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Chitosan nanocarriers-mediated delivery of double-stranded RNA *in planta*

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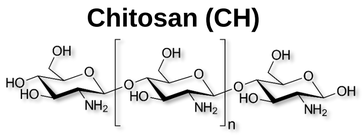
Introduction - Nowadays agricultural research is dealing with innovative approaches for an eco-sustainable management of crops. One of the most interesting innovations is the application of nanomaterials as shuttles for the efficient transport of biological agents aiming to protect plants from biotic or abiotic stresses [1]. At the same time, the study of alternative defense methods based on molecular techniques is raising interest, including the exploitation of the RNA-interference (RNAi) mechanism [2]. We tried to combine the two technologies, using chitosan (a natural and environment-friendly polymer) nanoparticles (NPs) to be used as carriers for dsRNA sequences. The nucleotide sequence used was total RNA obtained from a transformed *E. coli* strain able to synthetize the dsRNA of GFP-protein [3]. Here earlier results are shown, concerning (1) the synthesis and characterization of two types of NPs and their functionalization with dsRNAs, (2) the evaluation of their retention capacity and (3) a preliminary test of leaf distribution of the most promising NPs, doped with fluorescein-isothiocyanate (FITC) to allow visualization through confocal microscopy.

Experimental design & Results

A. Filtration of CH stock solution (0.2 µm filter)

Chitosan (CH)



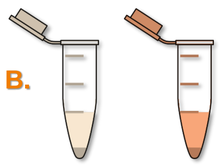
MW: 100 000 – 300 000 $gmol^{-1}$

B. Oxidative degradation treatment of CH stock solution for 24h (6% H_2O_2) [4]

1. NPs synthesis
Ionic gelation
&
Functionalization
20 µg/mL dsRNAs

2. Sonication
3 pulses of 15 sec
50 W

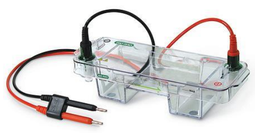
A. **NPsF** **dsRNA-NPsF**



B. **NPsD** **dsRNA-NPsD**

Particle sizer/ζ potential Analyzer

Dynamic Light Scattering (DLS) & Electrophoresis (Agarose 1.5%, 60 V x 100 min)



Mini-Sub Cell®GT Agarose Gel Electrophoresis System

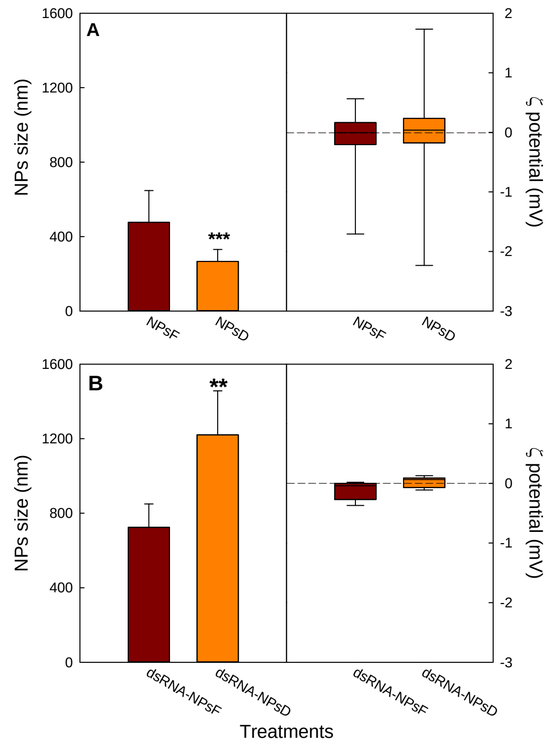


Fig.1 Size and ζ potential of NPsF and NPsD before (**A**) and after (**B**) their functionalization with dsRNAs. Data are means ± SD (n = 6). The significance of the applied t test is *P* = 0.000 (**A**) and *P* = 0.017 (**B**). NPsD are not suitable for functionalization since they increase their size much more than NPsF.

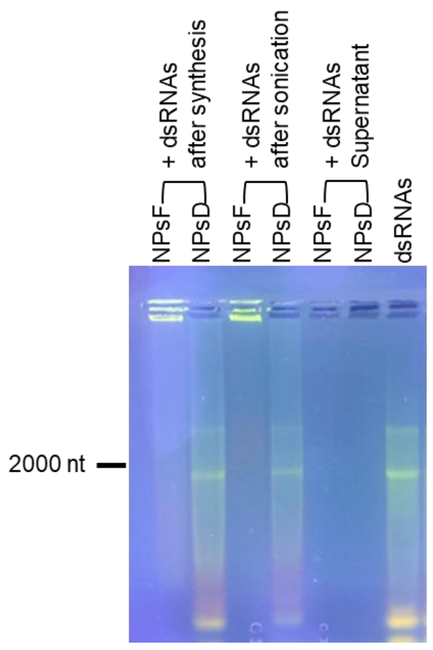


Fig.2 Electrophoretic evaluation of the dsRNAs retention capacity of NPsF and NPsD. Only NPsF are able to retain dsRNAs under electric field. Sonication does not cause loss of material, as shown by the absence of signal in the supernatant.

0.5 mL per leaf

Nicotiana benthamiana L.
single leaf treatment

NPsF functionalized with FITC (fluorescein-isothiocyanate) **resuspended in 0.1% EIA** (Ethoxylated isodecyl alcohol)

Confocal microscopy analisys (Leica TCS SP8)

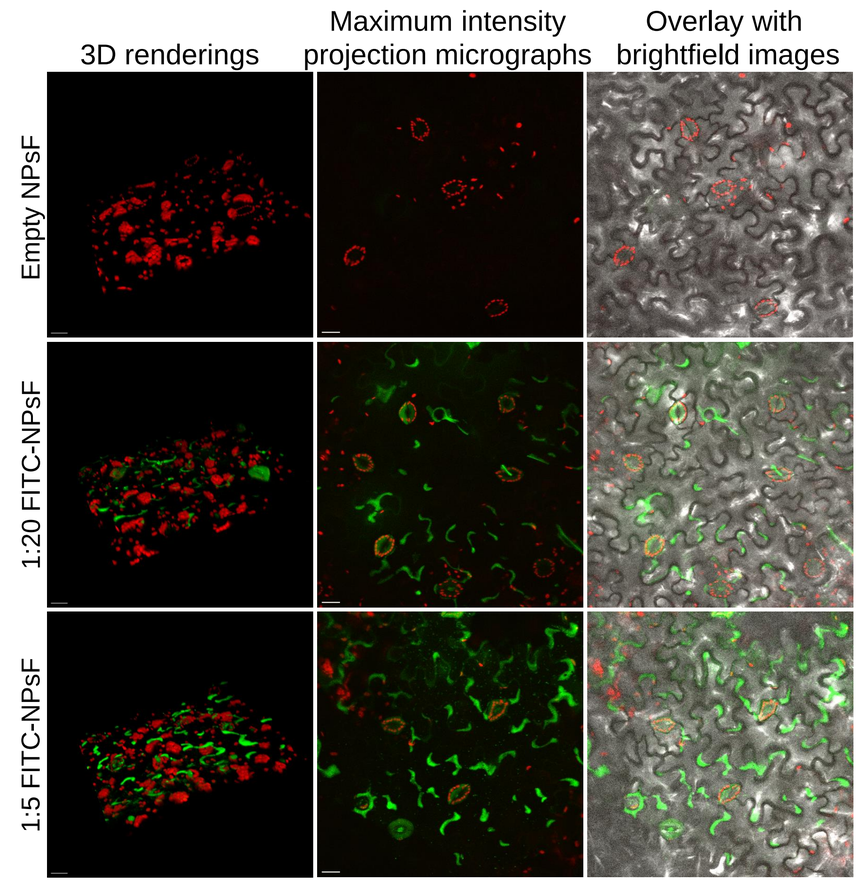


Fig.3 Confocal microscopy analysis of abaxial surface of *N. benthamiana* leaves sprayed with FITC-NPsF resuspended in 0.1% EIA. Green channel: FITC; red channel: chlorophyll autofluorescence. Scalebar 20 µm. NPsF are able to adhere on the leaf surface in a dose-dependent manner and concentrate mainly along the tangential cell walls of the epidermis.

Conclusions – The results revealed how the different treatments applied to the chitosan polymer, aiming to reduce its molecular weight [4], led to obtain NPs with different characteristics. Empty NPsF, even though characterized by a larger hydrodynamic diameter compared to NPsD, turned out to be the best choice both in terms of post-functionalization dimensions and dsRNAs retention. For this reason, NPsF have also been used for a first application test on leaf, showing their adhesion capacity when formulated with a wetting agent commonly used in agriculture. These findings are promising for future studies concerning the application on whole plants of NPs loaded with nucleotide sequences for triggering RNAi against plant pathogens.

References [1]Worrall *et al.*, 2018 doi:10.3390/agronomy8120285 [2]Giudice *et al.*, 2021 doi: 10.1111/pbi.13605 [3]Nerva *et al.*, 2020 doi:10.3390/biom10020200 [4]Zhao & Wu, 2006 doi.org/10.1016/S1872-2040(07)60015-2