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In vitro and *in vivo* toxicity evaluation of plant virus nanocarriers

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ABSTRACT

The use of biological self-assembling materials, plant virus nanoparticles in particular, appears very intriguing as it allows a great choice of symmetries and dimensions, easy chemical and biological engineering of both surface and/or internal cavity as well as safe and rapid production in plants. In this perspective, we present an initial evaluation of the safety profile of two structurally different plant viruses produced in *Nicotiana benthamiana* L. plants: the filamentous Potato virus X and the icosahedral Tomato bushy stunt virus. *In vitro* haemolysis assay was used to test the cytotoxic effects, which could arise by pVNPs interaction with cellular membranes, while early embryo assay was used to evaluate toxicity and teratogenicity *in vivo*. Data indicates that these structurally robust particles, still able to infect plants after incubation in serum up to 24 h, have neither toxic nor teratogenic effects *in vitro* and *in vivo*. This work represents the first safety-focused characterization of pVNPs in view of their possible use as drug delivery carriers.

1. Introduction

After more than twenty years of academic and industrial research, nanometer-sized carriers, although still in early stages, are becoming a reality in the biomedical field, especially in vaccinology, drug delivery, and diagnostic. Different nanoparticle-based therapeutic and diagnostic agents are under preclinical and clinical evaluation and some of them are already on the market [1]. The most exploited and investigated nanoparticles (NPs) are those based on organic (*e.g.*, lipids, polymers) and inorganic (*e.g.*, metal, metal oxide) materials [2,3]. Nonetheless, due to the extremely complex features that an ideal nano-delivery system needs to achieve clinical application, none of these carriers is immune from drawbacks (*e.g.*, difficult large-scale manufacturing, poor long-term stability, *in vivo* toxicity) [4,5].

In this scenario, the crosspollination between biotechnology, nanoscience, nanotechnology, pharmaceutics and biomedicine is shifting the attention to bioinspired and/or bioengineered nanocarriers [6]. Nanoparticles derived from natural macromolecules self-assembly are considered extremely promising. Among the various bio-building blocks, self-assembling proteins have unique features as they can form supramolecular structures giving rise to symmetrical nano-objects [7]. These so called 'protein cages' can be made of ferritin-like proteins, chaperons, heat shock proteins and, most notably, viral proteins [8,9]. The majority of virus nanoparticles (VNPs) display a spherical or a rod shape, and can be surprisingly diverse in terms of symmetries, dimensions and structure related properties. Additional intriguing features include the inherent monodispersity and extreme flexibility in terms of engineering strategies, the latter allowing the effective modification of the exposed surfaces and/or the exploitation of the internal cavity for cargo storage. For these reasons, VNPs have been proposed since the dawn of nanotechnology as carriers for targeted drug delivery, vaccinology, and imaging [10]. In this context, plant VNPs (pVNPs) are attracting increasing attention. Several plant viruses have been exploited for biomedical applications and successfully tested in animal models [11]. Plants, such as the *Solanacea Nicotiana benthamiana* L., relative of common tobacco, allow a convenient and easily scalable production of pVNPs that, when outside their specific plant host, become protein nanoshells unable to replicate.

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In this study, we have focused on two distinct pVNPs-based systems: Potato virus X (PVX), the type member of the genus *Potexvirus* (*Alphaflexiviridae* family), and Tomato bushy stunt virus (TBSV), the prototype member of the genus *Tombusvirus* (*Tombusviridae* family). Both viruses have a monopartite single stranded, positive-sense RNA genome. PVX is a flexible filamentous virus of ~500 nm in length and 15 nm in diameter, constituted by approximately 1300 units of a unique coat protein (CP). TBSV is a spherical virus of about 30 nm in diameter with 180 copies of the CP self-assembled to form a $T = 3$ icosahedral symmetry.

Both systems have been extensively explored as nanocarriers for drug targeting to tumour cells. CP genetic engineering can be used to incorporate moieties for the covalent binding of exogenous molecules (chemical derivatization strategy) or to display peptide sequences that bind selectively to a specific receptor overexpressed on cancer cells (genetic engineering strategy). For instance, PVX and TBSV CP have been engineered to display heterologous polypeptide at their N- or C-terminus respectively [12–14]. Internal sequence regions generating external structure loops following CP folding have been used for the same purpose with Cowpea mosaic virus [15].

The peculiar virus features have allowed the development of two different methods for spherical pVNPs loading: reversal viral pore gating (also termed infusion) and *in vitro* controlled reassembly [11]. By choosing the strategy in accordance with the proper viral system, a vast plethora of compounds can be loaded into the virus core.

Reversal viral pore gating relies on reversible conformation changes of capsid structure that generate pores. These pores allow the diffusion into the viral core of molecules that are entrapped upon the recovery of the native structure. This loading method is very efficient but is limited to molecules smaller than the pore size. It was estimated that about 900–1000 doxorubicin molecules could be encapsulated in red clover necrotic mosaic virus. This loading density (number of doxorubicin molecule/particle volume) is comparable to that of Doxil®, an approved liposomal doxorubicin formulation [16,17].

In vitro controlled reassembly contemplates viral particle disassembling and reassembling in the presence of the material to be encapsulated. This method has allowed the encapsulation of macromolecules (*e.g.*, nucleic acids, proteins, synthetic polymers) [18,19] and gold NPs [20].

Protein based NPs such as PVX and TBSV are considered ideal in terms of biocompatibility, because their biodegradability should prevent toxicity due to accumulation in the body. Plant viruses have evolved to infect plant hosts, and to this aim have developed infection strategies totally different from those adopted by animal viruses. Due to the lack of specific receptors for recognition and entry into animal cells, they cannot infect human cells. Nonetheless, even if they behave in these systems as unreplicative and biologically safe nano-objects, a possible intrinsic toxicity cannot be underestimated.

This work represents a first step for the safety evaluation of PVX and TBSV in view of their forthcoming use as functionalized nanocarriers. *In vitro* haemolysis assay has been employed to test cytotoxic effects while early embryo assay (EEA) was used to evaluate toxicity and teratogenicity *in vivo*.

2. Materials and methods

2.1. Production and molecular characterization of pVNPs

PVX and TBSV propagation in *N. benthamiana* L. plants as well as extraction and purification from plant tissues were performed as previously described [12–14]. Protein concentration (*i.e.*, CP concentration) in each preparation was determined using the bicinchoninic acid protein assay kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) and further verified through 12.5% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of several dilutions of purified pVNPs together with known quantities of bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO, USA) for the comparison of the intensities of Coomassie-stained bands. Purity was verified through SDS-PAGE and silver staining. The viral yield was calculated referring to the grams of fresh leaves tissue used for the extraction.

2.2. Structural characterization of the particles

NPs morphology was characterized by transmission electron microscopy (TEM) (Philips EM 400T, Eindhoven, The Netherlands). A drop of the purified pVNP suspension was placed onto the surface of a 200 mesh formvar® coated copper grid (TAAB Laboratories Equipment Ltd., Aldermaston, UK) previously treated with a poly-L-lysine solution. After 3 min, the pVNPs suspension was drawn off and replaced with a drop of negative staining agent (2% w/v phosphotungstic acid, pH 6.5). After 2 min, the stain drop was drawn off and the sample dried at room temperature (RT). Carbon nanoparticles (CNPs) (Sigma Aldrich Co., Saint Louis, MO, USA) and single wall carbon nanotubes (SWCNTs) [21] were suspended under sonication in ultrapure water and ethanol, respectively, and placed on 200 mesh formvar® coated copper grids. The samples were dried at RT and observed without negative staining.

Particles size was evaluated by dynamic light scattering (DLS), using a Nicomp 380 autocorrelator (PSS Inc., Santa Barbara, CA, USA) equipped with a Coherent Innova 70-3 (Laser Innovation, Moorpark, CA, USA) argon ion laser and a Peltier device for temperature control. The scattered light was detected at 90° from the incident light. TBSV samples were prepared by dispersing 250 μg of viral particles in 250 μL of acetate buffer (50 mM, pH 5.2) while CNPs (nominal particle size determined by TEM < 50 nm; Sigma Aldrich Co., Saint Louis, MO, USA) were dispersed in ultrapure water. Analyses were performed at 20 °C, for 15 min, in triplicate.

2.3. Incubation of pVNPs in murine serum and plants infection

Whole blood was collected from C57BL/6J mice (Harlan Laboratories, Inc., Indianapolis, IN, USA) and allowed to clot by incubation at RT for 15 min. The clot was pelleted through centrifugation at 2000 × *g* for 10 min at 4 °C and the overlying serum collected. pVNPs were diluted in serum at a concentration of 400 ng/μl and incubated at 37 °C for 0.5, 2, 4 and 24 h. In control samples pVNPs were diluted and incubated with phosphate buffered saline (PBS; 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 150 mM NaCl, pH 7.2). At each time interval, an aliquot of pVNPs was collected and used to infect *N. benthamiana* L. plants, as described above (with 20, 2 or 0.2 μg/leaf). As additional control, plants were infected with 20 μg/leaf of pVNPs diluted in PBS or serum but not incubated at 37 °C, to test if serum *per se* could hamper viral infectivity.

Plants were daily controlled to monitor the onset of infection symptoms.

2.4. Haemolysis assay

Human blood was drawn in S-monovettes (Sarstedt, Nümbrecht, Germany) containing EDTA-K_3 as anti-coagulant. Red blood cells (RBCs) were washed three times with 0.9% NaCl by centrifugation at 1200 × *g*, RT, no brake for 10 min and then suspended in Dulbecco's PBS Ca^{2+} and Mg^{2+} Free (DPBS CMF) (1:10). Two hundred μl of the RBCs suspension (corresponding to about 9×10^7 cells) were distributed in triplicate into Eppendorf tubes containing 800 μl of 10% Triton X-100 (positive control), DPBS CMF