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Drug Delivery

Genome-Free Viral Capsids as Multivalent Carriers for Taxol Delivery**

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Drugs used in chemotherapy act predominantly by targeting the mechanisms of cell division,[1] and to an extent affect cancer cells preferentially because of their unusually high proliferation rates. Unfortunately, many healthy cells are also affected by these treatments, and the resulting side effects cause substantial discomfort to the patient. Emerging methods seek to focus the delivery of drugs on cancer tissue by targeting specific characteristics found in solid tumors.[2] Macromolecular drug delivery, which involves the attachment many small-molecule drugs and targeting groups to large structures, forms a promising subset of these approaches.[3] Prolonged circulation time is observed for many of these delivery vehicles because of their increased size,[4] as well as a degree of passive targeting through the enhanced permeation and retention effects due to the unique characteristics of tumor vasculature.[5] As an additional benefit, macromolecules are large enough to display multiple copies of active targeting ligands to enhance binding avidity[6] and can also provide multiple cargo-attachment sites to increase the amount of payload that can be delivered.

Many macromolecules are currently being studied for use as drug carriers. Polymer therapeutics are based on a wide variety of materials, such as poly(ethylene glycol) (PEG)[7] or hydroxypropyl methacrylate;[8] therefore, carriers with a wide range of delivery behaviors can be designed. Dendritic polymers offer similar properties, with the added advantages of controlled polydispersity and globular shape.[9] Liposomal[10] and micellular[11] systems feature high cargo loadings but can be difficult to store and can suffer from irregular release and distribution profiles. Inorganic nanoparticles[12] and carbon nanotubes[13] both have unique properties as delivery vehicles but must undergo thorough testing to evaluate their biocompatibility. Protein-based systems, such as recently used serum albumin,[14] are inherently biocompatible; however, most known systems do not exhibit any form of active targeting. As a notable exception, thermophilic heat-shock protein cages have been modified to house up to 24 doxorubicin molecules and display targeting peptide inserts on their exterior surface.[15]

As a flexible alternative for the containment of an increased amount of drug cargo, we report herein the attachment of a water-soluble derivative of the chemotherapeutic agent taxol to viral capsids and demonstrate the cytotoxicity of the drug–capsid conjugates in cell culture. In combination with available methods for the installation of peptide[16] and aptamer-based[17] targeting groups on the external surface of the capsids, this approach could provide a new series of drug carriers that could be directed to a variety of specific tissue types.

The assembled capsid of bacteriophage MS2 provides an attractive scaffold for elaboration as a drug-delivery vehicle.[18] In the self-assembly process, 180 copies of the MS2 protein monomer come together to form a hollow sphere of 27 nm in diameter with 32 pores. These 2 nm wide pores enable the installation of small molecules on the interior surface without disassembly. The capsid protein assembles in *Escherichia coli* after recombinant expression[16] to yield noninfectious genome-free particles with adjustable amino acid composition. The capsid structure is inherently monodisperse, which is an important advantage for the controlled biodistribution of drug-delivery vehicles.

Through the covalent modification of specific amino acid residues on each of the MS2 protein monomers, it is possible to attach many copies of small molecules to the two surfaces of the MS2 shell. Our research group has previously reported the attachment of magnetic resonance imaging contrast agents,[19] positron emission tomography (PET) radioisotopes,[20] and fluorescent dyes[21,17] to the interior of intact capsids. We have modified the exterior surface with peptides[16] and DNA aptamers[17] that can target specific receptors on cancer tissue, as well as PEG chains that can protect the protein shell from antibody binding.[21] Preliminary animal studies monitored by PET demonstrated the longer lifetime of capsid-bound small molecules in comparison to that of the imaging agents themselves.[20] For drug-delivery purposes, drug molecules would be attached to the interior surface of the capsid with the expectation that the cargo would be shielded from degradation and from nonspecific interactions with healthy tissue.

The strategy for interior modification was based on cysteine alkylation. The wild-type MS2 coat protein contains two cysteine residues near the exterior surface of the capsid; however, these residues were previously observed to exhibit diminished reactivity as a result of their poor solvent accessibility.[17] Therefore, an amino acid on the interior of the capsid was mutated to a cysteine residue (N87C) to provide a sulfhydryl group for the attachment of cargo. The

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drug we chose to attach to the MS2 capsid was taxol, a potent chemotherapeutic agent used in current treatment of breast, lung, and ovarian cancers.[22] Owing to its prohibitively low water solubility, taxol must be administered over long periods of time as an infusion containing a toxic detergent.[23] Although the attachment of taxol to a water-soluble protein carrier would increase the solubility of the drug during delivery, it was first necessary to solubilize it in water to enable protein bioconjugation.

With this requirement in mind, we designed a linker with three functionalities: a site for attachment to the protein, a charged functional group to increase water solubility, and a cleavable linkage to taxol (Figure 1). To synthesize the linker, we first installed a maleimide group on the ε amino group of a mono-Boc-protected lysine derivative by treatment with maleic anhydride, followed by activation with *N*,*N*′-dicyclohexylcarbodiimide (DCC) and cyclization in the presence of *N*-hydroxysuccinimide (NHS).[24] In the same step, the carboxylic acid group of the lysine core was converted into the NHS ester to give **2**. Treatment of the activated acid with taurine to install a sulfonate group as a water-solubilizing agent was followed by liberation of the lysine α amino group with trifluoroacetic acid (TFA) for attachment to taxol. To install a suitable attachment group, we transformed the 2′-OH group of taxol into a carboxylic acid functionality with succinic anhydride according to a previously described procedure.[25] The linker **4** was attached to the resulting carboxylic acid under amide-bond-forming conditions in the presence of HATU. After solid-phase extraction with an anion-exchange resin, the crude product **6** was purified by reversed-phase (RP) HPLC and used as a 2 mM solution in phosphate buffer (10 mM, pH 7).

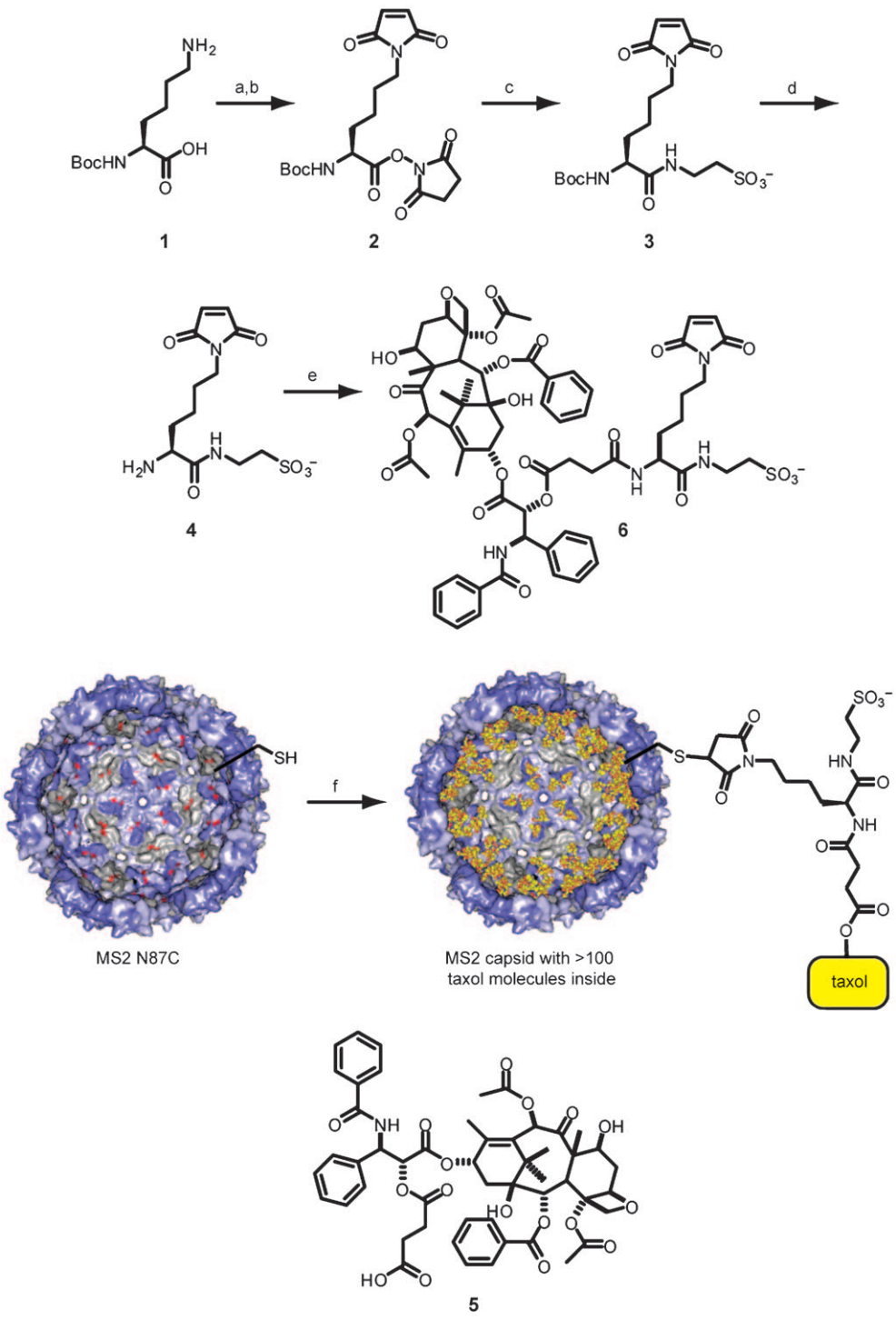

Figure 1. Synthesis of taxol–MS2 conjugates: a) maleic anhydride, DMF, 2 h, room temperature; b) NHS, DCC, DMF, room temperature, 12 h; c) taurine, DIPEA, DMF/H_2O (4:1), room temperature; d) TFA, CH_2Cl_2, 0 °C, 1 h; e) **5**, HATU, DIPEA, DMF, room temperature, 4 h; f) **6**, phosphate buffer (10 mM, pH 7), room temperature, 1 h. Boc = *tert*-butoxycarbonyl, DIPEA = *N*,*N*-diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, TFA = trifluoroacetic acid.

The reaction of the maleimide group of **6** with the sulfhydryl group of N87C MS2 was carried out at room temperature in phosphate buffer (10 mM) at pH 7. Compound **6** (5 equiv) was added to N87C MS2, and the reaction mixture was left to stand for 1 h. The use of buffers with higher pH values resulted in detectable degradation of the taxol component. The modified capsids were separated from small molecules by size-exclusion chromatography (SEC). ESIMS (Figure 2a) of the capsid monomers showed significant levels of single modification, whereas no modification of wild-type MS2 was observed under the same reaction conditions (see Figure S1 in the Supporting Information). Thus, the modification is most likely entirely confined to the cysteine residue introduced on the capsid interior. For analysis purposes, reversed-phase HPLC (Figure 2b) was used to separate modified and unmodified MS2 monomers. Integration of the tryptophan fluorescence peaks showed up to 65% modification of the MS2 monomer proteins, which translated to approximately 110 molecules of taxol per capsid, or approximately 2% loading by weight.

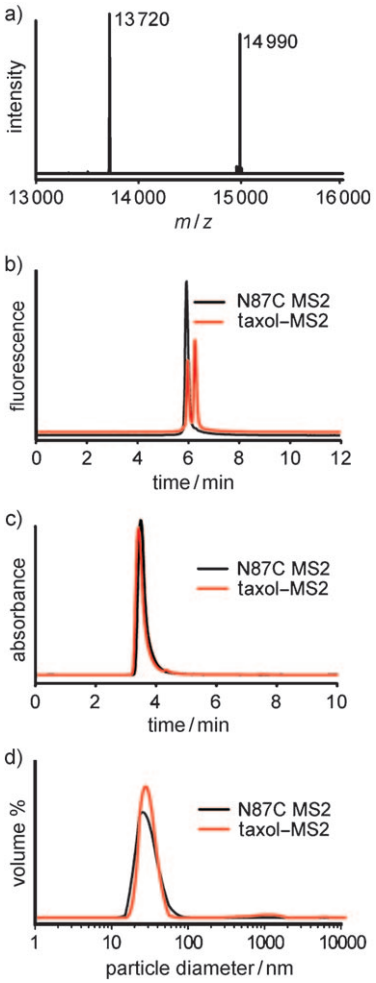

Figure 2. Analysis of taxol–MS2 conjugates. a) ESIMS reconstruction of N87C MS2 and the taxol–MS2 conjugate. Expected mass of N87C MS2: *m/z* 13719 $[M+H]^+$; expected mass of taxol–MS2: *m/z* 14987 $[M+H]^+$. b) Reversed-phase HPLC was used to quantify the modification level on the basis of the increased retention caused by the hydrophobic taxol group (fluorescence: $\lambda_{ex} = 280$ nm, $\lambda_{em} = 330$ nm). c) Size-exclusion chromatography (absorbance at 280 nm) and d) dynamic light scattering analysis of N87C MS2 and taxol–MS2 confirmed that the capsids remained assembled after modification.

The attachment of so many hydrophobic molecules to the capsid could potentially disturb the stability of the protein assembly. However, size-exclusion chromatography (Figure 2c) indicated that the protein remained as intact capsids. TEM imaging (see Figure S2 in the Supporting Information) confirmed the presence of assembled capsids, and dynamic light scattering (DLS) measurements (Figure 2d) showed that the modified MS2 capsids and wild-type MS2 were of comparable diameter. Thermal denaturation experiments (also monitored by DLS) indicated that the capsids were stable up to 64 °C (see Figure S4 in the Supporting Information). The modified capsids remained soluble in phosphate buffer at 200 μM (based on the MS2 monomer).

Antibody-binding experiments were performed to confirm the presence of taxol on the interior of N87C MS2 capsids. Western blot analysis (Figure 3a) showed the presence of taxol only on the N87C MS2 mutants. No signal was observed for wild-type capsids that had been exposed to maleimide **6**, which provided further evidence that the native cysteine residues in the MS2 coat protein were unreactive. We confirmed the internal location of the taxol molecules by performing an immunodot blot on intact versus GnHCl-denatured MS2 capsids (Figure 3b). Similar results were observed for the intact and denatured capsids when anti-MS2 antibodies (α-MS2) were used to assay for the presence of MS2 protein, whereas the use of anti-taxol antibodies (α-taxol) led to significantly different signal intensities for intact and denatured capsids. Presumably, the taxol molecules on the interior of the capsid are blocked from interaction with the antibodies, which are not able to fit through the capsid pores to access the interior surface. These molecules are only able to interact with the antibodies upon protein denaturation and capsid disassembly.

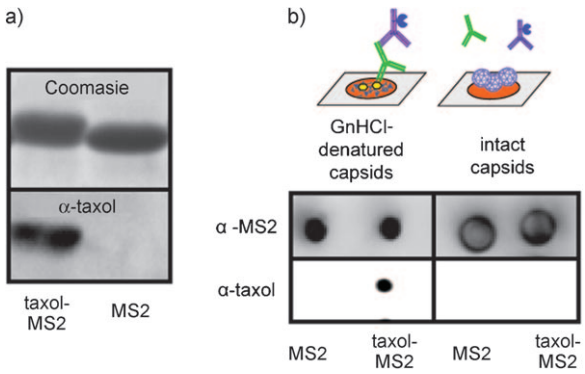

Figure 3. Antibody analysis of taxol–MS2 conjugates a) Western blot analysis indicated the presence of taxol on MS2 capsid monomers. b) Immunodot blot analysis showed different responses to taxol molecules attached to monomers denatured with guanidine hydrochloride (GnHCl; left) and inside assembled capsids (right).

To determine capsid stability under physiological conditions, we incubated samples of MS2 internally labeled with Oregon Green 488 maleimide (to facilitate detection) for

6 days at 37 °C in phosphate-buffered saline (PBS), 10 % fetal bovine serum (FBS), and cell-culture media. In each case, SEC analysis revealed that more than 80 % of the initial protein sample remained as assembled capsids (see Figure S5a in the Supporting Information). Thus, little disassembly is to be expected in the absence of cellular targets.

A taxol-release profile was also determined at pH 7.4. Samples of MS2–taxol conjugates were incubated in 10 % FBS for 5 days. At 24 h intervals, samples were taken, and the remaining capsids were isolated by selective precipitation and SEC. RP HPLC analysis of the resulting material enabled quantification of the amount of taxol that had been released (see Figure S5b in the Supporting Information).[26] These experiments indicated a 1.9 day half-life for the linker under these conditions.

A cell-viability assay was used to test the effect of MS2 and taxol–MS2 on cancer cells in vitro (Figure 4; see also Figure S3 in the Supporting Information). The release of taxol

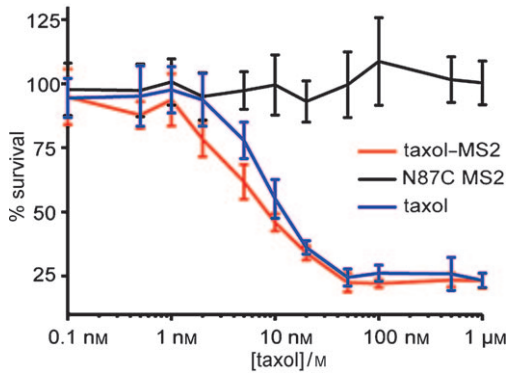

Figure 4. Cell-viability assay with an MCF-7 cell line. Cell survival was calculated relative to an untreated control 5 days after the initial treatment by measuring the fluorescence resulting from treatment with Alamar Blue. Taxol–MS2 conjugates were delivered as a solution in phosphate buffer (10 mM), and free taxol was applied as a solution in dimethyl sulfoxide (< 1 %)/phosphate buffer. Error bars show the standard deviation for six replicate experiments.

was expected to occur via hydrolysis of the ester bond at the 2′-OH position.[27] MCF-7 breast-cancer cells were assayed by using Alamar Blue dye, a fluorescent indicator of cellular metabolic processes.[28] Unmodified N87C MS2 was found to have no noticeable effect on MCF-7 viability. However, taxol-containing MS2 caused a significant lowering of cell viability; the effect was comparable to that observed upon the administration of an equivalent amount of taxol solubilized with dimethyl sulfoxide. Virtually identical results were obtained after incubation times of 3 and 5 days. Although it is not known how efficiently the untargeted capsids are taken up by pinocytosis, the drug-release experiments suggest that a decreased amount of taxol would be available through hydrolysis alone over the 3 day time period. Instead, the observation of equivalent toxicity to that of free taxol suggests that the cells may be playing an active role in the cleavage process. Whether or not this hypothesis is true, the addition of targeting groups that bind the drug conjugates to specific cell types would be expected to focus this activity only on the relevant cancer tissue.

In conclusion, a water-soluble derivative of the chemotherapeutic drug taxol was synthesized with bioconjugation functionality and attached to MS2 viral capsids. The modified capsids remained in their capsid form and released taxol when incubated with MCF-7 cells. The resulting cell-viability levels were similar to those observed for free taxol in solution. We are presently investigating the use of linkers that undergo cell-specific cleavage between the cargo and the capsid, as well as the generation of MS2 capsids with both drug cargo and active targeting groups.

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