liposomal batch. The two solutions are then mixed together and frozen at -80°C overnight. The ELP were lyophilized and stored at -20°C until used.

Silk-fibroin-coated, emodin-loaded liposomes (SF-ELP)

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 minutes with gentle agitation. The mixture was then frozen at -80°C and lyophilized. To insolubilize the SF-coated liposomes, methanol was added and mixed for 10 minutes. The methanol was then removed by centrifugation and then allowed to evaporate in the hood until dry. Samples (SF-ELP) were stored at -20°C until used.

Transmission electron microscopy of ELP and SF-ELP

Samples of ELP and SF-ELP in saline were imaged using a JEM 1010 transmission electron microscope (TEM; JEOL USA Inc, Peabody, MA, USA) equipped with a digital camera. Liposomal samples were placed on a 100 mesh formvar-coated, carbon-coated, nickel grid treated with poly-L-lysine for 1 hour for liposome adherence. The samples were coated with 1% ammonium molybdate with 0.02% bovine serum albumin (BSA) in distilled water for 1 minute. Samples were blotted dry and imaged.

Emodin diffusion from coated and uncoated liposomes

Sodium chloride solution (0.9%; Sigma-Aldrich) was added to each liposomal emodin sample. Samples (10 μL) were removed at appropriate time points and diluted with 990 μL saline. Absorbance readings were then obtained immediately at 442 nm. Diffused emodin concentration was calculated from a standard curve of emodin in dimethyl sulfoxide (DMSO; Fisher Scientific, Pittsburgh, PA, USA). Coated and uncoated liposomes were kept at 37°C between time points with gentle mixing. Three samples were measured for each condition.

Keloid and normal fibroblast cell culture and maintenance

Keloid fibroblasts (passage 11–17; ATCC, Manassas, VA, USA) and normal human dermal fibroblasts (NHDF, passage 10–17; Cambrex Corp, East Rutherford, NJ, USA) were

maintained on Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin, streptomycin, and glutamine solution at humidified 37°C and 5% CO_2 .

Adhesion of coated and uncoated ELP

Keloid (27 380 cells/ cm^2) and normal (66 667 cells/ cm^2) fibroblasts were seeded into 2-well chamber coverslips (Fisher Scientific, Pittsburgh, PA, USA) and incubated overnight. Cell seeding density was increased for normal cells due to the fact that keloid fibroblasts have a larger contact area with the surface. Medium was aspirated and cells were rinsed once with phosphate buffered saline (PBS). An aliquot (0.5 mL [2.86 mg/mL emodin]) of either SF-ELP or ELP was added to each well and incubated with cells for 10 minutes. The liposomes were removed and the cells rinsed 3 times with PBS to remove non-adherent liposomes. Phenol red free DMEM (0.5 mL) was added and cells were imaged on an Olympus IX70 fluorescent microscope (40 \times oil magnification; Melville, NY, USA) equipped with a Hamamatsu CCD camera (C5810 color chilled 3CCD camera; Hamamatsu City, Japan). All images had a 0.386-second exposure time (minimum 6 images per chamber). Fluorescent intensity was measured using IPLabs imaging software (Scanalytics, Fairfax, VA, USA) and normalized to cell number per image.

Survival of keloids and fibroblasts after exposure to ELP and SF-ELP

MTS assay (Promega, Madison, WI, USA) was performed to assess survival rates of cells after exposure to emodin. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is bio-reduced by cells into a formazan product, which is detectable by absorbance and is directly proportional to the number of living cells in culture. Keloids (5000 cells/well) and normal fibroblasts (5000 cells/well) were seeded into 96-well plates and incubated overnight. Medium was aspirated and 200 μL of 100 μM emodin loaded in coated or uncoated liposomes was added for 4 or 24 hours. The liposomes were removed and 100 μL fresh media was added with 20 μL MTS reagent. The formazan product was allowed to develop for 1 hour and the absorbance was read at 490 nm on a MRX plate reader (Dynex Technologies, Chantilly, VA, USA). Absorbance was compared with that of untreated cells and the sample number per treatment was 5.

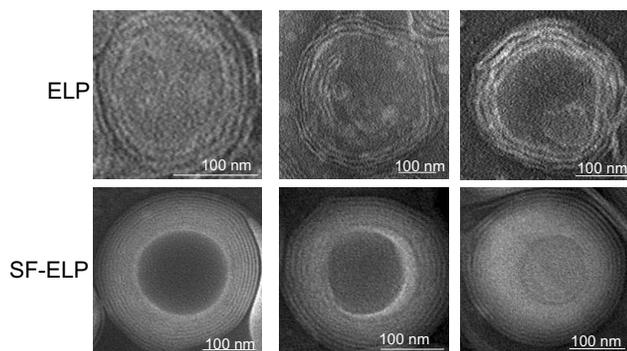


Figure 1 A representative collage of TEM images of emodin-loaded liposomes (ELP) and silk-fibroin-coated, emodin-loaded liposomes (SF-ELP), showing a distinct difference in the characteristics of coated and uncoated liposomes.

Statistical analysis

Data sets were compared using two-tailed, unpaired t tests. *p* values of less than 0.05 were considered significant.

Results and discussion

Coated and uncoated liposome structure

TEM images of SF-ELP and ELP are shown in Figure 1. Liposome diameters of ELP and SF-ELP were 232.2 ± 28.4 nm and 316.6 ± 43.0 nm, respectively. There was no significant difference in the overall diameter of the liposomes before and after SF coating. Overall lamellae thickness for ELP and SF-ELP was 26.2 ± 2.5 nm, and 80.5 ± 6.5 nm, respectively. Coating of ELP with SF increased lamellae thickness significantly ($p < 0.0001$). Lamellae morphology of ELP appeared loosely stacked. The SF polymer coated around the existing lamellae of ELP in a highly organized and refined manner. Thickness of the individual fine rings around SF-ELP was 4.2 ± 0.9 nm, significantly less ($p < 0.05$) than that of the rings around ELP (9.8 ± 2.1 nm) (Table 1).

Contribution of lamella size to overall liposome diameter was determined by calculating the ratio of lamella thickness to diameter (Figure 2). Lamellae contributed to 25% of the overall diameter of ELP, and 55% to the overall diameter of

Table 1 Structural characteristics of emodin-loaded liposomes (ELP) and silk-fibroin-coated, emodin-loaded enzymes (SF-ELP)

	ELP	SF-ELP
Liposome diameter	232.2 ± 28.4 nm	316.6 ± 43.0 nm
Lamellae thickness	26.2 ± 2.5 nm	80.5 ± 6.5 nm*
Lamella thickness	9.8 ± 2.1 nm	4.2 ± 0.9 nm†
Lamellae structure	Loosely stacked	Highly organized

* $p < 0.0001$, † $p < 0.05$ compared with ELP.

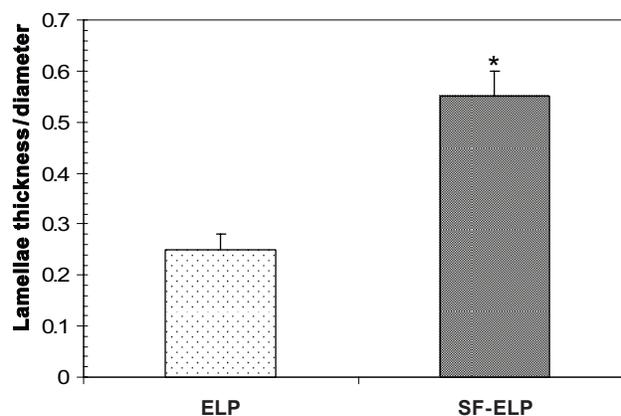


Figure 2 The ratio of the lamellae thickness to the overall diameter of the liposome shows that the silk fibroin-coated, emodin-loaded liposomes (SF-ELP) lamella contributed a significantly higher proportion to the overall diameter of the liposome than the emodin-loaded liposomes (ELP) lamella. * $p < 0.0001$ compared with ELP. Error bars in Figures 2, 3, 5, and 6 are standard error of the mean.

SF-ELP. Silk fibroin coating increased the overall lamellae thickness significantly ($p < 0.0001$) and contributed to over half the overall diameter of SF-ELP.

Emodin release kinetics

Emodin release from ELP and SF-ELP was quantified using a standard curve and UV-Vis spectroscopy (Figure 3). Initial release at time zero was similar from both ELP and SF-ELP. ELP released twice the initial amount within the first 4 hours, while emodin from the SF-ELP increased slightly over the same amount of time. Emodin release curve showed that approximately 70% of the emodin was released from ELP by 6 days, whereas only about 50% was released from SF-ELP. The rate of release of emodin from ELP was faster than from SF-ELP. Drug release from polymeric systems

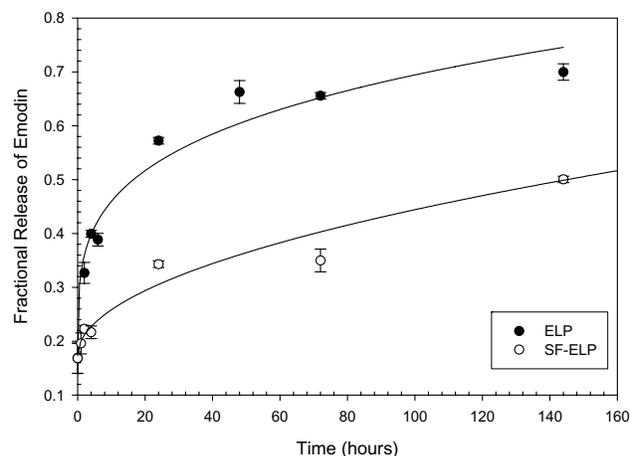


Figure 3 Emodin diffusion from emodin-loaded liposomes (ELP) and silk fibroin-coated, emodin-loaded liposomes (SF-ELP).

has been described by the power law (Equation 1) (Siepmann and Peppas 2001).

$$\frac{M_t}{M_\infty} = kt^n \quad (1)$$

Where M_t and M_∞ are the absolute amounts of the drug at any time t and at infinite time or the same as the initial drug loading concentration, respectively. k is a constant dependent on the structural and geometric characteristics of the liposome and n is the exponent dependent on the release mechanism. In this study, for ELP k is 0.169/hour and n is 0.25, and for SF-ELP k is 0.0267/hour and n is 0.50.

The results from both the structural composition studies and release kinetics describe how emodin is released differently from ELP and SF-ELP. The release mechanism from ELP involves the swelling of the liposomal lamellae and then quick diffusion of emodin. The swelling of the DMPC lamellae of ELP was shown in the TEM images where the lamellae are loosely organized. The mechanism of release of emodin from SF-ELP was purely diffusion and the rate of diffusion was slower than from ELP. The tight packing of the lamellae in a close-knit sphere indicates that the SF coating organized the liposomal geometry. There was no swelling of SF-ELP since the SF coating restricted the abrupt swelling of the liposomal lamellae, where the hydrophobic drug is trapped. In contrast, ELP appeared to swell before emodin release. The release of emodin from SF-ELP was probably slow due to the steric hindrance and barrier provided by the SF coating.

Adhesive targeting

To observe if the SF coating increased adhesion of the liposomes to target cells, ELP and SF-ELP were added to normal and keloid fibroblasts, which express normal and high levels of RTK, respectively. To verify the keloids were chronic wound cells, an immunoassay was used to detect overexpression of transforming growth factor beta 1 (TGF- β 1). The keloids expressed nearly a 3-fold increase in TGF- β 1 expression over normal fibroblasts (keloids: 83.4 ± 27.8 pg/mL, normal: 30.8 ± 5.9 pg/mL). To assess adhesion of the liposomes, cells were exposed to ELP or SF-ELP for 10 minutes, rinsed, and the remaining liposomes measured by fluorescence microscopy (Figure 4). The higher intensity of emodin found on normal and keloid fibroblasts indicates increased retention SF-ELP to cell surface compared with ELP ($p < 0.01$; Figure 5). Even higher retention of SF-ELP on keloid fibroblast indicates that SF is targeting the keloid fibroblast over normal fibroblast ($p < 0.05$; Figure 5).

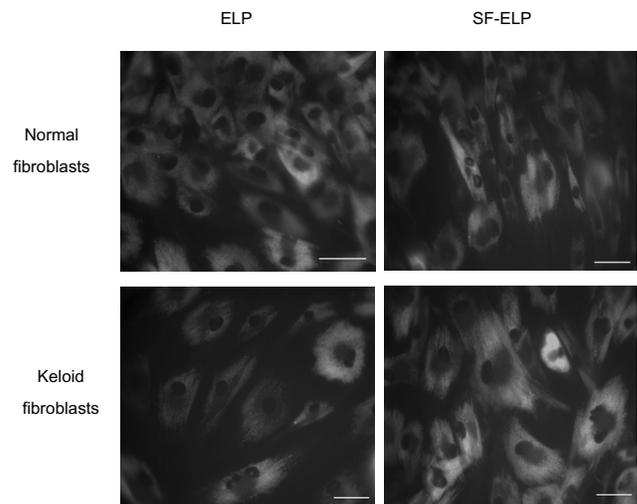


Figure 4 Representative images of normal and keloid fibroblast when exposed to emodin-loaded liposomes (ELP) or silk fibroin-coated, emodin-loaded liposomes (SF-ELP). Normal and keloid fibroblasts were more fluorescent when exposed to SF-ELP. Fluorescence is due to the natural fluorescence of emodin. Scale bars represent 50 μ m.

The targeting of SF to keloid fibroblast can result from a number of factors. First, SF is a fibrous protein polymer that supports cell adhesion similar to that of adhesion to collagen, even though it may not have the same adhesion domains as provided by collagen (Inouye et al 1998; Unger, Peters, Wolf et al 2004; Unger, Wolf, Peters et al 2004). Keloids cells synthesize approximately 20 times more collagen than normal unscarred skin (Rockwell et al 1989). Second, SF has good binding affinity to glycoproteins and proteoglycans (Chen et al 1997). It has been shown that keloid scars have abundant mucopolysaccharides compared with normal scars (Rockwell et al 1989; Ehrlich et al 1994;

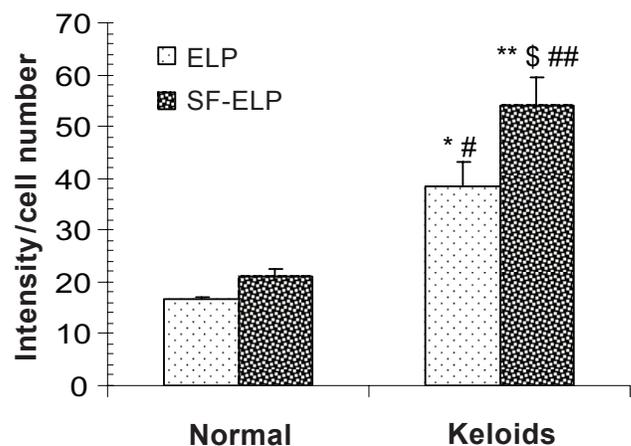


Figure 5 Fluorescent intensity measurements normalized by the number of cells per image. Cells treated with silk fibroin-coated, emodin-loaded liposomes (SF-ELP) fluoresced more than with emodin-loaded liposomes (ELP), indicating that more coated liposomes adhered to the cells. * $p < 0.01$, ** $p < 0.001$ compared with ELP on normal fibroblasts; # $p < 0.01$, ## $p < 0.001$ compared with SF-ELP on normal fibroblasts; \$ $p < 0.05$ compared with ELP on keloid fibroblasts.

Niessen et al 1999). Third, the organized geometry of the coated liposomal surface could influence its architecture and mechanical properties, thus influencing interaction and attachment with the tumor-like cells. Therefore, the SF coating retains more drug within the liposome so that when administered in vivo, quick leaching of the drug is reduced, allowing enough time for the targeted cells to absorb the drug.

Emodin efficacy and specificity

To assess the efficacy of the drug after coating the liposomes, keloids and normal fibroblasts were exposed to SF-ELP and ELP and survival rate was determined using a MTS assay. Survival rate of keloids decreased to 93% and then to 75% after exposure to ELP after 4 hours and 24 hours, respectively (Figure 6). However, the normal fibroblasts continued to proliferate. When keloids were exposed to SF-ELP over the same time scale, survival rate was 79% and 57% at 4 hours and 24 hours, respectively ($p < 0.05$ compared with ELP at 4 hours, $p < 0.001$ compared with ELP at 24 hours). Survival rate of normal fibroblasts exposed to SF-ELP also decreased, but to a lesser degree. This study showed the SF coating process did not affect the emodin efficacy. In addition, it demonstrates that emodin-loaded liposomes coated with SF increased efficacy and specificity of emodin when applied to tumor-like cells from the keloids. The specificity of SF-ELP is two part and is provided by the drug emodin and the SF coating. As previously stated,

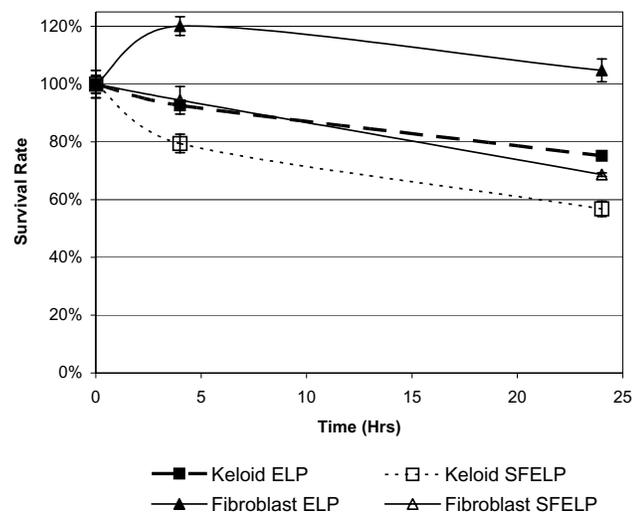


Figure 6 Survival rate of keloid and normal fibroblasts after exposure to emodin-loaded liposomes (ELP; 100 μ m emodin), or silk-fibroin-coated, emodin-loaded liposomes (SF-ELP; 100 μ m emodin). SF-ELP significantly decreased survival rate on keloid fibroblasts after 4 hours ($p < 0.05$) and 24 hours ($p < 0.001$) compared with exposure to ELP. Normal fibroblasts survival rate decreased in the presence of SF-ELP but to a lesser extent.

keloids overexpress RTK and are targeted by emodin, while the overexpression of the muco-polysaccharides over the cell surface would have attraction for the SF (Berman and Bielely 1995; Mancini and Quaife 1962).

Conclusions

Silk-fibroin-coated, emodin-loaded liposomes (SF-ELP), compared with uncoated, emodin-loaded liposomes (ELP), decreased emodin release rate but increased adhesion targeting to keloids, chronic wound cells. The definition of targeting in this paper addresses an interaction of the SF with cells' pericellular coating and not necessarily a particular receptor–ligand combination. Additional targeting is achieved by emodin, which targets the RTK. The goal of the SF coating is to bring the drug in close proximity to the targeting cell, and as the drug is released the drug binds to the targeting receptor. Silk fibroin coating overcomes the major shortcoming of any nanoparticle, which is the probability of a nanoscale entity colliding with a large cell surface in the correct vicinity and remaining in place. This occurs due to limitations of Brownian motion. The adhesiveness or attraction of the SF to the outer muco-polysaccharide layer increases the probability of the nanoparticle attaching to the cell surface as it comes in contact of the cell. In reality, the SF could be used for any drug coating, because the localization of the drug particle in combination with the specificity of the drug for the tumor is what makes it unique.

Acknowledgments

We thank Dr S Hudson of North Carolina State University for the donation of the raw silk (Korean Sericulture Institute). In addition, we thank the High Resolution Electron Microscopy Facility (HREM; Cancer Center Core Grant CA16672) for TEM imaging. The Whitaker Foundation, UT Center for Biomedical Engineering, Pharmaceutical Development Center, Goodwin Foundation, and the Commonwealth Foundation for Cancer Research at the University of Texas M.D. Anderson Cancer Center provided funding for this study.

References

- Altman GH, Diaz F, Jakuba C, et al. 2003. Silk-based biomaterials. *Biomaterials*, 24:401–16.
- Berman B, Bielely HC. 1995. Keloids. *J Am Acad Dermatol*, 33(1): 117–23.
- Chen X, Li W, Yu T. 1997. Conformation transition of silk fibroin induced by blending chitosan. *J Polym Sci, Part B: Polym Phys*, 35:2293–6.

- Claxton D, Newman RA, Lopez-Berestein G. Compositions and methods related to lipid:emodin formulations. US Patent 20 050 008 664. 2003.
- Ehrlich HP, Desmoulière A, Diegelmann RF, et al. 1994. Morphological and immunochemical differences between keloid and hypertrophic scar. *Am J Pathol*, 145(1):105–13.
- Falini G, Weiner S, Addadi L. 2003. Chitin-silk fibroin interactions: relevance to calcium carbonate formation in invertebrates. *Calcif Tissue Int*, 72:548–54.
- Inouye K, Kurokawa M, Nishikawa S, et al. 1998. Use of Bombyx mori silk fibroin as a substratum for cultivation of animal cells. *J Biochem Biophys Methods*, 37:159–64.
- Jain RK. 2001. Delivery of molecular and cellular medicine to solid tumors. *Adv Drug Deliv Rev*, 46:149–68.
- Mancini RE, Quaife JV. 1962. Histogenesis of experimentally produced keloids. *J Invest Dermatol*, 38:143–81.
- Megeed Z, Haider M, Li D, et al. 2004. In vitro and in vivo evaluation of recombinant silk-elastin like hydrogels for cancer gene therapy. *J Control Release*, 94:433–45.
- Niessen FB, Spauwen PH, Schalkwijk J, et al. 1999. On the nature of hypertrophic scars and keloids: a review. *Plast Reconstr Surg*, 104:1435–58.
- Rockwell WB, Cohen IK, Ehrlich HP. 1989. Keloids and hypertrophic scars: a comprehensive review. *Plast Reconstr Surg*, 84:827–37.
- Roden L, Koerner T, Olson C, et al. 1985. Mechanisms of chain initiation in the biosynthesis of connective tissue polysaccharides. *Fed Proc*, 44:373–80.
- Santin M, Motta A, Freddi G, et al. 1999. In vitro evaluation of the inflammatory potential of the silk fibroin. *J Biomed Mater Res*, 46:382–9.
- Siepmann J, Peppas NA. 2001. Modeling of drug release from delivery systems based on hydroxypropyl methylcellulose (HPMC). *Adv Drug Deliv Rev*, 48:139–57.
- Unger RE, Peters K, Wolf M, et al. 2004. Endothelialization of a non-woven silk fibroin net for use in tissue engineering: growth and gene regulation of human endothelial cells. *Biomaterials*, 25:137–46.
- Unger RE, Wolf M, Peters K, et al. 2004. Growth of human cells on a non-woven silk fibroin net: a potential for use in tissue engineering. *Biomaterials*, 25:1069–75.
- Yeo J-H, Lee K-G, Lee Y-W, et al. 2003. Simple preparation and characteristics of silk fibroin microsphere. *European Polymer Journal*, 39:1195–9.
- Zhang L, Chang CJ, Bacus SS, et al. 1995. Suppressed transformation and induced differentiation of HER-2/neu-overexpressing breast cancer cells by emodin. *Cancer Res*, 55:3890–6.
- Zhang L, Lau YK, Xi L, et al. 1998. Tyrosine kinase inhibitors, emodin and its derivative repress HER-2/neu-induced cellular transformation and metastasis-associated properties. *Oncogene*, 16:2855–63.
- Zhang L, Lau Y-K, Xia W, et al. 1999. Tyrosine kinase inhibitor emodin suppresses growth of HER-2/neu-overexpressing breast cancer cells in athymic mice and sensitizes these cells to the inhibitory effect of paclitaxel. *Clin Cancer Res*, 5:343–53.
- Zhou CZ, Confalonieri F, Jacquet M, et al. 2001. Silk fibroin: structural implications of a remarkable amino acid sequence. *Proteins*, 44:119–22.

