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Biodistribution, pharmacokinetics, and blood compatibility of native and PEGylated tobacco mosaic virus nano-rods and -spheres in mice

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ABSTRACT

Understanding the pharmacokinetics, blood compatibility, biodistribution and clearance properties of nanoparticles is of great importance to their translation to clinical application. In this paper we report the biodistribution and pharmacokinetic properties of tobacco mosaic virus (TMV) in the forms of $300 \times 18\ nm^2$ rods and 54 nm-sized spheres. The availability of rods and spheres made of the same protein provides a unique scaffold to study the effect of nanoparticle shape on *in vivo* fate. For enhanced biocompatibility, we also considered a PEGylated formulation. Overall, the versions of nanoparticles exhibited comparable *in vivo* profiles; a few differences were noted: data indicate that rods circulate longer than spheres, illustrating the effect that shape plays on circulation. Also, PEGylation increased circulation times. We found that macrophages in the liver and spleen cleared the TMV rods and spheres from circulation. In the spleen, the viral nanoparticles trafficked through the marginal zone before eventually co-localizing in B-cell follicles. TMV rods and spheres were cleared from the liver and spleen within days with no apparent changes in histology, it was noted that spheres are more rapidly cleared from tissues compared to rods. Further, blood biocompatibility was supported, as none of the formulations induced clotting or hemolysis. This work lays the foundation for further application and tailoring of TMV for biomedical applications.

Introduction

Nanoparticles hold great potential for clinical research and application for diagnosing and treating diseases (Parveen et al., 2012). Nanoparticles are used to deliver a high payload of cargo, such as imaging and therapeutic compounds, to specific sites of disease while avoiding healthy tissue. While receptor-specific ligands can direct these nanoparticles to target specific cells and tissues, a majority of the injected dose is cleared by the reticuloendothelial system (RES) and mononuclear phagocyte system (MPS) (Zhu et al., 2013). On the road toward clinical translation of any nanoparticle platform, a detailed understanding of the body's response to the nanoparticles is required; this will allow tailoring and optimizing biodistribution and clearance.

Viral nanoparticles (VNPs) are protein-based, nanoscale materials designed by nature to deliver cargos to cells; being natural experts at cargo delivery led to their study and application as drug and contrast agent delivery vehicles. There are a number of reasons that make VNPs excellent platforms for applications in biomedicine, including their biocompatibility, biodegradability, high monodispersity, and ease of production and functionalization. Platform simplicity and high processability are key components for clinical translation of nanoparticle platforms – VNPs offer this through their genetic engineering capabilities and simple purification protocols. A library of VNPs is available and currently under investigation for clinical applications. These include the plant viruses cowpea mosaic virus (CPMV) and potato virus X (PVX), as well as bacteriophages such as M13 and P22 (Yildiz et al., 2011). For example, it has been demonstrated that 30 nm-sized icosahdral CPMV target tumor cells and the inflamed endothelium in atherosclerotic plaques, based on its natural interactions with cell surface expressed vimentin (Steinmetz et al., 2011; Plummer et al., 2012). Also, targeting ligands specific to the vascular endothelial growth

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factor receptor-1 or gastrin-releasing peptide receptors have been used to re-direct CPMV to tumor endothelial cells and cancer cells in preclinical mouse models (Steinmetz et al., 2011; Brunel et al., 2010). Recent data indicate that in addition to tailoring the VNP surface chemistry (Wu et al., 2012), VNP shape can also be used as a handle to tailor biodistribution and tissue penetration properties (Gandra et al., 2012; Shukla et al., 2013; Lee et al., 2013). For example, we showed that filamentous PVX has enhanced passive tumor homing and deeper tissue penetration compared to icosahedral CPMV nanoparticles. While both platforms were cleared by MPS and accumulated in liver and spleen, differences were noted: PVX was mostly retained in the spleen, and CPMV in the liver (Shukla et al., 2013). However, it should be noted that besides the shape-derived differences, PVX and CPMV also differ in their protein make up. Therefore, in this work, we turned toward the evaluation of biodistribution and clearance of VNP-based materials of identical protein make up but different shape, specifically the rods and spheres, of tobacco mosaic virus (TMV).

TMV is a rod-shaped VNP measuring 300 nm in length, 18 nm in diameter and a 4 nm-wide interior channel; its structure is known to atomic resolution (Klug, 1999). This stiff rod-shaped nanoparticle has been utilized as a material for a variety of applications in nanotechnology (Soto and Ratna, 2010; Liu et al., 2012). Chemically and genetically engineered TMV particles have been developed and tested for applications as light harvesting systems (Miller et al., 2007, 2010), energy storage (Chen et al., 2010), sensing (Bruckman et al., 2010), cell growth (Kaur et al., 2010; Bruckman et al., 2008), magnetic resonance imaging contrast enhancement (Bruckman et al., 2013), and vaccine development (Koo et al., 1999; Fitchen et al., 1995; McCormick et al., 2006a, 2006b). Recently Atabekov et al. (2011) have shown that TMV can undergo thermal transition to form RNA-free spherical nanoparticles (SNPs). The thermal denaturation of TMV rods yields insoluble coat proteins that somewhat surprisingly associate with each other to assemble into highly stable SNPs. The size of the TMV SNPs can be tightly tuned through adjustment of the protein concentration: at higher concentration (10 mg/mL), up to 800 nm-sized SNPs are formed, and at lower concentrations (0.1 mg/mL), SNPs as small as 50 nm are formed (Atabekov et al., 2011). Further, it was shown that SNPs can be coated with a variety of polymers and proteins (Nikitin et al., 2011). Following electrostatic binding, formaldehyde can bond the proteins to the SNP surface. Protein-functionalized SNPs are under development for vaccines (Karpova et al., 2012).

To us, the TMV rods and SNPs provide an interesting platform to investigate the impact of shape on biodistribution and clearance. We would like to acknowledge that the TMV rods and spheres are not identical in their structural properties: while the TMV rod consists of a helical arrangement of precisely arranged coat proteins, the SNPs consist of aggregated, most likely denatured, coat proteins. Nevertheless, the TMV rods and SNPs are made up of the same proteins and zeta potential measurements support that the rods and spheres have comparable surface charge (see results). Therefore, we reasoned, that the TMV rod and SNP platform would be suitable tools for investigation of their *in vivo* properties. This is also important considering that several groups have turned toward the development of the platforms for potential applications in medicine and materials (see above).

Here, we report the study of TMV and SNP biodistribution, clearance, and blood compatibility in mice. The application of rods and spheres allow us to provide further insights into the impact of shape on the *in vivo* fate of nanoparticles. Further, we investigated the effect of PEGylation: native and PEGylated TMV rods were studied. PEG was applied because of its known properties to enhance solubility and biocompatibility of intravenously administered nanoparticles (Albanese et al., 2012).

Results

Properties of TMV-based nanorods and nanoparticles Cy5-TMV, PEG-Cy5-TMV, and Cy5-SNP

TMV was produced at yields of 4.5 mg pure TMV per 1 g infected plant material using a 1-day purification protocol (Leberman, 1966). Three different TMV-based nanoparticle formulations were produced for evaluation of biodistribution, pharmacokinetics, and blood compatibility in mice. Previously established bioconjugation and thermal transition protocols were used to produce dye-labeled TMV rods (Cy5-TMV), PEG_{2000}-coated dye-labeled TMV rods (PEG-Cy5-TMV), and dye-labeled TMV spheres (Cy5-SNPs) (Fig. 1). In brief, TMV rods were labeled with Cy5 (and PEG_{2000}) at exterior tyrosine residues (TYR139) using a two-step diazonium coupling and copper catalyzed azide alkyne cycloaddition (CuAAC) reaction protocol. Dye-labeled SNPs were obtained by heating interior Cy5-labeled TMV particles for 15 s at 96 °C (Bruckman et al., 2013). Interior labeling of the TMV rod is accomplished by labeling GLU97 and GLU106 with an alkyne handle followed by CuAAC chemistry protocols to introduce the fluorophores. (Bioconjugation methods for modification of SNPs are currently under development in our laboratory, however, not yet fully implemented. Therefore it was not possible to study PEGylated SNPs.)

The resulting Cy5-labeled (and PEGylated) TMV rods and SNPs were characterized for labeling efficiency, size, morphology, and surface properties using a combination of transmission and scanning electron microscopy (TEM and SEM), dynamic light scattering (DLS), UV-Vis absorbance, gel electrophoresis (SDS-PAGE), size exclusion chromatography (SEC), zeta potential, and MALDI-TOF MS. UV–vis absorbance confirmed the attachment of about 900 Cy5 dyes per Cy5-TMV and PEG-Cy5-TMV; this equates to a labeling efficiency of ~50%, meaning that every second Tyr residue was modified with a Cy5 dye (a single 300 nm-long TMV rod is comprised of 2130 identical copies of one coat protein). About 600 Cy5 dyes were conjugated to SNPs, meaning that 15% of the 4260 interior glutamic acids per TMV were modified. The lower labeling efficiency may be explained by steric hindrance and lower accessibility of the interior *versus* exterior surface. Based on MALDI-TOF MS and SDS-PAGE lane analysis (Fig. 2), we estimated coverage of PEG-Cy5-TMV with approximately 400 PEG_{2000} chains/TMV, which equates to a labeling efficiency of 20%.

TEM (Fig. 2) and SEM images (not shown) confirmed the structural integrity of the particle formulations. It should be noted that based on the length of the RNA-genome, fully-assembled TMV nanorods have a length of 300 nm (and a width of 18 nm with a 4 nm-wide interior channel). A distribution of lengths is observed in TEM imaging, which could be explained by the fact that intermediate states as well as broken TMV rods are observed in TEM imaging. SNPs were measured at 54 ± 14 nm based on TEM and 83 ± 6 nm based on DLS (Fig. 2). Based on the measured protein concentration and SNP size observed, we estimate that each SNP is formed by a single TMV rod. This is also in good agreement with the volume of a TMV cylinder and sphere composed of the same number of proteins.

Finally, the zeta potentials of Cy5-TMV, PEG-Cy5-TMV, and Cy5-SNP were determined at −18, −17, −10 mV, respectively (Fig. 2). The slightly lower zeta potential for SNPs is attributed to the loss of RNA following thermal transition.

Pharmacokinetics of TMV rods and SNPs differs

Plasma circulation half-life was determined in healthy Balb/c mice after intravenous bolus injection of 10 mg/kg Cy5-labeled (and PEGylated) TMV/SNP formulation (Fig. 3). The fluorescence of plasma spiked with known TMV and SNP concentrations was measured to establish a standard curve, which was used to