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A nanocarrier based on a genetically engineered protein cage to deliver doxorubicin to human hepatocellular carcinoma cells†

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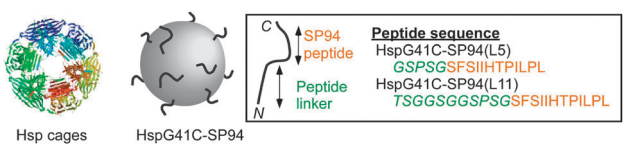
Herein, we report the preparation of genetically engineered protein cages (HspG41C-SP94), taken up selectively by human hepatocellular carcinoma (HCC) cells. An engineered protein cage–doxorubicin (DOX) conjugate was as cytotoxic as free DOX against HCC cells but much less cytotoxic against normal hepatocytes.

Drug delivery systems using drug carriers such as liposomes,[1] polymers,[2] micelles,[3] and proteins[4] have been extensively studied as a means to improve therapeutic effects and reduce undesired side effects of anticancer agents, which exhibit no selectivity toward cancer cells. Therefore, the specific delivery of therapeutic agents to targeted cells is a fundamental issue, and modification of drug carriers with peptide ligands that bind to specific receptors of targeted cells is one of the most attractive strategies.[5] However, conjugation of peptide ligands to the surface of drug carriers, especially polymeric drug carriers, through covalent bonds requires complicated multi-step chemical reactions. In contrast, naturally occurring proteins can be modified with peptide ligands much more easily using a genetic modification strategy.[6] In addition, naturally occurring proteins can have many advantages such as biodegradability, low toxicity, and ready availability.[4,6]

Here, we focused on a naturally occurring small heat shock protein (Hsp 16.5) produced by *Methanococcus jannaschii*. Hsp 16.5 forms a ''cage'' structure with an outer diameter of 12 nm and an inner diameter of 6.5 nm through self-organization of 24 subunit proteins.[7] The cage possesses eight pores with a diameter of 3 nm that link the interior and exterior environments,[7] enabling encapsulation of drugs and imaging agents in the cage's interior.[6,8] Furthermore, the presence of exposed C-terminal regions on the outer surface of the Hsp cage makes it easy to introduce peptide ligands through a genetic engineering approach.[7] Despite the cage's unique and well-defined structure, biocompatibility, and easy fabrication by a genetic methodology, its lack of cell specificity makes it difficult to use as a drug carrier. We have recently reported the preparation of Hsp cages with SP94 peptide ligands[9] bound to their surface *via* polyethylene glycol (PEG) linkers. These Hsp cages were taken up by human hepatocellular carcinoma (HCC) cells but not by other types of cells such as normal hepatocytes.[10] However, it was difficult to control the densities of SP94 peptides and PEG on the cage surface because of the required multi-step chemical reactions.

Herein we describe the simple preparation of a novel HCC-targetable Hsp cage (HspG41C-SP94) by a genetic engineering approach involving the addition of SP94 peptides to C-terminal regions exposed on the outer surface of the cage (Scheme 1). One of the advantages of a genetic engineering approach is the much easier acquisition of completely identical structures and chemical compositions (*e.g.* the number of peptide ligands). To assess the cell specificity of the engineered Hsp cages, we prepared fluorophore-labeled HspG41C-SP94 cages and investigated uptake by five cell types, including HCC cells and normal hepatocytes, using fluorescence microscopy.



Scheme 1 Schematic illustration of HCC-targeted DOX delivery using genetically engineered Hsp cages. Additional peptide sequences are genetically introduced at the surface of cages. SP94 (HCC-binding peptide): SFSIIHTPILPL; linker peptide: GSPSG or TSGGSGGSPSG.

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Furthermore, doxorubicin (DOX),[11] a well-known anticancer agent, was conjugated to the interior of HspG41C-SP94 cages (HspG41C-SP94–DOX). Cytotoxic effects of HspG41C-SP94–DOX, non-targeted HspG41C–DOX, and free DOX were compared using HCC cells and normal hepatocytes. The results suggest that this new HspG41C-SP94 cage will be a useful nanocarrier for targeted drug delivery to HCC cells and for dramatic reduction of undesired side effects toward normal hepatocytes.

Three types of engineered Hsp cages, including two HCC-targetable Hsp cages modified with SP94 peptides *via* linkers of different lengths (*i.e.* HspG41C-SP94(L5) and HspG41C-SP94(L11)) and a control Hsp cage (HspG41C), were prepared using an *E. coli* protein expression system (Scheme 1; see ESI† for amino acid sequences). To enable linkage of fluorophores and drugs, Gly41, located at the interior of native Hsp cages, was substituted with Cys through genetic modification. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 1(A)) and size-exclusion chromatography (SEC) analysis (Fig. S1, ESI†) showed that all cages were successfully obtained with sufficient purity. Also, all Hsp cages had a mean diameter of 10–20 nm, which was consistent with previous results,[7] showing that all genetically engineered Hsp formed cage structures (Fig. 1(B)).

For cellular uptake experiments, Hsp cages labeled with a fluorophore (Alexa488) were prepared by the Michael addition reaction of Alexa Fluor 488 C5 maleimide (Invitrogen) and Cys41 (interior of Hsp cages). Successful fluorophore modifications were confirmed by SDS-PAGE analysis (Fig. S2, ESI†). To determine whether HspG41C-SP94 cages were selectively taken up by HCC cells, the three types of fluorophore-modified Hsp cages were transfected to human HCC cells (Huh-7, HepG2, and Hep3B), normal rat hepatocytes (RLN-8), and human cervical carcinoma (HeLa) cells, and cellular uptake was observed 24 h later using fluorescence microscopy (Fig. 2(A) and Fig. S3, ESI†). Non-targeted HspG41C cages were taken up by all cell lines. On the other hand, both types of HspG41C-SP94 cages were taken up by HCC cells but not by RLN-8 cells or HeLa cells. The two types of HspG41C-SP94 cages showed comparable levels of uptake by Huh-7 cells and HepG2 cells. However, in the case of Hep3B cells, uptake of HspG41C-SP94(L11) cages was greater than uptake of HspG41C-SP94(L5) cages (Fig. S3, ESI†). Therefore, HspG41C-SP94(L11) cages were investigated further in subsequent experiments. Cellular uptake of HspG41C-SP94(L11) cages decreased 2- to 3-fold when HCC cells were pretreated with a 100-fold excess of SP94 peptides (Fig. 2(B) and (C)), showing that HspG41C-SP94(L11) cages were taken up by HCC cells expressing specific receptors for SP94. However, these receptors have not yet been identified. Also, both HspG41C-SP94(L11) and HspG41C cages taken up were localized in acidic organelles even at 48 h post-transfection (Fig. S4, ESI†). Moreover, HspG41C-SP94(L11) had no cytotoxic effect towards RLN-8 or Huh-7 cells (Fig. S5, ESI†). These characteristics (HCC targetability and biocompatibility) are important for the application of HspG41C-SP94 cages as nanocarriers for targeted drug delivery to HCC cells.

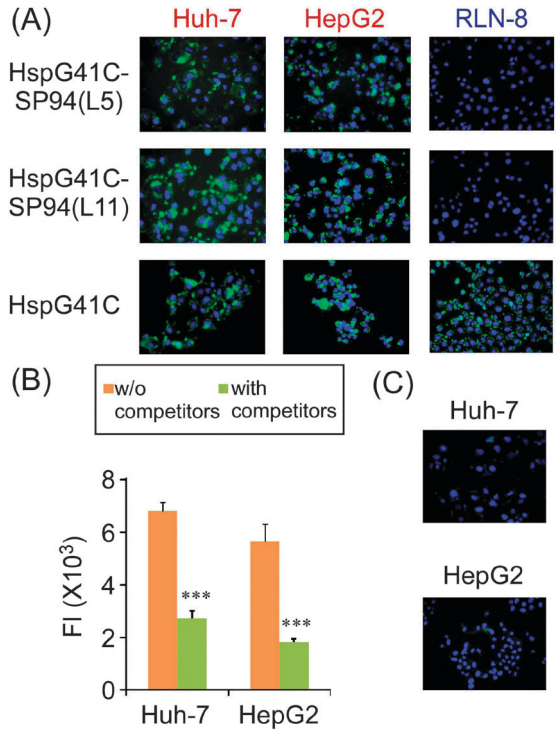

Fig. 2 (A) Fluorescence images of cellular uptake of Hsp cages. Green and blue represent Hsp cages and nucleus, respectively. (B and C) Uptake inhibition assay of fluorophore-labeled HspG41C-SP94(L11) cages with excess unlabeled HspG41C-SP94(L11). Data are means ± SEM of three independent experiments. ***, $p < 0.001$.

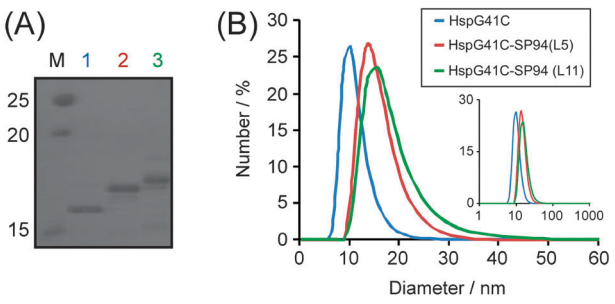

Fig. 1 (A) SDS-PAGE analysis of the Hsp cages. Lanes 1, 2, and 3 show HspG41C, HspG41C-SP94(L5), and HspG41C-SP94(L11), respectively. Protein was stained with Coomassie brilliant blue. (B) Size distributions of the Hsp cages. The inset shows a logarithmic scale for the *x*-axis.

We then investigated the use of HspG41C-SP94(L11) cages for DOX delivery to HCC cells. To this end, DOX-EMCH, a DOX analogue possessing a maleimide group and an acid-cleavable hydrazone bond, was synthesized according to a previous report[12] (detailed in ESI†). Two types of Hsp cage–DOX conjugates, HspG41C-SP94–DOX and HspG41C–DOX (negative control), were prepared by simple Michael addition reactions between the maleimide group of DOX-EMCH and Cys41 of the Hsp cage (detailed in ESI†). Based on the absorbance of DOX-EMCH (8030 cm^{-1} M^{-1}), HspG41C-SP94–DOX and HspG41C–DOX possessed comparable numbers of DOX-EMCH molecules (18 and 20 molecules of DOX-EMCH per cage, respectively). After the conjugation reactions, the SEC profiles (Fig. S6A, ESI†) and size distributions (Fig. S6B, ESI†) of the Hsp cages barely changed, showing that both types of Hsp cages maintained the cage structure even after conjugation reactions. Moreover, the release of

Table 1 IC_{50} values of DOX-conjugated Hsp cages and free DOX towards Huh-7 cells and RLN-8 cells

		IC_{50}/μM		
Cells	Time/h	HspG41C-SP94–DOX	Free DOX	HspG41C–DOX
Huh-7	24	>10	>10	>10
	48	8.7	5.0	10
RLN-8	24	9.0	0.63	3.5
	48	2.0	0.24	0.49

DOX from HspG41C-SP94–DOX was negligible (0.4%) after 24 h of incubation at pH 7.2, but increased up to 15% at pH 5.0, indicating that DOX can be released at acidic pH.

After transfection of HspG41C-SP94–DOX and HspG41C–DOX to RLN-8 cells, DOX fluorescence was observed in cells incubated with HspG41C–DOX but not in cells incubated with HspG41C-SP94–DOX (Fig. S7A, ESI†). In contrast, when the cage–DOX conjugates were added to Huh-7 cells, DOX fluorescence was observed at both cytosolic and nuclear regions (Fig. S7B and C, ESI†). These results showed that HspG41C-SP94–DOX cages were selectively taken up by HCC cells, which was consistent with the cellular uptake experiments (Fig. 2(A)). To assess the cytotoxicity of the two types of cage–DOX conjugates, the conjugates and free DOX were added to Huh-7 cells and RLN-8 cells, and cell viability was measured 24 and 48 h later using a CellTiter-Glo Luminescent Cell Viability Assay (Promega). Also, IC_{50} values (DOX concentrations that killed half the cells) were calculated and are summarized in Table 1. The IC_{50} value of free DOX after 48 h of treatment was 40 times lower for RLN-8 cells than for Huh-7 cells, showing that RLN-8 cells were much more sensitive to DOX than to Huh-7 cells. On the other hand, the IC_{50} value of HspG41C-SP94–DOX toward Huh-7 cells was comparable to those of free DOX and HspG41C–DOX. Generally, DOX–polymer and –protein conjugates reported in several previous studies exhibit a tendency to be much less cytotoxic (5 to 50 times) to target cells than to free DOX.[12] Thus, our data suggest that the HspG41C-SP94–DOX can selectively recognize target cells, without decreasing its cytotoxic capacity toward target cells.

Furthermore, HspG41C-SP94–DOX had a higher IC_{50} value than free DOX or HspG41C–DOX after 24 or 48 h of treatment. These results indicate that HspG41C-SP94–DOX is much less cytotoxic to RLN-8 cells, primarily because of lower uptake of HspG41C-SP94–DOX by these cells.

In summary, we have developed an HCC-targetable protein nanocarrier, HspG41C-SP94, by expressing the HCC-binding peptide SP94 on the surface of a naturally occurring Hsp cage. HspG41C-SP94 cages were selectively taken up by various types of HCC cells but not by normal hepatocytes. Uptake of fluorophore-labeled HspG41C-SP94 cages by HCC cells was inhibited by excess SP94 peptide, indicating that HspG41C-SP94 cages were taken up through specific receptors. Furthermore, selective capacity of a DOX-conjugated HspG41C-SP94 cage (HspG41C-SP94–DOX) to HCC cells dramatically reduced cytotoxicity towards RLN-8 normal hepatocyte cell lines, but maintained its cytotoxic effects against Huh-7 HCC cells. The HspG41C-SP94 cage will be a useful nanocarrier that can selectively deliver not only anticancer drugs for HCC treatment but also imaging agents for magnetic resonance imaging, radionuclide imaging, and fluorescence imaging for diagnosis of HCC.

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