Reduction-Triggered Doxorubicin Delivery by Self-Assembled Nanospheres of Lipoylated Caffeine

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This study reports a new amphiphilic bioconjugate (CAFF-LA) derived from the lipoylation of a hydroxyethyl derivative of caffeine. In water, CAFF-LA self-assembles into nanospheres with an average size of 155 nm, as evidenced from dynamic light scattering and electron microscopy studies. The nanospheres are stable in serum and could be disintegrated upon exposure to the reducing environment of dithiothreitol (DTT; 10 mM) and glutathione (GSH; 10 mM). These nanospheres easily encapsulate the chemotherapy medication, doxorubicin (DOX), and demonstrate an efficacious transport into doxorubicin-resistant cervical cancer (HeLa) cells, wherein a marked induction in apoptosis and significantly lower IC_{50} have been observed when compared to that of free drug. The in vitro assessment of cell viability and hemocompatibility present these nanospheres as potentially safe and efficient intracellular reduction stimulus-responsive drug-delivery vehicles.

Cancer incidence and mortality have become a global issue with continuously increasing and frightening number of new cases every year.[1] Chemotherapy is routinely recommended treatment for cancer, but it can lead to adverse side effects due to damage to healthy cells. Additionally, the emergence of multi drug resistance (MDR) in cancer cells resists the therapeutic effect of cytotoxic chemotherapy.[2] This, in turn, leads to the requirement of high and frequent dose regimen, further worsening the situation of many patients. The drug efflux via ATP-binding cassette (ABC) transporters is a well-known mechanism associated with the development of MDR in cancer.[3] The anti-cancer research community has contributed significantly in past decade which delivers hope to address this issue. The use of MDR inhibitors in chemotherapy offers a potential strategy for overcoming drug resistance. But, the lack of reliable response in clinical development due to their toxicity and drug interaction, limits their practical application.[3,4] The prevention of MDR development by small molecules has also been looked at as strategy to enhance efficacy of chemotherapy treatment.[4b] On the other hand, advances in nanotechnology have greatly influenced the design and development of drug-delivery systems.[5] These nanomedicines aim at improving the therapeutic index of drugs and deriving thereby maximum therapeutic benefits. The relative clinical success of nanomedicine platform fosters further development of safe and efficacious nanomedicines in order to achieve desirable therapeutic response. The stimuli responsive nanosystems have emerged as powerful and potential tools to raise the intracellular accumulation of drugs.[6] These are sensitive to specific endogenous (e.g., pH and redox potential) and/or exogenous (e.g., temperature and light) stimuli that trigger the release of the encapsulated drug.[6] Amongst these stimuli is the exploitation of relatively higher intracellular concentration (~2–10 mM) of glutathione (GSH) to that of extracellular environment (~2–10 μM), which can easily trigger the disorganization of nanocarriers involving disulfide linkages.[6c] In addition, the tumor cells are categorized with elevated levels of GSH which may serve as a gateway to tumor-specific drug delivery.[7] This understanding has been employed in developing various types of reduction responsive nanosystems such as liposomes,[8] polymeric nanoparticles/micelles,[9] niosomes,[10] dendrimers,[11] nanogels,[12] and metal-organic frameworks,[13] to effectively deliver the drug into cell cytoplasm. Scientific efforts are constantly on the rise to develop more biocompatible delivery systems to achieve desirable clinical success.[5b,14] The self-assembling conjugates derived from molecules of biological origin and which can respond to biological stimuli would certainly serve as a basis for developing biocompatible systems for efficacious drug delivery.[10,15] Therefore, we herein have synthesized a biocompatible amphiphilic conjugate from caffeine and lipoic acid that readily self-assembles into nanospheres in an aqueous solution. Caffeine is a globally consumed alkaloid of pharmacological importance with unequivocal antioxidant characteristics.[16] It is found in a variety of plant species including coffee beans, tea leaves and cocoa beans. Interestingly, caffeine has also been reported to possess dose-dependent anticancer activity and also to potentiate the cytotoxic effects of various anticancer drugs against different tumor types including the drug-resistant ones.[17] Moreover, caffeine is reported to inhibit drug efflux from tumor cells and thereby increases their antitumor effects.[17b] On the other hand, other moiety of the synthesized conjugate is universal antiox-

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idant, lipoic acid which possesses remarkable therapeutic potential and serves as an approved ingredient in many pharmaceutical products.[18] The lipoic acid based self-assemblies are prone to disorganization within the reducing environment of cell cytoplasm associated with the cleavage of disulfide linkage of its dithiolane ring which imparts redox responsive behavior to such systems for a favorable drug-delivery application.[10,15c,19] The present study investigates the self-assembly of this lipoylated caffeine bioconjugate for drug-delivery application.

The conjugate (Figure 1) was synthesized by using DCC (*N*,*N*′-dicyclohexyl carbodiimide)-induced coupling between lipoic acid and hydroxyethyl derivative of caffeine **1** as shown in supporting information (Scheme S1) and fully characterized using ^{1}H NMR, ^{13}C NMR, ESI-MS and FTIR. The caffeine derivative **1** mentioned herein was developed by appending hydroxyethyl group to caffeine using cross-dehydrogenative coupling with ethanol following C–H bond activation as reported previously (Scheme S2).[20] The hydroxy functional group on caffeine made in convenient to the coupling reaction with lipoic acid.

The thin-film hydration method was used to look into self-assembling process of CAFF-LA and the sample was analyzed by starting with scanning electron microscopic (SEM) study. It revealed the self-assembled structures to be nanospheres in the size range of 130–200 nm (Figure 2a). Very Interestingly, N-functionalized theophylline based polyelectrolyte also has been reported to produce similar spherical structures.[21] Few of these CAFF-LA nanospheres manifested porous morphology with different size and number of pores on their surface as shown in group image of four individual spheres (Figure 2b). The morphological analysis using transmission electron microscopy (TEM) substantiated the presence of nanospheres of CAFF-LA (Figure 2c). The atomic force microscopic (AFM) imaging further evidenced the spherical nanostructures (Figure 2d). The surface charge of nanospheres was determined by zeta potential measurement and was observed to be 14.8 ± 0.8 mV (Figure S7).

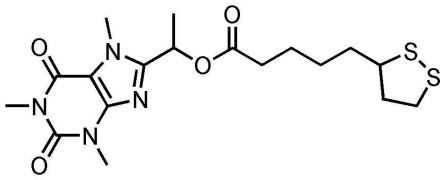

Figure 1. Molecular structure of the lipoylated caffeine derivative.

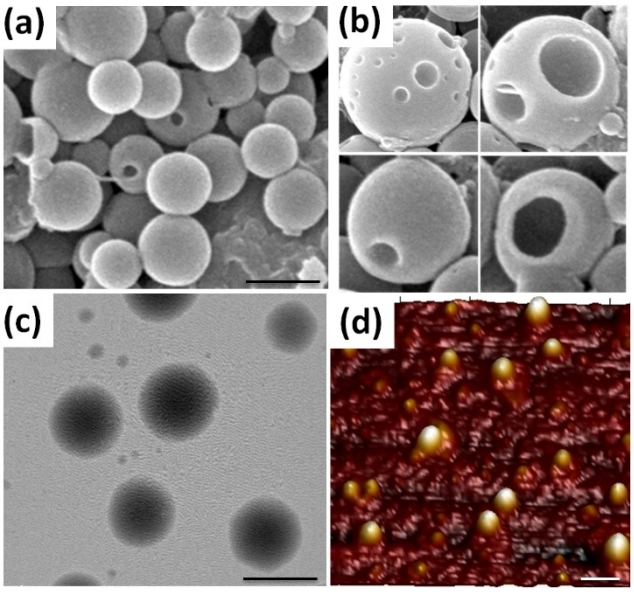

Figure 2. Morphological investigation of CAFF-LA self-assembly (0.50 mM). SEM images of self-assembled nanospheres of CAFF-LA (a) and of a few of those with porous surfaces (b). The CAFF-LA nanospheres as observed under TEM (c) and 3D AFM (d) analysis. Scale bars: 200 nm.

The hydrodynamic diameters of these nanospheres were also determined by means of dynamic light scattering (DLS) measurements which revealed them to be ~155 nm in size (Table 1 and Figure S8a). The low polydispersity index (PDI) values (~0.21) denoted nearly monodisperse population of nanospheres (Table 1).[22] In addition, no significant difference in the size of nanospheres was observed in 0.9% NaCl and 5% glucose solution (Table S1) which remarks on possible practical utility of these nanospheres. Subsequently, the DLS study was further extended to assess the redox sensitive behavior of the CAFF-LA nanospheres in the presence of reducing agent, dithiothreitol (DTT; 10 mM). A significant increase in mean hydrodynamic diameters (~295 nm) and PDI values (~0.7) of CAFF-LA was observed post DTT treatment (2 h) which signified the disorganization of nanospheres resulting from the reduction of disulfides of lipoic acid moieties (Table 1 and Figure S8b).[10,15c,19b,23]

This redox sensitive behavior of nanospheres was further exploited in a drug-delivery endeavor against cancer cells. For which, the well-known anticancer drug, doxorubicin (DOX) was encapsulated in nanospheres and the observed encapsulation efficiency (EE) was 52.23% as determined based on a calibration curve of DOX. These DOX loaded CAFF-LA nanospheres (DOX-CAFF-LA) were then examined for their stability against serum (fetal bovine serum; FBS) treatment. An overnight incubation (~12 h) of DOX-CAFF-LA with FBS revealed substantial retention (>85%) of encapsulated drug (Table 1). This result indicated that these DOX nanospheres do not show any detrimental drug release and may thus show good stability against serum proteins in circulation.[24] In contrast, the DTT (10 mM; 6 h) treatment of DOX-CAFF-LA resulted in nearly 76% release of DOX which evidenced the reducing environment induced disorganization of nanospheres leading to the release of drug.

Table 1. Evaluation of size of CAFF-LA nanospheres (DLS) and DOX release from DOX-CAFF-LA nanospheres.

Size [nm]/PDI		DOX release [%]		
−DTT	+DTT[a]	+DTT[b]	+GSH[c]	+FBS[d]
154.9 ± 4.3/ 0.21 ± 0.01	294.7 ± 11.1/ 0.71 ± 0.07	75.6 ± 4.8	53.1 ± 6.8	13.3 ± 1.5

[a] CAFF-LA + DTT (2 h). [b] DOX-CAFF-LA + DTT (6 h). [c] DOX-CAFF-LA + GSH (6 h). [d] DOX-CAFF-LA + FBS (12 h).

We also evaluated the redox sensitive release of DOX from nanospheres in the presence of biologically relevant redox active molecule, glutathione (GSH; 10 mM) which contributes to the relatively higher reducing environment of cell cytosol.[8,9a,10] The GSH induced ~53% release of DOX within 6 h (Table 1). This information helped in understanding the intracellular reduction responsive behavior of DOX-CAFF-LA. The reduction triggered delivery strategy may be relatively beneficent in order to elicit the specific antitumor therapeutic response as many different tumor types have been shown to possess elevated GSH levels and its association with chemotherapy resistance.[7a]

The intracellular internalization of DOX-CAFF-LA was then studied in MDA-MB-231 (human breast cancer) and HeLa (human cervical cancer) cells in comparison with that of free DOX using confocal microscopy. The free DOX treatment of both MDA-MB-231 and HeLa cells showed efficient internalization and nuclear entry of drug which is because of the well-known fact that free DOX enter cells via diffusion across cell membrane (Figure 3b and d). Interestingly, DOX-CAFF-LA mediated treatments also showed nuclear internalization of the drug against both the cell lines which demonstrated a reduction triggered release of drug (Figure 3a and c). The cellular internalization of DOX was further quantified using flow cytometry which revealed significant internalization of DOX by means of DOX-CAFF-LA treatments, however, remained slightly less than that of free DOX treatment (Figure S9).

We also evaluated the cellular internalization of DOX-CAFF-LA nanospheres against a DOX resistant version of HeLa cells (DR-HeLa). The drug resistance renders anticancer chemotherapeutics ineffective and remains a serious impediment to the achievement of therapeutic benefits in clinical cancer treatment.[2a] The P-glycoprotein (P-gp) and other transporters are overexpressed on cancer cell membranes and are adequately characterized to efflux different anticancer drugs out of the cells which results in chemotherapy failure.[3a] Therefore, we checked out the DOX accumulation inside DR-HeLa cells for the treatment of DOX-CAFF-LA in comparison with that of free DOX. Remarkably, a 24 h incubation of DOX-CAFF-LA with DR-HeLa cells resulted in substantial intracellular accumulation of DOX which was significantly greater than that observed for free DOX treatment as shown in confocal micrographs of Figure 4a and 4b. We then quantified the DOX fluorescence in DR-HeLa cells which showed that it was nearly 2.3 fold higher for DOX-CAFF-LA treatment (Figure S10). This higher intracellular DOX accumulation was further substantiated by flow cytometry analysis wherein cells treated with DOX-CAFF-LA showed relatively large positive shift than those treated with DOX alone as displayed in the histograms (Figure 4c) and thus, significantly higher geometric means of fluorescence intensity (gMFI) were recorded for DOX-CAFF-LA treatments (Figure S11a). The plausible explanation of this phenomenon is that during free DOX treatment, the drug molecules enter the cells through diffusion and become substrate for efflux transporters and are thereby

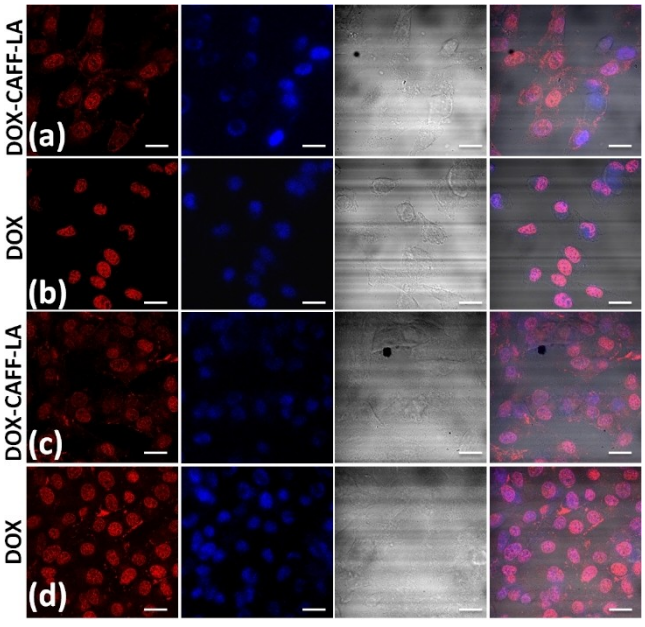

Figure 3. Confocal micrographs depicting cellular internalization of DOX-CAFF-LA (a and c) and free DOX (b and d) in MDA-MB-231 (a and b) and HeLa (c and d) cells at a concentration of 5.0 μg/mL for 4 h. Micrographs (left to right) represent DOX fluorescence (red), DAPI fluorescence (blue, nuclear stain), bright-field image and merged composite (scale bar: 20 μm).

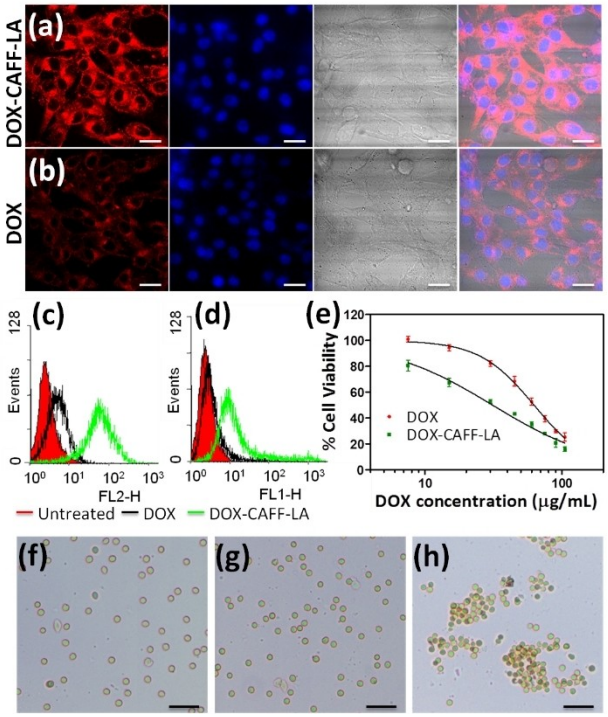

Figure 4. Confocal micrographs depicting cellular internalization of DOX-CAFF-LA (a) and free DOX (b) in DR-HeLa cells (15 μg/mL; 24 h). Micrographs (left to right) represent DOX fluorescence (red), DAPI fluorescence (blue, nuclear stain), bright-field image and merged composite (scale bar: 20 μm). Flow cytometry histograms for comparative c) DOX cellular internalization (10 μg/mL; 4 h) and d) annexin V binding (20 μg/mL; 72 h). e) Cytotoxicity data of DOX-CAFF-LA against DR-HeLa cells in comparison with free DOX (96 h) based on triplicates of three independent experiments (mean ± SEM; $n = 3$). Phase-contrast micrographs of f) untreated (normal saline) red blood cells (RBCs) and g) those treated with CAFF-LA (1.0 mM) and PEI (positive control; 1.0 mg/mL; scale bar: 50 μm).

cleared out. However, unlike free drug, the nanoencapsulated drug enters the cells via endocytosis and thus escapes recognition by these transporters.[25] Upon endocytosis, the drug is released in cytosol under the influence of redox stimulus which results in higher intracellular accumulation of drug. Therefore, such nano based delivery systems are of utmost importance for deriving clinically applicable outcomes in treatments against drug-resistant tumors especially those which lack therapeutic targets such as triple negative breast cancer wherein chemotherapy is a standard clinical practice.[26]

The in vitro cytotoxicity assessment against MDA-MB-231 cells demonstrated that toxicity of DOX-CAFF-LA was nearly comparable to that of free DOX (Figure S12a), whereas against HeLa, it was free DOX which was more cytotoxic than DOX-CAFF-LA (Figure S12b). In contrast to the cytotoxicity noted against drug sensitive cancer cells, significantly enhanced cytotoxicity was observed for DOX-CAFF-LA (IC_{50}: 31.13 μg/mL) treatment against DR-HeLa cells than DOX alone treatment (IC_{50}: 62.52 μg/mL; Figure 4e). The observed cytotoxicity profile was in line with the results of intracellular internalization of drug obtained from flow cytometry and confocal microscopy experimentations. In addition, no notable loss in cell viability was observed for the treatment of blank CAFF-LA nanospheres (Figure S13) which demonstrates promising practical utility of such bioresponsive aggregated nanostructures derived from small biomolecules. The pronounced enhancement in cytotoxicity against DR-HeLa cells by means of DOX-CAFF-LA treatment was also evaluated in terms of quantifying apoptosis induced by DOX.[10,27] The flow cytometric analysis of annexin binding to DR-HeLa cells treated with DOX-CAFF-LA revealed significantly higher number of apoptotic cells whereas no marked apoptosis was seen for free DOX treatments (Figures 4d and S11b). The observation could easily be attributed to rapid and enhanced intracellular accumulation of DOX by the treatment of DOX-CAFF-LA.

Lastly, hemocompatibility of these nanospheres was assessed following their incubation with human red blood cells (RBCs). Any untoward interaction of nanocarriers with RBCs leads to their lysis and the released amount of hemoglobin is quantified by recording UV/Vis spectra at a wavelength of 540 nm which in turn profiles the hemolytic action of nanocarriers.[15c,28] The CAFF-LA treatment did not evidence any detrimental hemolytic effect on RBCs at different concentrations studied (0.1 mM–2.0 mM) as shown in Figure S13. In addition, the visual investigation of RBC aggregation phenomenon post CAFF-LA treatment revealed no evident aggregation of RBCs which was comparable to untreated control and was in contrast to that observed for PEI treated (positive control) cells with clear sign of aggregation (Figure 4f–h). Therefore, it can be stated that these nanospheres which are derived from self-assembly of biological small molecules caffeine and lipoic acid demonstrate a safe interaction with blood cells and could serve as promising drug-delivery vectors for in vivo application.

In conclusion, we herein have attempted to develop biocompatible redox sensitive drug-delivery vector (nanospheres) originating from the self-assembly of an amphiphile derived from coupling of hydroxyethyl derivative of globally consumed antioxidant, caffeine with another powerful antioxidant of pharmacological importance, lipoic acid. The doxorubicin encapsulated nanospheres demonstrate adequate extracellular stability and prompt intracellular release of drug resulting in significant cytotoxic effects, particularly against the drug-resistant cells where drug alone treatment turns out to be ineffective. The hemocompatibility experiments sufficiently convince their safe interaction with blood cells which is suggestive of the practical utility of such systems in drug delivery.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Bioconjugate · caffeine · drug delivery · lipoic acid · self-assembly

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Taken in by caffeine: A lipoylated caffeine (CAFF-LA) derivative self-assembles in water into nanospheres that can be used as biocompatible vectors for the cellular delivery of doxorubicin (DOX). The DOX-loaded nanospheres (DOX-CAFF-LA) are redox-responsive in nature and show significantly higher intracellular accumulation of DOX against drug-resistant cancer cells than free DOX