Fluorescent Labelled Iron Oxide Nanoparticles for Tracing Their Uptake Behavior by Plant

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Abstract: The clarification on the transportation pathway of iron oxide nanoparticles in plants is prerequisite to profoundly understanding their biological mechanism toward plants. In this study, fluorescein isothiocyanate (FITC)-conjugated γ-Fe₂O₃ nanoparticles were prepared through a strategy of siloxane coupling. The crystalline structure and size of nanoparticles didn’t change after the FITC-conjugation process. Hence, such fluorescence labelled γ-Fe₂O₃ nanoparticles were used to investigate their uptake behavior by watermelon. So the entry and apoplastic pathway were disclosed. The results verified that the nanoparticles firstly penetrated into the root, and then migrated among cells from epidermis to endodermis.

Keywords: γ-Fe₂O₃ nanoparticles; Fluorescence labelling; uptake behavior; watermelon

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1. Introduction

Recently, it has drawn great concern to investigate the transportation pathway of nanomaterials and their accumulation behaviors in plants using the fluorescence labelling technology.¹⁻⁴ The obtained results contribute to profoundly understanding the biological effect and action mechanism of nanoparticles towards plants. For example, fluorescein isothiocyanate (FITC), a universal fluorescent molecule, was used to label CeO₂ nanoparticles.⁵ The blue fluorescent aggregates, which were assigned to FITC-conjugated CeO₂ nanoparticles, were observed in the cell walls of epidermis and cortex, suggesting an apoplastic pathway of CeO₂ nanoparticles in the corn roots.⁶ In addition, the labelling method of FITC was also used to investigate the uptake mechanism of carbon nanotubes by plant cells. Firstly, the intense fluorescence signal of single-walled carbon nanotubes (SWCNT) conjugating with FITC was observed in a kind of plant cell, the Bright Yellow cells (BY-2).⁷ It suggested that SWCNT could pass through the cell wall of plant. Moreover, the fluorescent signal of FITC-conjugated multiwalled carbon nanotubes (MWCNT) was observed in only a few endosomes of Catharanthus roseus protoplasts.⁸ The further study proved that the fluorescent intensity in protoplasts had almost no impact if such endosomal route was blocked, suggesting the entry of MWCNT into protoplasts complied with the mode of endosome-escaping uptake. The above study showed that FITC-labelling technology was very valuable for the uptake behavior of engineered nanomaterials in plant.

Iron plays an important role in the physiological activities of plants,⁹⁻¹¹ and is essential to the synthesis of chlorophyll. The lacking of iron element in the plants will directly result in the leaf yellowing of iron deficiency. In order to solve the shortcomings of traditional iron-supplementing method, such as low efficiency, great loss and so on, iron oxide nanoparticles are attempted to develop as a new agent for supplying iron in view of the fact that many previous reports have proved high-efficient uptake of nanoscaled particles by plants.⁸,⁹ According to this viewpoint, the iron oxide nanoparticles were used to cultivate the seeds of watermelon¹⁰ and mung bean.¹¹ It was found that iron oxide nanoparticles at proper concentrations could boost seed germination and root
growth together with the promotion of chlorophyll synthesis.\textsuperscript{[10, 11]} However, the mechanism of these positive biological effects remains unclear, and meanwhile, the nano-safety for the extensive application of iron oxide nanoparticles as an iron-supplement agent of plants is not verified. For this, the transportation pathway of iron oxide nanoparticles and their accumulation behaviors in plants should be clarified first of all.

In this work, the fluorescence labelling technology was used to explore the uptake behavior of iron oxide nanoparticles by watermelon and their transportation pathway and location in watermelon. Because the size of nanoparticles was directly relevant to its uptake by plants\textsuperscript{[10, 12]} as well as its transportation and location in plants, the process of fluorescence labelling was required not to interfere with the original size of nanoparticles. So the silane coupling agent was selected as an intermediate to conjugate the organic fluorescent species of FITC onto the surface of $\gamma$-Fe$_2$O$_3$ nanoparticles. The structure of FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles were characterized by Fourier transform infrared spectroscopy (FTIR), X-ray powder diffraction (XRD) and transmission electron microscope (TEM), and their optical property was evaluated by UV/Vis spectroscopy and fluorescence microscope. Moreover, the suspension containing FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles was used to cultivate the watermelon seedlings for revealing their uptake and transportation behaviors in watermelon root.

2. Methods

2.1. Materials

All the reactants and solvents were of analytical grade, and used as received. Iron (III) chloride hexahydrate (FeCl$_3$·6H$_2$O), iron (II) chloride tetrahydrate (FeCl$_2$·4H$_2$O), aqueous ammonia (NH$_3$·H$_2$O), oleic acid, muriatic acid, cyclohexane, methanol, ethanol, paraformaldehyde were purchased from Shanghai Chemical Regents Ltd. Co. (Shanghai, China). Fluorescein isothiocyanate (FITC), (3-aminopropyl) triethoxysilane (APTES), NP-40 and tetraethyl orthosilicate (TEOS) were purchased from Aladdin Regents Ltd. Co. (Shanghai, China). In addition, the watermelon seeds were kindly provided by Hubei Academy of Agricultural Sciences (Wuhan, China).

2.2. Preparation of $\gamma$-Fe$_2$O$_3$ nanoparticles

Firstly, the Fe$_2$O$_3$ nanoparticles were synthesized according to a traditional co-precipitation method.\textsuperscript{[13]} A solution with a molar ratio of Fe (III) vs. Fe (II) as 2:1 was prepared by mixing FeCl$_3$·6H$_2$O and FeCl$_2$·4H$_2$O in 200 mL distilled water. The solution was placed into a three-neck round-bottom flask equipped with a mechanical stirrer, reflux condenser and an inlet of nitrogen atmosphere, and then was stirred vigorously for 15 min under nitrogen atmosphere. Thereafter, the reaction temperature was heated up to 60 °C for another 30 min under vigorous stirring. Meanwhile, an approximately equal volume of 1 M aqueous ammonia solution was added to adjust the pH value. After cooling down to ambient temperature, Fe$_2$O$_3$ nanoparticles were collected, and then further purified by re-dispersing in ethanol and subsequent centrifuging at 8000 rpm for 5 min. This purification procedure was repeated five times. At last, the purified Fe$_2$O$_3$ nanoparticles were vacuum-dried at 60 °C for 24 h.

Subsequently, $\gamma$-Fe$_2$O$_3$ nanoparticles were prepared by the oxygenation process of the purified Fe$_2$O$_3$ nanoparticles. 0.2 mL of oleic acid was added into 200 mL suspension containing Fe$_2$O$_3$ nanoparticles with a concentration of 2 g/L, and then mechanically stirred for 6 h at 90 °C. At the same time, an amount of muriatic acid solution was added dropwise to adjust the pH as 2 ~ 3. After cooling down to ambient temperature, $\gamma$-Fe$_2$O$_3$ nanoparticles were separated using magnet, and then washed with distilled water. At last, $\gamma$-Fe$_2$O$_3$ nanoparticles were vacuum-dried.

2.3. Conjugation of FITC on the surface of $\gamma$-Fe$_2$O$_3$ nanoparticles

The process of conjugating FITC on the surface of $\gamma$-Fe$_2$O$_3$ nanoparticles was schematically illustrated in Figure 1.

Firstly, 10 mg of FITC and 48 mg of APTES were loaded into a dry round-bottom flask containing 2 mL cyclohexane, and then was mechanically stirred in the dark for 24 h. In this stage, the isothiocyanano group of FITC reacted with the amino group of APTES. On the other hand, 10 mg of $\gamma$-Fe$_2$O$_3$ nanoparticles were added into 2 mL cyclohexane containing NP-40, which was formulated by mixing 3 mL NP-40 and 80 mL cyclohexane, and then mechanically stirred for 5 min. Subsequently, 750 μL of the above solution and 0.65 mL of aqueous ammonium hydroxide solution were
added dropwise into the suspension of $\gamma$-Fe$_2$O$_3$ nanoparticles and mechanically stirred for 24 h. Thereafter, 0.3 mL of TEOS was added into the above suspension for another 24 h of hydrolysis. The reaction product was extracted by methanol, and then the methanol-rich phase containing the nanoparticles was collected. After reducing the solvent by rotary evaporator, the product was purified by washing with ethanol and centrifuging at 8000 rpm for 8 min several times. Finally, the sediment of FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles was vacuum-dried at 60°C for 12 h.

2.4. Characterization

Fourier-transform infrared spectroscopy (FTIR) spectra of the powdered $\gamma$-Fe$_2$O$_3$ nanoparticles and FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles were recorded on a FTIR 5700 spectrometer (Nicolet, USA) in the range of 4000 ~ 400 cm$^{-1}$ by a method of KBr pellets.

Ultraviolet-visible (UV-vis) absorption spectra were recorded on a UV-2450 spectrophotometer (SHIMADZU). The specimens were made by dissolving powder samples in 1×PBS and then were loaded with quartz cuvette.

X-ray diffraction (XRD) patterns of the powdered $\gamma$-Fe$_2$O$_3$ nanoparticles and FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles were recorded on a D/max-RB X-ray (Rigaku Denki, Japan) using Cu K$_\alpha$ radiation at 35 kV and 30 mA in the 2θ range of 10 ~ 80°.

Transmission electron microscope (TEM) photographs of $\gamma$-Fe$_2$O$_3$ nanoparticles and FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles were taken with a JEM100 electron microscope (JEOL, Japan) at an acceleration voltage of 100 kV. The TEM specimens were made by putting one drop of the nanoparticle suspension on a carbon-coated copper grid, and then immediately removed most of the liquid with filter paper followed by solvent evaporating.

2.5. Fluorescent observation of FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles in watermelon root

The suspensions containing FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles were firstly sonicated for 30 minutes, and hence supplemented water to give a concentration of 0.02 g/L. The watermelon seedlings were cultivated in the above suspension containing FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles at ambient temperature, and this solution was replaced once a day. When watermelon roots grew up to 5 cm, the watermelon seedlings were taken out of the cultivated suspension and thoroughly washed in 1×phosphate buffered saline (PBS). At last, the roots of watermelon seedlings, which were treated by FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles, were fixed by 4 vol% paraformaldehyde for 6 h, and then sectioned by a razor blade for the observation of fluorescent microscope.

The root sections of watermelon seedlings, which were treated by FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles, were observed on an Olympus BX60 fluorescence microscope using U-MWIG (330-385/400/420 nm), U-MWIB (460-490/505/515 nm), or U-MWIG filters (510-550/570/590 nm). Moreover, the fluorescence photographs were taken with a cooled CCD camera (1410E B0, Sensys Photometrics) using the Meta Morph software (Universal Imaging, version 4.6r5).

3. Results and Discussion

3.1 Structure of the FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles

Figure 2 shows the FTIR spectra of $\gamma$-Fe$_2$O$_3$ nanoparticles and FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles. In Figure 2a, $\gamma$-Fe$_2$O$_3$ nanoparticles showed the characteristic peak located at 635 cm$^{-1}$, and two peaks located at 579 cm$^{-1}$ and 441 cm$^{-1}$, that were assigned to the vibrations of Fe$^{3+}$-O$^2$ of tetrahedral and octahedral sites in lattice, respectively.[14, 15] When the surface of $\gamma$-Fe$_2$O$_3$ nanoparticles was modified by FITC via silane coupling, the vibration bands of Si-O-Si located at 1095 cm$^{-1}$ and 801 cm$^{-1}$ as well as Si-H located at 953 cm$^{-1}$ were appeared in Fig 2b while the band of Fe-O located at 579 cm$^{-1}$ was still distinguished. It indicated that FITC could be successfully conjugated on the surface of $\gamma$-Fe$_2$O$_3$ nanoparticles via Si-O bonds.[16]

![Figure 2. FTIR spectra of $\gamma$-Fe$_2$O$_3$ nanoparticles (a) and FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles (b).](image-url)

Moreover, the crystalline structure of $\gamma$-Fe$_2$O$_3$ nanoparticles after the process of FITC conjugation was verified by XRD. Figure 3 shows the XRD patterns of $\gamma$-Fe$_2$O$_3$ nanoparticles and FITC-conjugated $\gamma$-Fe$_2$O$_3$ nanoparticles. In Figure 3a, the diffraction peaks located at the 2θ of 30.0°, 35.3°, 43.0°, 53.4°, 56.9° and 62.5° were assigned to the planes of (220), (311), (400), (422), (511) and (440) in the cubic spinel of $\gamma$-Fe$_2$O$_3$ nanoparticles, respectively.[14, 17] After
conjugating FITC, all the locations appeared, which indicated that the FITC-conjugated γ-Fe₂O₃ nanoparticles inherited the crystalline character of γ-Fe₂O₃ nanoparticles. So the crystalline structure of γ-Fe₂O₃ nanoparticles was not interfered by the process of silane coupling. However, a large peak corresponding to the amorphous SiO₂ phase and a small peak due to γ-Fe₂O₃ were shown in Figure 3b.

3.2 Morphology and size of FITC-conjugated γ-Fe₂O₃ nanoparticles

Except for no changes of crystalline structure, the fluorescent study on the uptake behavior of γ-Fe₂O₃ nanoparticles by watermelon required the identical morphology and size for FITC-conjugated γ-Fe₂O₃ nanoparticles. Figure 4 depicted the TEM images of γ-Fe₂O₃ nanoparticles and of FITC-conjugated γ-Fe₂O₃ nanoparticles. As seen in Figure 4a, γ-Fe₂O₃ nanoparticles showed a spherical shape with a uniform size of ca. 17.7±3.9 nm. After FITC was conjugated on the surface of γ-Fe₂O₃ nanoparticles, its spherical shape was preserved (in Figure 4b), but the size slightly increased as an average diameter of 21.2±2.9 nm. No drastically increase in the size of nanoparticles was the key to reflect the credible uptake behavior by watermelon. Increasing its size also showed that γ-Fe₂O₃ nanoparticles were coated by amorphous SiO₂.

3.3 Optical properties of the FITC-conjugated γ-Fe₂O₃ nanoparticles

Figure 5 showed the fluorescence absorption and emission spectra as well as the fluorescence photograph for FITC-conjugated γ-Fe₂O₃ nanoparticles. From Figure 5a and 5b, the maximum excitation and emission wavelength of the FITC-conjugated γ-Fe₂O₃ nanoparticles were at 484 nm and 516 nm, respectively, which were in the range of maximum excitation and emission wavelength of the FITC. This result confirmed the conjugation of FITC onto the γ-Fe₂O₃ nanoparticles. In addition, the fluorescence photograph in Figure 5c clearly depicted the imaging effect of the FITC-conjugated γ-Fe₂O₃ nanoparticles in 1×phosphate buffered saline (PBS), indicating a good dispersion and that FITC was successfully conjugated on the surface of γ-Fe₂O₃ nanoparticles. The good dispersibility of nanoparticle suspension was very important to cultivate the plants because great aggregates would inhibit the uptake ability of plants.

3.4 Uptake behavior of the FITC-conjugated γ-Fe₂O₃ nanoparticles by watermelon

Because the fluorescent intensity of FITC in the range of 510-550 nm bandpass filter was greatly stronger than the autofluorescence of the root tissues of plants, their autofluorescence would not interfere with the uptake study of FITC-labelled nanoparticles by plants.[4] In this work, the fluorescence signals of the watermelon root section treated with FITC-conjugated γ-Fe₂O₃ nanoparticles could also be detected by fluorescence microscope. Figure 6 depicted the fluorescence photographs observed under the blue channel (510-550 nm) for the cross-sections of watermelon root and the section of watermelon epidermis treated with FITC-conjugated γ-Fe₂O₃ nanoparticles for 72 h, and the white bright dots in the photographs represented the FITC-conjugated γ-Fe₂O₃.
nanoparticles. As seen in Figure 6a and 6d, the FITC-conjugated γ-Fe$_2$O$_3$ nanoparticles could be found in the epidermis, endodermis and cortex of the watermelon root tissue, and a few of FITC-conjugated γ-Fe$_2$O$_3$ nanoparticles located at the neighborhood of xylem. Furthermore, the magnified images from the Figure 6a for the cross-section of watermelon root, i.e. Figure 6b and 6c, showed that most of FITC-conjugated γ-Fe$_2$O$_3$ nanoparticles existed in intercellular space and cell walls of cortex tissues. It suggested that γ-Fe$_2$O$_3$ nanoparticles could penetrate into the root of watermelon and migrate among cells from epidermis to endodermis via the apoplastic pathway. Consequently, the fluorescence observation by conjugating fluorescent species onto the surface of nanoparticles could provide the visualized proof to understand the uptake behaviors of nanoparticles by plants.

![Image 6](image6.png)

**Figure 6.** Fluorescence photographs observed under the blue channel (510-550 nm) for the cross-sections of watermelon root (a, b and c; Photograph b and c are the magnification defined by the squares in Photograph a), and the section of watermelon epidermis treated with FITC-conjugated γ-Fe$_2$O$_3$ nanoparticles for 72 h. (The white bright dots in the photographs represented FITC-conjugated γ-Fe$_2$O$_3$ nanoparticles.)

4. Conclusion

In this study, a fluorescent specie of FITC was conjugated onto the surface of γ-Fe$_2$O$_3$ nanoparticles by the method of silane coupling, and hence was use to investigate the uptake behavior of γ-Fe$_2$O$_3$ nanoparticles by watermelon. The XRD and TEM results showed almost no changes of crystalline, morphology and size of nanoparticles after FITC conjugation. Furthermore, the fluorescent excitation, emission and imaging of FITC-conjugated γ-Fe$_2$O$_3$ nanoparticles were also verified. In this case, the fluorescence imaging contributed to the visualization of nanoparticle uptake by plants. Hence, it was proven that γ-Fe$_2$O$_3$ nanoparticles could penetrate into the root of watermelon and migrate among cells from epidermis to endodermis via the apoplastic pathway. Briefly, this work opened a visualized method to observing the uptake behaviors of nanoparticles by plants as well as the transportation and accumulation in plants. It is beneficial to profoundly understand the biological effects and action mechanism of nanoparticles toward plants.

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