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Author	Family Name	**Cheli**
	Particle	
	Given Name	**Franco**
	Suffix	
	Organization	LENS – European Laboratory for Non-linear Spectroscopy, University of Florence
	Address	Florence, Italy
Corresponding Author	Family Name	**Falsini**
	Particle	
	Given Name	**Sara**
	Suffix	
	Division	Department of Biology
	Organization	University of Florence
	Address	Florence, Italy
	Email	sara.falsini@unifi.it
Author	Family Name	**Salvatici**
	Particle	
	Given Name	**Maria Cristina**
	Suffix	
	Organization	Institute of Chemistry of Organometallic Compounds (ICCOM)-Electron Microscopy Centre (Ce.M. E.), National Research Council (CNR)
	Address	Florence, Italy
Author	Family Name	**Ristori**
	Particle	
	Given Name	**Sandra**
	Suffix	
	Division	Department of Chemistry
	Organization	University of Florence
	Address	Florence, Italy
Author	Family Name	**Schiff**
	Particle	
	Given Name	**Silvia**

	Suffix	
	Division	Department of Biology
	Organization	University of Florence
	Address	Florence, Italy
Author	Family Name	**Corti**
	Particle	
	Given Name	**Emilio**
	Suffix	
	Division	Department of Biology
	Organization	University of Florence
	Address	Florence, Italy
Author	Family Name	**Costantini**
	Particle	
	Given Name	**Irene**
	Suffix	
	Organization	LENS – European Laboratory for Non-linear Spectroscopy, University of Florence
	Address	Florence, Italy
Author	Family Name	**Gonnelli**
	Particle	
	Given Name	**Cristina**
	Suffix	
	Division	Department of Biology
	Organization	University of Florence
	Address	Florence, Italy
Author	Family Name	**Pavone**
	Particle	
	Given Name	**Francesco Saverio**
	Suffix	
	Organization	LENS – European Laboratory for Non-linear Spectroscopy, University of Florence
	Address	Florence, Italy
Author	Family Name	**Papini**
	Particle	
	Given Name	**Alessio**
	Suffix	
	Division	Department of Biology
	Organization	University of Florence
	Address	Florence, Italy

Abstract	The microscopic visualization of nanoparticles in plants is crucial to elucidate the mechanisms of their uptake through the cell wall and plasma membrane and to localize the possible sites of their extracellular or intracellular accumulation. Lignin nanocarriers are polymeric hollow nanocapsules able to contain and transport several bioactive substances inside plant tissues. We describe here a method for the preparation of Fluorol Yellow 088-labeled lignin nanocapsules that allow their localization in plant organs and tissues by fluorescence microscopy.
Keywords (separated by '-')	Lignin nanocarriers - Nanocapsules - Fluorescence microscopy - Confocal microscopy - Two-photon microscopy - Scanning electron microscopy

Chapter 28

Fluorescent Labeling of Lignin Nanocapsules with Fluorol Yellow 088

Franco Cheli, Sara Falsini, Maria Cristina Salvatici, Sandra Ristori, Silvia Schiff, Emilio Corti, Irene Costantini, Cristina Gonnelli, Francesco Saverio Pavone, and Alessio Papini

Abstract

The microscopic visualization of nanoparticles in plants is crucial to elucidate the mechanisms of their uptake through the cell wall and plasma membrane and to localize the possible sites of their extracellular or intracellular accumulation. Lignin nanocarriers are polymeric hollow nanocapsules able to contain and transport several bioactive substances inside plant tissues. We describe here a method for the preparation of Fluorol Yellow 088-labeled lignin nanocapsules that allow their localization in plant organs and tissues by fluorescence microscopy.

Key words Lignin nanocarriers, Nanocapsules, Fluorescence microscopy, Confocal microscopy, Two-photon microscopy, Scanning electron microscopy

1 Introduction

Lignin nanocarriers are hollow polymeric nanocapsules (NCs) that can be used as devices to transport several bioactive substances. One of the advantages of lignin nanoparticles is that their chemical nature is natural (lignin is produced by land plants) and hence are less toxic than other types of nanoparticles [1] and completely biodegradable [2]. This aspect is fundamental if the use of nanoparticles is carried out releasing them in the environment in order to obtain a specific effect, particularly in agriculture [3, 4].

Several polymers (in particular starch, alginate, chitin, albumin, and cellulose) were tested to produce nanoparticles in plant science with the function of transporting bioactive compounds [5, 6]. Another polymeric β-glucan, chitosan, can be obtained by extraction from crustaceans chitin and shows antifungal activity [4], while alginate can be extracted from the epidermis of brown algae

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(Phaeophyceae) and can be used for building nanoparticles able to transport insecticides [7].

Lignin NCs contain nanocavities composed of a lignin shell matrix that traps the bioactive compounds also protecting them from degradation [8]. Such nanocavities may contain lipophilic substances that are normally difficult to transfer inside living organisms. Lignin is a plant-produced substance that may hence be considered particularly suitable for the delivery of lipophilic substances inside plants such as hormones, biocides, and insecticides [4, 5, 7], with the function of modulating growth and health status of the plant. Moreover, lignin is the main waste produced by paper and cellulose production [2, 9].

The preparation of lignin NCs can be obtained by engineering the solvent/co-solvent interface for polymer-based nanosystems with several possible protocols, depending on the chemical property of the inner core/cavity and the shell of the nanocapsule and the nature of the cargo to be carried [10].

The microscopic visualization of nanoparticles in plants is of fundamental importance to observe if they enter the plant, in which tissues they tend to accumulate, if they are able to penetrate the wall and plasma membrane, in which part of the cells they can be stored, and finally what an effect they have at the tissue and cell level, even possible toxicity, which is another relevant concern related to the use of NCs in agriculture [2].

The protocol we describe here allows the preparation of lignin NCs labeled with Fluorol Yellow 088, a lipophilic fluorescent dye that makes them observable in plant tissues and cells by fluorescence microscopy.

2 Materials

2.1 Samples and Chemicals

For all the solutions, use ultrapure Milli-Q filtered water.

1. Plants seeds from both monocots and dicots (*see* **Note 1**).
2. Commercially available Kraft lignin.
3. Olive oil.
4. 0.1% Fluorol Yellow 088 (FY088) stock solution: dissolve 0.1 g of FY088 in 100 mL of olive oil, as modified by Giuliani et al. [12].
5. 0.1-M phosphate buffer saline (PBS): add 9 g of NaCl to 500 mL of water under gentle agitation by magnetic stirrer; add 50 mL of PB (phosphate buffer 0.2-M, pH 7.4); bring the solution to a final volume of 1 L with water and adjust to pH 7.4 using 1-N NaOH.

2.2 Equipment

1. Ultrasonic processor (we used a Branson 450 Digital Sonifier).
2. Apparatus for dynamic light scattering measurements (we used Malvern Zetasizer Nano ZS, ZEN 1600 system).
3. Cryostat.
4. Scanning electron microscope (we used Gaia 3 Tescan s.r.o, Brno, Czech Republic) and sputtering system.
5. Bright-field and epifluorescence microscope (*see* **Note 2**).
6. Polydimethylsiloxane (PDMS)-coated petri dishes.
7. A custom-made two-photon fluorescence microscope (TPFM) was also used. We employed the apparatus described in Costantini et al. [13] that enable mesoscopic reconstruction of biological samples (*see* **Note 3**).

3 Methods

3.1 Preparation of Lignin Nanoparticles

1. Dissolve 1 g of Kraft lignin powder in 100 mL of 1% KOH, to obtain a lignin alkaline solution.
2. Prepare olive oil/acetone emulsions. (A) For empty nanoparticles, add olive oil to acetone dropwise, at a 1:1 (v/v) ratio; (B) for dye-loaded nanoparticles, add 500 mL of FY088 stock solution in olive oil to acetone dropwise, at a 1:1 (v/v) ratio.
3. Add 300 μL of olive oil/acetone emulsions to 3 mL of the lignin alkali solution.
4. Emulsify the oil/water phase by applying high-frequency ultrasounds, which facilitate the incorporation of oil or oil plus FY088 into the pre-existing lignin aggregates, thus forming the final NCs dispersion (Fig. 1) (*see* **Note 4**).

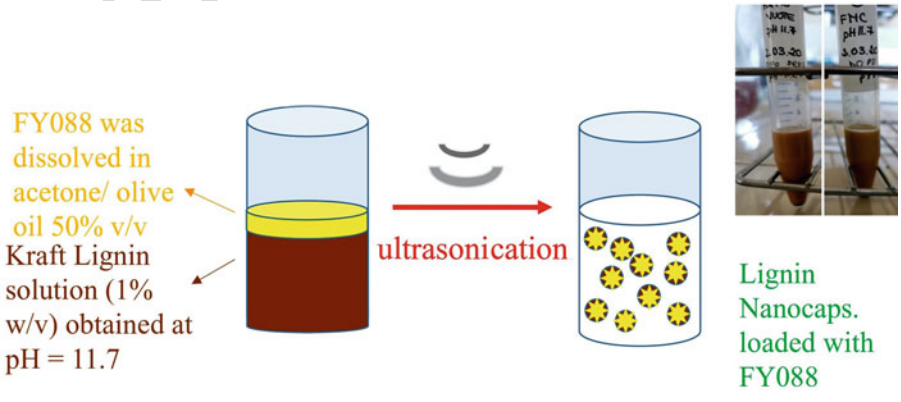

Fig. 1 Preparation of the fluorescent nanocapsules

The final pH of the lignin NCs loaded with FY088 (fNCs) is close to neutrality (6.8–7.2) that is a suitable range for organisms and can be applied to biological systems in vitro.

3.2 Physicochemical Characterization of Empty and FY088-Loaded NCs

For DLS measurements:

1. Dilute the samples 1:500 with water, to adjust the optical turbidity.
2. Perform the diameter measurements, and average the results over an adequate number of runs (*see* **Note 5**).

For the fluorescence emission measurements:

1. Dilute 100 μL of the stock solution (0.1% FY088 in oil) in 1.9 mL of olive oil, to prepare the FY088 0.005% working solution at the same concentration used for the nanoformulation.
2. Perform emission spectra recorded in the range 480–570 nm with fixed excitation wavelength (470 nm) and 125-nm/min acquisition time (Fig. 2) (*see* **Note 6**).

SEM observations (to be performed to investigate if the morphology of fNCs may be affected by dye loading):

1. Deposit the samples on a stub.
2. Dry in a vacuum.

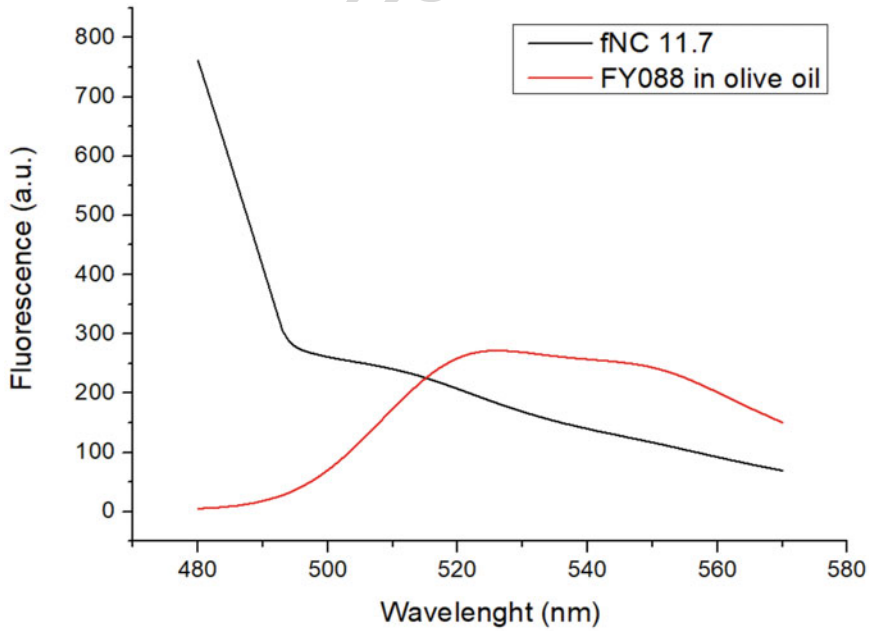

Fig. 2 Emission spectra of FY88 alone (green curve) and after encapsulation into the NCs at fixed excitation wavelength (470 nm)

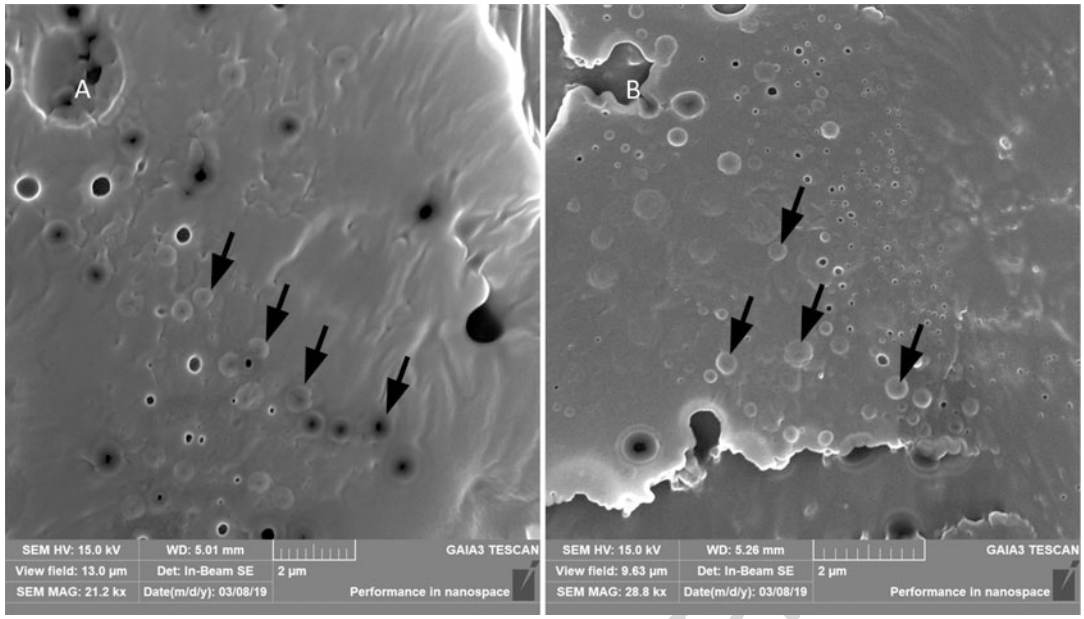

Fig. 3 SEM micrograph of (**a**) empty NCs (magnification: 21,200) and (**b**) fNCs (in average smaller than the empty ones; magnification: 28,800)

3. Coat with an ultrathin gold coating to enhance the contrast, thanks to the presence of an electrically conducting material.
4. Perform the NCs morphology observations (Fig. 3a, b) (*see* **Note 7**).

3.3 Fluorescence Microscope Observation

1. Grow the seedlings (we used *Eragrostis teff* and *Eruca sativa*) on wet paper, and make them in contact with the fNCs for 24 h. Dilute the final NCs dispersion 1:1 with water.
2. Cut 20- to 30-µM-thick sections by a cryostat, to obtain longitudinal and cross sections.
3. Place the sections on microscope slides in a drop of water, and gently coverslip.
4. Observe the sections in bright-field and epifluorescence microscopy using blue light excitation (450–480 nm).

Epifluorescence images showed that the fNCs tended to preferentially concentrate in some cells toward the middle of the root (Fig. 4a): at higher magnification (Fig. 4b), the mostly involved cells proved to be the xylem vessels (*see* **Note 8**).

3.4 Two-Photon Observation

1. A mode-locked Ti-Sapphire laser (Chameleon, 120 fs pulse width, 80-MHz repetition rate, Coherent, CA) operating at 900 nm was coupled into a custom-made scanning system based on a pair of galvanometric mirrors (LSKGG4/M, Thorlabs, USA).

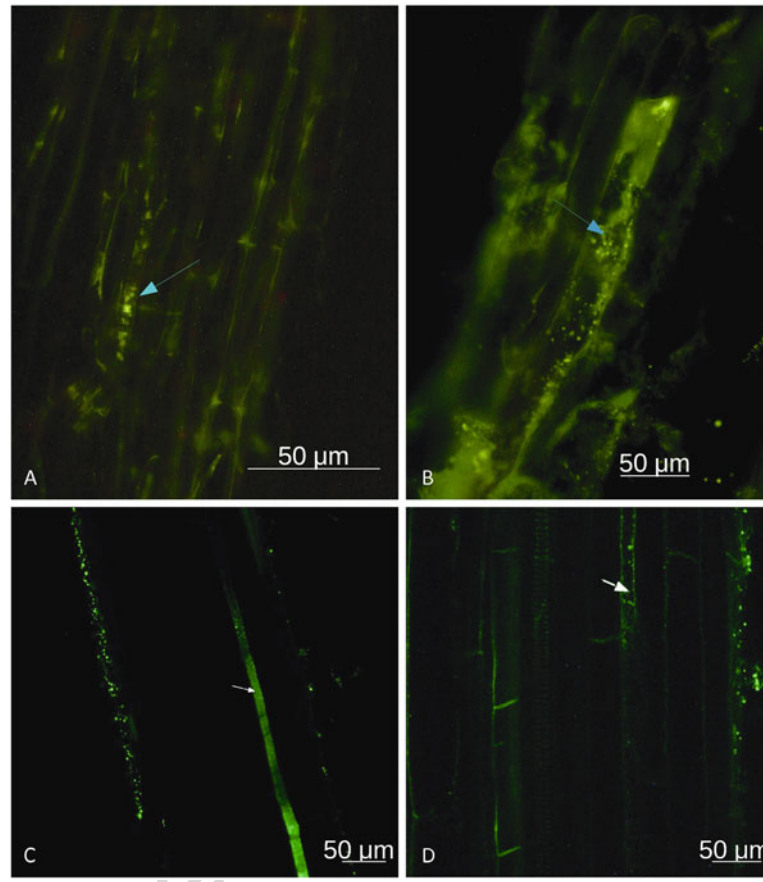

Fig. 4 (**a**) *Eragrostis teff* root, epifluorescence image: the fNCs (arrow) tended to concentrate preferentially in some cells close to the middle of the root (arrow). (**b**) *Eragrostis teff* root. Same as panel (**a**), but at higher magnification, the cells mostly involved appeared to be xylem vessels (arrow). (**c**) *Eruca sativa* root, two-photon microscope image: the fNCs lined the root epidermis (rhizodermis) and were recorded also in specific cell lines inside the root (white arrow). (**d**) *Eruca sativa* root, two-photon microscope image: the fNCs were also observed inside longitudinal lines of cell of the root central cylinder (white arrow)

2. The laser was focused onto the specimen by a refractive index tunable 25× objective lens (LD LCI Plan-Apochromat 25×/0.8 Imm Corr DIC M27, Zeiss, Germany).
3. The sample was mounted on a PDMS-coated petri dish using two pins that enable to immobilize the sample (see **Note 9**).
4. Emission filter of 530 ± 55 nm was used to detect the signal for Fluorol Yellow 088 (*see* **Notes 10** and **11**). The acquisition was performed using a FOV of 450 µM resulting in 1024 × 1024 pixels images that were saved as TIFF files.

   The reduction in noise with respect to epifluorescence was clearly observed both in the epidermis areas (Fig. 4c) and inside longitudinal lines of cell inside the root central cylinder (Fig. 4d).

4 Notes

1. Here we used Teff, *Eragrostis teff* (Zucc.) Trotter, belonging to the family Poaceae, and Arugula, *Eruca sativa* L. Cav., belonging to the family Brassicaceae. The seeds were left to germinate on wet paper and later hydroponically cultivated.
2. If available, confocal or two-photon fluorescence microscopy is especially appropriate due to their high-resolution and optical sectioning capability, which allow imaging the nanoconstructs through the tissue depth (as deep as about half the thickness of a seedling root, in the species we studied).
3. Two-photon fluorescence microscopy, thanks to the use of an infrared laser, allows excitation light to penetrate deep into tissues while offering high axial and lateral resolution.
4. This step was conducted in mild conditions in order to avoid possible damage of labile molecules [1]. Specifically, the apparatus power was kept at 200 W and 5 cycles of 3 min (1 s pulse on and 0.5 s pulse off) were used.
5. To measure the average diameter of the fNCs, we used a Malvern Zetasizer Nano ZS (ZEN 1600) apparatus, equipped with a He-Ne 633 nm, 4 mW laser, and backscattering optics at 173° detection. Each measurement was averaged over 11 runs and taken in duplicate. DLS measurements showed that the unlabeled NCs had a mean diameter of 204 ± 20 nm and a polydispersity index (PDI) of 0.35, while the fNCs had a mean diameter of 230 ± 20 nm with a moderate PDI (0.25).
6. The emission wavelength of the encapsulated FY088 is close to green (510 nm, Fig. 4) as indicated by the shoulder in the emission curve (Fig. 2), with a shift compared to FY088 in olive oil (520 nm, yellow), which confirms the association of this dye with the lignin in the NCs.
7. SEM micrographs showed that both empty (Fig. 3a) and loaded (Fig. 3b) NCs had a spherical shape with a homogeneous surface. When one or more size distributions are present in solution, SEM tends to evidence larger particles, while DLS reports on the statistical average over all the volume.
8. Noise in epifluorescence imaging can be reduced by averaging or moding a given number of images of the same field. Some microscope camera software can do the averaging during the acquisition, while for moding specific Python software should be used [1].
9. Roots were completely immersed in tap water during the acquisition.

10. The system was equipped with a closed-loop XY stage (U-780 PILine® XY Stage System, 135 × 85-mM travel range, Physik Instrumente, Germany) for the radial displacement of the sample and with a closed-loop piezoelectric stage (ND72Z2LAQ PIFOC objective scanning system, 2-mM travel range, Physik Instrumente, Germany) for the displacement of the objective along the z-axis. The fluorescence signal was collected by a GaAsP photomultiplier module (H7422, Hamamatsu Photonics, NJ).
11. The instrument was controlled by a custom software, written in LabView (National Instruments, TX) able to acquire 3D stacks through the depth of the sample by performing z-stack imaging with a voxel size of $0.44 \times 0.44 \times 2\ \mu M^3$ up to 180 μM of depth.

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