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A biodistribution study of two differently shaped plant virus nanoparticles reveals new peculiar traits

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ABSTRACT

Self-assembling plant virus nanoparticles (pVNPs) have started to be explored as nanometre-sized objects for biomedical applications, such as vaccine or drug delivery and imaging. Plant VNPs may be ideal tools in terms of biocompatibility and biodegradability endowed with a wide diversity of symmetries and dimensions, easy chemical/biological engineering, and rapid production in plants. Recently, we defined that icosahedral Tomato bushy stunt virus (TBSV) and filamentous Potato virus X (PVX) are neither toxic nor teratogenic. We report here the results of an interdisciplinary study aimed to define for the first time the biodistribution of unlabelled, unpegylated, underivatized TBSV and PVX by proved detecting antibodies. These data add new insights on the *in vivo* behaviour of these nano-objects and demonstrate that the pVNPs under scrutiny are each intrinsically endowed with peculiar properties foreshadowing different applications in molecular medicine.

1. Introduction

The research on nanometre-sized particles as carriers for vaccine or drug delivery or for diagnostic applications is a strongly trans-disciplinary and rapidly emerging field with enormous potential [1]. Considering the vastness of the topic, there is an urgent need to define reference methods to profile the major parameters governing the interaction of these "objects" with the biological matter and consequent effects [2]. The definition of features such as shape and size uniformity, manufacturing consistency, stability, toxicity and biodistribution of nanoparticles derived from a wide spectrum of materials is at the very beginning [3]. Plant virus nanoparticles (pVNPs) are also included in the list of nano-objects of interest [4,5]. In fact, pVNPs hold properties such as: i) ability to self-assemble in structurally simple scaffolds homogeneous in size and shape; ii) wide array of symmetries and dimensions; iii) ease of chemical and/or biological engineering on both external surface or internal cavity; iv) rapid and direct "manufacturing" in plants. Moreover, the biochemical composition of pVNPs and their infection strategy evolved to target the plant host make these nanostructures ideal in terms of biosafety, biocompatibility and biodegradability [6].

Tomato bushy stunt virus (TBSV) and Potato virus X (PVX) are among those pVNPs that have started to be characterized in view of a possible use in the pharmaceutical field [7]. These two plant viruses are ideal, highly ordered, multivalent scaffolds to be used for this purpose. TBSV is icosahedral with single-stranded positive-sense (ss(+)) RNA genome embedded in a capsid of about 30 nm in diameter made of 180 identical coat protein (CP) units. PVX has a very simple filamentous flexible structure of about 500 nm in length and 13 nm in diameter, made of a ss(+) RNA wrapped in approximately 1300 CP units [8,9].

We have established that TBSV can encapsulate small molecules and display polypeptides [8]. As for PVX, we demonstrated that chimeric particles genetically engineered to display peptides of interest for vaccine development are able to induce the activation of both antibody and cell-mediated immune responses without the need of adjuvant co-delivery [10,11].

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Recently, we have defined the effects resulting from TBSV and PVX interaction with different cell types. In fact, even if in the animal cell environment pVNPs are expected to behave as unreplicative and biologically safe nano-objects, intrinsic toxicity cannot be excluded. The results of haemolysis assays *in vitro* (to test possible cytotoxic effects deriving from pVNPs interaction with cellular membranes) and early chicken embryo assays (to evaluate toxicity and teratogenicity *in vivo*) clearly indicated that TBSV and PVX are neither toxic nor teratogenic [12].

We report here the results defining, for the first time, the biodistribution of unpegylated non-chimeric PVX and TBSV in healthy mice through a combination of enzyme linked immunosorbent assay (ELISA) and immunohistochemistry, highlighting effects on histo-architecture and peculiar interactions and localization within organs.

2. Methods

2.1. Plants infection and pVNPs purification

Vectors carrying the full-length cDNA copies of the ss(+) RNA genomes of TBSV (pTBSV-P) [13] and PVX (pPVX201) [14] are available. These vectors are useful instruments to perform genetic manipulations and/or induce/facilitate plant infection for pVNPs production. The pTBSV-P plasmid is the template for the synthesis of the full-length viral genome transcripts that are used to induce infection onset and TBSV particles production in plants. To this aim, the plasmid has been linearized with *Xma I* and *in vitro* transcribed using the MEGAscript_T7 High Yield Transcription kit (Ambion, Life technologies), following the instructions of the manufacturer.

The pPVX201 plasmid is directly used to induce the infection of plants as the transcription of the cDNA encoding the whole viral genome is under the control of the 35S promoter of Cauliflower Mosaic virus, constitutively active in plant cells.

Nicotiana benthamiana plants have been grown in controlled conditions (24 °C, 16 h light/8 h dark). At the age of 6 to 8 weeks the adaxial surface of two leaves/plant has been abraded with carborundum (Silicon carbide, VWR International) and inoculated with: (i) 20 μg/leaf of pPVX201 or (ii) approximately 2 μg/leaf of TBSV RNA. Virus propagation and pVNPs purifications were performed as previously described [8,15].

2.2. Denaturing gel electrophoresis

Virus concentrations (*i.e.* CP concentrations) in each preparation have been determined using the bicinchoninic acid protein assay kit (Pierce, Thermo Fisher Scientific) and further verified through 12.5% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. The gel analysis allowed also verifying the purity of the viral batches.

2.3. Transmission electron microscopy

Purified virus particles have been distributed on carbon/formvar film-coated 400 mesh copper grids (Electron Microscopy Sciences) and stained with 2% (w/v) uranyl acetate aqueous solution. Grids have been analysed with a JEM 1200 EXII (Jeol) transmission electron microscope and images acquired through a CCD Camera SIS Veleta (Olympus) at the Interdepartmental Centre of Electron Microscopy (University of Tuscia, Viterbo, Italy).

2.4. Animals and in vivo treatments

CD1 female mice were maintained on commercial pellet diet *ad libitum* with a 12 h light/dark cycle. At 8 weeks of age, mice were injected through the tail vein with saline alone or containing 200 μg of the purified TBSV or PVX, corresponding to about 134.6×10^{11} and 34.56×10^{11} virions, respectively (n = 12 for each treatment). Possible extra-venous injection, causing local blanching or tissue necrosis at the injection site immediately after injection and in the following days, was excluded. Moreover, after administration, mice were observed daily monitoring changes especially in skin and fur, eyes membranes, respiratory, circulatory, and autonomic and central nervous systems and behaviour patterns.

At 6, 24, 48 h and 7 days after the treatment, mice (n = 3 for each time point) were sacrificed. The blood was obtained from ophthalmic veins. The spleen, liver, lungs, kidneys and brain, were weighed and divided in two parts, one of which was fixed in 10% formalin for histopathological examination and the other flash frozen and stored to −80 °C for molecular analyses.

Additional groups of mice (n = 3 for each treatment), 24 h after injection with saline or with the pVNPs were perfused with saline before organs collection in order to flush blood from the circulatory system and to establish in which proportion the positivity to pVNPs in the different organs is influenced by the blood supply.

All experiments were conducted according to the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Institutional Animal Care and Use Committees of ENEA approved this study.

2.5. Histological analysis and immunohistochemistry

The formalin-fixed samples were embedded in paraffin wax by standard techniques, and sections (4 μm thick) analysed after staining with haematoxylin and eosin (Bio Optica). Any sign of histological impairment was examined using a light microscope. For immunohistochemical analyses, sections were opportunely pretreated before incubation with primary anti-TBSV (1:100) and anti-PVX (1:100) antibodies (Agdia). Antibody–antigen complexes were visualized using a horseradish peroxidase (HRP)-conjugated secondary antibody (Dako) and the DAB chromogen system (Dako). Negative controls were performed omitting primary antibodies.

2.6. RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA has been extracted from approximately 30 mg of each flash frozen organ using the NucleoSpin RNA isolation kit (Macherey-Nagel) and RT-PCRs have been performed using 1 μg of RNA as template with the RETROscript™ Reverse Transcription Kit (Ambion), following the instructions of the manufacturer. The cDNAs have been synthesized using random primers, while the PCR have been performed with: i) TBSV primers (TBSVFor 5′-GACATTTCGTGCAACTGG-3′ and TBSVRev 5′-AAGATCCAAGGACTCTGTGC-3′) specific for a *cp* gene region of 648 bp in length; ii) PVX primers (PVXFor 5′-CTGGGGAATCAATCACAGTGTTG-3′ and PVXRev 5′-CAGTCTAGCTCTGCTGATGCCGTTG-3′) specific for a *cp* gene region of 529 bp in length; iii) murine actin specific primers (ACTFor 5′-GCTACAGCTTCACCACCACA-3′ and ACTRev 5′-CATCGTACTCCTGCTTGCTG-3′) that amplify a 500 bp fragment.

2.7. Double antibody sandwich enzyme linked immunosorbent assays (DAS-ELISA) on murine organs extracts

For each experimental time interval (6, 24, 48 h and 7 days), after grinding to fine powder with liquid nitrogen and mortar and pestle, each organ sample (spleen, liver, lung, brain, kidney) has been extracted 1:2 w/v in bicin buffer (25 mM bicin, 150 mM NaCl, 1% Triton X-100, pH 7.6). Blood cells have been separated from serum by centrifugation at $500 \times g$ for 5 min, after incubation at room temperature for 30 min and on ice for 20 min. Proteins have