

Lipid–Peptide Vesicle Nanoscale Hybrids for Triggered Drug Release by Mild Hyperthermia *in Vitro* and *in Vivo*

Zahraa S. Al-Ahmady,[†] Wafa' T. Al-Jamal,[†] Jeroen V. Bossche,[†] Tam T. Bui,[‡] Alex F. Drake,[‡] A. James Mason,[‡] and Kostas Kostarelos^{†,*}

[†]Nanomedicine Lab, Centre for Drug Delivery Research, UCL School of Pharmacy, University College London, London WC1N 1AX, United Kingdom and

[‡]Institute of Pharmaceutical Science, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, United Kingdom

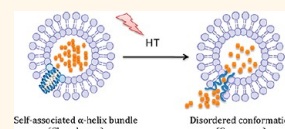
An important limitation in the treatment of cancer with chemotherapeutics is the inability to achieve therapeutically effective local drug concentrations avoiding healthy tissue damage.¹ Since the biological activity of most chemotherapeutic agents is concentration-dependent, specific amounts need to be delivered into cancer cells in order to achieve cytotoxic effects.² Several drug delivery systems have been developed to increase drug accumulation at the tumor site by taking advantage of the enhanced permeability and retention (EPR) effect of their malformed blood vessels³ or by utilizing the binding affinity for tumor-specific receptors.⁴

Liposomes are phospholipid-based delivery systems, clinically used for the transport of chemotherapeutics, and have been approved for various cancer indications. The clinical use of phospholipid-based delivery systems is based on reduced toxicity to normal tissues with a simultaneous increase in tumor accumulation.^{1,5,6} However, accumulation of drug-loaded liposomes in the tumor tissue does not necessarily lead to a higher drug bioavailability. Drug release from both conventional and “stealth” liposomes is a slow process that depends on passive leakage which determines therapeutic activity. This limitation can be overcome by designing responsive liposomes that will release the loaded drug in response to external stimuli.⁵

A promising external trigger for cancer therapy is thermal energy in the form of localized hyperthermia (HT). HT is an effective tool for the treatment of solid tumors either alone or in a combination with drugs or radiation therapy in a process termed thermoablation. Such local heating of the tumor to temperatures exceeding 50 °C for prolonged periods has a direct cytotoxic effect that leads to coagulative necrosis of

ABSTRACT The present study describes leucine zipper peptide–lipid hybrid nanoscale vesicles engineered by self-assembled anchoring of the amphiphilic peptide within the lipid bilayer. These hybrid vesicles

aim to combine the advantages of traditional temperature-sensitive liposomes (TSL) with the dissociative, unfolding properties of a temperature-sensitive peptide to optimize drug release under mild hyperthermia, while improving *in vivo* drug retention. The secondary structure of the peptide and its thermal responsiveness after anchoring onto liposomes were studied with circular dichroism. In addition, the lipid–peptide vesicles (Lp-peptide) showed a reduction in bilayer fluidity at the inner core, as observed with DPH anisotropy studies, while the opposite effect was observed with an ANS probe, indicating peptide interactions with both the headgroup region and the hydrophobic core. A model drug molecule, doxorubicin, was successfully encapsulated in the Lp-peptide vesicles at higher than 90% efficiency following the remote loading, pH-gradient methodology. The release of doxorubicin from Lp-peptide hybrids *in vitro* indicated superior serum stability at physiological temperatures compared to lysolipid-containing temperature-sensitive liposomes (LTSL) without affecting the overall thermo-responsive nature of the vesicles at 42 °C. A similar stabilizing effect was observed *in vivo* after intravenous administration of the Lp-peptide vesicles by measuring ¹⁴C-doxorubicin blood kinetics that also led to increased tumor accumulation after 24 h. We conclude that Lp-peptide hybrid vesicles present a promising new class of TSL that can offer previously unexplored opportunities for the development of clinically relevant mild hyperthermia-triggered therapeutic modalities.



KEYWORDS: temperature-sensitive liposomes · leucine zipper peptide · hyperthermia · doxorubicin · cancer · nanomaterials

the tissues. However, thermoablation is limited due to inhomogeneous heating of the margin areas leaving residual tumor cells.⁷ Besides the direct cytotoxic effect and augmentation/synergism with other chemotherapeutics, HT can be used to improve the efficacy of anticancer agents in two possible ways: (1) enhancing local drug release from thermo-responsive delivery systems,⁸ and (2) increasing drug accumulation to the tumor by increasing local blood flow and endothelial cell permeability.⁵

* Address correspondence to k.kostarelos@ucl.ac.uk.

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Temperature-sensitive liposomes (TSL) were first introduced by Yatvin in the late 1970s⁸ and have been followed by several contemporary types of TSL by exploiting diverse lipid chemistry and lipid phase behavior.^{9–11} Importantly, Needham *et al.* recently described a liposome system that shows an abrupt increase in drug release at the liposome transition temperature (T_m) by incorporating micelle-forming lysolipid molecules into the liposome bilayer. The proposed responsive mechanism suggests that at the T_m the lysolipids allow the stabilization of long-lasting pores at the boundaries between solid and liquid crystalline subdomains in the bilayer leading to rapid drug release.^{12,13} Such ultrafast release properties allow for extensive delivery within the heated tumor vasculature, provided that liposomes are injected just prior to or during hyperthermia application.¹⁴ This intravascular release approach is currently applied for LTSL liposomes and does not rely on the ability of the liposomes to extravasate into the heated tumor area.⁵ Alternatively, long-circulating liposomes can also be used to trigger drug release interstitially. For this approach, the tumor vasculature is first heated to enhance the extravasation into the tumor area, followed by the administration of long-circulating TSL that can extravasate into the tumor area. A second hyperthermia treatment then need to be applied to trigger drug release from the extravasated liposomes that are localized in close proximity to the tumor cells.⁵

Poly- and oligopeptides can be readily produced with a defined sequence and chain length offering better control of transition temperatures compared to general synthetic polymers.¹⁵ MacKay and Chilkoti have proposed that oligopeptides with repeated short sequences (<7 amino acids in length) can generate a highly ordered biopolymer with complex temperature-responsive properties that could provide new tools for engineering hyperthermia-mediated drug delivery systems.¹⁶ From the drug delivery point of view, two important properties of temperature-sensitive peptides should be considered: directionality and reversibility. Directionality usually refers to the self-association and dissociation changes of the peptide in response to heating. Reversibility describes whether or not the peptide secondary structure is retained upon cooling.¹⁶ Elastin-like polypeptides (ELPs) are examples of temperature-responsive peptides that have shown promising results in cancer therapy due to an ability to deposit and switch conformation in the heated tissues and tumors. ELPs have been incorporated into self-assembled nanoparticles encapsulating drug or directly conjugated to drug molecules.¹⁶ Aluri *et al.* and McFarlane *et al.* have described conformationally ordered peptides that can potentially be used in drug delivery systems by trapping therapeutics in assembled particulate drug carriers or in the form of switchable hydrogels to control the drug release at elevated temperature.^{15,17}

In the present work, we have investigated the engineering of previously characterized thermo-responsive liposome systems based on anchoring a temperature-sensitive amphiphilic peptide within their lipid bilayer (Lp-peptide). Our approach aims to combine the traditional temperature-responsive liposome system technology with the dissociative/unfolding properties of a leucine zipper sequence peptide to allow better control, modulation, and timing of drug release under mild hyperthermia while improving *in vivo* drug retention.

We have chosen a leucine zipper with the amino acid sequence [VSSLESKVSSLESKVSKLESKSKLESKVSKLESKVSSLESK]-NH₂ for its interesting ability to dissociate above its melting temperature (~40 °C) into a disordered conformation in the temperature range that is clinically attainable.¹⁶ The peptide presents two or more α -helices self-assembled by wrapping around each other to form a superhelix coiled-coil form of dimers or higher-order aggregates.^{18–20} Figure 1 illustrates the peptide structure. The molecular conformation of leucine zipper peptides and their self-association states have been discussed in several papers, and evidence has been produced indicating the formation of superhelices based upon dimers, trimers, and tetramers. However, the self-associated states are defined in the crystalline state rather than in solution or bound to liposomes.^{21–23} Leucine peptide sequences are characterized by heptad repeats (abcdefg) of 7 amino acids. At appropriate pH and temperature conditions, the naturally unfolded peptide self-associates, adopting an α -helix conformation that exposes the hydrophobic **a** and **d** residues on one side of the helix.¹⁹ Above the melting temperature, dissociation of the coiled-coil structure occurs, leaving disordered peptide monomers.²⁴ The ability to modulate the peptide transition temperature, conformational changes in response to heat, and its potential in the field of drug delivery^{16,20} make leucine zipper peptides an attractive component for the design of temperature-responsive delivery vesicles (Figure 1A).

Our results show that leucine zipper peptides successfully incorporate into lipid bilayers during liposome preparation without affecting the liposome morphology or size characteristics. In addition, Lp-peptide hybrids deliver high levels of doxorubicin (DOX) following localized mild hyperthermia, comparable to lysolipid-containing temperature-sensitive liposomes (LTSL). The leucine zipper peptide appears to be in an unfolded state at the higher temperatures required for drug release. A further (3-fold) increase in tumor accumulation was observed 24 h after HT termination. The engineered Lp-peptide hybrids incorporating a temperature-sensitive peptide provide a new class of clinically relevant thermosensitive liposomes suitable for *in vivo* applications.

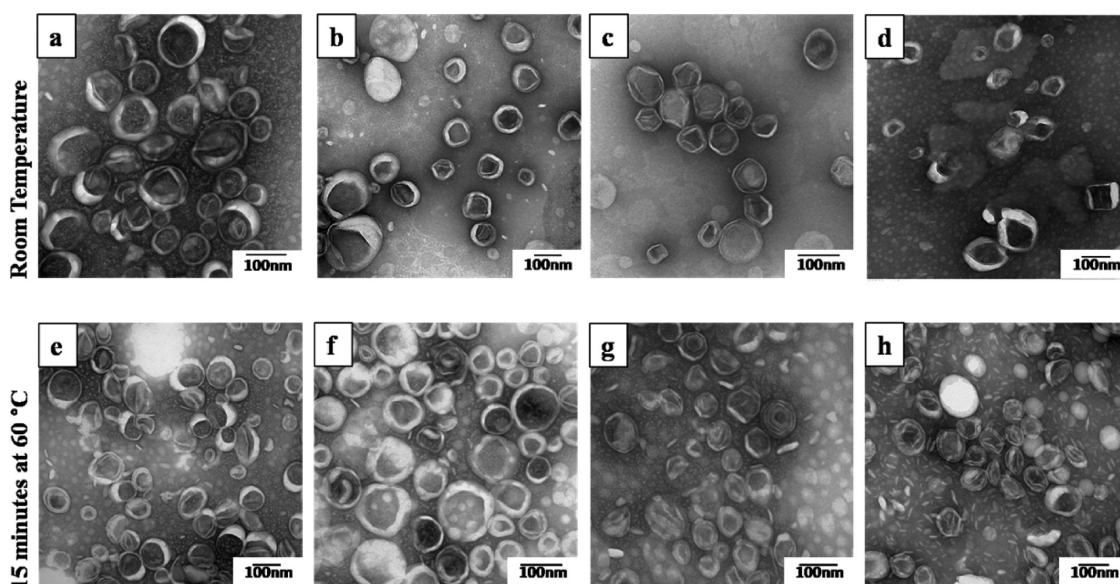


Figure 2. Structural elucidation of Lp-peptide hybrids. Transmission electron microscopy images at room temperature (top) and after 15 min incubation at 60 °C (bottom) of DPPC/DSPC/DSPE-PEG₂₀₀₀ (90:10:5) liposomes (a,e) and Lp-peptide hybrids at 600:1 (b,f), 200:1 (c,g), and 100:1 (d,h) lipid/peptide molar ratios.

The peptide conformation melting temperature (T_m) was determined from the global analysis software associated with the Chirscan spectrometer: $T_m = 46.3 \pm 2.3$ °C was found for the unbound peptide and $T_m = 40.95 \pm 0.1$ °C for Lp-peptide hybrids (Figure 3C,D). Reversibility of the peptide conformation was assessed by measuring CD spectra at 6 °C after cooling (Figure S2A,B). No change in the far-UV CD spectra of the unbound peptide was obtained on rapid cooling of the sample. No recovery of the α -helical structure was observed. Even after 4 h of incubation at 4 °C, a $[\Theta]_M(222 \text{ nm}) \sim -7000 \text{ deg cm}^2 \text{ dmol}^{-1}$ was observed with no further changes over 1 week (data not shown). Clearly, the α -helix conformation of the leucine zipper peptide is not reversible and stabilized by self-association. In contrast, CD spectra of the Lp-peptide hybrids indicated that the α -helix unfolding is reversible giving $[\Theta]_M(222 \text{ nm}) \sim -10\,000 \text{ deg cm}^2 \text{ dmol}^{-1}$ at 6 °C after rapid cooling to 6 °C. Taken together, the CD results confirmed that the leucine zipper peptide was anchored in the vesicle bilayer with an α -helical self-association status that is temperature reversible.

To investigate the effect of peptide anchoring on the phase transition of the lipid bilayer, differential scanning calorimetry (DSC) was used. Table 1 and Figure S3 show the DSC thermograms of the different vesicle systems studied, indicating that the incorporation of different molar ratios of peptide in the lipid bilayer did not substantially affect the phase transition temperature of the liposome (~ 42.5 °C). As expected, the DSC thermogram of LTSL showed a lower transition temperature of about 41.4 °C due to the incorporation of the MSPC lysolipid in the bilayer that led to slightly less ordered phospholipid molecule packing in the gel

phase.¹³ In order to compare the effect of peptide incorporation into the bilayer with that of cholesterol, we investigated vesicles containing cholesterol at a molar ratio of 200:1. Incorporating small amounts of CHOL in the lipid bilayer (200:1 lipid/CHOL molar ratio, equivalent to 0.5 mol %) did not affect the phase transition of the liposomes, which was similar to unmodified liposomes and Lp-peptide hybrids (Table 1 and Figure S3).

In order to obtain a better understanding of the interactions between the lipid bilayer and the anchoring peptide molecules, fluorescence anisotropy measurements were performed. Two different types of membrane-bound probes were used: DPH, the spectrum of which reflects perturbations in the hydrophobic region of the membrane, and ANS, which resides at the headgroup region and can better reflect changes in the lipid–water interfaces.²⁶ The temperature-dependent anisotropy curves of both DPH and ANS (Figure 4) show that both liposomes and Lp-peptide hybrid vesicles exhibited a gradual decrease in anisotropy values with increasing temperature. Around 42 °C, this reduction was dramatic since this was close to the phase transition temperature of the DPPC/DSPC/DSPE-PEG₂₀₀₀ (9:1:0.5) liposomes (~ 42.5 °C).²⁷

When the bilayer fluidity was monitored with DPH spectroscopy (Figure 4A), Lp-peptide hybrids showed higher anisotropic values below T_m , which was not observed with unmodified liposomes and liposomes containing cholesterol (Figure S4A). This indicated that, in the gel phase, DPH probe mobility was constrained by anchoring the peptide in the lipid bilayer. When the ANS probe was studied (Figure 4B), the opposite trend was observed. Lp-peptide hybrid vesicles showed a concentration-dependent reduction in the

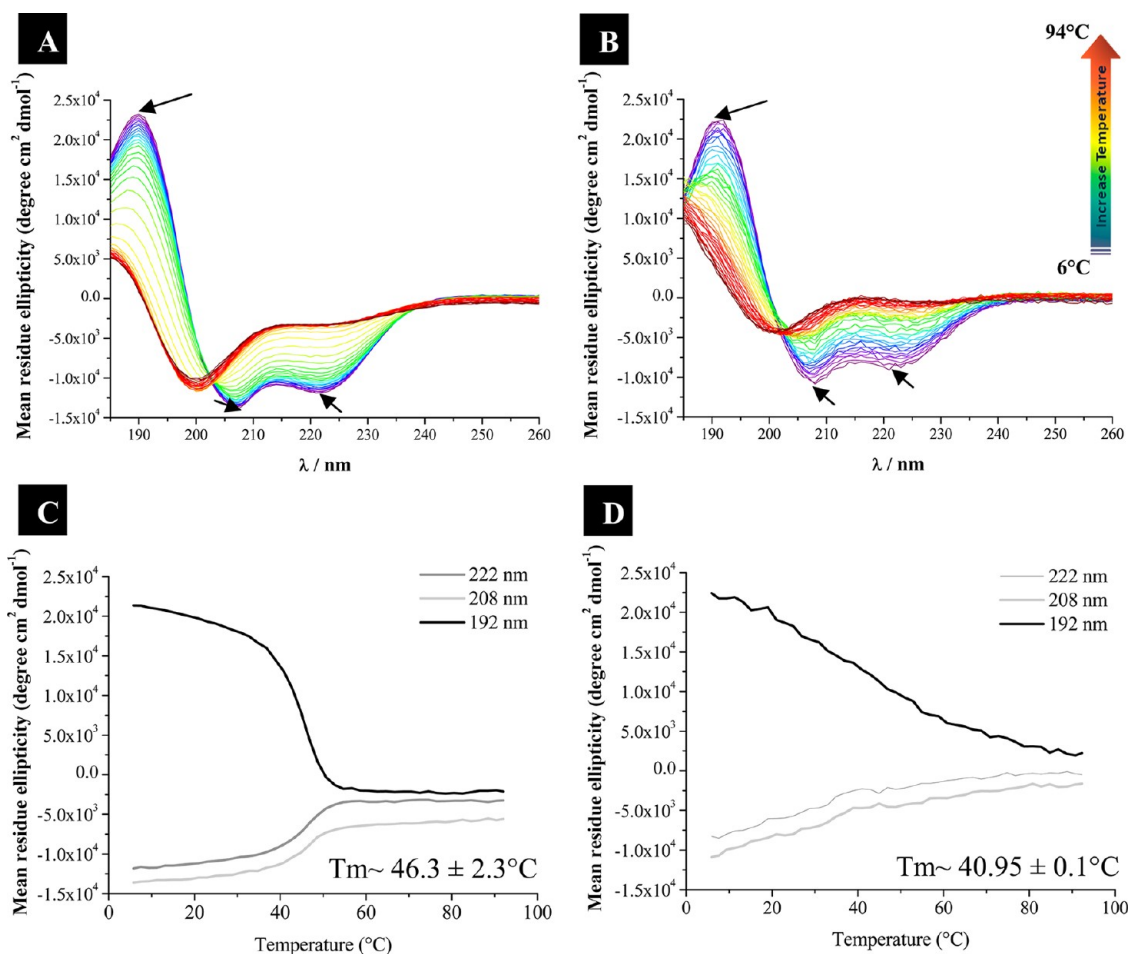


Figure 3. Temperature-dependent conformational changes of Lp-peptide hybrids. Far-UV CD spectra of (A) free leucine zipper peptide and (B) Lp-peptide hybrids (200:1 lipid/peptide). The peptide adopts an α -helix conformation at low temperature and becomes increasingly disordered as the temperature is raised, both in solution and in liposomes. The same color scheme was used for both graphs. Peptide melting temperatures of (C) free leucine zipper peptide and (D) Lp-peptide hybrids (200:1). The mean residue ellipticities ($\text{degree cm}^2 \text{dmol}^{-1}$) at the wavelength values characteristic of an α -helix peptide (222, 208, and 192 nm) were plotted as a function of temperature, and the transition temperature of the peptide was determined with the Applied Photophysics Global 3 analysis software for dynamic multimode spectroscopy.

ANS anisotropy values, indicating an increase in the ANS probe mobility in the presence of peptide at the bilayer interface. In contrast, the control liposome–cholesterol system showed higher anisotropic values below T_m compared with the liposomes and Lp-peptide hybrids (Figure S4B), indicating a bilayer rigidifying effect below the phase transition temperature. Overall, the fluorescence anisotropy studies showed Lp-peptide vesicle reduction in bilayer fluidity at the inner core below T_m (as observed with DPH anisotropy) while increasing the bilayer fluidity at the interface both below and above T_m (ANS anisotropy results). This was thought to indicate that the peptide interacts with both regions of the liposomal membrane rather than attaining only a superficial conformation (Figure 4C). The interaction of the peptide with the liposomal membrane was also studied by solid-state NMR studies using DPPC-d62 as the deuterated reporter lipid (Figure S5). Quadrupole echo spectra obtained for liposomes above the main phase transition revealed that the presence of peptide at

0.5 mol % and, more notably, at 1 mol % increased the DPPC-d62 acyl chain order parameters. The peptide was consequently responsible for a concentration-dependent ordering effect on DPPC lipids in the Lp-peptide hybrid system.

To evaluate the stability of the Lp-peptide hybrid vesicles after peptide anchoring and their temperature response, DOX was encapsulated inside liposomes and Lp-peptide hybrids using the ammonium sulfate gradient method.^{28–30} The incorporation of the peptide did not interfere with DOX loading, and the encapsulation efficiencies were >90% for all of the vesicles studied. Furthermore, the size and polydispersity did not change after DOX encapsulation (Table S1). DOX leakage was then studied over time at 37 °C in 50% CD-1 mouse serum to simulate *in vivo* conditions (Figure 5A,B). During the first hour, a significant improvement in drug retention was observed ($p < 0.05$) with only 10% drug leakage observed from liposomes and all Lp-peptide hybrids compared with over 60%

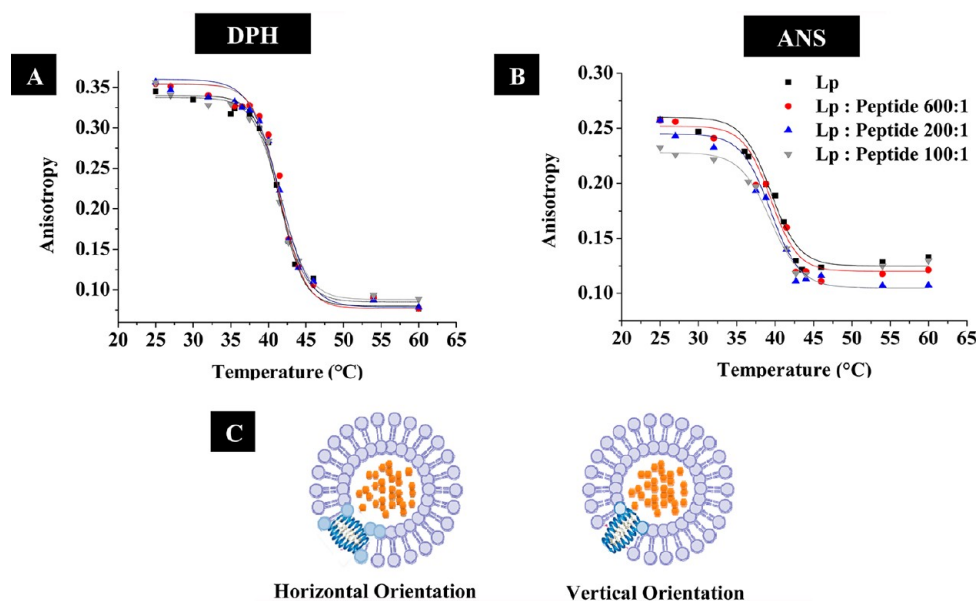


Figure 4. Effect of peptide anchoring on liposome fluidity and lipid packing. The fluorescence anisotropy of (A) DPH and (B) ANS incorporated into DPPC/DSPC/SDPE-PEG₂₀₀₀ (90:10:5) with and without peptide was measured as a function of temperature. (C) Two possible orientations of the self-associated leucine zipper peptides in the liposome bilayer.

release with LTSL and Lp-CHOL (Figure 5A and Figure S4C). The drug leakage profile of DPPC/DSPC/DSPE-PEG₂₀₀₀ liposomes was significantly improved ($p < 0.05$) by anchoring of the peptide in a concentration-dependent manner, with almost 50 and 60% of the encapsulated DOX retained in Lp-peptide hybrids at 200:1 and 100:1 lipid/peptide molar ratios (respectively) over 24 h (Figure 5B).

To study the effect of peptide anchoring on temperature responsiveness of the vesicles, DOX release was studied at 42 °C in 50% serum (Figure 5C). Almost 100% of DOX was released from LTSL in the first minute of incubation. In comparison, the drug release from DPPC/DSPC/DSPE-PEG₂₀₀₀ liposomes increased over time between 80 and 100% after 5 and 30 min, respectively. No significant difference in DOX release was observed between liposomes and Lp-peptide hybrids up to 200:1 lipid/peptide ratio; however, at 100:1 ratio, only 60% of DOX was released from Lp-peptide hybrids over 1 h. Similar to peptide anchoring, the presence of cholesterol did not affect the DOX release at 42 °C (Figure S4D). To evaluate the effect of even higher temperatures, DOX release from plain liposomes and Lp-peptide hybrids was studied by incubation at 45 and 50 °C. No significant differences in the drug release profile were observed compared to those at 42 °C (Figure S6). The drug release results indicated that bilayer anchoring of peptide up to a 200:1 lipid/peptide ratio did not preclude their responsiveness to temperature. Moreover, significant increase of drug retention of the Lp-peptide vesicles under physiological conditions (50% serum and 37 °C) was achieved. These findings highlighted the importance of peptide anchoring in the enhancement of drug

retention at very low molar ratios and in a different way to that of cholesterol-mediated bilayer rigidity. Therefore, Lp-peptide hybrid vesicles (200:1 ratio) were considered a candidate for further *in vivo* investigations.

To determine if the Lp-peptide hybrid vesicle behavior observed *in vitro* could be replicated *in vivo*, a pharmacokinetic study was carried out by loading the Lp-peptide hybrids (200:1 ratio) with ¹⁴C-DOX and measuring the drug level in the blood over time. Longer blood circulation of ¹⁴C-DOX was observed from Lp-peptide hybrids compared to liposomes without peptide. Interestingly, drug retention can be further improved by anchoring another leucine zipper peptide (peptide II) with a different amino acid sequence (VSSLESK)₆ (Figure S7), resulting in a higher overall hydrophobic character and transition temperature (~83 °C). In comparison to LTSL, Lp-peptide hybrids showed 50% blood retention of DOX 1 h after injection compared to only 10% from LTSL (Figure 6A). Last, the Lp-peptide hybrid vesicle pharmacokinetic profiles were established by determining the amount of DOX that accumulated in B16F10 melanoma tumors in C57BL/6 tumor-bearing mice. Tumor-bearing mice were injected intravenously with ¹⁴C-DOX-loaded LTSL and Lp-peptide hybrids (200:1), and localized hyperthermia was immediately applied by a standard water bath-based hyperthermia controlled at 43 °C. ¹⁴C-DOX was quantified in the tumors 1 and 24 h after injection and heat application. No significant difference was observed in the amount of DOX accumulated at the tumor site after 1 h of HT (immediately after vesicles injection) between the two groups tested. Interestingly, however, Lp-peptide hybrids (200:1) showed a 3-fold increase in DOX accumulation in the

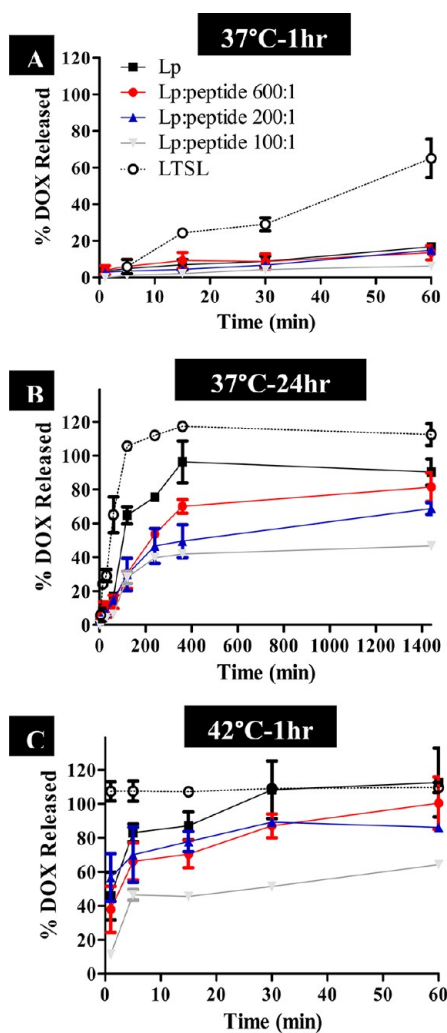


Figure 5. Serum stability and temperature sensitivity of Lp-peptide hybrids. The percentage of DOX release from unmodified liposomes, Lp-peptide hybrids, and LTSL in 50% CD-1 mouse serum at (A) 37 °C over 1 h, (B) 37 °C over 24 h, and (C) 42 °C over 1 h.

heated tumor compared to LTSL 24 h post-HT, and the difference was statistically significant ($p < 0.05$) (Figure 6B). ^{14}C -DOX quantification in the tumor was used to study the total drug tumor accumulation, and further work will be done to look at the bioavailable drug fraction and correlate that to the total drug accumulation in the tumor.

DISCUSSION

The combination of local hyperthermia with drug-loaded liposomes to trigger drug release is an attractive approach to enhance their chemotherapeutic activity to tumors. LTSL is the most developed TSL system today and has shown efficacious results in mice¹² and dogs³¹ and is now under clinical evaluation in humans.⁶ The inclusion of lysolipid results in burst-release kinetics upon heating but also leads to limited *in vivo* stability⁷ that may limit its application to targeting the tumor vasculature only.¹⁴

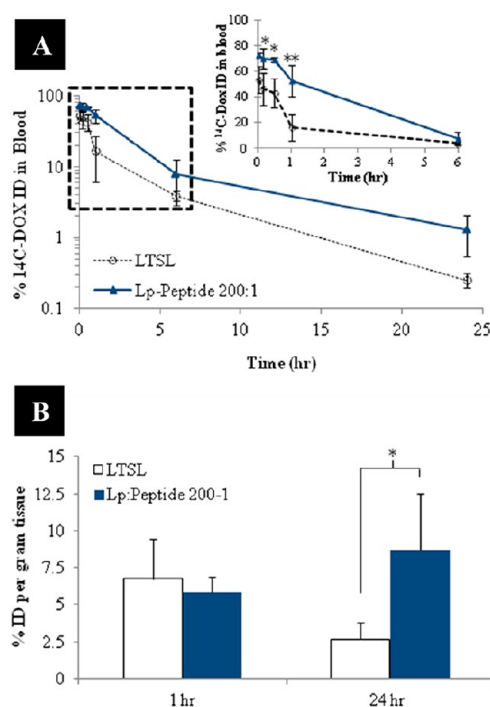


Figure 6. Blood clearance and tumor accumulation of ^{14}C -DOX-loaded LTSL and Lp-peptide hybrids (200:1) in tumor-bearing C57BL/6 mice after intravenous administration. (A) ^{14}C -DOX blood clearance profile after tail vein injection (inset: ^{14}C -DOX blood level up to 6 h). (B) Tumor accumulation of ^{14}C -DOX in B16F10 melanoma tumor at 1 and 24 h post-hyperthermia treatment (HT, 60 min). ^{14}C -Labeled DOX was analyzed in the organs by liquid scintillation counting ($n = 4 \pm \text{SD}$); * indicates $p < 0.05$ for the Lp-peptide system when compared with the LTSL control.

Interest in engineering peptide-modified delivery systems has increased with advances in biotechnology and genetically engineered biomaterials.³² Polypeptides provide multiple cues that can provide biologically specific interactions, environmental responsiveness with opportunities to direct self-assembly, and control over biodegradation. The majority of peptide-conjugated liposomes have been designed in an attempt to enhance therapeutic effectiveness and specificity using peptides as surface ligands to specifically target liposomes to tumors.³³ Examples include RGD^{34,35} and TAT-modified liposomes,³⁶ engineered by covalent linking of the peptide to the liposome surface. Another reason for engineering peptide-modified liposomes is to induce liposomal content release in response to enzymes³⁷ or pH changes as a result of the ability of peptides to aggregate and form pores in the lipid bilayer.^{38,39} The design of such hybrids involves either covalent linking to the liposome surface or peptide encapsulation inside liposomes or simple mixing.^{38,40}

In the present work, we engineered Lp-peptide hybrids by anchoring temperature-sensitive amphiphilic peptides into the lipid bilayer by self-assembly. To our knowledge, such peptide-modified liposomes have not been described previously. The Lp-peptide

hybrids at different peptide ratios maintained almost identical characteristics (mean vesicle diameter, surface charge, phase transition temperature, and morphology). In addition, the anchored temperature-sensitive amphiphilic peptides rigidified the lipid bilayers and improved the liposome serum stability. More importantly, Lp-peptide vesicles consisting of up to 200:1 lipid/peptide molar ratio exhibited high serum stability without significant changes in the overall vesicle temperature sensitivity at 42 °C. Recently, DOX-loaded liposomes covalently modified with the thermosensitive peptide ELP have been described and shown to have enhanced cellular uptake in tumor cells after heating at the transition temperature of the peptide as a result of peptide dehydration at the liposomal surface.⁴¹ Despite enhancing cellular uptake with ELP, its temperature sensitivity was not enough to enhance drug release from the liposomes after heating at 42 °C as these liposomes were not temperature-responsive.⁴¹

Circular dichroism has been shown to be capable of monitoring peptide secondary structure in a variety of environments, including vesicles.⁴² Here, CD revealed that the leucine zipper peptide retained its secondary structure and temperature sensitivity after anchoring into liposomes. However, the CD spectra of the Lp-peptide system as a function of temperature showed a less cooperative change upon melting compared with unbound peptide, which can be explained based on the stabilization effect imparted to the embedded peptide molecules by the adjacent lipid molecules. Interestingly, CD spectra after cooling indicated rapid and completely reversible conformational changes when anchored within the lipid bilayer compared with a slow and incomplete refolding of the unbound peptide.

The incorporation of leucine zipper peptides in liposomes was further studied by DSC and fluorescence anisotropy. No significant overall change in T_m was observed (Table 1) despite previous studies reporting that peptide incorporation in the lipid bilayer can result in increased transition temperature.^{43,44} DPH anisotropy data showed that the incorporation of the peptide in the bilayer may be associated with a decrease in the fluidity of the hydrophobic core at temperatures below the vesicle T_m , in agreement with Sospedra *et al.*²⁶ At the same time, ANS anisotropy measurements indicated increased membrane fluidity in the region of headgroup moieties (that ANS interacts with)⁴⁵ when peptide was incorporated. Therefore, the indications from both anisotropy probes suggested that the peptide interacted with both the hydrophobic and hydrophilic regions of the lipid membrane. However, determination of the exact orientation of the peptide will require further work. Solid-state NMR of chain perdeuterated lipids incorporated in the Lp-peptide hybrid system revealed a dose-dependent ordering effect of the peptide on the DPPC lipid acyl

chains. Overall, our results were in agreement with others showing that peptide incorporation can have a rigidifying effect on the lipid bilayer without affecting their transition temperature.²⁶

Incorporation of peptides within the lipid membrane could “rigidify” the lipid bilayer as has been demonstrated previously by fluorescence anisotropy studies of other peptides interacting with lipid vesicles, such as viscotoxin A3 and laminin.^{26,43,46} However, limited information exists on the impact of such effects on drug release from the vesicles. DOX leakage at 37 °C indicated that the permeability of the liposome bilayer below T_m decreased in serum after peptide incorporation in a concentration-dependent manner, while at higher temperatures (where an ordering effect of the peptide was readily detected), DOX release was substantially reduced when incorporated at 1 mol %. These data are consistent with a tightening effect in phospholipid packing that can improve liposome stability and suggest that an optimal peptide-to-lipid ratio is required to ensure liposome stability does not compromise drug release under hyperthermic conditions.⁴⁷

In order to gain better understanding of the effect of peptide anchoring on lipid bilayer fluidity and the release rate of encapsulated drug molecules, bilayer incorporation of an equivalent molar ratio of cholesterol was studied. Cholesterol is commonly used to modulate the release rate from liposomes and increase their *in vitro* and *in vivo* stability by protecting against serum destabilization effects.^{48,49} It is well-documented that incorporation of small amounts of cholesterol into phosphatidylcholine liposome (less than 20 mol %) decreases T_m by approximately 0.24 °C/mol.⁵⁰ However, no change in T_m was detected when we included 0.5 mol % of cholesterol (Table 1), and at Lp-CHOL (200:1), higher DOX leakage was obtained compared with both unmodified liposomes and Lp-peptide under physiological conditions. The inclusion of the temperature-sensitive peptide at low molar ratios was sufficient to stabilize the lipid bilayer, providing a superior stabilizing effect when compared with cholesterol at the same molar ratio.

Despite the low percentage of the temperature-sensitive peptides incorporated in the lipid bilayer, the Lp-peptide hybrid vesicles showed superior serum stability compared to liposomes of the same lipid composition without the peptide, LTSL, and liposomes containing the same cholesterol content. Our *in vivo* results were consistent with *in vitro* release data. The rate of ¹⁴C-DOX clearance from the blood compartment observed with Lp-peptide 200:1 hybrids was much lower compared with LTSL, indicating higher serum stability. The loss of lysolipid component of LTSL at 37 °C is known to significantly affect drug retention at body temperature and leads to rapid drug clearance from the blood.⁵¹ The improved pharmacokinetics of Lp-peptide vesicles led to significantly increased tumor

accumulation of these DOX-loaded hybrids after 24 h of injection. The higher stability in blood observed for the Lp-peptide hybrids resulted in continuous accumulation of the drug in the tumor even when hyperthermia was terminated, as previously observed with liposomes and nanoparticles.^{52,53}

High level of doxorubicin was delivered to the tumor following localized mild hyperthermia application which was comparable to lysolipid-containing temperature-sensitive liposomes. Besides a further (3-fold) increase in tumor accumulation 24 h after heat application suggested the Lp-peptide hybrids can be suitable for both intravascular and interstitial approach depending on the timing between the liposome administration and hyperthermia application. Further studies are currently taking place to explore this in more detail.

The correlation between the peptide temperature sensitivity, the change in its conformation in response to heating, and the leakage of DOX from the hybrid vesicles is rather complex and not yet fully understood. Although the presence of peptide in the vesicles does not interfere with the thermo-responsive properties of the individual components, the expected enhancement in DOX release after peptide incorporation was not observed. One possible explanation may be that the peptide conformational changes are not

sufficient to enhance the release process. Alternatively, additional rearrangements of the peptide in the bilayer can reduce its cooperative unfolding that was expected to be responsible for the triggering process. We speculate that the thermal responsiveness of Lp-peptide hybrid vesicles can be further optimized by better understanding and reconfiguring the interaction of the peptide within the lipid bilayer.

CONCLUSION

This study illustrated the engineering of self-assembled hybrid vesicles consisting of lipid and amphiphilic peptide molecules. The hybrid Lp-peptide vesicles retained the temperature sensitivity of both the peptide and the liposomes and did not interfere with either the liposome formation or the effective DOX loading. Anchoring of the self-associated α -helical, temperature-sensitive peptides into lipid bilayers significantly enhanced the hybrid vesicle serum stability *in vitro* and *in vivo* without affecting their thermo-responsive character. Improved serum stability also led to higher tumor drug accumulation following hyperthermia in tumor-bearing animals. Lp-peptide hybrid vesicles present a promising new class of TSL that may offer previously unexplored opportunities for the development of clinically relevant, hyperthermia-triggered therapeutic modalities.

MATERIALS AND METHODS

Materials. Leucine zipper peptide was purchased from Peptide Synthetics (Peptide Protein Research Ltd., Hampshire, UK). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (MSPC), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000 (DSPE-PEG₂₀₀₀) were kind gifts from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (CHOL), 8-anilino-1-naphthalenesulfonic acid (ANS), Triton-X-100, chloroform, methanol, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Tris base, hydrogen peroxide solution 30%, and doxorubicin hydrochloride were obtained from Sigma (UK). 1,6-Diphenyl-1,3,5-hexatriene (DPH) (Invitrogen, UK), Soluene-350, and doxorubicin HCL [14-14C] were bought from PerkinElmer (USA), scintillation cocktail high-performance scintisafe gel (Fisher). 1,2-Dipalmitoyl(d62)-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL) was a kind gift from Dr. Richard Harvey. Chemical substances and solvents were used without further purification.

Preparation of Liposomes. Liposomes were prepared by a thin lipid film hydration method followed by extrusion.^{54,55} Briefly, lipids of different types were dissolved in a chloroform/methanol mixture (4:1) in a round-bottom flask. To engineer liposome-peptide hybrids, the respective amount of peptide dissolved in methanol was added to the lipid mixture before formation of the lipid film. After evaporation of the organic solvents, the lipid film was hydrated with 1 mL of HBS (20 mM HEPES, 150 mM NaCl) at 60 °C to achieve a final lipid concentration of 5 mM. Following hydration, small unilamellar liposomes were obtained by extrusion at 60 °C through 800 and 200 nm polycarbonate filters 5 times each followed by 11 times extrusion through 100 nm membranes using a mini-extruder (Avanti Polar Lipids, Alabaster, AL). Liposome size and surface charge were measured by using Zetasizer Nano ZS (Malvern, UK, He-Ne laser).

Visualization by Transmission Electron Microscopy. Fifty microliters of the liposome or liposome-peptide hybrids was added onto a gold grid, and excessive material was removed using a filtered paper. These were stained with 1% aqueous uranyl acetate. Samples were allowed to dry in air and then imaged using a CM120 BioTwin electron microscope (Philips/FEI).

Circular Dichroism Studies. CD thermal scan measurements were performed on a Chirascan spectrometer (Applied Photophysics, Leatherhead, UK) supplied with a thermoelectric temperature control system. Temperature-dependent conformational changes were measured for leucine zipper peptide solutions (20 μ M) in Tris amine buffer (5 mM) and for liposome-peptide hybrids (200:1 lipid/peptide molar ratio, total lipid concentration 4 mM). Measurements were performed in Tris buffer at pH \approx 8.8 as this pH was close to the pH used for DOX loading experiments. CD spectra of the samples were recorded from 260 to 180 nm using a 0.5 mm cuvette at 6 °C before starting the thermal scan. The temperature sensitivity was then tested by increasing the temperature from 6 to 94 °C at 1 °C/min heating rate and 2 °C/step. At the end of the thermal scan, the sample was equilibrated at 94 °C and the CD spectrum was recorded. Once the thermal scan is completed, the samples were cooled to 6 °C and equilibrated for 15 min before recording the CD spectra. Data analysis was performed using Applied Photophysics Chirascan software, and the transition temperatures were determined with Global 3 analysis software for dynamic multimode spectroscopy.

Solid-State NMR. For solid-state NMR, samples with the lipid composition 200:1 and 100:1 mol/mol DPPC-d62/DSPC/DSPE-PEG₂₀₀₀ (90:10:5) with or without peptide were prepared. A total of around 4.2 mg lipids per sample were dissolved and mixed together with the peptide in chloroform/methanol mixture and dried under rotor-evaporation through 100 nm membranes in order to remove all organic solvent, the lipid films were exposed to vacuum overnight. The films were then rehydrated with 4 mL of 250 mM ammonium sulfate (pH 8.5) at 60 °C. Samples were

subjected to five rapid freeze–thaw cycles for further sample homogenization, generating multilamellar vesicles, and then centrifuged at 21 000*g* for 30 min at room temperature. The pellets, containing lipid vesicles and associated peptides, were transferred to Bruker 4 mm MAS rotors for NMR measurements. Lipid vesicles were also prepared in this way in the absence of peptide.⁵⁶

²H quadrupole echo experiments⁵⁷ for samples containing DPPC-d62 were performed at 61.46 MHz on a Bruker Avance 400 NMR spectrometer using a 4 mm MAS probe, spectral width of 100 kHz and with recycle delay, echo delay, acquisition time, and 90° pulse lengths of 0.25 s, 100 μs, 2.6 ms, and 3 μs, respectively. The samples were maintained at different temperatures (41, 42, 43, 45, and 50 °C). During processing, the first 10 points were removed in order to start Fourier transformation at the beginning of the echo. Spectra were zero filled to 1k points, and 50 Hz exponential line broadening was applied. Smoothed deuterium order parameter profiles were obtained from symmetrized and dePaked ²H NMR powder spectra of DPPC-d62 using published procedures^{57–59} Order parameters were averaged along the length of the acyl chain and plotted as a function of temperature.⁵⁶

Differential Scanning Calorimetry Measurements. In order to determine the phase transition temperatures of the liposomes, 20 μL samples of liposome suspension (10 mM) were placed in (T zero hermetic aluminum) pans sealed with lids. Samples were then thermally scanned from 30 to 60 °C at 1 °C/min heating rate using differential scanning calorimetry (Q2000 differential scanning calorimeter, TA Instruments, USA).

Fluorescence Anisotropy Measurements. Liposomes and liposome–peptides were prepared then further diluted to 0.025 mM and divided into two 4 mL aliquots. DPH solution in tetrahydrofuran (0.8 mM, 2.5 μL) or an aqueous ANS solution (10 mM, 4 μL) was mixed with the liposomes at 500:1 lipid/DPH or 25:1 lipid/ANS. To allow the probes to be incorporated, the samples were shaken at room temperature for 2 h then left overnight before starting measurements. Fluorescence polarization was then measured by a LS-50B fluorimeter (PerkinElmer) equipped with automated polarizer and thermostatic cell holder connected to a water bath to control the sample temperature. For the DPH experiment, the anisotropy measurements were carried out at an excitation slit of 10 nm and emission slit of 5 nm and excitation and emission wavelengths of 361 and 425 nm, respectively. ANS anisotropy was measured at excitation and emission slits of 10 and 395 nm and 476 nm excitation and emission wavelengths, respectively. Measurements were started at 25 °C, and then temperature increased gradually up to 60 °C. Sigmoidal curve fitting of the experimental points were performed using Origin software. The samples were equilibrated for at least 6 min after each temperature change. Fluorescence anisotropy was then measured automatically by the fluorimeter based on the following equation.⁶⁰

$$r = \frac{I_{V_V} - GI_{V_H}}{I_{V_V} + 2GI_{V_H}} \quad (1)$$

where *r* is the fluorescence anisotropy and *I_{V_V}*

 and *I_{V_H}* are the emission intensity excited with vertically polarized light and measured with emission polarizer oriented in a parallel or perpendicular direction to the plane of excitation, respectively. *G* is an instrument specific factor calculated to correct the instrument polarization,⁶¹ which is equal to *I_{H_V}* / *I_{H_H}*, and obtained by measuring the vertically and horizontally polarized emission intensities after excitation with horizontally polarized light.

DOX Loading and Release Experiments. For DOX loading, an ammonium sulfate gradient method was used.⁶² Liposomes were hydrated with 250 mM ammonium sulfate (pH 8.5) at 60 °C followed by extrusion and then flushed with N₂ gas and kept in the refrigerator for annealing overnight. Exchanging the external unencapsulated ammonium sulfate was performed by gel filtration through a Sepharose CL-4B column (15 cm × 1.5 cm) (Sigma, UK) equilibrated with HBS, pH 7.4. Doxorubicin hydrochloride (5 mg/mL) was added to the liposome suspensions at 1:20 DOX: Lipids mass ratio in respect to the original total lipid concentration. Subsequently, samples were incubated for 90 min at 37 °C in the case of LTSL and 2 h at 39 °C

for the rest of the formulations. Following the incubation, free non-encapsulated DOX was removed by gel filtration through a Sepharose CL-4B column as described above. DOX encapsulation efficiency (EE) was calculated by comparing the total fluorescence intensity of DOX post- and pre-gel filtration, diluted to the same final lipid concentration,²⁵ %EE = *I*(*t*)_{post-column} / *I*(*t*)_{pre-column} × 100, where *I*(*t*) is the fluorescence intensity of the liposome suspension after adding 2 μL of Triton-X-100 (10% in HBS).

DOX release was measured by taking advantage of the fluorescence quenching process. When doxorubicin is encapsulated inside the liposomes, its concentration is very high, resulting in self-quenching of its fluorescence signal. When the ambient temperature exceeded the liposome transition temperature, DOX was released from the liposomes and its concentration is diluted, resulting in an increase in its fluorescence intensity, which is used to monitor its release. Release experiments were performed over 1 h at different temperatures in 50% CD-1 mouse serum (Sera Laboratories International, UK). For liposome stability studies, DOX release in 50% serum was continued at 37 °C overnight. At different time points, 50 μL samples were withdrawn and further diluted to 200 μL with HBS (pH 7.4) and measured at 480 nm excitation wavelength and 595 nm emission wavelength (slit 15/20 nm) in a quartz cuvette using PerkinElmer luminescence fluorimeter (LS50B). The intensity of the fluorescence signals were then normalized and the % of DOX release was calculated as DOX release % = [*I*(*t*) – *I*(*t*)₀] / [*I*(*t*)₀ – *I*(*t*)₀],⁶³ where *I*(*t*) is the fluorescence intensity of individual samples at different time points, *I*(*t*)₀ is the background fluorescence intensity of liposome samples after purification, and *I*(*t*) is the fluorescence intensity liposome suspension after the addition of 2 μL of 10% Triton-X-100 in HBS (pH 7.4).

Animals and Tumor Models. Five to six week old C57BL/6 mice (15–20 g) were purchased from Harlan (UK Limited, U.K). Animal procedures were performed in compliance with the UK Home Office Code of Practice for the Housing and Care of Animals used in Scientific Procedures. Mice were housed in groups of five with free access to water and kept at temperature of 19–22 °C and relative humidity of 45–65%. Before performing the procedures, animals were acclimatized to the environment for at least 7 days. B16F10 melanoma was established by subcutaneous injection of 2.5 × 10⁵ B16F10 melanoma cells in a volume of 20 μL of PBS into the right lower leg using 26G needles. The tumor volume was estimated by measuring three orthogonal diameters (*a*, *b*, and *c*) with calipers; the volume was calculated as (*a* × *b* × *c*) × 0.5 mm³. The experiments were performed when the tumor volume reached 200–400 mm³.

Blood Circulation and Tumor Accumulation of ¹⁴C-DOX-Loaded Liposomes. In order to check for the stability of the liposomes *in vivo*, Lp-peptide 200:1 and LTSL liposomes were prepared in 25 mM (total lipid concentration) as described earlier and loaded with radiolabeled ¹⁴C DOX (equivalent to 0.2 uCi/dose) as a drug label. C57BL/6 mice (*n* = 4) were anesthetized by inhalation of isoflurane and injected *via* the tail vein with 200 μL of the liposome suspension (equivalent to 2.5 μmol of lipids/200 μL, DOX 5 mg/kg) in HBS. At different time points, the mice were bled by tail vein puncture and 70 μL of blood was collected using a 70 μL heparinized capillary tube. Blood withdrawn did not exceed 10% of the mouse blood volume per day. The mice were killed after 1 h and 24 h by cervical dislocation.⁵⁵ The total radioactivity in the blood was calculated based on the assumption that the total blood volume is accounting 7.3% of the total body weight.⁶⁴

The amount of ¹⁴C-DOX accumulated in the tumor in response to heat treatment was also quantified. Local hyperthermia was applied immediately after injection and maintained for 60 min by immersing the tumor-bearing leg in a water bath stabilized at 43 °C. Animals were anesthetized by inhalation of isoflurane, and the body temperature of the mice was monitored with a rectal thermocouple. A fan and a heating pad were used to maintain the body temperature at 36–37 °C. At 1 and 24 h post-injection, the mice were killed and the tumors were excised. The results were represented as the percentage of the injected dose (%ID) per gram tissue.

Radioactivity Measurements of Blood and Tissues. Radioactivity measurements were carried out as previously described.⁶⁵

Blood and whole tumor samples were transferred to 20 mL scintillation vials and solubilized with 1 mL of Soluene-350 tissue solubilizer (PerkinElmer, UK) and shaken overnight at 55 °C. Samples were decolorized before adding the scintillation cocktail by adding 0.3 mL of 30% H₂O₂ and isopropyl alcohol as an antifoaming agent. Samples were shaken at 55 °C for 1–3 h to expel H₂O₂ before adding the scintillation cocktail. Samples were then mixed with 20 mL of Optiphase “Safe” scintillation cocktail (Fisher Scientific, UK) acidified with 0.7% (v/v) glacial acetic acid to eliminate any chemiluminescence and counted in an LS6500 multipurpose scintillation counter (Beckman, USA).

Statistical Analysis. For testing the statistical significance of the data, Graph Pad Prism software was used. Both two-tailed unpaired student *t* test and one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test were used and *p* values <0.05 considered significant.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Physicochemical characterization of DOX-loaded Lp-peptide hybrids; hydrodynamic diameter and zeta-potential of the DPPC/DSPC/DSPE-PEG₂₀₀₀ liposomes with and without the peptide; thermal reversibility of unbound leucine zipper peptide and Lp-peptide hybrids (200:1); differential scanning calorimetric scan of unmodified DPPC/DSPC/DSPE-PEG₂₀₀₀ liposomes, Lp-peptide hybrids, Lp-CHOL, and LTSL; effect of CHOL on liposome fluidity, lipid packing, and DOX release; solid-state NMR study of leucine zipper temperature-sensitive peptide effect on the average order parameters of the DPPC lipid acyl chains as a function of temperature; temperature sensitivity of Lp-peptide hybrids at 45 and 50 °C; wheel diagram amino acid arrangement of leucine zipper peptide II (VSSLESK)₆, its temperature-dependent conformational changes with and without liposomes and blood profile of ¹⁴C-doxorubicin-loaded liposomes with and without peptide and peptide II in C57BL/6 mice after intravenous administration without hyperthermia. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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