Thermal Cycling Enhances the Accumulation of a Temperature-Sensitive Biopolymer in Solid Tumors

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Abstract

The delivery of anticancer therapeutics to solid tumors remains a critical problem in the treatment of cancer. This study reports a new methodology to target a temperature-responsive macromolecular drug carrier, an elastin-like polypeptide (ELP) to solid tumors. Using a dorsal skin fold window chamber model and intravital laser scanning confocal microscopy, we show that the ELP forms micron-sized aggregates that adhere to the tumor vasculature only when tumors are heated to 41.5°C. Upon return to normothermia, the vascular particles dissolve into the plasma, increasing the vascular concentration, which drives more ELPs across the tumor blood vessel and significantly increases its extravascular accumulation. These observations suggested that thermal cycling of tumors would increase the exposure of tumor cells to ELP drug carriers. We investigated this hypothesis in this study by thermally cycling an implanted tumor in nude mice from body temperature to 41.5°C thrice within 1.5 h, and showed the repeated formation of adherent microparticles of ELP in the heated tumor vasculature in each thermal cycle. These results suggest that thermal cycling of tumors can be repeated multiple times to further increase the accumulation of a thermally responsive polymeric drug carrier in solid tumors over a single heat-cool cycle. More broadly, this study shows a new approach—tumor thermal cycling—to exploit stimuli-responsive polymers in vivo to target the tumor vasculature or extravascular compartment with high specificity.

Introduction

The treatment of cancer with anticancer agents is typically limited by toxic side effects in normal tissues. The goal of drug delivery in the treatment of solid tumors is to increase the concentration of an anticancer agent within a tumor while limiting systemic exposure, thereby reducing normal tissue toxicity and increasing overall therapeutic efficacy (1, 2). Numerous drug delivery technologies have been developed to accomplish this goal, including liposomes (3), micelles (4), antibody-directed enzyme prodrug therapy (5), photodynamic therapy (6), affinity targeting (7), and macromolecular drug carriers (8, 9), the class of drug carriers that are the focus of this study.

Macromolecular drug carriers typically consist of high–molecular weight polymers (>10 kDa) that are linked to a therapeutic agent and target solid tumors either “passively,” based on molecular weight and charge, or “actively,” due to a specific affinity or stimulus (9–11). The passive targeting of solid tumors by macromolecular carriers occurs through the enhanced permeability and retention effect (12–15), which is caused by the increased vascular permeability of tumors relative to normal tissues and a slower rate of clearance due to a lack of functional lymphatics (16). Combined, these two features of solid tumors result in the increased accumulation of macromolecules in tumors as compared with low–molecular weight drugs (12, 17). In addition to the enhanced permeability and retention effect, macromolecular drug carriers are an attractive drug delivery vehicle because they have longer plasma half-lives, reduced normal tissue toxicity, activity against multiple drug-resistant cell lines, and greater solubility than free drug (8, 9). These attributes often result in the better efficacy of macromolecular drug carriers as compared with low molecular weight drugs (8, 9).

Recently, stimuli-responsive, “smart” polymers have been investigated for drug and gene delivery because these polymers offer the opportunity to modulate their physicochemical properties within the tumor microenvironment (18–23), and therefore, enhance the delivery of their therapeutic payload within a specific tumor compartment. We are interested in a class of thermally responsive macromolecules called elastin-like polypeptides (ELPs) as drug carriers because ELPs combine the passive targeting afforded by the enhanced permeability and retention effect with active thermal targeting using externally focused hyperthermia (19, 24). ELPs are artificial repetitive polypeptides that undergo an inverse temperature phase transition (also called a lower critical solution temperature transition) (24) that can be repeated multiple times to further increase the accumulation of a thermally responsive polymeric drug carrier in solid tumors over a single heat-cool cycle. More broadly, this study shows a new approach—tumor thermal cycling—to exploit stimuli-responsive polymers in vivo to target the tumor vasculature or extravascular compartment with high specificity.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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focused hyperthermia. Then the tumor temperature is reduced to normal body temperature and the ELP particles dissolve into the plasma. Because the transvascular flux is proportional to the concentration difference across the endothelium, the higher vascular concentration in the tumor selectively drives more ELP into the extravascular compartment where the cancer cells reside. We show that this process can be repeated multiple times to pump more ELP into the extravascular compartment with each thermal cycle. We believe that these results are significant for two reasons: (a) they elucidate the mechanism for tumor accumulation of smart polymers and (b) provide a novel strategy for tumor cell targeting that allows the spatiotemporal distribution of a polymeric drug carrier to be precisely controlled within the tumor microenvironment to enhance its overall accumulation, as compared with passive delivery of the same macromolecule.

Materials and Methods

Synthesis of ELPs. The recombinant synthesis of the ELP drug carriers from a plasmid-borne synthetic gene in Escherichia coli (31), and their purification by inverse transition cycling (ITC; refs. 32, 33) has been described previously. Two different ELPs were used in these studies, and the molecular weight of each ELP was controlled by their sequence (34) and molecular weight (18). ELP1 is a 59.4 kDa polypeptide comprised of 150 pentapeptide repeats (Xaa-Gly-Val-Pro-Gly) and was designed to be the thermally sensitive carrier with a Tt of −30°C by incorporation of Val, Ala, and Gly residues in a 5:2:3 ratio at the guest residue position (Xaa). ELP2 is a 61.1 kDa thermally insensitive control polypeptide comprised of 160 pentapeptide repeats and was designed to have a Tt significantly greater than the hyperthermic tumor temperature of 41.5°C (Tt = −70°C) by incorporation of Val, Ala, and Gly in a 1:8:7 ratio at the guest residue position. Both ELPs also had a short NH2-terminal “leader” (Met-Ser-Lys-Gly-Pro-Gly) and COOH-terminal “tailer” (Tyr-Pro) sequence (31).

Labeling of the ELP with fluorescent reporter molecules. The ELPs were labeled with succinimidyl ester derivatives of rhodamine, Alexa Fluor 488, and Alexa Fluor 546 (Invitrogen). The ELPs have two primary amines, one at the NH2 terminus and one from the Lys residue in the leader sequence, which are reactive sites for conjugation using N-hydroxysuccinimide ester chemistry. In a typical conjugation reaction, a 150 μmol/L solution of ELP was prepared in 100 mmol/L of sodium bicarbonate buffer (pH 8.4) and reacted with ~5-fold molar excess of the N-hydroxysuccinimide derivative of the fluorophore dissolved in DMSO (10 mg/mL) for 1 h at room temperature. The ELP-fluorophore conjugate and free ELP were separated from unreacted fluorophore by ITC (35) as follows: NaCl was added to the reaction mixture to a final concentration of 1.33 mol/L to aggregate the ELP by depressing its Tt below the solution temperature (the Tt of the ELP is dependent on cosolutes; ref. 35). This solution was centrifuged at 16,1 krcf at room temperature for 10 min. The supernatant was discarded and the pellet was suspended in cold PBS, and centrifuged at 16,1 krcf at 4°C for 5 min to remove any insoluble matter. The resulting supernatant was then further purified by size exclusion chromatography with a PD-10 column (Amersham Biosciences) to ensure that all free fluorophore was removed. The purified conjugate was then concentrated by ITC and stored at −20°C in PBS at a concentration of ~500 μmol/L, until further use. The conjugation reactions resulted in final labeling ratios between 0.3 and 1.0 (fluorophore/ELP) depending on the fluorophore.

Cell line. Human squamous cell carcinoma (FaDu) cells were maintained as monolayers in tissue culture flasks containing minimal essential medium supplemented with Earle’s salts, 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B (Gibco). The cultures were grown at 37°C with 5% CO2 in air.

Dorsal skin fold window chamber. All animal experiments were done in accordance with Duke University’s Institutional Animal Care and Use Committee. Nude mice (BALB/c nu/nu) were anesthetized with a cocktail of ketamine and xylazine (10:1 w/w, 100 mg/kg ketamine, i.p.) and prepared for window chamber implantation using common surgical techniques. A 1-cm diameter circular incision was made in the dorsal skin fold, over which a titanium chamber was surgically implanted. A single cell suspension of FaDu cells was injected into the opposing fold of skin (1 × 105 cells in 5 μl of RPMI medium; Gibco). A circular glass coverslip was placed over the incision through which the tumor and its associated vasculature were later visualized. Studies were done 7 to 11 days after the cell injection, when the tumors reached 2 to 3 mm in diameter.

Image acquisition. Nude mice (BALB/c nu/nu) with implanted dorsal fold window chambers were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and positioned laterally recumbent on a custom-designed microscope stage. The tail vein was cannulated for i.v. administration of experimental agents or anesthesia if required. The window chamber was connected to a heating fixture on the microscope stage to control the window chamber temperature and limit translation in x, y, and z (z corresponds to the axial direction). A circulating water bath controlled the heating fixture temperature, which was calibrated to maintain the tumor temperature at a temperature of 41.5°C (hyperthermia) or 37°C (normothermia). Fluorescence images were acquired with a LSM 510 laser scanning confocal microscope (Zeiss).

Data for quantitative analysis were obtained with a two-channel method, in which one channel was used to define the tumor vasculature and the other channel was used to image the distribution of the ELPs in the tumor. These images were acquired with an in-plane resolution of 512 × 512 pixels with a field of view of 460 μm, and an optical slice thickness of 7.1 μm. For each time point, a volume or z-stack was collected from ~100 μm of tumor tissue with a step interval that was half the optical slice thickness. In a typical experiment, 0.5 mg of fluorescein-labeled anionic 2 MDa dextran (Invitrogen) was injected i.v. to identify a representative tumor field and to define the tumor vasculature. A background image was collected and then a rhodamine-labeled ELP was injected i.v. at a dose of 1.0 mg/20 g body weight (target plasma concentration, 15 μmol; n = 4–6). Two-channel multitrack scanning of a z-stack began 20 s after ELP-rhodamine administration. Therefore, the zero time point reported herein corresponds to 2 to 3 min after ELP injection because a volume of data was collected for each time point (20–30 images per time point, 2 to 3 min per volume acquisition, 21 time points, total imaging time was ~65 min).

Images for illustrative purposes were taken with the circulating water bath turned off during imaging as the circulating water in the heating fixture caused slight vibrations, and compromised image clarity. A target plasma concentration of 30 μmol/L (dose = 2.0 mg/20 g body weight) was used for the ELP-Alexa conjugates because Alexa 488 did not depress the Tt of ELP1 as much as rhodamine (18). In general, the illustrative images were taken with a higher in-plane resolution and a slower scan speed to improve image quality.

Image analysis. Three-dimensional vascular masks were created for each time point using the “3D for LSM” program (Zeiss) and a five-step custom image processing algorithm designed to define continuous tumor vasculature from the fluorescein channel data (17). Image analysis was done using custom-designed MATLAB software (MathWorks) on 16-bit TIFF format images. The data were corrected for motion to ensure that the same volume of tumor tissue was consistently analyzed throughout the experiment. Vascular and extravascular fluorescence intensities were determined by subtracting the background intensity, applying the vascular mask to the corresponding data for each time point, and dividing the intensity in each compartment by the initial vascular intensity for each tissue absorption because the same slices (i.e., images at a consistent depth) were analyzed. Data were reported from five slices centered on the tumor vasculature.

The immobile ELP particles were isolated using Otsu’s method (36), which selects a threshold value to minimize the intraclass variance of the positive and nonpositive pixels. The ELP particle mask was combined with the vascular mask to isolate the vascular ELP particles. Particle number, size, area, and intensity were calculated for each image and reported from the same five slices as the tumor compartment analysis. Relative particle
mass was calculated by multiplying the particle's intensity by the particle's area and normalizing by the maximum particle mass for each animal to control for interanimal variability and more clearly identify the kinetics of particle formation.

**Statistical analysis.** The tumor compartment accumulation data were evaluated using a repeated measures ANOVA with treatment group as the factor and a Fisher's partial least-squares difference (PLSD) post hoc test. P < 0.05 were considered significant (Statview, SAS Institute). Data were reported as mean ± SE and each mouse was treated as an individual sample.

**Results and Discussion**

**Thermally responsive ELP accumulated in vascular particles.** To examine the effect of the phase transition of ELP on its accumulation within tumors, a thermally sensitive ELP1-Alexa 488 conjugate (green) and a thermally insensitive ELP2-Alexa 546 conjugate (red) were coinjected into the same mouse. Representative images of the tumor using intravital laser scanning confocal microscopy (ILSCM) are shown in Fig. 1. Initially, the tumor was not heated and the imaging variables were selected such that the fluorescence intensity of ELP1 and ELP2 were roughly equal, producing a yellow color within the lumen of the tumor blood vessels. Then the tumor was heated to 41.5°C, and bright green aggregates were clearly visible within the tumor, indicating that the phase transition of the thermally sensitive ELP1 was triggered in vivo, in agreement with a previous study (24). In contrast, no red particles were observed, which confirmed that ELP2, with a T_c that was much higher than 41.5°C, did not undergo its phase transition in the heated tumor. The aggregates of ELP1 grew in number and size during ~30 min of hyperthermia and then dissolved when the tumor temperature was returned to 37°C, which was below ELP1’s T_c. The more intense, albeit diffuse green color at the end of the experiment suggested that the thermally sensitive ELP1 accumulated in solid tumors to a greater extent than the thermally insensitive ELP2 due to the effect of its phase transition.

A series of high-magnification images were acquired in a separate experiment to investigate the location of these intense ELP1 particles that were formed in a heated tumor. In these experiments, only a thermally responsive ELP1-rhodamine conjugate (red) was injected i.v. along with 2 MDa dextran that was labeled with fluorescein (green) to visualize the tumor vasculature. It is clearly evident in Fig. 2A that the thermally responsive ELP1 particles (red) were adherent to the tumor vasculature (green). In general, the ELP1 particles adhered to the more tortuous segments, branching points, and angiogenic sprouts of the tumor vasculature in which the basement membrane is likely to be exposed. The ELP may adhere to these segments due to nonspecific hydrophobic interactions between the ELP and hydrophobic patches and/or specific interactions with extracellular matrix constituents such as elastin (see Supplemental Fig. S1). The exposure of basement membrane is normally confined to pathologic states such as that found in tumors (37, 38), which may provide another level of tumor specificity, in addition to targeted hyperthermia, for the ELP drug delivery system. It is important to note that the adherent ELP1 particles have a fluorescence intensity that is ~20 times the tumor vascular intensity after ~20 min of hyperthermia (mean, 20.8 ± 3.3; n = 9). This high degree of localization to the tumor vascular endothelium provides a powerful rationale to use stimuli-responsive smart polymers for antivascular therapy.

The distribution of ELP1 and ELP2 after cessation of the hyperthermia treatment is shown in Fig. 2B. A high concentration of ELP1 (green) and ELP2 (red) was still found in the tumor blood vessel lumen even 1 h after administration, and the more intense green color in the extravascular compartment suggests a greater tumor accumulation of the thermally sensitive ELP1 compared with ELP2, which is consistent with Fig. 1. ELP1 was heterogeneously distributed throughout the interstitium and seemed to percolate around cells, indicated by the low–fluorescence intensity round objects, through the extracellular matrix. The endothelial barrier was evident in these images by the lack of fluorescence intensity surrounding the lumen of the tumor blood vessels. This lack of signal intensity ranged from ~0.5 to 5 μm and was consistent with the reported endothelial cell thickness in tumors in which the larger measurement was probably across the nucleus (37).

**ELP extravascular accumulation and vascular kinetics.** We hypothesized that the ELP particles dissolve into the plasma upon cooling, which creates a large, transient concentration spike in the vascular compartment. The increased vascular concentration should create a steeper transvascular concentration gradient that would drive more ELP into the extravascular compartment. This extravascular accumulation would be in addition to the accumulation of ELPs that did not form large aggregates on the tumor vasculature, but instead leaked out into the extravascular compartment during the hyperthermia treatment (see Fig. 1).

![Figure 1](image-url) Images of thermally sensitive ELP1 (green) and thermally insensitive ELP2 (red) in a solid tumor before, during, and after a hyperthermia treatment. ELP1 and ELP2 were i.v. administered (ELP1 and ELP2 were labeled with Alexa 488 and Alexa 546, respectively; dose = 2.0 mg/20 g body weight; target plasma concentration = 30 μmol/L) directly before the first time point. Images are maximum projections in the z-direction of ~50 μm of tumor tissue and imaging variables were selected such that the vascular intensity of ELP1 and ELP2 was balanced producing a yellow color. The tumor was not heated during the first image (0 min).

Subsequently, the tumor was heated to 41.5°C, which is indicated above the image and then cooled to 37°C for 10 min prior to the 40 min image. Bar, 100 μm for all images.
hyperthermia. This enhanced rate of extravasation for ELP1 during the hyperthermia treatment may be due to: (a) an increased residence time in the vasculature, which increases the vascular concentration, (b) a reduction in ELP1’s apparent radius, which increases its vascular permeability, (c) active transport of ELP1 across the endothelium, and (d) additional routes of transvascular transport such as through the endothelial cell membranes, which are accessible to more hydrophobic molecules such as ELP1 above its $T_c$ (41). We cannot rule out that some fraction of this increase may arise from scattering of the fluorescence emission from the vascular ELP1 particles into the extravascular compartment, although we note that in preliminary experiments, it was found that only 0.4% of the vascular signal normally scattered into the extravascular compartment.

Upon cooling, an abrupt increase in the rate of extravasation was observed for the thermally sensitive ELP1 but not for the thermally insensitive ELP2, and led to a 2-fold increase in the degree of extravascular enhancement for ELP1 over ELP2 (i.e., ELP1’s intensity divided by ELP2’s intensity) within 15 min of cooling. The extravascular intensity at 60 min after one cycle of hyperthermia was 2.8- and 1.6-fold greater for ELP1 with hyperthermia than ELP1 under normothermia and ELP2 with hyperthermia, respectively ($P < 0.05$, Fisher's PLSD).

These results can be understood by a simplified form of the Kedem-Katchalsky equation shown below that describes the rate of solute transport ($J_a$) across a tumor blood vessel wall (41, 42).

$$ J_a = P_{app} S A C $$

(A)

In this equation, the apparent permeability ($P_{app}$) is used to reflect that there may be an unknown influence of convective

To test this hypothesis, we quantified the distribution of ELPs in solid tumors using the dorsal skin fold window chamber, ILSCM and a suite of customized image analysis tools. The extravascular accumulation of ELP, shown in Fig. 3, is expressed as a percentage of the initial vascular intensity ($\% V_t - 0$). ELP1 exhibited modest accumulation in the extravascular compartment under normothermic conditions. The thermally insensitive ELP2 with hyperthermia had an enhanced accumulation over ELP1 without heat, most likely due to the physiologic effects of hyperthermia, such as increased perfusion and vascular permeability (39, 40). During the hyperthermia treatment, the thermally sensitive ELP1 initially accumulated in the extravascular compartment at a slightly greater rate than ELP2 (see accumulation data from 0 to 30 min in Fig. 3). These results confirm that the ELP exists in two populations in heated tumors: an immobile fraction that exists as ELP micro-particles that adhere to the tumor vessels, and a freely mobile fraction that escapes into the extravascular compartment during

Figure 2. Images of ELP1 particle localization under hyperthermia (A) and distribution of ELP1 and ELP2 1 h after injection under normothermia (B). A, the tumor was heated to 41.5°C, and rhodamine-labeled ELP1 (red) was i.v. injected (dose = 1.0 mg/20 g body weight; target plasma concentration = 15 μmol/L). After 20 min of hyperthermia, 0.5 mg of fluorescein-labeled 2 MDa dextran (green) was i.v. administered to define the vasculature and a z-stack was acquired. The image is shown as a maximum projection of ~ 5 μm of tumor tissue in the z-direction. B, a single image is shown (optical slice thickness = 1.16 μm) 1 h after i.v. injection of ELP1 (green) and ELP2 (red; ELP1 and ELP2 were labeled with Alexa 488 and Alexa 546, respectively; dose = 2.0 mg/20 g body weight; target plasma concentration = 30 μmol/L). The tumor was heated to 41.5°C for the first 30 min, then cooled to 37°C. The imaging variables were selected such that the vascular intensity of ELP1 and ELP2 was balanced producing a yellow color. Bar, 50 μm.

Figure 3. Extravascular accumulation of ELP without, during, and after a hyperthermia treatment as a function of time. Data were normalized by the initial vascular intensity for each animal and expressed as a percentage of vascular intensity at $t = 0$ min ($\% V_t - 0$). The tumor was not heated for the ELP1 normothermia control (*). The tumor was heated to 41.5°C for the first 45 min and then cooled to 37°C for the remaining 15 min for ELP1 hyperthermia (5) and ELP2 hyperthermia (6). ELP1 hyperthermia was significantly greater than ELP1 normothermia from 3 to 60 min (not shown on graph, $P < 0.05$, Fisher’s PLSD). ELP2 is significantly greater than ELP1 normothermia from 42 to 60 min (not shown on graph, $P < 0.05$, Fisher’s PLSD). * $P < 0.05$, Fisher’s PLSD ELP1 with hyperthermia versus ELP2 with hyperthermia. Points, means; bars, SE (n = 4–6).
transport, \( S \) is the surface area of the endothelium, and \( \Delta C \) is the concentration difference across the vessel wall. According to Eq. A, the increased slope in Fig. 3 must be due to a greater concentration difference across the endothelium, as \( P_{app} \) and \( S \) should be similar between the hyperthermic experimental groups. These results clearly confirm the hypothesis that dissolution of ELP microparticles in the tumor blood vessel drives more ELP across the tumor vascular wall into the extravascular compartment in which the tumor cells reside.

The localization of the ELPs in the vascular compartment was next investigated to prove that the increased transvascular flux and extravascular accumulation of ELP1 was due to the dissolution of the vascular ELP1 particles in the plasma when the tumor was cooled back to normal body temperature after one cycle of hyperthermia. The vascular fluorescence intensity was divided into two groups: (a) immobile vascular ELP1 particles and (b) freely mobile ELP1 as shown in Fig. 4A. The intensity of the freely mobile ELP1 fraction decreased throughout the 45 min of hyperthermia, as would be expected due to renal clearance of soluble ELP (43, 44). In contrast, the relative mass of ELP1 in the vascular particles increased during the hyperthermia treatment due to the nucleation and growth of vascular ELP1 particles (see Fig. 4B). Upon cooling, the ELP1 particles dissolved rapidly, accompanied by an increase of \( \sim 20\% \) in the vascular concentration of mobile ELP1. This increased vascular concentration, in turn, drives more mobile ELP across the endothelial barrier into the extravascular compartment as observed in Fig. 3.

These results clearly suggested that tumor thermal cycling may be a promising method to enhance the accumulation of ELP in the extravascular compartment. The feasibility of multiple tumor thermal cycles was next investigated with ILSCM. The formation of ELP particles within the vasculature through three heat-cool cycles is shown in Fig. 5. These ELP particles formed rapidly and grew in number and size during each round of hyperthermia with an average diameter of 2.5 \( \mu \)m (range, 1–12 \( \mu \)m) at the end of each hyperthermia treatment. The mass of ELP within the particles during the hyperthermia treatment was equal to the initial vascular mass of the soluble ELP prior to the formation of ELP microparticles (mean, 95.6 \( \pm \) 16.3% of the initial vascular mass; range, 35.4–195.6%; \( n = 9 \)). This finding suggests that instantaneous dissolution of the ELP microparticles could potentially double the vascular concentration. Further quantification of the ILSCM images revealed that 83% and 75% of the first cycle’s particle mass formed during the second and third cycles, respectively. The large amount of ELP mass stored in the ELP microparticles in each thermal cycle is an important observation which is relevant for future therapeutic application of this methodology, because it is essential that the vascular ELP particles contain sufficient mass to create a large enough spike in the vascular concentration when the particles dissolve to drive soluble ELP from the vascular compartment into the extravascular space.

**Spatiotemporal control of ELP tumor accumulation and its application to drug delivery.** The spatiotemporal control of the localization of ELP within a tumor as a function of hyperthermia suggests two complementary strategies for their effective utilization with thermal targeting. The first strategy simply exploits the hyperthermia-triggered formation of ELP microparticles that adhere to the tumor vasculature as highly disseminated drug depots within the tumors. The formation of highly disseminated ELP microparticles in the tumor vasculature is potentially useful in two delivery scenarios. In the first scenario, the formation of ELP microparticles provides a powerful rationale for vascular targeting with a single thermal cycle. For example, the ELP could be labeled with a radionuclide that emits short-penetration (55–70 \( \mu \)m) high linear energy transfer \( \alpha \)-particles in order to concentrate the radiation dose to within a few cell diameters of the tumor endothelium, and thereby selectively irradiate the tumor vasculature (45–47) while sparing adjacent tissue. In this strategy, the hyperthermia treatment duration should be sufficiently long to deliver an effective antivascular dose to the tumor endothelium (1–2 h; refs. 45, 48). To our knowledge, this is a new strategy to control the spatiotemporal distribution of a macromolecule in the complex tumor microenvironment.

In a second scenario, the goal is to deliver an ELP-chemotherapeutic conjugate to a tumor cell. Therefore, tumor thermal cycling is likely to be the preferred strategy, as it will concentrate the ELP-drug conjugate within the extravascular compartment in which cancer cells reside by pumping the conjugate out of the tumor vasculature in each heat-cool cycle. During each thermal cycle,
upon heating to 41.5°C, the ELP microparticles that are formed in the tumor vasculature will act as local microdeposits of the polymer-drug conjugate that are disseminated throughout the tumor vasculature. Turning off the hyperthermia treatment will result in the immediate dissolution of these microdeposits, causing a transient increase in the vascular concentration of a freely mobile ELP-chemotherapeutic conjugate, which drives more drugs across the tumor blood vessel wall into the extravascular space. The selective targeting of the tumor cell population in the extravascular compartment by tumor thermal cycling should increase therapeutic efficacy by concentrating the anticancer therapeutic in the tumor while limiting systemic exposure. We note that we do not know of any other macromolecular drug carriers whose transport can be manipulated to selectively increases its tumor vascular concentration after systemic administration.

Finally, macromolecular drug carriers must cross many successive transport barriers within a tumor. Some of these barriers prefer hydrophilic molecules (e.g., interstitial transport) whereas other barriers are more easily breached by hydrophobic molecules (e.g., cell membrane). For example, if the site of therapeutic action is the cancer cell nucleus, then it would be beneficial to have a hydrophilic molecule to cross the endothelial barrier and interstitium, but then a hydrophobic molecule to cross the cell membrane. After entering the cell, transport would be more rapid through the cytosol by reducing the hydrophobic character of the molecule, and then once again, switching it back to a hydrophobic molecule to cross the nuclear envelope. At a molecular level, tumor thermal cycling toggles the ELP between a hydrophilic and hydrophobic state, and the dynamic modulation of the hydration of ELPs may well facilitate its transport across these successive transport barriers at the cellular level. This hypothesis is supported by a previous study, in which we showed that a thermally responsive ELP exhibits an ~2-fold increase in cellular uptake under hyperthermic conditions when compared with a thermally insensitive ELP (30).

**Conclusion.** In summary, we have shown a new methodology, tumor thermal cycling, for the targeted delivery of thermally responsive macromolecular drug carriers to solid tumors. Tumor thermal cycling creates and "embeds" micron-sized aggregates of the polymer-drug conjugate throughout the tumor vasculature by applying heat; turning off heat dissolves the particles, creating a steep transvascular concentration gradient that "pumps" the ELP-drug conjugate out of the vasculature into the tumor extravascular space. This process can be repeated numerous times to further enhance tumor accumulation with each heat-and-cool cycle. Although we have shown this methodology with one class of thermally responsive carriers, we believe that this methodology can be extended to other stimulus-responsive polymers to target the tumor vasculature or extravascular compartment with high spatiotemporal precision.

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