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Development of elastin-like polypeptide for thermally targeted delivery of doxorubicin

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ABSTRACT

The chemotherapeutic drug doxorubicin (Dox) is widely used as an antitumor agent in hematological malignancies and solid tumors. However, one of the limitations of its clinical use is that systemic administration of an effective dose of Dox results in nonselective cardiac toxicity and myelosuppression. In order to minimize this nonspecific toxicity, Elastin-like polypeptide (ELP) was examined for its ability to serve as a macromolecular carrier for thermally targeted delivery of Dox. The ELP-based doxorubicin delivery vehicle (Tat-ELP-GFLG-Dox) consists of: (1) a peptide derived from the HIV-1 Tat protein to facilitate its cellular uptake, (2) ELP to allow thermal targeting, and (3) the lysosomally degradable glycylphenylalanylleucylglycine (GFLG) spacer and a cysteine residue conjugated to a thiol reactive doxorubicin derivative. Cytotoxicity of Tat-ELP-GFLG-Dox in MES-SA uterine sarcoma cells was enhanced 20-fold when aggregation of ELP was induced with hyperthermia. The ELP delivered doxorubicin displayed a cytoplasmic distribution and induced temperature dependent caspase activation.

1. Introduction

The efficacy of traditional cytotoxic chemotherapy drugs is limited by their adverse effects on non-cancerous tissue. Only a small fraction of the administered dose of drug reaches the tumor site, while the rest of the drug is distributed throughout the body. This causes undesirable damage to normal tissue when used in doses required to eradicate cancer cells, resulting in a limited therapeutic index. Use of the topoisomerase II poison doxorubicin (Dox) is limited by the induction of myelosuppression and cardiotoxicity [1]. Site-specific drug delivery vehicles would make chemotherapy more effective and less toxic by increasing the amount of drug reaching the intended target.

A commonly used approach to address the issue of drug delivery to solid tumors is to attach the drug to a macromolecular carrier. Soluble polymeric carriers are attractive for systemic drug delivery because polymer–drug conjugates preferentially accumulate in tumors due to their enhanced microvascular permeability and retention [2–5] and exhibit significantly lower systemic toxicity compared to free drug [6–8]. Studies have shown that water-soluble polymer carriers can overcome multidrug resistance [9–12]. The most compelling evidence for the advantages of using polymer–drug conjugates

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Abbreviations: aa, amino acid; BSA, bovine serum albumin; DIC, differential interference contrast; Dox, doxorubicin; ELP, elastin-like polypeptide; FBS, fetal bovine serum; HPMA, *N*-(2-hydroxypropyl)methacrylamide; M_W, molecular weight; PBS, phosphate-buffered physiological saline; Tat, cell penetrating peptide derived from HIV-1 Tat protein; T_t, transition temperature

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over free chemotherapeutic agents for the treatment of cancer comes from extensive preclinical and clinical studies by Kopecek and colleagues on the use of *N*-(2-hydroxypropyl)-methacrylamide (HPMA) copolymers as drug carriers [13,14].

Elastin-like polypeptide (ELP) is a protein comprised of a five amino acid repeat (XGVPG, where X is any amino acid except proline). ELPs are attractive as polymeric carriers for drug delivery because they undergo an inverse temperature phase transition [15,16]. Below a characteristic transition temperature (T_t), ELPs are structurally disordered and highly solvated. But, when the temperature is raised above their T_t, they undergo a sharp (2–3 °C range) phase transition, leading to desolvation and aggregation of the biopolymer [15,16]. This process is fully reversible when the temperature is lowered below T_t.

The phase transition of these polypeptides may be exploited for use in drug delivery by applying focused, mild hyperthermia to the tumor site. Systemically injected ELP will remain soluble and freely circulate at normal body temperature. However, at localized sites where hyperthermia is applied to raise the tissue above the ELP's T_t, the polypeptide will aggregate and accumulate [17]. Attachment of drugs to ELP offers the capability to specifically deliver these drugs to the desired tissue by focused application of externally applied hyperthermia. The use of hyperthermia has an added advantage of increasing vessel permeability [18–20]. The ELP-based drug delivery system described here combines the advantages of macromolecular delivery, hyperthermia, and thermal targeting.

A previous study demonstrated the ability of ELP to deliver doxorubicin into the cell cytoplasm and induce cytotoxicity, but with no significant enhancement in cell toxicity in response to heat [21]. Since the ultimate goal of drug delivery by ELP is to thermally target the chemotherapeutic, it is imperative that cytotoxicity be enhanced in response to temperature increase. In this study, a thermally responsive drug carrier was generated by modifying the sequence of ELP to include additional targeting features, and the result was a drug delivery vector that achieved a 20-fold enhancement of cell killing in response to hyperthermia.

The thermally responsive ELP polypeptide was modified with the addition of a cell penetrating peptide derived from the HIV-1 Tat protein (Tat) and by employing a cleavable tetrapeptide linker for attachment of the drug to the macromolecule. The Tat peptide is known to facilitate transport of large molecules across the plasma membrane [22], and a previous study demonstrated its ability to enhance ELP uptake by an endocytic mechanism [23]. In addition, the ELP contains a tetrapeptide glycylphenylalanylleucylglycyl (GFLG) linker and a C-terminal cysteine residue. The thiol of the terminal cysteine is used for attachment of drugs, and the GFLG linker can be cleaved by lysosomal proteases of the cathepsin family [24], resulting in intracellular drug release. For this study, a thiol reactive derivative of doxorubicin (WP936) containing a maleimido moiety linked to the C-3′ amino group was designed and synthesized. WP936 was attached to the C-terminal cysteine residue of the ELP carrier (Tat-ELP-GFLG-Dox). The Dox delivery construct exhibited a T_t of 40 °C, and its cellular uptake was enhanced by both the Tat peptide and hyperthermia. Dox delivered by the ELP vector accumulated in the cell cytoplasm. The ELP-delivered Dox was cytotoxic to MES-SA uterine sarcoma cells, and the toxicity was enhanced 20-fold by the application of hyperthermia. The ELP-delivered Dox induced apoptosis by caspase activation in a temperature dependent manner.

2. Materials and methods

2.1. Synthesis of WP936, the thiol reactive doxorubicin derivative

N-succinimidyl ester of 6-maleimidocaproic acid was prepared as shown in Fig. 1A. The mixture of 6-maleimidocaproic acid (211 mg, 1 mmol) and sodium carbonate (50.3 mg, 0.5 mmol) in water (10 ml) was prepared and stirred at room temperature until all acid was dissolved. Water was evaporated to dryness. Residue was dissolved in methanol (10 ml), toluene (25 ml) was added, and solvents were evaporated to dryness. Addition and evaporation of the mixture of methanol and toluene was repeated three times. The obtained white powder of sodium salt (Fig. 1, (**1**)) was dissolved in ice-cold DMF (2 ml). *N*,*N*-dissucinimidyl carbonate (281.8 mg, 1.1 mmol) was added and the reaction mixture was stirred at 0 °C for 1 h. The reaction mixture was diluted with dichloromethane (25 ml), washed with water (3 × 15 ml), and dried over sodium sulfate. Inorganic salts were filtered off, solvents were evaporated to dryness, and residue was purified by column chromatography (SilicaGel 60, Merck) using dichloromethane as eluent, to give 184 mg of **2**, yield 60%.

The mixture of doxorubicin (hydrochloride salt) (38.4 mg, 0.065 mmol), (**2**) (30 mg, 0.08 mmol), diisopropylethylamine (28 μl, 0.16 mmol) and DMF (1 ml) was prepared and stirred at room temperature (Fig. 1B), while progress of the reaction was monitored by TLC (chloroform:methanol:ammonia = 85:15:2). After 40 min, the reaction was completed. The reaction mixture was diluted with dichloromethane (2 ml) and precipitated with hexanes (50 ml). The obtained solid was separated from solvents by centrifugation. The product was separated by column chromatography (SilicaGel 60, Merck) using chloroform, chloroform:methanol 98:2, 95:5 as eluents. Fractions containing WP936 were pooled together, evaporated to dryness, dissolved in chloroform (1 ml) and precipitated with hexanes (25 ml). The obtained solid was dried under vacuum to give 24 mg of WP936 (yield 60%). The ^{1}H NMR spectrum was obtained for WP936 and was in agreement with the proposed structure.

^{1}H NMR ($CDCl_3$, δ) ppm: 14.0, 13.25 (2s, 1H ea, 6,11-OH), 8.04 (dd, 1H, J = 7.6 Hz, J = 0.7 Hz, H-1), 7.72 (dd, 1H, J = J = 8.4 Hz, H-2), 7.4 (d, 1H, J = 8.1 Hz, H-3), 6.67 (s, 2H, CH maleimid), 5.82 (d, 1H, J = 8.6 Hz, NH), 5.50 (d, 1H, J = 3.5 Hz, H-1′), 5.38 (bs, 1H, H-7), 4.76 (s, 2H, 14-CH_2), 4.56 (s, 1H, 9-OH), 4.16 (q, 1H, J = 6.1 Hz, H-5′), 4.08 (s, 3H, OMe), 3.63 (bs, 1H, H-4), 3.49 (t, 2H, J = 7.1 Hz, CH_2-linker), 3.28 (dd, 1H, J = 18.8 Hz, J = 1.8 Hz, H-10), 3.24 (d, 1H, J = 18.8 Hz, H-10), 3.02 (m, 1H, H-3′), 2.34 (d, 1H, J = 14.7 Hz, H-8), 2.17 (dd, 1H, J = 14.7 Hz, J = 4.1 Hz, H-8), 2.12 (dd, 2H, J = 7.2 Hz, J = 2.4 Hz, CH_2 from linker), 1.83 (dd, 1H, J = 12.7 Hz, J = 5.5 Hz, H-2′e), 1.78 (ddd, 1H, J = J = 12.7 Hz, J = 4.1 Hz, H-2′a), 1.63–1.55 (m, 4H, CH_2 from linker), 1.29 (d, 3H, J = 6.1 Hz, H-6'), 1,30–1.25 (m, 2H, CH_2 from linker).

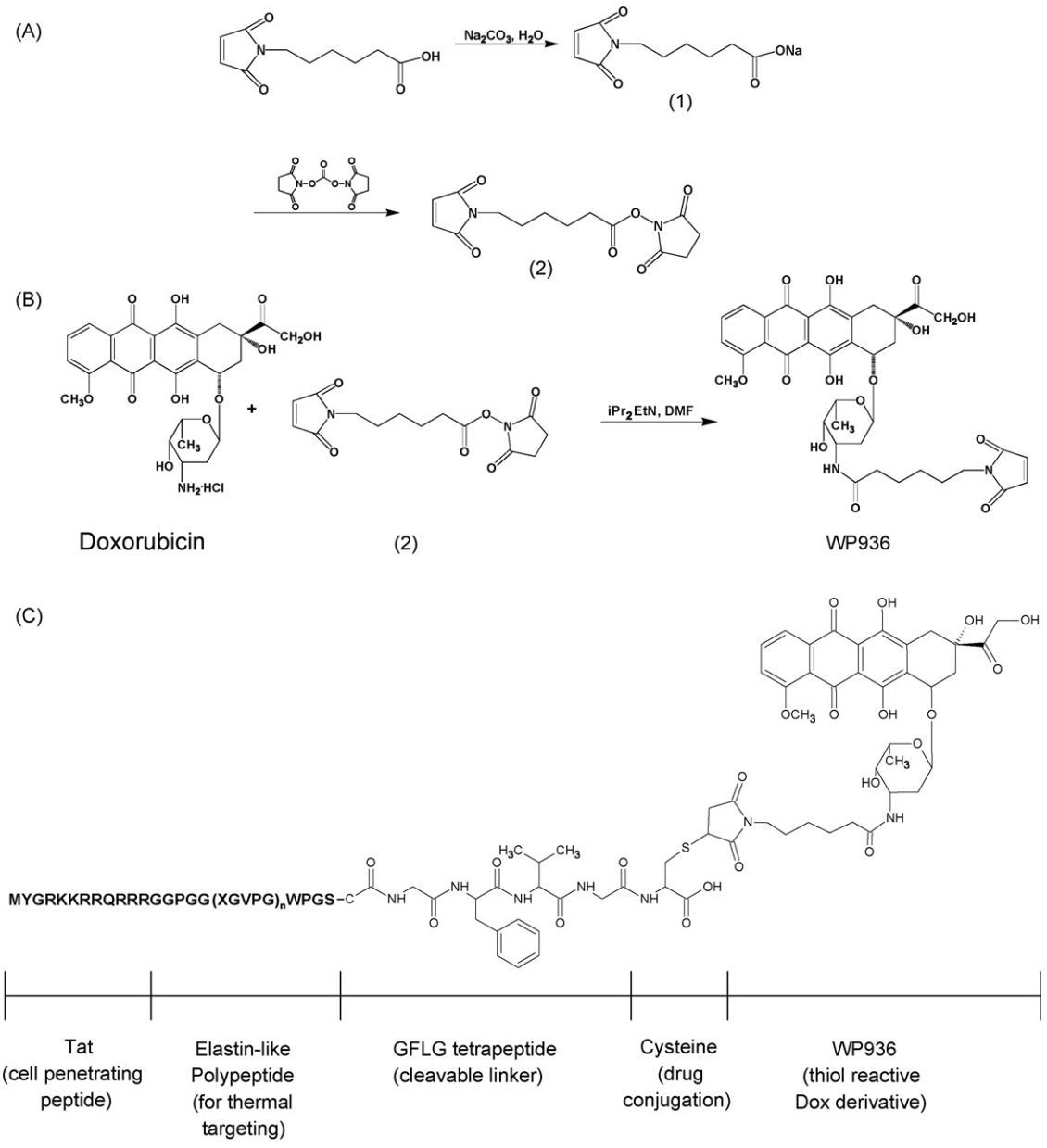

Fig. 1 – Synthesis of *N*-succinimidyl ester of 6-maleimidocaproic acid (A). Synthesis of WP936, a thiol reactive derivative of doxorubicin (B). Schematic of the doxorubicin carrier polypeptide (C). The amino acid sequence of the Tat cell penetrating peptide and the ELP polypeptide are shown in single letter amino acid codes, and the chemical structure of the GFLG linker with the cysteine-conjugated WP936 is shown.

Anal. Elem. For $C_{37}H_{40}N_2O_{14}\cdot 2H_2O$ Calcd. C, 57.51; H, 5.74; N, 3.63, Found: C, 57.90; H, 5.42; N, 3.60.

2.2. *Expression and purification of ELP*

The ELP1 and ELP2 gene sequences were graciously supplied by Dr. Chilkoti. Tat-ELP-GFLG and other constructs used in this study were generated as described previously [25]. All ELP constructs were expressed in *E. coli* using the hyperexpression system [26] and purified by inverse transition cycling [25]. Briefly, the *E. coli* strain BLR(DE3) containing the Tat-ELP-GFLG construct in the pET 25b expression vector was grown for 24 h at 37 °C in Circle Grow media (Q-Biogene, Irvine, CA). The cells were lysed by sonication and, after precipitation of nucleic acids, Tat-ELP-GFLG was precipitated from the soluble lysate by increasing the sodium chloride concentration and raising the temperature above the ELP T_t. The protein was collected by centrifugation, and this process was repeated until the desired purity was obtained as assessed by a single band on an SDS-PAGE gel.

2.3. *Conjugation of ELP with WP936*

A solution of ELP (ELP-GFLG or Tat-ELP-GFLG) was diluted to 100 μM in 50 mM Na_2HPO_4. Tris-(2-carboxyethyl)phosphine (TCEP; Molecular Probes, Eugene, OR) was added to a 10-fold

molar excess. WP936 was slowly added while mixing to a final concentration of 100 μM and incubated with continuous stirring overnight at 4 °C. Unreacted label was removed by three inverse transition cycles [25] into PBS. Efficiency of labeling on the single cysteine residue was assessed by UV–vis spectrophotometry (modified from Ref. [21]). The typical molar label to protein ratio was 0.83 ± 0.15.

2.4. Characterization of the transition temperature

The temperature induced aggregation of the proteins was characterized by monitoring absorbance at 350 nm while increasing the temperature. For initial analysis of Dox-labeled ELPs, solutions containing 10 μM protein in PBS were heated or cooled at a constant rate of 1 °C/min in a temperature-controlled multicell holder in a UV–vis spectrophotometer (Cary 100, Varian instruments). The analysis was repeated with Tat-ELP1-GFLG-Dox at concentrations ranging from 1 μM to 30 μM in cell culture media in order to determine the concentration dependence of the phase transition under experimental conditions. Absorbance data was converted to percentage of the maximal absorbance for each curve, and the T_t was defined as the temperature at which the OD_{350} reached 50% of the maximum turbidity. The concentration dependence of the T_t was fit using a logarithmic equation.

2.5. Cell culture and polypeptide treatment

MES-SA uterine sarcoma cells (ATCC, Manassas, VA) were grown as a monolayer in 75 cm^2 tissue culture flasks and passaged every 3–5 days. MES-SA cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml amphotericin B (Invitrogen, Carlsbad, CA). Cultures were maintained at 37 °C in a humidified atmosphere + 5% CO_2. For experiments, cells were removed from tissue culture flasks by brief treatment with 0.05%, v/v trypsin-EDTA (Invitrogen), plated in six well plates (300,000 cells/well for flow cytometry, 25,000 cells/well for proliferation) and allowed to grow for 24 h. Cells were treated with media containing polypeptides for 1 h at the indicated temperature, rinsed, and replaced with fresh media. In order to eliminate variability due to variation in labeling levels among different proteins or labeling batches, concentrations for cell treatments were always based on the Dox concentration as judged by the absorbance peak at 495 nm.

2.6. Polypeptide uptake

Cells were treated with 20 μM Tat-ELP1-GFLG-Dox, Tat-ELP2-GFLG-Dox, or control polypeptides lacking the Tat sequence as described above. Duplicate plates were treated for 1 h at 37 °C and 42 °C. Cells were rinsed with PBS and harvested using nonenzymatic cell dissociation buffer (Invitrogen), centrifuged for 2 min, and resuspended in 0.5 ml PBS. Total uptake of the doxorubicin labeled polypeptides was measured using the intrinsic Dox fluorescence by a Cytomics FC 500 flow cytometer (Beckman Coulter, Fullerton, CA). Forward versus side scatter gating was used to remove cell debris from the analysis, and Dox fluorescence was measured using FL3. Each histogram was a unimodal peak (n = 5000 cells), and the peak mean was normalized to propidium standard beads.

2.7. Laser scanning confocal fluorescence microscopy

MES-SA cells were plated on 22 mm^2 cover slips at approximately 50% confluence and treated as described above with Tat-ELP-GFLG-Dox. Cells were rinsed with PBS at the indicated times, fixed with paraformaldehyde (PFA, 2%, v/v) and visualized using a TCS SP2 laser scanning confocal microscope with a 100× oil immersion objective (Leica, Wetzlar, Germany). PMT voltages were lowered for imaging of cells treated at 42 °C during image acquisition to maximize image resolution and intensity. Therefore, the difference in image intensity between cells treated at 37 °C and 42 °C does not represent the actual difference in the amount of polypeptide in the cells.

2.8. Cytotoxicity assay

Cells were plated and treated for 1 h at 37 °C and 42 °C as described above with Tat-ELP1-GFLG-Dox, Tat-ELP2-GFLG-Dox, or control polypeptides lacking the Tat sequence or unlabeled with Dox. Cells were rinsed and harvested 72 h after treatment by trypsinization, collected by centrifugation, and resuspended in isotonic buffer. Cell number was determined using a Coulter counter and expressed as a percentage of untreated cells.

2.9. Apoptosis assays

For differential interference contrast (DIC) microscopy, MES-SA cells were plated onto 22 mm^2 coverslips at 50% confluence. Cells were treated as described above with the indicated protein, rinsed with PBS, and mounted onto glass slides. The unfixed cells were imaged immediately using a Zeiss Axiovert 200 DIC microscope with a 40× oil immersion objective and a Coolsnap HQ camera.

Caspase activation was measured using a carboxyfluorescein FLICA polycaspase assay (Immunochemistry Technologies, Bloomington, MN). Cells were treated as described above with the indicated protein, collected by trypsinization, and stained for 2 h at 37 °C with the carboxyfluorescein FLICA reagent as described by the manufacturer. Cells were rinsed twice and analyzed for caspase activation by flow cytometry using a Cytomics FC 500 flow cytometer (n = 5000 cells). Forward versus side scatter gating was used to eliminate cell debris from the analysis, and a histogram of fluorescein fluorescence (channel FL1) was bimodal with peaks for caspase positive and caspase negative cells. The percentage of caspase positive cells was determined from the histograms and expressed as an average of three experiments.

2.10. Data fitting and statistical analysis

The concentration dependence of the T_t was fit with a logarithmic equation using Microsoft Excel. Dose–response curves were fit using exponential or sigmoid equations as applicable using Microcal Origin in order to determine the 50% inhibitory concentration (IC_{50}). The thermal targeting index was calculated for each construct by dividing the IC_{50} at 37 °C

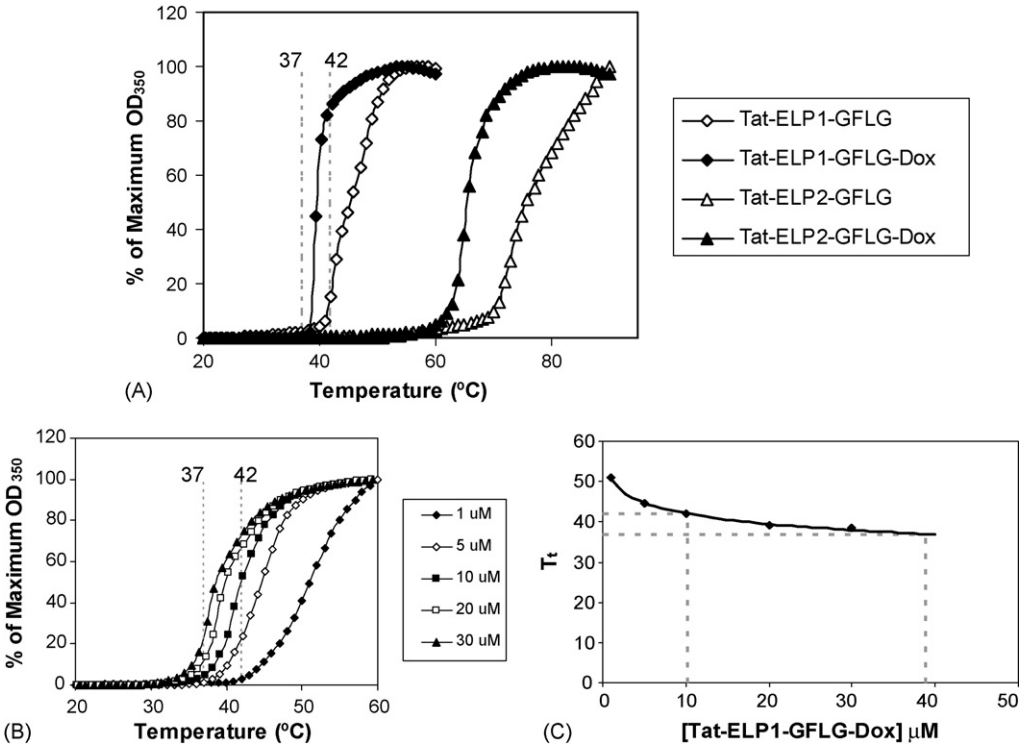

Fig. 2 – Thermal properties of Dox delivery constructs. The turbidity of a 10 μM solution of labeled and unlabeled Tat-ELP-GFLG in PBS was monitored while the temperature was increased at a rate of 1 °C/min (A). The effect of ELP concentration on the T_t under cell culture conditions was determined by repeating the turbidity assay using concentrations of Tat-ELP1-GFLG-Dox ranging from 1 to 30 μM in cell culture media (B). Optical density (OD) data is converted to a percentage of the OD at 60 °C for each curve in order to view all concentrations on the same scale. The midpoint of the phase transitions in (B) were plotted versus the protein concentration and fit using a logarithmic equation (R^2 = 0.9942) in order to determine the concentration range in which the T_t lies between 37 °C and 42 °C (C).

by the IC_{50} at 42 °C. The significance of the thermal targeting index was assessed by comparing the normothermia group with the hyperthermia group using a paired Student's *t*-test in Microsoft Excel. *p*-Values of less than 0.05 were considered statistically significant. Polypeptide uptake and caspase activation were analyzed for statistical differences using one-way ANOVA analyses with post hoc Scheffe's tests for pair-wise comparisons of treatment groups. The statistical significance level was $p < 0.05$.

3. Results

3.1. *Design, synthesis, and conjugation of Tat-ELP-GFLG and WP936*

The ELP drug delivery vector is composed of the pentapeptide repeat XGVPG, and it is designed to undergo a phase transition when the temperature is raised above its characteristic T_t. Two versions of the Dox delivery vector were made using two different ELP repeats. The ELP1-based construct contains 150 pentapeptide repeats (M_W = 59.1 kDa) with the guest position comprised of the amino acids V, G, and A in a 5:3:2 ratio. ELP1 was designed to have a T_t just above physiologic temperature for use as a thermally targeted vector. A second polypeptide was constructed including ELP2, which contains 160 XGVPG repeats (M_W = 61 kDa), where X is represented by V, G, and A in a 1:7:8 ratio. ELP2 has a similar molecular weight to ELP1, but it does not undergo its phase transition during the mild hyperthermia used in this study. Thus it serves as a non-thermally responsive control for the effects of hyperthermia [17]. Tat-ELP-GFLG was designed to include the Tat cell penetrating peptide [22] to allow cellular entry of the macromolecular construct, and the GFLG tetrapeptide linker was included to allow intracellular drug release by lysosomal proteases [24]. The C-terminal amino acid of the vector is a cysteine, which provides a chemically reactive thiol group for drug conjugation. WP936 is a thiol reactive Dox derivative obtained by conjugating a maleimido moiety via a linker with the amino group at the C-3′ position (Fig. 1A and B). WP936 can be covalently attached to the cysteine sulfur by nucleophilic addition. The labeling reaction resulted in an average molar ratio of Dox:protein of 0.83 ± 0.15. The amino acid sequence and chemical structure of WP936 covalently bound to the sulfur atom of the C-terminal cysteine is shown in Fig. 1C.

3.2. *Thermal properties of the Dox delivery vector*

In order to apply ELP-based polypeptides for drug delivery, a suitable temperature transition must be attained. The

drug-labeled ELP should undergo its phase transition between 39 °C and 42 °C, a temperature range sufficiently above normal body temperature to prevent unwanted systemic aggregation. This temperature range is preferred for clinical applications of hyperthermia because it minimizes the incidence of edema and necrosis in healthy tissue surrounding a heated tumor [27]. Elastin-like polypeptide is ideally suited as a thermally targeted drug carrier because the T_t can be easily manipulated by varying the molecular weight or the amino acid composition of the guest residue X in the XGVPG sequence using simple molecular biology techniques [28]. The T_t of Tat-ELP-GFLG-Dox was assessed by monitoring the turbidity of a 10 μM solution of the polypeptide while heating in PBS. The T_t of Tat-ELP1-GFLG was found to be 46 °C. However, labeling the polypeptide with Dox caused a significant downshift in the T_t (Fig. 2A), and as a result, Tat-ELP1-GFLG-Dox had an ideal phase transition for drug delivery (T_t = 40 °C). The polypeptide was soluble and the solution was clear at physiological temperature (37 °C), but when the temperature was raised to the hyperthermia temperature (42 °C), the polypeptide aggregated and the solution approached its maximum turbidity. Tat-ELP2-GFLG-Dox is a polypeptide similar in size to Tat-ELP1-GFLG-Dox, but a different ELP moiety is incorporated. Tat-ELP2-GFLG-Dox aggregates at a temperature significantly above the hyperthermia temperature (T_t = 65 °C), and it serves as a control to distinguish effects of the ELP phase transition from nonspecific hyperthermia-induced effects.

The phase transition of ELP is inversely related to the concentration of ELP and is also influenced by the concentration of other co-solutes. Therefore, in order to test the concentration range in which the Tat-ELP1-GFLG-Dox T_t is in the desired temperature range of 37–42 °C, the turbidity assay was repeated with various concentrations of Tat-ELP1-GFLG-Dox in cell culture media containing 10% FBS (Fig. 2B). The T_t was determined for each curve, and a plot of T_t versus polypeptide concentration revealed an inverse relationship which was best fit using a logarithmic function (Fig. 2C). This analysis showed that the midpoint of the phase transition was in the desired temperature range for concentrations between 10 μM and 39 μM. It should be noted, however, that some aggregation does occur at 42 °C for concentrations lower than 10 μM. Therefore, some thermal effect may be seen for cells treated under these conditions. The Tat-ELP-GFLG-Dox concentration is an important characteristic for thermal targeting because if the concentration of the ELP carrier is too high, it will undergo its phase transition at normal physiological temperature.

3.3. Cellular uptake of ELP-delivered doxorubicin

MES-SA uterine sarcoma cells were used in this study to evaluate the efficiency of ELP-mediated cellular delivery of doxorubicin. MES-SA cells were incubated with 20 μM Tat-ELP-GFLG-Dox or a control polypeptide which lacks the Tat sequence for 1 h at either 37 °C or 42 °C. After harvesting the cells with non-enzymatic cell dissociation buffer to prevent degradation of polypeptide attached to the cell surface, the non-fixed cells were analyzed by flow cytometry to measure the Dox fluorescence. This assay provides information about the total amount of Dox associated with the cells, both internalized and bound to the cell membrane. Fig. 3 shows that in the absence of the Tat peptide, low levels of ELP were associated with the cells at either temperature. However, cell association and uptake of Tat-ELP1-GFLG-Dox was increased over three-fold as compared to ELP1-GFLG-Dox when cells were treated at 37 °C ($p < 0.05$). In addition, hyperthermia further enhanced the association and uptake of Tat-ELP1-GFLG-Dox. Cellular fluorescence was increased over two-fold when the MES-SA cells were treated with Tat-ELP1-GFLG-Dox at 42 °C rather than 37 °C ($p < 0.05$). Levels of Tat-ELP2-GFLG-Dox, the non-thermally responsive control polypeptide, were unaffected by treatment with hyperthermia. This supports the hypothesis that the additional cellular association observed with Tat-ELP1-GFLG-Dox and hyperthermia is due to the polypeptide phase transition and not to nonspecific effects of hyperthermia.

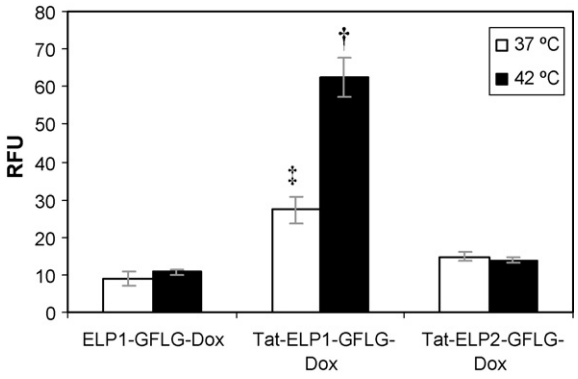

Fig. 3 – Cellular uptake of Dox labeled ELP. MES-SA uterine sarcoma cells were incubated with Dox labeled constructs (20 μM in cell culture media) for 1 h at 37 °C or 42 °C. The Dox fluorescence intensity was determined using flow cytometry and is expressed in relative fluorescence units (RFU). The data represents the mean of at least three experiments (error bars, S.E.M.). ‡Significant difference compared with ELP1-GFLG-Dox at 37 °C. †Significant difference compared with Tat-ELP1-GFLG-Dox at 37 °C ($p < 0.0001$, one way ANOVA, $p < 0.05$, Scheffe's test).

3.4. Cellular distribution

To confirm that Tat-ELP-GFLG-Dox was internalized and not merely attached to the cell exterior, the cellular distribution of the ELP-delivered drug was examined by confocal fluorescence microscopy. MES-SA cells were treated for 1 h at 37 °C and 42 °C with 10 μM Tat-ELP1-GFLG-Dox. The cells were rinsed and analyzed immediately after treatment and 24 h later using the intrinsic Dox fluorescence. Taking a confocal section through the center of the cells confirmed internalization of Tat-ELP1-GFLG-Dox immediately after treatment, with the drug accumulating in the plasma and nuclear membranes as well as in a punctate pattern in the cytoplasm (Fig. 4A, top, left). When Tat-ELP1-GFLG-Dox treatment was combined with hyperthermia, the membrane and cytoplasmic distribution was still present, but the protein was present in large

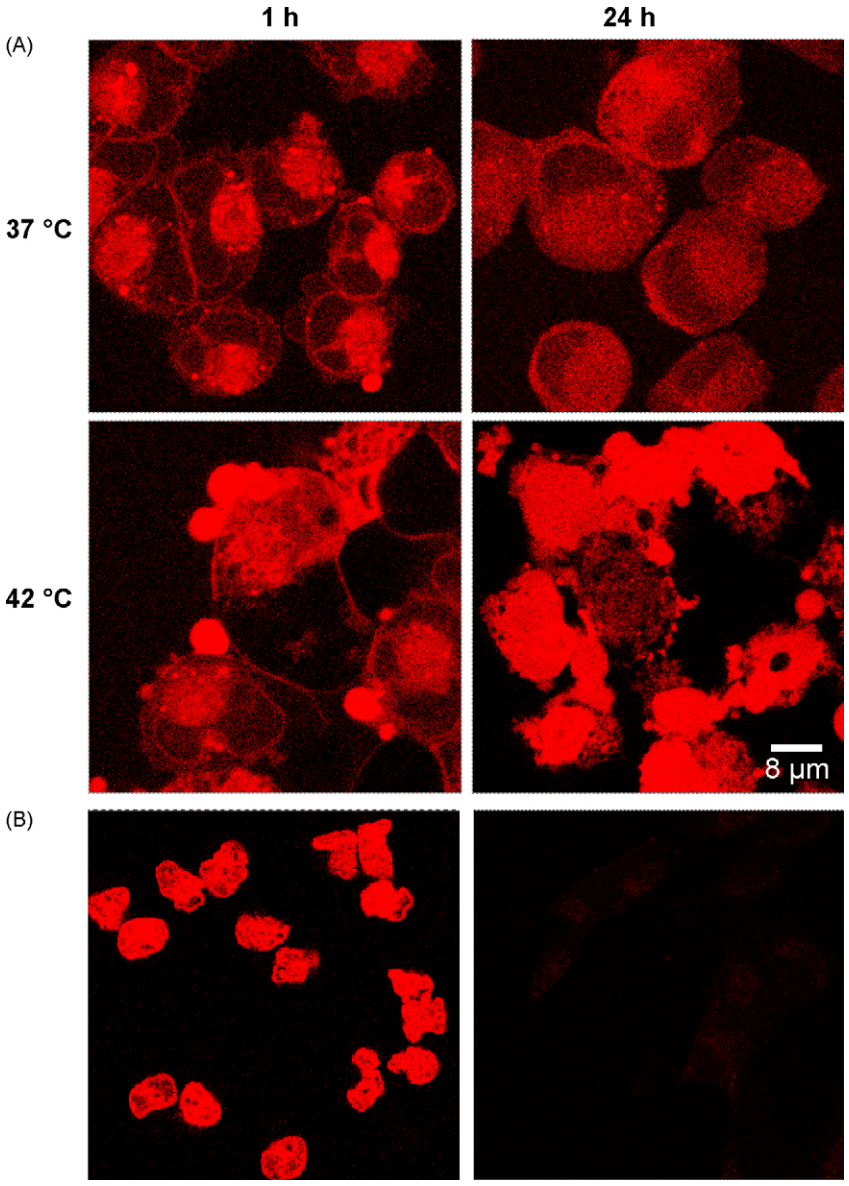

Fig. 4 – Intracellular localization. Confocal fluorescence images of MES-SA cells were collected 1 h and 24 h after a 1 h exposure to Tat-ELP1-GFLG-Dox (10 μM in cell culture media) at 37 °C or 42 °C (A). PMT voltages were decreased for collection of 42 °C images. Therefore, the image intensity does not reflect the actual level of Dox in the cells. Control cells were treated with free Dox (10 μM in cell culture media) (left panel) or untreated to check for autofluorescence (right panel) (B).

aggregates and the cells had begun to show hallmarks of apoptosis including rounding and membrane blebbing (Fig. 4A, bottom, left). Twenty-four hours after treatment, the ELP-delivered Dox still remained in the cell cytoplasm, and it displayed a more diffuse distribution (Fig. 4A, top, right). The cells observed 24 h after Tat-ELP1-GFLG-Dox treatment combined with hyperthermia again showed accumulation of the large ELP-drug aggregates in a diffuse cytoplasmic distribution and extensive apoptosis (Fig. 4A, bottom, right). In contrast to the ELP-delivered doxorubicin, the free drug was found exclusively in the cell nucleus (Fig. 4B, left panel). Untreated cells had no signal under the imaging settings used, ruling out any contribution to the images from autofluorescence (Fig. 4B, right panel).

3.5. *Cytotoxicity of ELP-delivered Dox*

The growth-inhibitory activity of Tat-ELP-GFLG-Dox was evaluated in the MES-SA uterine sarcoma cell line. MES-SA cells were treated for 1 h at 37 °C or 42 °C with various concentrations of Tat-ELP-GFLG-Dox; ELP-GFLG-Dox, which lacks the Tat cell penetrating peptide, the Tat-ELP-GFLG polypeptide without Dox; or free Dox. After the 1 h treatment, the cells were rinsed and fresh media was replaced for 72 h. Cells remaining after 72 h were rinsed, collected by trypsinization, and counted using a Coulter counter. Fig. 5A shows that Tat-ELP1-GFLG-Dox was cytotoxic at 37 °C, but high concentrations were needed. However, when Tat-ELP1-GFLG-Dox was applied in combination with hyperthermia, the

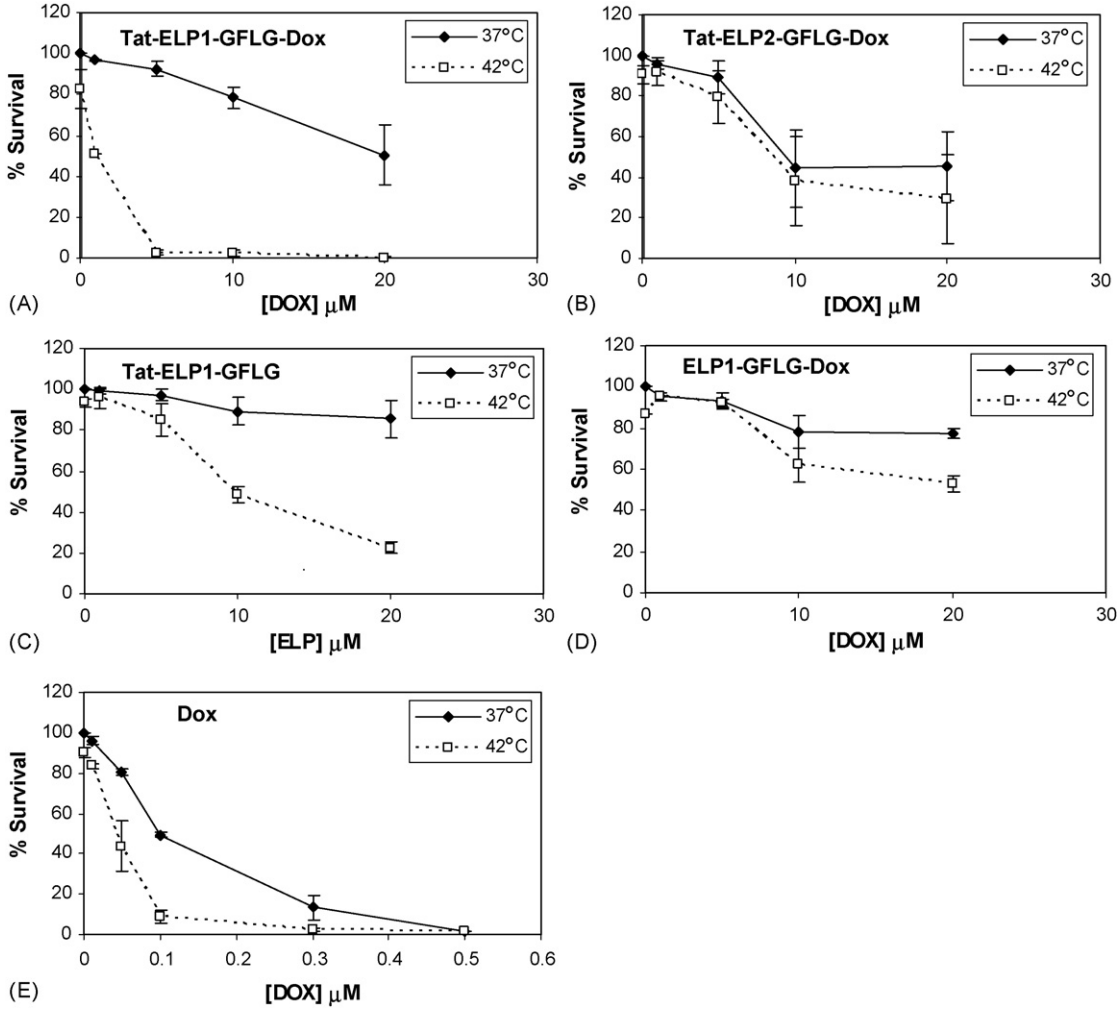

Fig. 5 – Cell proliferation. MES-SA cells were exposed to varying concentrations of Tat-ELP1-GFLG-Dox (A), Tat-ELP2-GFLG-Dox (B), Tat-ELP1-GFLG (C), ELP1-GFLG-Dox (D), or free Dox (E) for 1 h at 37 °C or 42 °C, and cells were counted after 72 h. The data represent an average of at least three experiments (bars, S.E.M.).

cytotoxicity was greatly enhanced. Tat-ELP2-GFLG-Dox was also cytotoxic (Fig. 5B), but the toxicity was not affected by hyperthermia and was similar to Tat-ELP1-GFLG-Dox at 37 °C. The toxicity of the unlabeled Tat-ELP-GFLG protein was negligible when treated below the T_t. However, Tat-ELP1-GFLG did show some toxicity at high concentrations when treated at 42 °C, but this nonspecific toxicity did not approach the toxic levels observed with the Dox labeled protein (Fig. 5C). ELP1-GFLG Dox exhibited very poor potency at either treatment temperature, which is consistent with its poor cellular uptake in the absence of the Tat peptide (Fig. 5D). Free Dox was more cytotoxic than the polymer-delivered drug (Fig. 5E), which reflects its passive cellular entry and is consistent with other studies of Dox bound to synthetic polymers [29,30]. The Dox toxicity was also affected by hyperthermia, which is likely due to enhanced diffusion of the drug into the cells.

For direct comparison of the cytotoxic effects of the Dox delivery constructs, each data set was fitted to an exponential or sigmoid equation to determine the IC_{50} value. Additionally, the thermal targeting index was calculated by dividing the IC_{50} of each polypeptide at 37 °C by its IC_{50} at 42 °C, and presented in Table 1. This calculation allows analysis of the enhancement of polypeptide toxicity by its hyperthermia induced phase transition. The IC_{50} of free Dox was about 150 times lower than the Tat-ELP-GFLG delivered Dox. The Dox IC_{50} was enhanced by heat treatment, but the temperature enhancement was only 2.3-fold ($p = 0.02$). Some toxicity was noted with the Tat-ELP1-GFLG protein that does not contain Dox, especially when aggregation was induced with heat. However, the level of cell killing was ten-fold less potent than Tat-ELP1-GFLG-Dox. ELP1-GFLG-Dox, which is thermally responsive but lacks the Tat cell penetrating peptide, showed a slight thermal effect, but the potency was two-fold lower at 37 °C and 15-fold lower at 42 °C than Tat-ELP1-GFLG-Dox. In fact, under hyperthermia conditions, Tat-ELP1-GFLG is more toxic than ELP1-GFLG-Dox. Tat-ELP1-GFLG-Dox was cytotoxic at 37 °C, but relatively high concentrations were needed to achieve significant cell killing. However, the IC_{50} was reduced 20-fold when Tat-ELP1-GFLG-Dox treatment was combined with hyperthermia ($p = 0.04$). The 20-fold enhancement of cytotoxicity induced by hyperthermia and Tat-ELP1-GFLG-Dox treatment results from a combination of the toxicities of the

Table 1 – IC_{50} values for all ELP-doxorubicin constructs

Construct	IC_{50} (37 °C) (μM ± S.E.M.)	IC_{50} (42 °C) (μM ± S.E.M.)	Thermal targeting index, ($IC_{50}^{37\,°C}/IC_{50}^{42\,°C}$)
Dox	0.130 ± 0.01	0.058 ± 0.02	2.3†
Tat-ELP1-GFLG	45.1 ± 12*	10.1 ± 0.36	4.47†
ELP1-GFLG-Dox	44 ± 8*	15.4 ± 3.9	2.85†
Tat-ELP1-GFLG-Dox	21.9 ± 8.2	1.1 ± 0.1	19.9†
Tat-ELP2-GFLG-Dox	18.3 ± 11.4	17.3 ± 12.8	1.1

IC_{50} values for each construct. Data from Fig. 5 was fit to calculate the IC_{50} value for each construct with treatment at 37 °C or 42 °C. The thermal targeting index was calculated by dividing the IC_{50} value for each construct at 37 °C by its IC_{50} value with 42 °C treatment. †Significant difference between the 37 °C and 42 °C IC_{50} ($p < 0.05$, Student's *t*-test). *IC_{50} extrapolated from curve fitting because the required concentration was unreachable.

Tat peptide and Dox. The Tat-ELP2-GFLG-Dox IC_{50} was unaffected by the hyperthermia treatment, indicating that the thermal enhancement of toxicity observed with the ELP1-based construct was due to enhanced cellular delivery by aggregation of the polymer.

3.6. *Induction of apoptosis*

As mentioned above, hallmarks of apoptosis were noted in the cell morphology after treatment with Tat-ELP1-GFLG-Dox, especially in combination with hyperthermia. This effect was documented by treating MES-SA cells adhered to glass cover slips with each construct and imaging them by DIC microscopy. Cells were treated for 1 h as described above, then immediately mounted on slides and imaged without fixation. Fig. 6A shows representative images of each treatment population. Untreated cells were spread onto the glass, and morphological features such as nuclei and endocytic vesicles were readily visible. This morphology was not altered by simply exposing the cells to hyperthermia. When the cells were treated with Tat-ELP1-GFLG, their morphology was unaltered with treatment below the T_t, and treatment above the T_t resulted in slight rounding and minor blebbing in only a small portion of the population. Cells treated with ELP1-GFLG-Dox showed some hallmarks of apoptosis, including rounding and membrane blebbing. However, such effects were minor and limited to a fraction of the cells. Treatment with Tat-ELP1-GFLG-Dox at 37 °C caused more extensive rounding and blebbing. When cells were treated with Tat-ELP1-GFLG-Dox at 42 °C, normal cell morphology was completely destroyed. All cells examined were rounded with no discernable nucleus and fragmented chromosomes. Tat-ELP2-GFLG-Dox induced minor blebbing in a proportion of the population with no heat effect, similar to Tat-ELP1-GFLG-Dox at 37 °C. Free Dox also caused noticeable rounding and membrane blebbing, but not to the extent of Tat-ELP1-GFLG-Dox at 42 °C.

The mechanism by which Tat-ELP1-GFLG-Dox induced apoptosis was investigated by determination of caspase activity. MES-SA cells were treated as described above, cells were harvested after the 1 h exposure, and caspase activity was detected using a fluorescent caspase inhibitor and flow cytometric analysis. A histogram of fluorescence versus cell number revealed two peaks, one with bright fluorescence which includes cells with positive caspase activity and one with lower fluorescence comprised of caspase negative cells (Fig. 6B). Cell numbers under each peak were quantified and averaged to determine the percentage of the cell population in which caspases were activated under each treatment condition. Caspase activity induced by hyperthermia alone, by Tat-ELP1-GFLG, or by ELP-GFLG-Dox was similar to control levels (Fig. 6C). When cells were treated at 37 °C, Tat-ELP1-GFLG-Dox induced apoptosis slightly above control levels. However, when the treatment temperature was raised to 42 °C, Tat-ELP1-GFLG-Dox induced caspase activation in a significantly higher proportion of the population. In fact, the percent of caspase positive cells was enhanced almost 4 fold when cells were treated with Tat-ELP1-GFLG-Dox at 42 °C versus 37 °C ($p < 0.05$). Apoptosis induction by Tat-ELP2-GFLG-Dox was not enhanced by hyperthermia, supporting the hypothesis that the higher levels of apoptosis seen with Tat-ELP1-GFLG-Dox and hyperthermia were due to accumulation of the polypeptide as a result of the ELP phase transition. Free Dox induced apoptosis under the conditions tested ($p < 0.05$), but the levels were not affected by hyperthermia.

4. Discussion

It has been shown previously that the endocytic uptake of a thermally responsive ELP is significantly enhanced by the thermally triggered phase transition of the polypeptide [31]. This observation was confirmed in this study where it was shown that cellular uptake of the ELP-delivered Dox was enhanced by both the Tat peptide and hyperthermia. Interestingly, cellular uptake of ELP1-GFLG-Dox was not significantly enhanced by heat treatment in the absence of the Tat peptide, even though both constructs undergo their phase transition in the 37–42 °C window. This result differs from previous reports of ELP1 labeled with fluorescein [25,31], in which as much as a two-fold enhancement of cellular uptake with hyperthermia was seen in several different cell lines. A possible explanation is that the fluorescein dye is less hydrophobic than Dox, and therefore the large aggregates of ELP1-GFLG-Dox may have a reduced affinity for the charged cell surface. Clearly, however, the addition of the Tat peptide increases the affinity of the large ELP aggregates for the cell membrane, as the uptake of Tat-ELP1-GFLG-Dox was increased three-fold at 37 °C and six-fold at 42 °C as compared to ELP1-GFLG-Dox.

The cellular Dox levels resulting from Tat-ELP-GFLG-Dox treatment were much lower than what results from treatment with free doxorubicin under similar conditions (data not

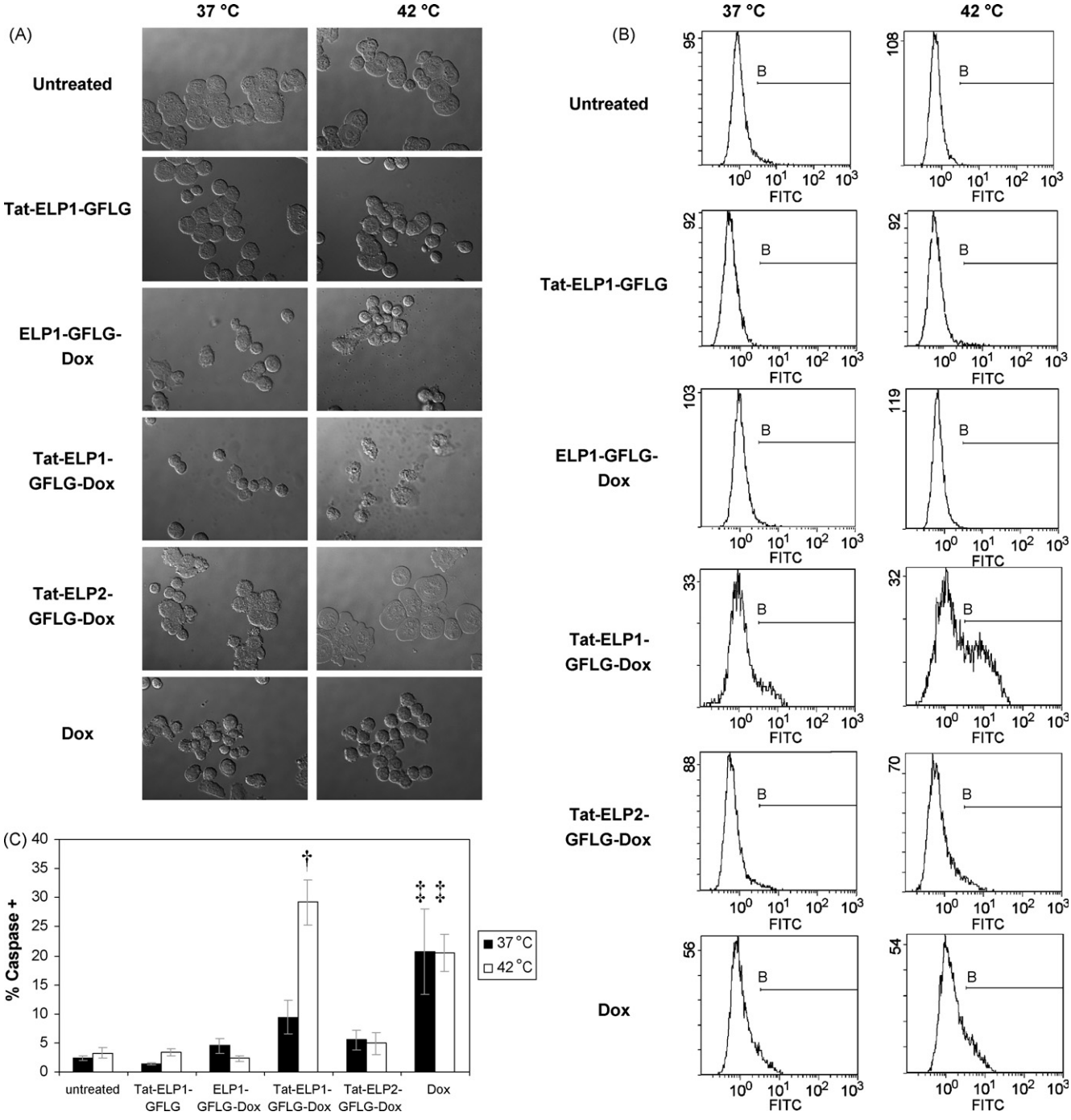

Fig. 6 – Induction of apoptosis by ELP-delivered Dox. DIC images of MES-SA cells were collected after treatment for 1 h with each construct (10 μM) at 37 °C or 42 °C (A). Histograms of caspase activation in MES-SA cells were obtained after the 1 h treatment (B). The percentage of cells with active caspases was measured from the histograms and averaged (C). Data represent the average of at least 3 experiments (bars, S.E.M.). ‡Significant difference compared with untreated controls at 37 °C. †Significant difference compared with Tat-ELP1-GFLG-Dox at 37 °C ($p < 0.0001$, one was ANOVA, $p < 0.05$, Scheffe's test).

shown). This reflects the disparate mechanisms by which the two molecules gain cellular entry. Free Dox is sufficiently small and hydrophobic to gain access to the intracellular environment by passive diffusion [32], while Tat-ELP-GFLG-Dox relies on an active transport mechanism for cellular entry [23]. This contrast in cellular uptake mechanisms of free and polymer bound Dox has been observed previously with HPMA delivered Dox [33]. In the case of HPMA–Dox, the endocytic uptake is an advantage, leading to an alternative toxicity mechanism involving lipid peroxidation in cell membranes [29], enhanced tumor activity in vivo [34], lowered systemic toxicity [34], and the ability to overcome multidrug resistance [12].

The data presented here demonstrate that the Tat-ELP-GFLG delivered Dox does not localize to the nucleus. This is in contrast to the free drug, which localizes entirely in the nucleus where it serves as an intercalator and topoisomerase II poison. Similar to HPMA delivered Dox, Tat-ELP-GFLG delivered Dox displayed a cytoplasmic distribution. The cause of this altered distribution may be the endocytic mode of cellular entry used by Tat-ELP-GFLG [23], the failure of Dox to be released from the ELP carrier, or the modifications made to the Dox structure. Interestingly, the Tat-ELP-GFLG-delivered Dox is still cytotoxic regardless of its lack of nuclear localization, suggesting an alternate mechanism of toxicity for free and Tat-ELP-GFLG delivered Dox [21]. Tat-ELP-GFLG-Dox also induced caspase activation more effectively than free Dox in spite of its less potent cytotoxicity, adding additional support to the hypothesis that the two forms of the drug are acting through different mechanisms. It has been shown that HPMA conjugated Dox causes alterations in plasma membrane permeability [30], induces lipid peroxidation [29], and induces both caspase dependent apoptosis [35] and necrosis [32]. Experiments are underway to determine the mechanism of cytotoxicity by Tat-ELP-GFLG delivered Dox.

In the in vitro assay used in this study, free Dox was found to be much more potent than the ELP-delivered Dox. However, the cytotoxicity of the two agents cannot be compared directly because of the different levels of drug in the cells. In fact, a previous study using HPMA delivered Dox found that, when corrected for intracellular drug levels, the cytotoxicity of polymer-delivered Dox was comparable to free Dox [29]. Because of the complexities of drug pharmacokinetics and tissue distribution in vivo, the in vitro potency is a poor predictor of a drug's therapeutic value, especially when comparing a polymer-bound drug to the free drug. HPMA–Dox conjugates have been shown to have in vitro IC_{50}s in the micromolar range [12,30], much higher than the free Dox IC_{50}, but these HPMA–Dox conjugates have shown promise in preliminary clinical evaluations [36]. The IC_{50} values of HPMA–Dox and Tat-ELP-GFLG-Dox are comparable, indicating that a therapeutic concentration of Tat-ELP-GFLG-Dox is achievable.

A previous study by Dreher et al. reported cytotoxicity with ELP delivered Dox in a squamous cell carcinoma line (FaDu) [21]. However, they did not observe enhancement of the cytotoxic effect with hyperthermia. Several differences between the Dreher et al. work and this exist. First, Dreher et al. used an acid-labile hydrazone linker for conjugation of the drug to ELP, which contrasts with the protease-cleavable linker used here. Second, the site of modification of Dox with the reactive moiety was different. Dreher et al. used a multistep synthesis process to first introduce a reactive moiety into ELP, then attached Dox via the C-13 carbonyl group [37]. In this study, doxorubicin was modified by addition of a maleimide group via a linker to the amine nitrogen at C-3′, allowing conjugation to a unique cysteine residue in a single step. Third, Dreher et al. used the squamous cell carcinoma line (FaDu), while in this study, MES-SA uterine sarcoma cells were used. However, it is unlikely that these differences led to the enhancement of cytotoxicity by hyperthermia observed in this study. It is more likely that the thermally induced cytotoxicity reported here is due to the experimental conditions and to modification of ELP by addition of the cell penetrating peptide. In the Dreher et al. study, cells were continuously exposed for 24 h or 72 h to ELP-Dox with hyperthermia applied only during the first hour, which would diminish the influence of hyperthermia. In the study presented here, the cells were exposed to both drug and hyperthermia for only 1 h in order to highlight the influence of hyperthermia treatment. During this time, the hyperthermia causes the protein to aggregate, and the polypeptide aggregates accumulate on the surface of the cell. As a result, there is an increase in the amount of polypeptide bound to the cells and eventually internalized by the cells when compared to soluble polypeptides at the normothermic temperature. The thermal enhancement observed in this study was also aided by the use of the Tat peptide. Some cytotoxicity was observed with the unlabeled Tat-ELP-GFLG polypeptide, which is possibly due to the highly charged polymer's interaction with and disruption of the plasma membrane [38]. The exact mechanism for this toxicity is under further evaluation. However, cell killing by Tat-ELP-GFLG-Dox is not due simply to the toxicity of Tat-ELP-GFLG, as the unlabeled protein was an order of magnitude less potent than the Dox labeled construct and induced no caspase activation in the absence of Dox in MES-SA cell line. The Tat peptide enhanced the cellular delivery of the large protein aggregates, as ELP1-GFLG-Dox only showed a thermal targeting index of 2.85 compared to an index of 19.9 for Tat-ELP1-GFLG-Dox.

This study demonstrates the use of ELP as a thermally targeted drug carrier for the intracellular delivery of doxorubicin. The Dox delivery construct underwent its phase transition at 40 °C, and its internalization by cells was enhanced by both the use of a cell penetrating peptide and by its hyperthermia-induced phase transition. The ELP vector delivered Dox to the cell cytoplasm and killed cells by induction of apoptosis through a caspase dependent mechanism. The cytotoxicity was enhanced 20-fold when treatment was combined with hyperthermia. This work provides initial proof of principle for the use of ELP as a thermally targeted delivery system for doxorubicin in vitro, and it opens the door for the future evaluation of the efficacy of ELP as a potential drug carrier in vivo.

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