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Tobacco mosaic virus-based protein nanoparticles and nanorods for chemotherapy delivery targeting breast cancer

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ABSTRACT

Drug delivery systems are required for drug targeting to avoid adverse effects associated with chemotherapy treatment regimes. Our approach is focused on the study and development of plant virus-based materials as drug delivery systems; specifically, this work focuses on the tobacco mosaic virus (TMV). Native TMV forms a hollow, high aspect-ratio nanotube measuring 300 × 18 nm with a 4 nm-wide central channel. Heat-transformation can be applied to TMV yielding spherical nanoparticles (SNPs) measuring ~50 nm in size. While bioconjugate chemistries have been established to modify the TMV rod, such methods have not yet been described for the SNP platform. In this work, we probed the reactivity of SNPs toward bioconjugate reactions targeting lysine, glutamine/aspartic acid, and cysteine residues. We demonstrate functionalization of SNPs using these chemistries yielding efficient payload conjugation. In addition to covalent labeling techniques, we developed encapsulation techniques, where the cargo is loaded into the SNP during heat-transition from rod-to-sphere. Finally, we developed TMV and SNP formulations loaded with the chemotherapeutic doxorubicin, and we demonstrate the application of TMV rods and spheres for chemotherapy delivery targeting breast cancer.

1. Introduction

In the United States, approximately 300,000 women will be diagnosed with breast cancer this year and more than 40,000 of those will die from the disease. Chemotherapy has limited success due to adverse effects such as congestive heart failure [1–5]. Nanotechnologies have improved treatment because nanoparticle delivery systems offer better safety and drug efficacy. Many different classes of nanomaterials are undergoing development and some approaches have advanced to clinical use. Examples include liposomal formulations Doxil® or albumin-based formulation Abraxane®. Currently available nanoparticle-delivery systems increase safety of the treatment; however they often do not increase efficacy due to inefficient carrier tissue penetration and drug release [6]. Therefore, more research is required to develop and assess novel drug delivery system and strategies. Our research is focused on the development and application of nanomaterials derived from plant viruses manufactured through farming in plants.

Mammalian virus-based nanoparticles for gene therapy and oncolytic virotherapy are undergoing clinical trials [7–9], so the potential of virus-based materials for medical applications has been recognized. There are many novel virus-based materials in the pipeline, with plant viruses typically considered safer in humans than mammalian viruses [10]. Plant viruses do not infect or replicate in mammals. They can be administered at doses of up to 100 mg (10^{16} particles) per kg body weight without clinical toxicity [11,12]. We have shown that biomaterials derived from the plant virus, tobacco mosaic virus (TMV), can be delivered intravenously and do not induce hemolysis or coagulation [13]. Further, the protein-based carriers exhibit rapid tissue clearance (hours) from non-target organs [13]. The pharmacokinetics of plant viruses is tunable through PEGylation achieving half-lives of minutes-to-hours [14]. Like other nanomaterials, unmodified TMV is moderately immunogenic, but the immunogenicity of protein-based carriers [15,16], such as plant viruses [17], can be attenuated by polymer coating. These properties make this biodegradable platform a promising strategy for drug delivery.

Viruses have naturally evolved to deliver cargos to specific cells and tissues; we seek to repurpose this natural ability to deliver cargos for drug delivery targeting cancer. Advantages of the bio-inspired drug delivery system include the production through molecular farming in

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plants, which is highly scalable and therefore provides a realistic nanotechnology for translation. For example, Medicago Inc. already produces several virus-like particles in plants at pharmaceutical scale [18–21]. The protein-based materials are genetically encoded, therefore these materials offer a high degree of reproducibility and monodispersity; each particle is a clone of another; this degree of quality control is still challenging to achieve with synthetic systems.

TMV was the first virus to be discovered more than 100 years ago and it has served as a research tool for structural biology and virology since [22]. TMV-based materials are undergoing development for diverse applications as battery electrodes and light harvesting systems [23,24], as well as tissue engineering [25], molecular imaging [26], vaccines [27], and as demonstrated in this work, drug delivery. Native TMV forms high aspect ratio rods measuring 300 × 18 nm with a 4 nm-wide central channel. Each particle consists of a ssRNA genome encapsidated into 2130 identical copies of a coat protein unit. The length can be tailored through a bottom-up RNA-templated self-assembly approach and TMV shape-switching into spherical nanoparticles can be achieved through thermally-induced process [13,28,29].

While the development pipeline for nanoparticle platform technologies continues to progress rapidly, the fundamental nanomaterial–cell interactions have been studied only in a limited way and its relationship to drug efficacy is largely unknown. Size, shape, composition and surface chemistry of the nanocarrier impact its biodistribution, cell interaction, and intracellular trafficking. Some fundamentals are understood: mammalian cell membranes are negatively charged, therefore positively charged nanomaterials interact more strongly compared to negatively charged counterparts [30–32]. PEGylation is an accepted strategy to camouflage nanoparticles and inhibit (or reduce) cell binding or uptake; and receptor targeting is an effective strategy to enhance cell binding and/or induce tissue-specificity [33]. Based on the abundance of spherical nanomaterials, the effect of size on endocytosis is well understood: through competition between hydrodynamic driving force and receptor diffusion kinetics, the optimum radius for cellular uptake lies at r = 30 nm (viruses were used as model systems in several of these studies) [30,31,34–40]. Nevertheless, data remain elusive with regard to understanding the effects of nanoparticle shape: while some data indicate that higher aspect ratio (AR5) materials show faster uptake kinetics [31,32]. Others reported the opposite: spheres and low aspect ratio objects have enhanced cell interaction compared to high aspect ratio nanorods [41–44]. The TMV and SNP platforms provide a tool set of different-shaped protein-based nanoparticles and their side-by-side studies are expected to provide further insights into the design space 'shape' and how to utilize it in the design of next-generation drug delivery systems.

In the present studies, we considered TMV rods and spheres as a platform for drug delivery. While the chemistries on TMV rods are well-established, enabling functionalization of the interior or exterior surface with a variety of payloads [28,45,46]; bioconjugate chemistries have not yet been established using the SNP platform technology. The availability of chemical modification procedures would enable functionalization of SNPs with targeting ligands and medical cargo for molecular imaging [29,45] or drug delivery (this paper), as well as for vaccine development [47]. A recent study showed that SNPs could be non-covalently modified through adsorption of foreign proteins and epitopes on the SNP surface, however, these modifications are non-specific and driven through electrostatic and hydrophobic interactions [47]. The availability of bioconjugate chemistry would streamline the functionalization of SNPs through targeted, covalent approaches and also enable functionalization with small cargos such as contrast agents and therapeutics. Chemical modification strategies would therefore complement the 'modification by adsorption' techniques. In addition to covalent labeling techniques, we present encapsulation techniques, where the cargo is loaded into the SNP during heat-transition from rod-to-sphere. Finally, we demonstrate the application of TMV rods and spheres for chemotherapy delivery targeting breast cancer.

2. Experimental section

2.1. Materials

Unless otherwise noted, chemicals and materials were obtained from Fisher Scientific.

2.2. TMV nanomanufacturing

2.2.1. Propagation

TMV was propagated in *Nicotiana benthamiana* plants and recovered, with a yield of 5 mg TMV per gram infected leaf material, using established extraction methods [28]. The concentration of TMV from plant extracts was determined by UV/vis spectroscopy ($\varepsilon_{260\ nm} = 3.0\ mg^{-1}\ mL\ cm^{-1}$) and virus integrity was verified by TEM imaging.

2.2.2. Thermal transition of TMV rods into spherical nanoparticles (SNPs)

Thermal transition from TMV rods to SNPs was carried out by heating TMV rods (0.3 mg mL^{-1} in H_2O) for 60 s at 96 °C using a Peltier thermal cycler and then recovering concentrated particles by centrifugation at 160,000 ×*g* for 1 h. The methods were as we previously described [29,48].

2.3. TMV and SNP bioconjugation chemistry

2.3.1. Lysine modification

Lysine reactivity of SNPs was tested with Sulfo-Cyanine5 NHS ester (Lumiprobe). Sulfo-Cy5 NHS (1, 2, 5, 10, 20, and 40 M equivalents per coat protein (eq)), in 0.1 M potassium phosphate buffer pH 7.0 was added to 1 mg/mL SNPs at room temperature overnight. The reaction was purified by ultracentrifugation at 160,000 ×*g* for 1 h and analyzed (see below).

2.3.2. Cysteine modification

SNP cysteine reactivity was tested using Sulfo-Cyanine5 maleimide (Lumiprobe). Sulfo-Cy5 maleimide (1, 2, 5, 10, 20, and 40 M equivalents per coat protein (eq)), in 0.1 M potassium phosphate buffer pH 7.0 was added to 1 mg/mL SNPs at room temperature overnight. The reaction mix was purified by ultracentrifugation at 160,000 ×*g* for 1 h and analyzed (see below).

2.3.3. Glutamic/aspartic acid modification

Carboxylic acids were activated using hydroxybenzotriazole (HOBt) and ethyldimethylpropyl carbodiimide (EDC) to form amide bonds with the primary amine from Cyanine5 amine (Lumiprobe). Here, HOBt:EDC:amine ratio was set at 1:2.5:5, the molar equivalents tested were: 0.625:1.25:2.5, 1.25:2.5:5, 2.5:5:10, 5:10:20, and 10:20:40. SNPs (1 mg/mL) were reacted with HOBt:EDC:amine overnight at room temperature in 0.1 M HEPES buffer pH 7.0. The reaction was purified by ultracentrifugation at 160,000 ×*g* for 1 h and analyzed (see below).

2.3.4. Conjugation of Cy5 or doxorubicin to TMV and SNP

For drug delivery studies, doxorubicin hydrochloride (DOX, Indofine Chemical Company) and for imaging studies amine-Cyanine5 (Cy5) was conjugated to TMV and SNP targeting carboxylic acid functional groups on the TMV/SNP; TMV or SNP (1 mg/mL) was reacted with HOBt:EDC:DOX (10:20:40 eq per CP) overnight at room temperature in 0.1 M HEPES buffer pH 7.0. The reaction was purified by ultracentrifugation at 160,000 ×*g* for 1 h and analyzed (see below).

2.3.5. Biotin conjugation to SNPs

For lysine modification, SNPs (1 mg/mL in 0.1 M potassium phosphate buffer pH 7.0) were added to NHS-biotin (LifeTechnologies, 20217) at 20 eq and reacted overnight at room temperature. For cysteine modification, SNPs (1 mg/mL in 0.1 M potassium phosphate buffer