Selective attachment and release of a chemotherapeutic agent from the interior of a protein cage architecture†

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The antitumor agent doxorubicin was covalently bound and selectively released in a pH dependent manner from the interior surface of a genetically modified small heat shock protein (Hsp) cage.

Nature provides many nanometer scale protein cage architectures, including viral capsids, ferritins, and heat shock proteins. These cages are comprised of multiple subunits assembled into precisely defined symmetric structures. Protein cage architectures provide three surfaces (interior, exterior and subunit interfaces) to which one can impart function by design. Atomic resolution structural information, in conjunction with genetic and chemical modification, allow the rational incorporation of small molecules, peptides or metallic particles at precise locations on the protein cage.[1–6] Protein cages have been chemically derivatized and utilized as spatially constrained reaction vessels for materials synthesis, thus establishing their ability to serve as chemical building blocks.[1–3,7–10]

The objective of this work is to use a protein cage architecture for controlled encapsulation and release of a chemotherapeutic agent. Other types of nanometer scale drug delivery systems exist including micelles, silica nanoparticles, liposomes, hydrogel dextran nanoparticles, and antibody or peptide targeted therapeutics.[11–14] Protein cages offer potential advantages including the ability to incorporate peptides and/or small molecules at precise locations on the cage in a spatially selective manner. Their interior cavities potentially allow them to encapsulate a large amount of therapeutic agent. Additionally, the bioavailability of cargo housed on the interior can be limited until release is selectively triggered by external stimuli.

We demonstrate the ability to house and selectively release the antitumor agent doxorubicin (adriamycin) from the interior of a small heat shock protein (Hsp) cage from the hyper-thermophilic archeaon, *Methanococcus jannaschii* (Fig. 1). This cage assembles into a 24 subunit spherical architecture with 4:3:2 symmetry.[15,16] The assembled protein cage is 12 nm in diameter and has large (3 nm diameter) pores at the three-fold axes allowing free exchange between the interior and bulk solution.[15,16] The Hsp cage is a robust platform for chemical modification, stable up to ~70 °C and in a pH range of 4–11.[1,17] The introduction of unique cysteine residues on its interior surface provides reactive thiol groups for spatially selective attachment of small molecules (HspG41C).[1] In previous work, we showed that small molecules could be selectively bound to the interior and exterior surfaces of the Hsp cage. Full occupancy of the 24 interior thiols was achieved.[1]

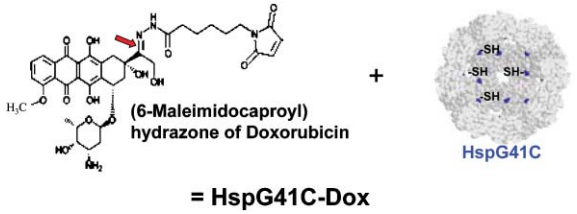

Fig. 1 Reaction schematic: the (6-maleimidocaproyl) hydrazone of doxorubicin[18] was attached to the 12 nm diameter heat shock protein cage. The (Hsp) cage was genetically modified to have 24 interiorly exposed cysteines (blue) (space filling model – interior view). At pH below 5.0 acid hydrolysis results in the release of bioactive doxorubicin (arrow).

The (6-maleimidocaproyl) hydrazone derivative of doxorubicin[18] (Mal-Dox) was linked to the interior surface of the HspG41C protein cage *via* coupling of the maleimide and thiol functionalities (Fig. 1). HspG41C cages (2.5 mg mL^{-1}, 151.5 μM subunit) were reacted with an excess (3 molar equivalents) of Mal-Dox (454.5 μM) in HEPES (100 mM, pH 6.5) for 1 hour at room temperature. Immediately following the reaction, derivatized cages were separated from free Mal-Dox by size exclusion chromatography (Superose 6, Amersham-Pharmacia) (Fig. 2(A)). Co-elution of HspG41C protein cages (280 nm) and doxorubicin (495 nm) after 21 min indicates that doxorubicin is associated with the protein cages. Both transmission electron microscopy (TEM) (Leo 912 AB) and dynamic light scattering (Brookhaven 90 Plus) analysis confirm that HspG41C-Dox maintains the 12 nm diameter of underivatized Hsp cage (Figs. 2(B) and S1 (ESI†)).

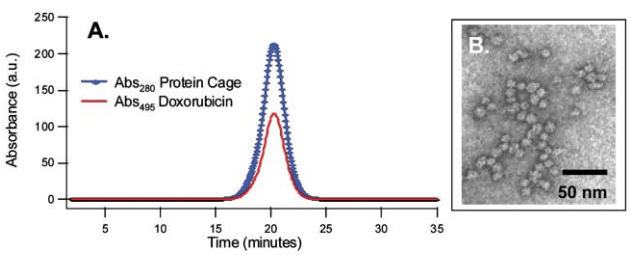

Fig. 2 (A) Size exclusion chromatography elution profile of HspG41C reacted with a 3-fold (3×) molar excess of (6-maleimidocaproyl) hydrazone of doxorubicin per subunit. The profile illustrates the co-elution of doxorubicin (Abs 495 nm) and Hsp cage (Abs 280 nm). (B) TEM of HspG41C cages containing doxorubicin (stained).

† Electronic supplementary information (ESI) available: Fig. S1: Dynamic light scattering data. See http://www.rsc.org/suppdata/cc/b4/b413435d/

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The covalent linkage of doxorubicin to HspG41C is demonstrated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Fluorescence imaging of an SDS-PAGE gel reveals a mobility shift of doxorubicin (excitation 488 nm; emission 520 nm) bound to HspG41C compared to that of free doxorubicin (Fig. 3). Subsequent staining of the protein bands with Coomassie blue illustrates the co-migration of the HspG41C subunit band with the fluorescent (doxorubicin) band indicating their covalent linkage (Fig. 3(A), (B): lane 1). The HspG41C-Dox conjugate migrates slightly slower than underivatized HspG41C due to its increased molecular weight (Fig. 3(A), (B): lanes 1 and 2).

Covalent attachment of doxorubicin to all 24 HspG41C subunits within the cage was confirmed by liquid chromatography/electrospray mass spectrometry (LC/MS) analysis (Esquire 3000, Bruker). HspG41C-Dox (10 μl, 0.5 mg mL^{-1}) was injected onto a C8 column and eluted with a H_2O–acetonitrile gradient; both solvents contained 2% acetic acid at pH 2.3. Analysis of the single LC peak by electrospray mass spectrometry detected two protein components (Fig. 4). Deconvolution of the electrospray mass spectrum detected both the complete *HspG41C subunit + linker + doxorubicin* (experimental mass 17 251; calculated mass 17 249) and *HspG41C subunit + linker* without the doxorubicin (experimental mass 16 725; calculated mass 16 724). This result is consistent with partial acid hydrolysis of the hydrazone linkage at pH below 5.0. The detection of only two protein components, both at similar concentrations, is indicative of complete labelling of HspG41C with Mal-Dox; no unlabeled HspG41C was detected (Fig. 4).

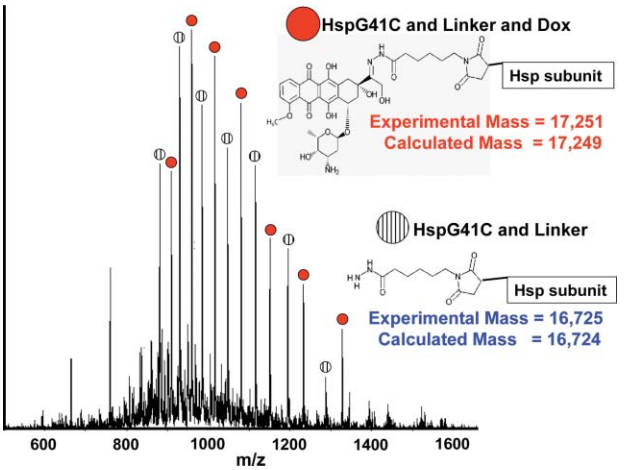

Fig. 4 Deconvolution of the above electrospray mass spectrum illustrates the presence of two protein components: the complete *HspG41C subunit + linker + doxorubicin* (red) (experimental mass 17 251; calculated mass 17 249) and *HspG41C subunit + linker* (blue) without the doxorubicin moiety (experimental mass 16 725; calculated mass 16 724).

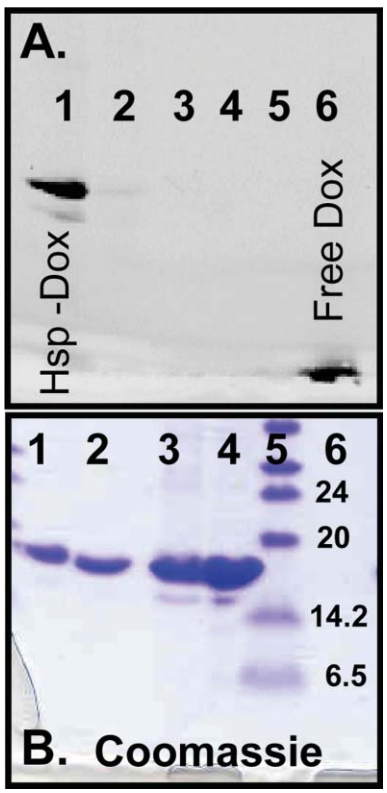

Fig. 3 SDS-PAGE of doxorubicin (Dox) linked to HspG41C imaged by fluorescence (A) and the same gel Coomassie stained (B). Lanes: 1. HspG41C-Dox; 2. WtHsp-Dox; 3. HspG41C; 4. WtHsp; 5. Molecular weight standards (kDa); 6. Dox. Analysis of HspG41C reacted with the (6-maleimidocaproyl) hydrazone of dox illustrates covalent linkage of dox to the protein subunit (A, B: lane 1) whereas after identical reaction conditions WtHsp does not show labeling (A, B: lane 2). The gel illustrates the shift in mobility of dox when linked to HspG41C subunit (compare A: lanes 6 and 1) as well as a shift in mobility of HspG41C-Dox (B: lane 1) compared to unlabeled HspG41C (B: lane 3). The lower band visible in lanes A. 1 and B. 1, 3, 4 was confirmed by mass spectrometry to be an Hsp degradation product still containing position 41.

Quantitative analysis by absorbance spectroscopy also indicates that 24 doxorubicin molecules are linked to the 24-subunit HspG41C cage. The concentration of HspG41C protein in purified doxorubicin derivatized cage preparations was determined by subtracting a normalized doxorubicin spectrum from the labelled Hsp spectrum (Hsp: $\varepsilon = 9322\ M^{-1}\ cm^{-1}$).[16] The concentration of doxorubicin covalently attached to HspG41C was determined from the absorbance maxima at 495 nm (Mal-Dox: $\varepsilon = 8030\ M^{-1}cm^{-1}$).[19] Control reactions utilizing the wild type (wt) Hsp cage, lacking thiols, did not show appreciable labelling with Mal-Dox (Fig. 3(A): lane 2).

We quantified the selective release of doxorubicin from HspG41C protein cages through hydrolysis of the hydrazone linkage under acidic conditions.[18,19] Doxorubicin release studies were performed at pH 4.0, 4.5, and 5.0 (37 °C). After incubation for times ranging from 0.25 to 24 h, doxorubicin labelled HspG41C was separated from free doxorubicin by chromatography (Micro Bio-Spin P30 Columns, Bio-Rad). The amount of doxorubicin that remained associated with the HspG41C protein was quantified by absorbance spectroscopy. As shown in Fig. 5, 50% of the doxorubicin bound to the interior of HspG41C was released after 1.5, 3.9 and 5.1 h at pH 4.0, 4.5 and 5.0, respectively. Acid triggered release of doxorubicin from other systems has been shown effective both *in vivo* and *in vitro*.[13,19] Our data shows the potential for doxorubicin release from Hsp cages under biologically relevant (lysosomal) conditions.

We have demonstrated the ability to load, quantify, and selectively release doxorubicin from a heat shock protein cage. Housing a therapeutic agent within the interior of the Hsp cage

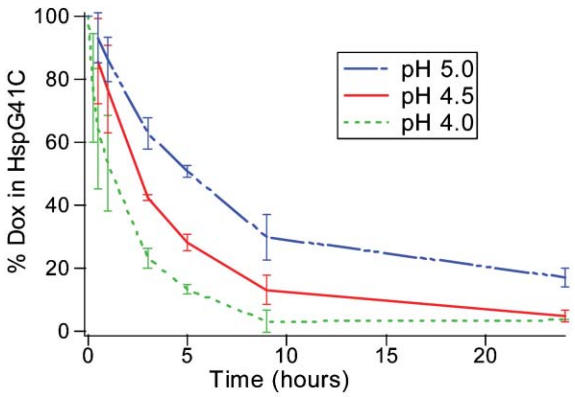

Fig. 5 Acid triggered release of doxorubicin from HspG41C cages: 50% of the doxorubicin bound to the interior of HspG41C cages was released after 1.5, 3.9 and 5.1 h at pH 4.0, 4.5 and 5.0, respectively.

restricts its bioavailability until release. These results illustrate the potential of protein cage architectures to serve as versatile drug delivery systems.

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