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Nanoharvesting of bioactive materials from living plant cultures using engineered silica nanoparticles

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Abstract

Plant secondary metabolites are valuable therapeutics not readily synthesized by traditional chemistry techniques. Although their enrichment in plant cell cultures is possible following advances in biotechnology, conventional methods of recovery are destructive to the tissues. Nanoharvesting, in which nanoparticles are designed to bind and carry biomolecules out of living cells, offers continuous production of metabolites from plant cultures. Here, nanoharvesting of polyphenolic flavonoids, model plant-derived therapeutics, enriched in *Solidago nemoralis* hairy root cultures, is performed using engineered mesoporous silica nanoparticles (MSNPs, 165nm diameter and 950 m²/g surface area) functionalized with both titanium dioxide (TiO₂, 425mg/g particles) for coordination binding sites, and amines (NH₂, 145 mg/g particles) to promote cellular internalization. Intracellular uptake and localization of the nanoparticles (in Murashige and Skoog media) in hairy roots were confirmed by tagging the particles with rhodamine B isothiocyanate, incubating the particles with hairy roots, and quenching bulk fluorescence using trypan blue. Nanoharvesting of biologically active flavonoids was demonstrated by observing increased antiradical activity (using 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay) by nanoparticles after exposure to hairy roots (indicating general antioxidant activity), and by the displacement of the radio-ligand [³H]-methyllycaconitine from rat hippocampal nicotinic receptors by solutes recovered from nanoharvested particles (indicating pharmacological activity specific to *S. nemoralis* flavonoids). Post-nanoharvesting growth suggests that the roots are viable after nanoharvesting, and capable of continued flavonoid synthesis. These observations demonstrate the potential for using engineered nanostructured particles to facilitate continuous isolation of a broad range of biomolecules from living and functioning plant cultures.

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Declaration of competing interest

John Littleton, D. Trent Rogers and Jatinder Sambi are employees of Naprogenix Inc., and John Littleton owns stock in the company.

Appendix A. Supplementary data

Supplementary materials include procedures for silica nanoparticle synthesis and functionalization, nanoparticle characterization, fluorescence imaging of nanoparticle uptake in *S. nemoralis* hairy roots, and images from the study of viability of *S. nemoralis* hairy roots after exposure to nanoparticles.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.msec.2019.110190>.

Keywords

Therapeutics; Nanoharvesting; Engineered mesoporous silica; Nanoparticles; Cellular internalization; Phytotoxicity

1. Introduction

Plant cells are capable of synthesizing valuable secondary metabolites, in the form of small organic molecules that are potential and proven therapeutics that cannot readily be made by traditional synthetic chemistry [1–3]. Recent progress has been made in increasing the yields of target metabolites in plant cell cultures with genetic or environmental modification. Genetic sequencing and manipulation of biosynthetic pathways provide a vast resource of potential therapeutic agents [4–8]. It is also possible to select mutant plant cells for survival on the basis of their overproduction of bioactive metabolites to generate a population of mutants with a specific pharmacological phenotype [9]. Flavonoids, a class of secondary metabolites known to be active antioxidants with medicinal, therapeutic and pharmacological applications, have recently been enriched in hairy root cultures by genetic transformation [10–15]. However, these potential therapeutics are usually present in low concentrations, making recovery and purification expensive and complicated. The shortage of commercially viable separation technologies is a main bottleneck in the discovery and application of plant metabolites [16–20]. The conventional method of recovery of natural products from plant cells is to harvest the whole tissues and chemically recover the targeted materials from macerated tissues, usually by solvent extraction. Besides the destruction of the expensive genetically modified plant cell cultures, the activity of labile biomolecules can be reduced depending on the solvent used for the extraction process [21]. Thus, new techniques are required to continuously isolate metabolites from living plant cultures without whole tissue harvesting.

Nanoharvesting, in which nanoparticles are used to carry biomolecules out of living plant cells, can provide continuous harvesting from living and functioning source plants. This is the reverse process of the delivery of bioactive materials (drugs, nucleic acids, etc.) to cells [22–24], but is guided by the same principles in that a nanoparticle must gain entry to the cell with minimal toxicity. Kurepa et al. [25] reported the use of nonporous 2.8 ± 1.4 nm TiO_2 nanoparticles to harvest quercetin-derived flavonoids directly from *Arabidopsis thaliana* plants based on a high degree of chelation of these molecules by TiO_2 [26,27], but this is the only report of nanoharvesting to date.

While they are effective in nanoharvesting, nonporous TiO_2 nanoparticles lack the high surface area desirable for adsorption, and mesoporous TiO_2 nanoparticles are not readily synthesized as a robust, stable platform [28,29]. Additionally, requirements for stabilization of small nanoparticles limit the functional groups that can be utilized, and the recovery of 3 nm TiO_2 particles from solution is difficult due to their small size [30]. To overcome these limitations, TiO_2 can be dispersed on mesoporous silica nanoparticles (MSNPs) using post-synthesis TiO_2 functionalization [31–34]. MSNPs, which include both nanosized and nanostructured silica particles, have high surface area (~ 1000 m²/g) and tunable pore size,

and the silica surface can be easily modified with other functional groups as necessary. Moreover, MSNPs can be synthesized with a magnetic metal oxide core, which provides the opportunity for facile recovery of the particles using an external magnetic field [35].

Although MSNPs are broadly applied in catalysis, chromatography, and biomolecule loading and cellular delivery [36–41], hydroxyl-terminated bare silica has limited, nonspecific affinity for most plant metabolites. Thus, to exploit the surface properties of MSNPs for nanoharvesting, functionalization of silica with a metabolite binding group such as TiO_2 is necessary. Previous efforts to incorporate TiO_2 in MSNPs have primarily focused on the adsorption and/or photo-degradation of organic compounds using large-size silica nanoparticles and non-spherical silica mesostructures [32,33,42–45], which are not suitable for cellular internalization. In contrast, we recently demonstrated facile TiO_2 functionalization of MSNPs (average particle diameter ~ 170 nm), where TiO_2 was deposited inside the mesopores by hydrolysis of a TiO_2 precursor [46]. TiO_2 functionalized MSNPs exhibited a high capacity for the model flavonoid quercetin, over 100 times greater than functionalized nonporous silica. A solvent-based ligand displacement method was developed for flavonoid recovery after binding, and quercetin was found to retain most of its antiradical activity throughout the adsorption and desorption steps. Hence, these particles are hypothesized to be capable of isolating flavonoids in high quantity if internalized in flavonoid-rich cells during nanoharvesting, and to provide for the recovery of active flavonoids.

In addition to binding and releasing active flavonoids, an ideal nanoharvesting process should also provide for efficient uptake of nanoparticles into plants with minimal toxicity. Internalization of MSNPs by plant cells were primarily investigated for gene/protein delivery using MSNP carriers [39,47–49], where valuable guidance can be found for the design of nanoparticles for internalization. The interaction of the particles with plant cells and the ability to extract metabolites depends on the particle shape, size, surface area, adsorption capacity, degree of aggregation, and bulk pH and ionic strength [50]. Particle size and surface chemistry are two critical properties for nanoparticle cellular uptake. Nanoparticle uptake is also dependent on the plant species [51,52], and the uptake mechanisms are still under investigation. Endocytotic uptake in root cells is believed to occur through a variety of mechanisms sometimes associated with nutrient uptake [53,54]. Nanoparticles smaller than the pores in plant cell walls (5–20 nm) can directly pass into the cell membrane. Larger particles can be internalized through endocytosis or direct penetration [39,48]. Further, surface functionalization of MSNPs with amine ($-\text{NH}_2$) groups is often used to promote cell membrane penetration through charge interactions, to enhance their colloidal stability, and thus - facilitate their internalization by living cells [55,56]. Amine groups not only provide positive charge for lipid cell membrane penetration during uptake [54,57], but also act as binding sites for fluorescent molecules [58], which are used for visualization of cellular uptake via fluorescence imaging.

Nanoharvesting also requires that the internalized nanoparticles be recovered from the living plant cells. Internalized particles can escape from cellular systems using vesicle fusion with cell membranes or through direct membrane penetration (probably due to electrostatic interactions) [59,60]. Moreover, plant cells must remain viable and capable of re-

synthesizing the target biomolecule (i.e., flavonoids) after nanoparticle uptake and release to maintain a continuous nanoharvesting system. Prolonged interaction between particles and plants has been reported to create toxicity in some cases [61]. The effect of silica nanoparticle concentration on the cell viability of *A. thaliana* plant root cells was studied by Slomberg and Schoenfisch, who observed that particles between 14 and 200 nm in diameter are taken up and not phytotoxic at concentrations up to 1 mg/mL [62]. *A. thaliana* roots were also found to be viable after 7 days of uptake of anatase TiO₂ nanoparticles [25,63]. The toxicity of MSNPs functionalized with TiO₂ and/or amines to hairy root cultures is unknown.

In this study, the application of high surface area engineered MSNPs are reported for the nanoharvesting of polyphenolic flavonoids from living *Solidago nemoralis* hairy roots. Dual surface functionalization (with TiO₂ and amines) is used to facilitate metabolite binding and cellular uptake. The accessibility and order of mesopores of the MSNPs (designed with 2.8 nm diameter pores for loading and 170 nm diameter to permit uptake) after surface functionalization are studied by scanning electron microscopy (SEM), X-ray diffraction (XRD) and nitrogen adsorption. Quercetin is used as a model flavonoid to evaluate the adsorption capacity and the subsequent recovery of active biomolecules from particle surfaces using ligand displacement (in 20% w/v ethanolic citric acid solution). Cellular uptake and localization of engineered nanoparticles fluorescently labeled with rhodamine B isothiocyanate (RITC) from Murashige and Skoog (MS) media by *S. nemoralis* hairy root cultures are visualized using fluorescent microscopy, using trypan blue (TPB) to quench extracellular fluorescence [64,65]. Nanoparticles from the hairy root cultures are isolated using centrifugation. Antiradical activity and radio-ligand displacement activity ([³H]-methyllycaconitine) - from rat hippocampal nicotinic receptor membranes (characteristics of *S. nemoralis* flavonoids [66]) are measured to demonstrate the presence of active surface-bound flavonoids and flavonoids recovered in solution. Hairy roots are re-cultured following nanoharvesting to examine continued cell viability and the ability of the cultures to re-synthesize flavonoids, as measured by antiradical activity of root extracts. All of these studies provide proof of concept that engineered MSNPs represent a promising platform for *in situ* recovery of small molecule drug candidates from living plant cell tissues, and thus are a viable route to advance the concept of nanoharvesting.

2. Materials and methods

2.1. Chemicals and reagents

Tetraethyl orthosilicate (TEOS, 99%), sucrose (grade I, 99.5%), 2-(N-morpholino) ethanesulfonic acid hydrate (MES hydrate, 99.5%) and H₂O₂ (35 wt% in water) were purchased from Acros Organics; Pluronic F127 (tri-block copolymer, bio-grade), quercetin (> 95%), trypan blue (TPB, 0.4% in 0.81% NaCl and 0.06% K₂HPO₄), titanium (IV) ethoxide (TEO, technical grade), (3-Aminopropyl)triethoxysilane (APTES, 99%), ethylenediaminetetraacetic acid (EDTA, 99%), citric acid (CA, 99.5%), nicotine (99.5%), sodium azide (NaN₃, 99%), phenylmethanesulfonyl fluoride (PMSF, 98.5%), phosphate buffer saline tablets (PBS, pH 7.4) and rhodamine B isothiocyanate (RITC, mixed isomers) from Sigma-Aldrich; cetyltrimethylammonium bromide (CTAB, 99.8%) from MP

Biomedicals; [^3H]-methyllycaconitine (^3H -MLA) from American Radiolabeled Chemicals; 2,2-diphenyl-1-pi-crylhydrazyl (DPPH, 95%) and fluorescamine from Alfa-Aesar; NaOH pellets (97%) from EMD Millipore; Acetone (99.5%) from BDH analytical; Ti reference solution (1000 ppm in 10% HCl), ultrapure deionized ultra-filtrated (DIUF) water, methanol (HPLC grade, 99.9%), ethanol (200-proof), 36 N H₂SO₄ (95–98% in water), tris-HCl buffer (molecular biology grade, 99%), NaH₂PO₄ (certified ACS grade), 12 N HCl (ACS grade) and 29.3 wt% NH₄OH solution from Fisher Scientific; and Murashige and Skoog (MS) media supplemented by vitamin B5 and antibiotic cefotaxime sodium (~95%) from PlantMedia (BioWorld, Dublin, OH, USA).

2.1.1. Solidago nemoralis hairy roots—Hairy roots were generated from stem explants of seedlings using *Agrobacterium rhizogenes* mediated genetic transformation reported earlier in detail [15]. After *Agrobacterium* treatment, explants were transferred onto half-strength MS media in agar plates supplemented with 400 mg/L cefotaxime and 3% sucrose, where hairy roots were generated within 2 to 3 weeks. Roots were then excised and maintained in continuous culture on MS media supplemented with 250 mg/L cefotaxime and 3% sucrose.

2.1.2. Animals—Adult, male Sprague-Dawley rats (body weight approximately 200–225 g) were purchased from Harlan Laboratories (Indianapolis, IN, USA). Handling, care and use of animals were performed according to the National Institute of Health Guide for Care and Use of Laboratory Animals after the approval of all protocols by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky.

2.1.3. Engineered silica nanoparticles—Functional mesoporous silica nanoparticles were synthesized using a surfactant templated sol-gel process and post-synthesis grafting with TiO₂ and amine, successively, using techniques previously established in literature [46,67–69]. Some of the particles were tagged with fluorescent RITC for visualization after internalization by the hairy roots [70,71]. The detailed procedures for particle synthesis, functionalization, and fluorescent group attachment are provided in the Supplementary materials (Section A.1 of Appendix A).

2.2. Material characterization

A Hitachi S-4300 Scanning Electron Microscope (SEM) was used to examine the particle morphology. Particles were mounted onto a 15 mm aluminum stub using double sided carbon tape, excess materials were blown off with dry N₂, and the samples were stored in a desiccator for 24 h. Prior to SEM analysis, the particles were coated with conductive Au–Pd alloy using an Emscope SC400 sputtering system. Average and standard deviation of particle diameters were calculated using 20 random particles with ImageJ Software. Surface characterization was performed from nitrogen adsorption conducted at –196 °C using a Micromeritics TriStar 3000. Samples were degassed at 135 °C for 4 h under flowing N₂ gas before analysis. The specific surface area, average pore diameter and pore size distribution were estimated using the Brunauer, Emmett and Teller (BET) isotherm and by the method of Barrett, Joyner and Halenda (BJH), respectively. Micropore volume and external surface area were estimated using the comparative adsorption method described by Jaroniec et al.

[72,73]. Fourier transform infrared (FTIR) spectroscopy was conducted by a Thermo Nicolet Nexus 470 spectrometer with a deuterated triglycine sulfate (DTGS) detector. 0.5 g of anhydrous KBr and particles (0.5–1.0wt%) were crushed with a mortar-pestle, and some of this powder was pressed into a pellet for transmission analysis with N₂ purging. XRD was performed using a Bruker-AXS D8 Discover diffractometer with a Cu K_α source ($\lambda = 1.54 \text{ \AA}$) at 0.5°/min for 2 θ from 1.5 to 6°. XRD samples were prepared in a powder sample holder and tapped flat with a spatula.

2.2.1. TiO₂ quantification—The amount of TiO₂ on the particle surface was determined using an H₂SO₄/H₂O₂ assay [31,74]. 25 mg of TiO₂-functionalized particles were mixed vigorously with 25 mL of 2 M H₂SO₄ for 20 min at 90 °C and then the solution was filtered through a PTFE (0.02 μm) syringe filter. 1 μL of H₂O₂ was added to 1 mL of the resulting filtrate, and after 10 min, the absorbance was measured using a BioTek plate reader (Winooski, VT) at 407 nm in a 96 well plate. The absorbance was calibrated using samples prepared using a Ti reference solution.

2.2.2. Amine quantification—The amount of amine groups on the functionalized particle surface was determined by a previously reported fluorescamine assay after particle dissolution [75,76]. 30 mg of particles were dissolved over an 8 h period in 30 mL of 0.02 M NaOH at room temperature under vigorous stirring. 100 μL of this solution and 1.0 mL of 1.0 mM fluorescamine in acetone were mixed with 2.0 mL of PBS solution at pH 7.4. The emitted fluorescence intensity of this solution was measured at 480 nm after excitation at 366 nm using a Varian Cary Eclipse fluorescent spectrophotometer. The calibration curve was prepared using known amounts of APTES.

2.3. Adsorption and recovery of the flavonoid quercetin

Quercetin adsorption onto particles was measured using the method of Schlipf et al. [74]. For each measurement, 25 mg of particles was pre-wetted under vortex mixing with 1 mL of ethanol for 24 h in a 2 mL tube, centrifuged for 5 min at 17,000 $\times g$ (AccuSpin Micro 17, Fisher Scientific), and the supernatant was discarded. Then, 1 mL of quercetin solution in ethanol (0.05–10 mg/mL) was added to the pre-wetted particles for vortex mixing (24 h) in the dark. The particles were centrifuged again and 200 μL of supernatant was analyzed using the plate reader. A calibration curve was plotted using known concentrations of quercetin, and was used to find the amount of adsorbed flavonoid on to the particle surface by the solution depletion method. For quercetin recovery, quercetin-loaded particles were re-suspended in 1 mL of recovery solvent (20% w/v ethanolic CA). After 24 h of vortex mixing, the particles were centrifuged and the supernatant was analyzed in a plate reader.

2.4. Nanoparticle uptake in hairy roots

Hairy roots cultures which overproduce flavonoids, obtained from stem explants of seedlings using *A. rhizogenes* induced genetic transformation, were studied in continuous culture for a period of 4–6 weeks [15]. Roots were periodically checked for flavonoid content indicated by radical scavenging activity, and only those found to be overproducing flavonoids were used for nanoharvesting experiments. Sterile nanoparticles of desired functionalization were sonicated in MS media for 1 h to make a uniform 10 mg/mL stock solution, which was

diluted to different concentrations (1 µg/mL, 10 µg/mL, 100 µg/mL, 1 mg/mL and 2.5 mg/mL) for root uptake experiments. Growing portions of *Solidago nemoralis* hairy roots were cut from agar plate cultures and rinsed carefully with sterile water. Roughly 500 mg of hairy roots were placed in sterile Nalgene™ centrifuge tubes and 10 mL of a desired nanoparticle solution and the antibiotic cefotaxime (200 mg/L) were added, followed by vortex shaking in the dark for 48 h. Uptake experiments for each concentration of nanoparticles were performed in triplicate.

2.4.1. Fluorescence imaging of nanoparticle uptake—Fluorescence microscopy of RITC tagged amine-functionalized nanoparticles was performed using a Nikon Ti-U inverted microscope. Roots exposed to fluorescent nanoparticle solutions were submerged in trypan blue (TPB) solution (0.04%) for 10 min and then rinsed with water. Roots with and without TPB treatment were sliced in petri dishes using razor blades, and smashed gently onto glass slides with cover slips before bright field and fluorescence imaging. For some of the roots, a drop of TPB solution was added on top of the sliced roots and the images were taken when the roots were in TPB solution.

2.5. Nanoparticle separation and flavonoid recovery

After 48 h exposure, nanoparticles were recovered from hairy roots by centrifugation (7000 rpm) for 15 min. Whole roots were then separated and kept in sterile conditions for viability studies. The remaining solution was centrifuged again at high speed (17,000 rpm) and the pellets re-suspended in 1 mL of ethanol for particle activity measurements. Flavonoids were recovered from the particle surface by suspending nanoharvested particles in ethanolic CA solution (20% w/v) followed by 24 h of vortex shaking (see Section 2.3).

To verify that the centrifugation step was not responsible for antioxidant activity on recovered particles, one experiment was performed in which hairy roots and particles were centrifuged for 15 min (without prior incubation in the vortex mixer).

2.6. Hairy root extract preparation

Solidago nemoralis hairy roots extract was prepared using the method discussed elsewhere [4]. Briefly, roots were removed from the cultures, rinsed with growth medium, flash frozen in liquid nitrogen, and lyophilized for 24 h and stored at –80 °C. For extraction, 100 mg/mL lyophilized tissue was shaken overnight in extraction solvent (0.1 M HCl in methanol) and the extracts were filtered to remove remaining plant material and dried in a Labconco rotary evaporator (Kansas City, MO, USA) under reduced pressure and stored at –80 °C.

2.7. Activity determination

2.7.1. Radical scavenging activity (RSA)—For RSA determination, 100 µL of the flavonoid solution in ethanol (0–0.5 mg/mL) was mixed with 1 mL of DPPH solution (0.1 mg/mL in ethanol) in a 2 mL vial. The vials were covered immediately and after 30 min the absorbance was measured at 517 nm. The initial DPPH absorbance was measured by adding 100 µL of ethanol with 1 mL of DPPH solution, and RSA was calculated from the percent decrease in DPPH absorbance. RSA of flavonoids recovered by ethanolic CA was determined using the same procedure. For RSA of particle-bound flavonoids, particles with

a known amount of bound flavonoids (Section 2.3) were dispersed in 1 mL of ethanol by sonication and 100 μ L of the solution was added to DPPH.

For RSA measurement of plant extracts, 50 μ L of 2 mg/mL extract solution in methanol was added to 450 μ L of tris-HCl buffer and 50 μ L of the resultant solution were added to a 96 well plate in triplicate. 100 μ L of 0.1 mM DPPH solution in methanol was added to each well and the plate was then kept in the dark for 30 min. Absorbance was measured at 517 nm and RSA of root extracts calculated from the percent decrease in DPPH absorbance.

2.7.2. Specific radio-ligand binding displacement activity—Radio-ligand binding displacement activity of nanoharvested particles and their eluents was performed by displacing ^3H -MLA from rat hippocampal membranes [66,77]. Membranes were prepared from freshly harvested hippocampal tissues from adult, male Sprague-Dawley rats by homogenizing the tissues in sucrose buffer (0.32 M sucrose with 50 mM NaH_2PO_4 , 0.1 mM EDTA, 0.1 mM PMSF and 0.01% w/v NaN_3) at pH 7.4 using a glass homogenization tube and Teflon pestle. The homogenate was washed two times at $1000\times g$ for 10 min and the supernatants re-centrifuged at $50,000\times g$ for 20 min. The pellet was re-suspended in ice-cold buffer and the protein content was determined by the Pierce Method in Bicinchoninic Acid Kit (Sigma-Aldrich). Final protein content was adjusted to 3 mg/mL using extra buffer solution and samples were frozen at -80°C before use. Individual samples were screened for displacement activity in quadruplicate against 2 nM solution of ^3H -MLA. 100 μ L of samples containing nanoparticles or nanoparticle eluents were added in quadruplicate to a 96 well plate containing membranes (1 mg/mL protein content). After 15 min of incubation, ^3H -MLA was added and the plate incubated for further 2 h, before harvesting onto a 96 well GF/B filter array and rapidly washed 3 times with 350 μ L of 50 mM tris-HCl buffer (pH 7.4) and dried overnight. Finally, 35 μ L of scintillation fluid (Microscint 20, Packard Inc.) was added to each filter and the plate was counted using a scintillation counter (Packard TopCount NXT). Nonspecific binding in the presence of 300 μ M nicotine was subtracted from total binding to find specific binding.

2.8. Viability and flavonoid synthesis of hairy roots after nanoparticle exposure

Viability of *Solidago nemoralis* hairy roots after exposure to particles was studied by re-culturing them (at least 10 roots for each nanoparticle type and concentration) on agar plates containing MS media supplemented by 3% sucrose and 250 mg/L cefotaxime. Re-cultured root growth and viability were observed after every week up to four weeks. To measure the flavonoid re-synthesis capability of nanoharvested roots, extracts were prepared in triplicate after regrowth and RSA of the extracts measured and compared to a control (roots not treated with nanoparticles).

3. Results and discussion

3.1. Nanoparticle characterization

Spherical MSNPs (approximately 170 nm diameter) were synthesized by a modified Stober method using CTAB, TEOS and Pluronic F127 as structure directing agent, silica precursor, and dispersant, respectively. Complete removal of the organic surfactants was confirmed by

FTIR analysis by the disappearance of the peak corresponding to CH₂ stretching (2800–3000 cm⁻¹) after acidic ethanol wash (Fig. A.1 in Supplementary materials) [46], thus preventing any toxic effects of surfactants from impacting cell viability. Surface characteristics (BET area, average pore diameter and BJH pore volume) of the dried particles were determined using nitrogen adsorption and are presented in Table 1. Average pore diameter (2.76 ± 0.23 nm) falls in the expected range of values obtained from the literature when CTAB is used as a templating agent [78]. The BET specific surface area and BJH pore volume were 953 ± 51 m²/g and 1.21 ± 0.09 cm³/g for non-functionalized MSNPs, confirming their potential as high surface area platforms for biomolecule adsorption. In a previous study, MSNPs were functionalized with a range of TiO₂ amounts via the hydrolytic condensation of TEO, and optimum capacity for the model flavonoid quercetin (in mg quercetin/g particle) was observed at a TiO₂ incorporation of 425 ± 9.2 mg/g particles [46]. This sample is labeled MSNPT in Table 1. TEM and STEM imaging of these particles shows that there is a tradeoff between the creation of binding sites with by TiO₂ functionalization in the mesopores of ordered cubic MSNPs and the blocking of pores with increased TiO₂ functionalization, where the sample with the greatest quercetin loading (MSNPT) has titanium both inside the pores and at the particle surface [46]. The average nanocrystal size for MSNPT is larger than the average pore diameter from nitrogen adsorption, which suggests that nanocrystallites form only on the particle outer surface, and that TiO₂ in the pores is amorphous (a suitable form for adsorption). Reduction in pore diameter occurred not only due to TiO₂ deposition but also due to restructuring and contraction of silica matrices (indicated by reduced d-spacing) resulting from the solvothermal functionalization process. The presence of uniformly distributed amorphous TiO₂ (confirmed by wide angle XRD [46]) was also evident from peak broadening and reduced contrast in low angle XRD patterns after TiO₂ loading, consistent with other literature for TiO₂ incorporation in mesoporous silica [31,33,79].

Both MSNPs and MSNPTs were functionalized by amine (-NH₂) groups using an aminosilane precursor, APTES, resulting in materials referred to as MSNPA and MSNPTA, respectively. For MSNPA and MSNPTA, the amount of amine on the particle surface (presented in Table 1) corresponds to 0.0038 mmol/m² and 0.0012 mmol/m² on bare silica and TiO₂-functionalized silica, respectively. Based on the reported area of an amine group in monolayer coverage (50 Å² surface area/amine functional group) [75], the particle coverage of amines corresponds to 114% and 37% of monolayer coverage for MSNPA and MSNPTA, respectively. The reduced monolayer coverage on TiO₂ (MSNPTAs) is probably due to smaller surface area and lower pore size of MSNPTs compared to bare MSNPs. After amine functionalization, XRD peaks were broadened and less resolved (Fig. A.2), but this effect was not as drastic as after TiO₂ functionalization [46]. MSNPs retain their mesostructure after amine functionalization, which is in agreement with literature observation for other types of mesoporous silica [80,81].

Similarly, MSNPs retained their spherical shape after TiO₂ and/or amine functionalization (Fig. 1) and functionalization did not measurably affect particle diameter (Table 1). The effect of functionalization on the surface properties of MSNP was studied using nitrogen sorption isotherms, BJH pore size distribution, and α_s -plots, as shown in Fig. A.3. The nitrogen sorption isotherm for MSNPs is a type IV isotherm with clear capillary

condensation, which indicates a well-ordered mesostructure [67]. For all functionalized particles, the capillary condensation step was not evident, consistent with reduced mesopore volume in the presence of TiO_2 and amine groups. The pore size distribution of MSNPs showed a clear peak at around 2.2 nm, but for all types of functionalized particles, the peak sharpness (Fig. A.3b) and the average pore diameter decreased, as reported in Table 1. Prior studies of TiO_2 [33,42,79] or amine [82,83] loading onto mesoporous silica are consistent with our observations, where surface area, pore size and pore volume decreased after functionalization. MSNPTAs are less common, and in this case showed an additional surface area reduction (approximately 50%) relative to the starting MSNPTs. The standard reduced adsorption (α_s) plots (Fig. A.3c) [72,73] show no micropore formation (pores < 2 nm). The external surface areas were also measured from α_s -plots to be 137.2 m²/g for MSNP, and were reduced to 120.8 m²/g, 77.9 m²/g and 65.3 m²/g for MSNPT, MSNPA and MSNPTA, respectively. The gradual reduction in surface area and pore diameter and the absence of microporosity after both functionalization steps are consistent with monolayer-like coverage in the pores, providing surfaces appropriate to provide positive charge (to promote cell uptake) and adsorption sites for biomolecule binding.

3.2. Nanoparticle uptake in hairy roots and hairy root viability

Plant cells are inherently different from animal cells in that they have rigid cell walls. It has been proposed that the cell wall may place a size limit on nanoparticle uptake based on the size of naturally occurring pores [48,53,61]. Silica nanoparticles with much larger size (up to 200 nm [62]) than the pores were shown to be internalized by plant cells, but the penetration mechanism through plant cell walls is still unknown. Enlargement of pores or new pores/channel creation after the disruption of cell wall proteins and polysaccharides is speculated to be the pathways for nanoparticle entry [48]. The exact uptake mechanism of nanoparticles through the cell membrane is also still unknown or disputed; endocytosis, direct penetration and pore creation by nanoparticles have all been proposed as mechanisms for nanoparticle uptake in plant cells [39,51,53]. Direct penetration occurs for strongly positively charged nanoparticles and after that the nanoparticle can localize in lysosomes, endosomes, cytoplasm, mitochondria, endoplasmic reticulum and cell nuclei [54]. Although nanoparticle transport through a cell membrane reaches equilibrium given sufficient time [54], smaller particles are transported faster than larger ones [84]. Bare and/or amine-functionalized silica nanoparticles (< 200 nm) or TiO_2 nanoparticles (< 140 nm) were reported to be internalized by plant cells [39,62,85,86], but the effect of combined amine and TiO_2 functionalization on internalization of nanoparticles is unknown. Thus, MSNPTAs were tagged with RITC for visualization of uptake in hairy roots by fluorescence microscopy. To confirm internalization, fluorescence of RITC on particles outside of cells [65] was quenched by trypan blue (TPB) added after uptake and fluorescence microscopy was performed, by rinsing off TPB from the roots [87] and also without rinsing [65].

At concentrations of 100 µg/mL MSNPTA, fluorescent images (Fig. 2) showed evidence for nanoparticle internalization. The bright field images (Fig. 2a and c) show clear outlines of cells with green chloroplasts inside. The corresponding fluorescence image (Fig. 2b) of the root cells after 24 h of nanoparticle exposure showed bright fluorescence, which remained after TPB treatment (Fig. 2d), indicating nanoparticle association and partial internalization

by the cells. In contrast, the images of root cells not exposed to nanoparticles and TPB (Fig. A.4b) showed no fluorescence. The faint red fluorescence observed when TPB solution was added to the roots without any nanoparticle treatment (Fig. A.4d) is attributed to the auto-fluorescence of TPB in contact with outer cell membranes [87,88].

The fluorescent image presented in Fig. 2b shows some nanoparticles in aggregates, also visible in less quantity after TPB treatment (Fig. 2d). These particles were observed more closely to verify internalization and localization in magnified fluorescence images with or without TPB (Fig. 3). Nanoparticles were found in clusters as well as scattered throughout the cells (Fig. 3a). After TPB addition and rinsing, images of the roots (Fig. 3b) still show intracellular localization, but the reduction in the number of fluorescent spots indicates some particles (or clusters) were quenched. Particle entry into the plant cells and cluster formation inside the cells has been reported based on particle surface chemistry (hydrophobicity) [89]. For MSNPs, Torney et al. found endocytosis clusters inside tobacco plant protoplasts [49], whereas Chang et al. reported direct penetration (energy independent) for amine functionalized MSNPs into *Arabidopsis* plant roots [39]. It is also possible that TPB entered and quenched RITC in some of the viable cells, which can explain reduced intracellular fluorescence after TPB treatment. When particles enter plant cells, the cell membrane structures can be disrupted, increasing the cell wall permeability [53], and allowing TPB entry.

At a higher particle concentration (1 mg/mL), the fluorescent images were too bright for individual clusters to be seen (Fig. A.5 in Supplementary materials). After TPB addition, almost all of the cells were still very brightly fluorescent, suggesting a higher amount of particle internalization, which indicates a concentration-dependent equilibrium in particle uptake. Finally, because imaging in the presence of a TPB-containing solution has been used to confirm particle internalization [90], fluorescent images were collected after adding a drop of TPB solution to the roots to ensure quenching of extracellular particles (Fig. A.6). The bright field images in TPB solution are bluish in color suggesting cells are completely covered with TPB solution. The corresponding fluorescent images appear similar to those for which TPB was rinsed before imaging. Since extracellular particle fluorescence quenching by TPB is very fast (< 2 min [87]), images taken after rinsing the TPB solution provided additional evidence for particle internalization and localization.

Another requirement for the nanoharvesting process is to not only isolate flavonoids from hairy root cultures but also to maintain viable roots capable of regrowth. Uptake and translocation of engineered nanomaterials in vascular plants, and associated phytotoxicity and cytotoxicity were discussed by Miralles et al. [53], where several factors were identified as contributors to toxicity, such as nanoparticle surface characteristics and possible metal ion leaching. They advise that viability should be studied case-by-case. TiO₂ nanoparticles have been reported to inhibit plant leaf growth and transpiration by reducing root water transport, to delay photosynthesis by acting as photocatalysts, and to disrupt cell function and microtubule networks by generating reactive oxygen species (ROS) [52,91,92]. Cell membrane disruption during the uptake process can also lead to cellular dysfunction [54].

The viability of hairy roots exposed to 2.5 mg/mL nanoparticle concentration, the same experimental conditions used for nanoharvesting experiments, was demonstrated over a period of two weeks after particle exposure and reculturing in petri dishes containing agar (Fig. 4). The results are consistent with the absence of an effect on root growth after exposure to nanoparticles. This is shown in Fig. A.7 (Supplementary materials), where data are shown for hairy roots treated with 0 (control), 10 µg/mL, and 1 mg/mL nanoparticle solutions after 0, 2 and 4 weeks of re-culturing. Root viability is similar to the control over the concentration range tested.

3.3. Nanoparticle recovery and flavonoid activity measurements

Genetically modified *S. nemoralis* hairy roots synthesize polyphenolic flavonoids that are detectable from their radical scavenging activity RSA (antiradical activity using DPPH assay), a broad measure of their ability to reduce oxidative stress [93]. Also, flavonoids from *S. nemoralis* have demonstrated specific bioactivity in their ability to displace ³H-MLA from nicotinic receptors [66]. After the nanoharvesting process, nanoparticles were recovered from the solution in contact with the hairy root cultures by centrifugation. Particles were analyzed for RSA directly. Particles were also washed with ethanolic citric acid (CA) and the activity of flavonoids recovered in the solution was tested. We have previously demonstrated ethanolic CA for the effective recovery of flavonoids from TiO₂-functionalized mesoporous silica [46], presumably by ligand displacement of the chelated flavonoid. CA is ubiquitous in nature (found blood plasma at ~1 mM) and has tremendous advantages over other extraction agents for its excellent biocompatibility. For these reasons, CA is widely used in living systems for mineral dissolution and detoxification [94]. Moreover, the citrate ion provides very good surface binding, enhances water solubility and reduces aggregation [95], and as a result can increase the bio-availability of hydrophobic flavonoids.

RSA was measured directly from solutions containing suspended MSNPT or MSNPTA particles recovered from the nanoharvesting process (Fig. 5). Both MSNPT and MSNPTA particles were used in nanoharvesting, where the positive charge of the amine groups (in MSNPTAs) is expected to be necessary for localization in the cell. Additional control experiments included RSA measurements for particles exposed only to the media, and particles exposed to media in which hairy roots have been kept (but not incubated with hairy roots directly). All particles were vortexed in their respective solution (media only, media after 24 h of incubation with hairy root cultures (leftover media), and media in which particles and hairy roots were incubated together for 24 h). For MSNPTAs, nanoparticles exposed to roots showed a statistically significant increase in activity compared to particles exposed to leftover solution after shaking with roots and control (no roots), consistent with the color change of the particles after exposure to roots (Fig. A.8 in Supplementary materials). This color change suggests that active metabolites bound to the particles. For MSNPTs, no significant activity difference was found among particles exposed to roots, particles exposed to leftover solution, and particles exposed only to media (Fig. 5). Thus, internalization of the nanoparticle, which was mediated by the amine functionalization on the particle surface, is necessary for nanoharvesting of these flavonoids to occur. Flavonoids in plants are localized throughout plant cells, with quercetin derivatives accumulating in the

plasma membrane, but also found in the nucleus [96]. The mechanism of nanoparticle uptake and release are currently under investigation, but positively charged particles have previously been shown to be taken up by direct penetration of cell membranes [54,57]. After particles are internalized, flavonoids bind to the TiO_2 groups of the particles [46], presumably through complexation of their cis-diol group with Ti, and then are carried out of the cells during particle expulsion.

Several additional controls were investigated to demonstrate that uptake of particles in the hairy root cultures was necessary to observe RSA. Centrifugation of particles in the presence of hairy roots (without incubation of the roots and particles) did not result in any increase in particle activity (data not shown), suggesting that prolonged interaction during vortex shaking is necessary to allow particles to enter and leave cells. Roots were also subject to vortex shaking and centrifugation in the absence of nanoparticles. No measurable RSA activity was found in the supernatant of any of the experiments conducted, which proves that metabolites were not secreted by the roots and subsequently bound to the particles in solution. Metabolites chelating to MSNPTA particles inside of cells is consistent with the elemental analysis of internalized NaYF_4 nanoparticle clusters, which provided evidence for phosphate attachment to the particle surface [97]. Similarly, silica nanoparticles were shown to adsorb nutrients from the cellular environment [62], thus making the nutrients unavailable to plant cells. Regarding potential mechanisms of particle recovery from the hairy root cultures, there is a strong tendency in cells with internalized nanoparticles towards cell membrane regeneration and repair, leading to the spontaneous direction of some of the endocytosed materials towards the cell membrane [98]. Also, endosomal and recycling pathways often gradually reduce the intracellular concentration of internalized materials [98,99]. Particles inside endocytotic vesicles can be removed by fusion with the cell membrane [59] or can become free in the cytoplasm after endosomal escape, which is reported to happen within 12 h for endocytosed mesoporous silica nanoparticles [100]. These free particles, along with those that directly penetrate the cell membrane, can be steadily exocytosed from the cellular interior by secretory vesicles formation and fusion with the cell membrane [101]. Exocytosis, like endocytosis, reaches thermodynamic equilibrium, but is faster for smaller nanoparticles [54,102]. More than 50% of internalized nanoparticle were observed to be transported to the cell periphery and subsequently expelled out of mammalian cells within 1 to 2 h [102,103].

To show that biologically selective compounds could be nanoharvested using MSNPTAs (since both amine and TiO_2 are required for nanoharvesting), we demonstrated that eluted flavonoids specifically bind $\alpha 7$ nicotinic receptors in rat hippocampal cell membranes. Root extracts from the genetically modified *S. nemoralis* hairy roots used in this study were previously shown to contain specific flavonoids that selectively bind $\alpha 7$ nicotinic receptors [66]. Binding to $\alpha 7$ receptors is presented as the percent displacement of ^3H -MLA from hippocampal membrane and compared to the binding of only ^3H -MLA without adding anything (as control). Non-specific binding was measured in the presence of excess nicotine and subtracted from total binding to find the specific binding. Specific binding of ^3H -MLA in the presence of extract or nanoharvested compounds (nanoparticle and recovered in solution) is presented as percent of specifically bound ^3H -MLA in the control. The percentage of specifically bound ^3H -MLA (Fig. 6a) decreased in the presence of increasing

plant extract concentration, showing that flavonoids present in the extract have nicotinic receptor activity as predicted for specific flavonoids from *S. nemoralis* [66].

MSNPTAs exposed to hairy roots show a high degree of displacement of ^3H -MLA, but nanoparticles that were not exposed to roots or exposed to only leftover solution show no statistically significant displacement of ^3H -MLA compared to control (Fig. 6b), suggesting that polyphenolic flavonoids active towards $\alpha 7$ nicotinic receptors are present on only the particle surface that was able to enter into and bind metabolites from hairy roots. The displacement is the same as a solution containing 0.16mg/mL of root extract (Fig. 6a). Similarly, the average displacement by compounds recovered in solution (ethanolic CA) from nanoparticles exposed to roots was higher compared to that of the nanoparticles not exposed to roots or exposed to root left-over solution only (Fig. 6b), although the differences are not as significant as when the activity is measured directly from the particles. The single washing step by ethanolic CA may not have been sufficient to remove all the flavonoids from the particle surface [46].

3.4. Flavonoid re-synthesis potential of roots after nanoharvesting

We have shown that *S. nemoralis* hairy roots remain viable and capable of regrowth after exposure to 2.5 mg/mL MSNPs, irrespective of the type of functionalization (TiO_2 and/or amine). To determine whether nanoharvested roots have the ability to synthesize new flavonoids, we made extracts of nanoharvested roots after 2, 10 and 17 days of re-culturing. Extracts from fresh roots were prepared to measure concentration-dependent RSA, which shows RSA increases with increasing extract concentration (data not shown). RSAs of roots exposed to nanoparticles are presented in Fig. 7, where roots exposed to MSNPT (TiO_2 only) and MSNPTA (TiO_2 and amine) along with control roots (no particles) were re-cultured. After 2 days of exposure, roots exposed to MSNPTA (but not MSNPT) gave an extract showing less RSA activity, suggesting that a fraction of the metabolites were nanoharvested. This again supports our hypothesis that both amine and TiO_2 functional groups are required for flavonoid removal from plant cells. The root extract activity remained roughly the same after 10 days, which probably is due to the readjustment of roots to the new culturing environment. 17 days after exposure, roots exposed to MSNPTA regained their original activity, and their RSA was statistically the same when compared to control roots and roots exposed to MSNPT. The regaining of RSA activity in the root extracts indicates flavonoid re-synthesis after nanoharvesting using MSNPTAs. Thus, *in situ* product removal from plant culture, hypothesized as one way to increase the rate of production of secondary metabolites by biosynthesis in plants [20], has been demonstrated.

4. Conclusions

The use of engineered mesoporous silica nanoparticles in a nanoharvesting process, which is broadly applicable for several classes of biomolecules, was demonstrated for *Solidago nemoralis* plant hairy root cultures. MSNPs with uniform, well-defined pores were successfully synthesized and functionalized with amines and TiO_2 to promote both cellular nanoparticle uptake and polyphenolic compound binding, respectively.

The first requirement for nanoharvesting, the uptake of functionalized nanoparticles in hairy roots was demonstrated by fluorescence microscopy of RITC-tagged MSNPTA. To further demonstrate that particles are internalized within root cells, the fluorescence of extracellular particles was quenched by trypan blue (TPB) solution. The next requirements for nanoharvesting were demonstrated by showing that the particles could be readily recovered after hairy root uptake, and that the roots were viable and capable of synthesis of new flavonoids after nanoharvesting, as demonstrated by post-uptake growth studies and plant extract activity analysis. Finally, to demonstrate that the compounds recovered on the surface of the MSNPTAs are active flavonoids, the increase in radical scavenging activity (RSA) was analyzed after nanoharvesting. Nanoparticles exposed to roots and their eluents in ethanolic citric acid solution were also tested for biological activity, where both particles and recovered compounds were able to displace radiolabeled ^3H -MLA from rat hippocampal cell membrane nicotinic receptors. ^3H -MLA displacement from nicotinic receptors proves that the nanoharvested compounds are polyphenolic flavonoids that exhibit the type of bioactivity found in plant extracts.

The ability of particle-bound flavonoids from *S. nemoralis* to bind to nicotinic receptors on synaptic membranes suggests a new direction for treatment, where particles after nanoharvesting can be directly applied for biomolecule delivery to their cellular targets [66]. Furthermore, mesoporous silica layers can be coated onto magnetic nanoparticles to create functional magnetic core-shell particles, for enhanced recovery [35,104]. The magnetic core will facilitate particle recovery from living plant cells, while the TiO_2 functionalized mesoporous silica coating can act as a high surface area adsorbent in separation processes. The synthesized engineered particles are expected to represent a platform technology for the isolation and delivery of a broad range of therapeutic biomolecules in living tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. De Luca V, Salim V, Atsumi SM, Yu F, Mining the biodiversity of plants: a revolution in the making, *Science* 336 (6089) (2012) 1658–1661, 10.1126/science.1217410. [PubMed: 22745417]
- [2]. Harvey AL, Natural products in drug discovery, *Drug Discov. Today* 13 (19–20) (2008) 894–901, 10.1016/j.drudis.2008.07.004. [PubMed: 18691670]
- [3]. Kirakosyan A, Kaufman PB, *Recent Advances in Plant Biotechnology*, Springer US, New York, USA, 2009, 10.1007/978-1-4419-0194-1.
- [4]. Littleton J, Rogers T, Falcone D, Novel approaches to plant drug discovery based on high throughput pharmacological screening and genetic manipulation, *Life Sci.* 78 (5) (2005) 467–475, 10.1016/j.lfs.2005.09.013. [PubMed: 16274700]

- [5]. Georgiev M, Pavlov A, Bley T, Hairy root type plant in vitro systems as sources of bioactive substances, *Appl. Microbiol. Biotechnol* 74 (6) (2007) 1175–1185, 10.1007/s00253-007-0856-5. [PubMed: 17294182]
- [6]. Li JW-H, Vederas JC, Drug discovery and natural products: end of an era or an endless frontier? *Science* 325 (5937) (2009) 161–165, 10.1126/science.1168243. [PubMed: 19589993]
- [7]. Oksman-Caldentey K-M, Inzé D, Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites, *Trends Plant Sci.* 9 (9) (2004) 433–440, 10.1016/j.tplants.2004.07.006. [PubMed: 15337493]
- [8]. Roessner U, Luedemann A, Brust D, Fiehn O, Linke T, Willmitzer L, Fernie AR, Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems, *Plant Cell* 13 (1) (2001) 11–29, 10.1105/tpc.13.1.11. [PubMed: 11158526]
- [9]. Brown DP, Rogers DT, Gunjan SK, Gerhardt GA, Littleton JM, Target-directed discovery and production of pharmaceuticals in transgenic mutant plant cells, *J. Biotechnol.* 238 (2016) 9–14, 10.1016/j.jbiotec.2016.09.007 [PubMed: 27637316]
- [10]. Murakami A, Ashida H, Terao J, Multitargeted cancer prevention by quercetin, *Cancer Lett.* 269 (2) (2008) 315–325, 10.1016/j.canlet.2008.03.046. [PubMed: 18467024]
- [11]. Chen T-J, Jeng J-Y, Lin C-W, Wu C-Y, Chen Y-C, Quercetin inhibition of ROS-dependent and -independent apoptosis in rat glioma C6 cells, *Toxicology* 223 (1–2) (2006) 113–126, 10.1016/j.tox.2006.03.007. [PubMed: 16647178]
- [12]. Del Follo-Martinez A, Banerjee N, Li X, Safe S, Mertens-Talcott S, Resveratrol and quercetin in combination have anticancer activity in colon cancer cells and repress oncogenic microRNA-27a, *Nutr. Cancer* 65 (3) (2013) 494–504, 10.1080/01635581.2012.725194. [PubMed: 23530649]
- [13]. Hirpara KV, Aggarwal P, Mukherjee AJ, Joshi N, Burman AC, Quercetin and its derivatives: synthesis, pharmacological uses with special emphasis on antitumor properties and prodrug with enhanced bio-availability, *Anti Cancer Agents Med. Chem.* 9 (2) (2009) 138–161, 10.2174/187152009787313855.
- [14]. Perez-Vizcaino F, Duarte J, Flavonols and cardiovascular disease, *Mol. Asp. Med* 31 (6) (2010) 478–494, 10.1016/j.mam.2010.09.002.
- [15]. Gunjan SK, Lutz J, Bushong A, Rogers DT, Littleton J, Hairy root cultures and plant regeneration in *Solidago nemoralis* transformed with *Agrobacterium rhizogenes*, *Am. J. Plant Sci.* 4 (2013) 1675–1678.
- [16]. Verpoorte R, Exploration of nature's chemodiversity: the role of secondary metabolites as leads in drug development, *Drug Discov. Today* 3 (5) (1998) 232–238, 10.1016/S1359-6446(97)01167-7.
- [17]. Littleton J, The future of plant drug discovery, *Expert Opin. Drug Discovery* 2 (5) (2007) 673–683, 10.1517/17460441.2.5.673.
- [18]. Heilmann J, New Medical Applications of Plant Secondary Metabolites, *Annual Plant Reviews Volume 39: Functions and Biotechnology of Plant Secondary Metabolites*, Wiley-Blackwell, 2010, pp. 348–380, 10.1002/9781444318876.ch5.
- [19]. Koehn FE, Carter GT, The evolving role of natural products in drug discovery, *Nat. Rev. Drug Discov* 4 (3) (2005) 206–220, 10.1038/nrd1657. [PubMed: 15729362]
- [20]. Wilson SA, Roberts SC, Recent advances towards development and commercialization of plant cell culture processes for synthesis of biomolecules, *Plant Biotechnol. J.* 10 (3) (2012) 249–268, 10.1111/j.1467-7652.2011.00664.x. [PubMed: 22059985]
- [21]. Do QD, Angkawijaya AE, Tran-Nguyen PL, Huynh LH, Soetaredjo FE, Ismadji S, Ju Y-H, Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila cromatica*, *J. Food Drug Anal* 22 (3) (2014) 296–302, 10.1016/j.jfda.2013.11.001. [PubMed: 28911418]
- [22]. Slowing II, Vivero-Escoto JL, Wu C-W, Lin VSY, Mesoporous silica nanoparticles as controlled release drug delivery and gene transfection carriers, *Adv. Drug Deliv. Rev* 60 (11) (2008) 1278–1288, 10.1016/j.addr.2008.03.012. [PubMed: 18514969]
- [23]. Kanasty R, Dorkin JR, Vegas A, Anderson D, Delivery materials for siRNA therapeutics, *Nat. Mater* 12 (11) (2013) 967–977, 10.1038/nmat3765. [PubMed: 24150415]

- [24]. Luo D, Saltzman WM, Synthetic DNA delivery systems, *Nat. Biotechnol* 18 (1) (2000) 33–37, 10.1038/71889. [PubMed: 10625387]
- [25]. Kurepa J, Nakabayashi R, Paunesku T, Suzuki M, Saito K, Woloschak GE, Smalle JA, Direct isolation of flavonoids from plants using ultra-small anatase TiO₂ nanoparticles, *Plant J. Cell Mol. Biol* 77 (3) (2014) 443–453, 10.1111/tj.12361.
- [26]. Costa D, Savio L, Pradier CM, Adsorption of amino acids and peptides on metal and oxide surfaces in water environment: a synthetic and prospective review, *J. Phys. Chem. B* 120 (29) (2016) 7039–7052, 10.1021/acs.jpcc.6b05954. [PubMed: 27366959]
- [27]. Thomas AG, Syres KL, Adsorption of organic molecules on rutile TiO₂ and anatase TiO₂ single crystal surfaces, *Chem. Soc. Rev* 41 (11) (2012) 4207–4217, 10.1039/C2CS35057B. [PubMed: 22517475]
- [28]. Vivero-Escoto JL, Chiang Y-D, Wu K, Yamauchi Y, Recent progress in mesoporous titania materials: adjusting morphology for innovative applications, *Sci. Technol. Adv. Mater* 13 (1) (2012) 013003, 10.1088/1468-6996/13/1/013003. [PubMed: 27877467]
- [29]. Masolo E, Meloni M, Garroni S, Mulas G, Enzo S, Baró M, Rossinyol E, Rzeszutek A, Herrmann-Geppert I, Pilo M, Mesoporous titania powders: the role of precursors, ligand addition and calcination rate on their morphology, crystalline structure and photocatalytic activity, *Nanomaterials* 4 (3) (2014) 583–598, 10.3390/nano4030583. [PubMed: 28344237]
- [30]. Faure B, Salazar-Alvarez G, Ahniyaz A, Villaluenga I, Berriozabal G, De Miguel YR, Bergström L, Dispersion and surface functionalization of oxide nanoparticles for transparent photocatalytic and UV-protecting coatings and sunscreens, *Sci. Technol. Adv. Mater* 14 (2) (2013) 023001, 10.1088/1468-6996/14/2/023001. [PubMed: 27877568]
- [31]. Busuioc AM, Meynen V, Beyers E, Mertens M, Cool P, Bilba N, Vansant EF, Structural features and photocatalytic behaviour of titania deposited within the pores of SBA-15, *Appl. Catal. A* 312 (0) (2006) 153–164, 10.1016/j.apcata.2006.06.043.
- [32]. Acosta-Silva YJ, Nava R, Hernández-Morales V, Macías-Sánchez SA, Pawelec B, TiO₂/DMS-1 disordered mesoporous silica system: structural characteristics and methylene blue photodegradation activity, *Microporous Mesoporous Mater.* 170 (2013) 181–188, 10.1016/j.micromeso.2012.11.027.
- [33]. Signoretto M, Ghedini E, Trevisan V, Bianchi CL, Ongaro M, Cruciani G, TiO₂–MCM-41 for the photocatalytic abatement of NO_x in gas phase, *Appl. Catal. B* 95 (1–2) (2010) 130–136, 10.1016/j.apcatb.2009.12.019.
- [34]. Singh R, Bapat R, Qin L, Feng H, Polshettiwar V, Atomic layer deposited (ALD) TiO₂ on fibrous nano-silica (KCC-1) for photocatalysis: nanoparticle formation and size quantization effect, *ACS Catal.* 6 (5) (2016) 2770–2784, 10.1021/acscatal.6b00418.
- [35]. Liu Z, Li M, Yang X, Yin M, Ren J, Qu X, The use of multifunctional magnetic mesoporous core/shell heteronanostructures in a biomolecule separation system, *Biomaterials* 32 (21) (2011) 4683–4690, 10.1016/j.biomaterials.2011.03.038. [PubMed: 21481453]
- [36]. Li Z, Barnes JC, Bosoy A, Stoddart JF, Zink JJ, Mesoporous silica nanoparticles in biomedical applications, *Chem. Soc. Rev* 41 (7) (2012) 2590–2605, 10.1039/C1CS15246G. [PubMed: 22216418]
- [37]. Schlipf DM, Rankin SE, Knutson BL, Pore-size dependent protein adsorption and protection from proteolytic hydrolysis in tailored mesoporous silica particles, *ACS Appl. Mater. Interfaces* 5 (20) (2013) 10111–10117, 10.1021/am402754h. [PubMed: 24063730]
- [38]. Liberman A, Mendez N, Trogler WC, Kummel AC, Synthesis and surface functionalization of silica nanoparticles for nanomedicine, *Surf. Sci. Rep* 69 (2–3) (2014) 132–158, <https://doi.org/10.1016/j.surfrep.2014.07.001>. [PubMed: 25364083]
- [39]. Chang F-P, Kuang L-Y, Huang C-A, Jane W-N, Hung Y, Y.-i.C. Hsing, C.-Y. Mou, A simple plant gene delivery system using mesoporous silica nanoparticles as carriers, *J. Mater. Chem. B* 1 (39) (2013) 5279–5287, 10.1039/C3TB20529K.
- [40]. Hartono SB, Phuoc NT, Yu M, Jia Z, Monteiro MJ, Qiao S, Yu C, Functionalized large pore mesoporous silica nanoparticles for gene delivery featuring controlled release and co-delivery, *J. Mater. Chem. B* 2 (6) (2014) 718–726, 10.1039/C3TB21015D.

- [41]. Schlupf DM, Zhou S, Khan MA, Rankin SE, Knutson BL, Effects of pore size and tethering on the diffusivity of lipids confined in mesoporous silica, *Adv. Mater. Interfaces* 4 (9) (2017) 1601103, 10.1002/admi.201601103.
- [42]. Zaccariello G, Moretti E, Storaro L, Riello P, Canton P, Gombac V, Montini T, Rodriguez-Castellon E, Benedetti A, TiO₂-mesoporous silica nanocomposites: cooperative effect in the photocatalytic degradation of dyes and drugs, *RSC Adv.* 4 (71) (2014) 37826–37837, 10.1039/C4RA06767C.
- [43]. Beyers E, Biermans E, Ribbens S, De Witte K, Mertens M, Meynen V, Bals S, Van Tendeloo G, Vansant EF, Cool P, Combined TiO₂/SiO₂ mesoporous photocatalysts with location and phase controllable TiO₂ nanoparticles, *Appl. Catal. B* 88 (3–4) (2009) 515–524, 10.1016/j.apcatb.2008.10.009.
- [44]. Ye M, Zhang Q, Hu Y, Ge J, Lu Z, He L, Chen Z, Yin Y, Magnetically recoverable core-shell nanocomposites with enhanced photocatalytic activity, *Chem. Eur. J* 16 (21) (2010) 6243–6250, 10.1002/chem.200903516. [PubMed: 20401878]
- [45]. Dong W, Sun Y, Hua W, Yao Y, Zhuang G, Lv X, Ma Q, Zhao D, Preparation of secondary mesopores in mesoporous anatase-silica nanocomposites with unprecedented-high photocatalytic degradation performances, *Adv. Funct. Mater* 26 (6) (2016) 964–976, 10.1002/adfm.201504001.
- [46]. Khan MA, Wallace WT, Islam SZ, Nagpure S, Strzalka J, Littleton JM, Rankin SE, Knutson BL, Adsorption and recovery of polyphenolic flavonoids using TiO₂-functionalized mesoporous silica nanoparticles, *ACS Appl. Mater. Interfaces* 9 (37) (2017) 32114–32125, 10.1021/acsami.7b09510. [PubMed: 28825464]
- [47]. Martin-Ortigosa S, Peterson DJ, Valenstein JS, Lin VS-Y, Trewyn BG, Lyznik LA, Wang K, Mesoporous silica nanoparticle-mediated intracellular Cre protein delivery for maize genome editing via *loxP* site excision, *Plant Physiol.* 164 (2) (2014) 537–547, 10.1104/pp.113.233650. [PubMed: 24376280]
- [48]. Nair R, Varghese SH, Nair BG, Maekawa T, Yoshida Y, Kumar DS, Nanoparticulate material delivery to plants, *Plant Sci.* 179 (3) (2010) 154–163, 10.1016/j.plantsci.2010.04.012.
- [49]. Torney F, Trewyn BG, Lin VS-Y, Wang K, Mesoporous silica nanoparticles deliver DNA and chemicals into plants, *Nat. Nanotechnol* 2 (2007) 295–300, 10.1038/nnano.2007.108. [PubMed: 18654287]
- [50]. Klaine SJ, Alvarez PJJ, Batley GE, Fernandes TF, Handy RD, Lyon DY, Mahendra S, McLaughlin MJ, Lead JR, Nanomaterials in the environment: behavior, fate, bioavailability, and effects, *Environ. Toxicol. Chem* 27 (9) (2008) 1825–1851, 10.1897/08-090.1. [PubMed: 19086204]
- [51]. Rico CM, Majumdar S, Duarte-Gardea M, Peralta-Videa JR, Gardea-Torresdey JL, Interaction of nanoparticles with edible plants and their possible implications in the food chain, *J. Agric. Food Chem* 59 (8) (2011) 3485–3498, 10.1021/jf104517j. [PubMed: 21405020]
- [52]. Wang S, Kurepa J, Smalle JA, Ultra-small TiO₂ nanoparticles disrupt microtubular networks in *Arabidopsis thaliana*, *Plant Cell Environ.* 34 (5) (2011) 811–820, 10.1111/j.1365-3040.2011.02284.x. [PubMed: 21276012]
- [53]. Miralles P, Church TL, Harris AT, Toxicity, uptake, and translocation of engineered nanomaterials in vascular plants, *Environ. Sci. Technol* 46 (17) (2012) 9224–9239, 10.1021/es202995d. [PubMed: 22892035]
- [54]. Mu Q, Jiang G, Chen L, Zhou H, Fourches D, Tropsha A, Yan B, Chemical basis of interactions between engineered nanoparticles and biological systems, *Chem. Rev* 114 (15) (2014) 7740–7781, 10.1021/cr400295a. [PubMed: 24927254]
- [55]. Slowing II, Trewyn BG, Giri S, Lin VY, Mesoporous silica nanoparticles for drug delivery and biosensing applications, *Adv. Funct. Mater* 17 (8) (2007) 1225–1236, 10.1002/adfm.200601191.
- [56]. Graf C, Gao Q, Schütz I, Noufele CN, Ruan W, Posselt U, Korotianskiy E, Nordmeyer D, Rancan F, Hadam S, Vogt A, Lademann J, Haucke V, Rühl E, Surface functionalization of silica nanoparticles supports colloidal stability in physiological media and facilitates internalization in cells, *Langmuir* 28 (20) (2012) 7598–7613, 10.1021/la204913t. [PubMed: 22524440]
- [57]. Wong MH, Misra RP, Giraldo JP, Kwak S-Y, Son Y, Landry MP, Swan JW, Blankshtein D, Strano MS, Lipid exchange envelope penetration (LEEP) of nanoparticles for plant engineering:

a universal localization mechanism, *Nano Lett* 16 (2) (2016) 1161–1172, 10.1021/acs.nanolett.5b04467. [PubMed: 26760228]

- [58]. Jung H-S, Moon D-S, Lee J-K, Quantitative analysis and efficient surface modification of silica nanoparticles, *J. Nanomater* (2012), 10.1155/2012/593471.
- [59]. Sakhtianchi R, Minchin RF, Lee K-B, Alkilany AM, Serpooshan V, Mahmoudi M, Exocytosis of nanoparticles from cells: role in cellular retention and toxicity, *Adv. Colloid Interf. Sci* 201–202 (2013) 18–29, 10.1016/j.cis.2013.10.013.
- [60]. Strobel C, Oehring H, Herrmann R, Förster M, Reller A, Hilger I, Fate of cerium dioxide nanoparticles in endothelial cells: exocytosis, *J. Nanopart. Res* 17 (5) (2015) 206, 10.1007/s11051-015-3007-4. [PubMed: 25972759]
- [61]. Ma X, Geiser-Lee J, Deng Y, Kolmakov A, Interactions between engineered nanoparticles (ENPs) and plants: phytotoxicity, uptake and accumulation, *Sci. Total Environ* 408 (16) (2010) 3053–3061, 10.1016/j.scitotenv.2010.03.031. [PubMed: 20435342]
- [62]. Slomberg DL, Schoenfisch MH, Silica nanoparticle phytotoxicity to *Arabidopsis thaliana*, *Environ. Sci. Technol* 46 (18) (2012) 10247–10254, 10.1021/es300949f. [PubMed: 22889047]
- [63]. Kurepa J, Paunesku T, Vogt S, Arora H, Rabatic BM, Lu J, Wanzer MB, Woloschak GE, Smalle JA, Uptake and distribution of ultrasmall anatase TiO₂ alizarin red S nanoconjugates in *Arabidopsis thaliana*, *Nano Lett.* 10 (7) (2010) 2296–2302, 10.1021/nl903518f. [PubMed: 20218662]
- [64]. Cowen T, Haven AJ, Burnstock G, Pontamine sky blue: a counterstain for background autofluorescence in fluorescence and immunofluorescence histochemistry, *Histochemistry* 82 (3) (1985) 205–208, 10.1007/bf00501396. [PubMed: 2581921]
- [65]. Sharma RI, Schwarzbauer JE, Moghe PV, Nanomaterials can dynamically steer cell responses to biological ligands, *Small* 7 (2) (2011) 242–251, 10.1002/sml.201001518. [PubMed: 21213389]
- [66]. Lutz JA, Kulshrestha M, Rogers DT, Littleton JM, A nicotinic receptor-mediated anti-inflammatory effect of the flavonoid rhamnetin in BV2 microglia, *Fitoterapia* 98 (2014) 11–21, 10.1016/j.fitote.2014.06.012. [PubMed: 24972350]
- [67]. Kim T-W, Chung P-W, Lin VSY, Facile synthesis of monodisperse spherical MCM-48 mesoporous silica nanoparticles with controlled particle size, *Chem. Mater.* 22 (17) (2010) 5093–5104, 10.1021/cm1017344.
- [68]. Lee JE, Lee N, Kim H, Kim J, Choi SH, Kim JH, Kim T, Song IC, Park SP, Moon WK, Uniform mesoporous dye-doped silica nanoparticles decorated with multiple magnetite nanocrystals for simultaneous enhanced magnetic resonance imaging, fluorescence imaging, and drug delivery, *J. Am. Chem. Soc* 132 (2) (2009) 552–557, 10.1021/ja905793q.
- [69]. Pan L, He Q, Liu J, Chen Y, Ma M, Zhang L, Shi J, Nuclear-targeted drug delivery of TAT peptide-conjugated monodisperse mesoporous silica nanoparticles, *J. Am. Chem. Soc* 134 (13) (2012) 5722–5725, 10.1021/ja211035w. [PubMed: 22420312]
- [70]. Gartmann N, Brühwiler D, Controlling and imaging the functional-group distribution on mesoporous silica, *Angew. Chem. Int. Ed.* 48 (34) (2009) 6354–6356, 10.1002/anie.200902436.
- [71]. Hu H, Zhou H, Du J, Wang Z, An L, Yang H, Li F, Wu H, Yang S, Biocompatible hollow silica microspheres as novel ultrasound contrast agents for *in vivo* imaging, *J. Mater. Chem* 21 (18) (2011) 6576–6583, 10.1039/C0JM03915B.
- [72]. Jaroniec M, Kruk M, Olivier JP, Standard nitrogen adsorption data for characterization of nanoporous silicas, *Langmuir* 15 (16) (1999) 5410–5413, 10.1021/la990136e.
- [73]. Sayari A, Liu P, Kruk M, Jaroniec M, Characterization of large-pore MCM-41 molecular sieves obtained via hydrothermal restructuring, *Chem. Mater* 9 (11) (1997) 2499–2506, 10.1021/cm970128o.
- [74]. Schlupf DM, Jones CA, Armbruster ME, Rushing ES, Wooten KC, Rankin SE, Knutson BL, Flavonoid adsorption and stability on titania-functionalized silica nanoparticles, *Colloids Surf. A Physicochem. Eng. Asp* 478 (2015) 15–21, 10.1016/j.colsurfa.2015.03.039.
- [75]. Schlupf DM, Rankin SE, Knutson BL, Selective external surface functionalization of large-pore silica materials capable of protein loading, *Microporous Mesoporous Mater.* 244 (2017) 199–207, 10.1016/j.micromeso.2016.10.023.

- [76]. Ritter H, Nieminen M, Karppinen M, Bruhwiler D, A comparative study of the functionalization of mesoporous silica MCM-41 by deposition of 3-aminopropyl-trimethoxysilane from toluene and from the vapor phase, *Microporous Mesoporous Mater.* 121 (1–3) (2009) 79–83, 10.1016/j.micromeso.2009.01.006.
- [77]. Brown DP, Rogers DT, Pomerleau F, Siripurapu KB, Kulshrestha M, Gerhardt GA, Littleton JM, Novel multifunctional pharmacology of lobinaline, the major alkaloid from *Lobelia cardinatis*, *Fitoterapia* 111 (2016) 109–123, 10.1016/j.fitote.2016.04.013. [PubMed: 27105955]
- [78]. Kim M-H, Na H-K, Kim Y-K, Ryoo S-R, Cho HS, Lee KE, Jeon H, Ryoo R, Min D-H, Facile synthesis of monodispersed mesoporous silica nanoparticles with ultralarge pores and their application in gene delivery, *ACS Nano* 5 (5) (2011) 3568–3576, 10.1021/nn103130q. [PubMed: 21452883]
- [79]. Suligoj A, Stangar UL, Ristic A, Mazaj M, Verhovsek D, Tusar NN, TiO₂-SiO₂ films from organic-free colloidal TiO₂ anatase nanoparticles as photocatalyst for removal of volatile organic compounds from indoor air, *Appl. Catal. B* 184 (2016) 119–131, 10.1016/j.apcatb.2015.11.007.
- [80]. Ayad MM, Salahuddin NA, El-Nasr AA, Torad NL, Amine-functionalized mesoporous silica KIT-6 as a controlled release drug delivery carrier, *Microporous Mesoporous Mater.* 229 (2016) 166–177, 10.1016/j.micromeso.2016.04.029.
- [81]. Ezzeddine Z, Batonneau-Gener I, Pouilloux Y, Hamad H, Saad Z, Kazpard V, Divalent heavy metals adsorption onto different types of EDTA-modified mesoporous materials: effectiveness and complexation rate, *Microporous Mesoporous Mater.* 212 (2015) 125–136, 10.1016/j.micromeso.2015.03.013.
- [82]. Feliczak-Guzik A, Jadach B, Piotrowska H, Murias M, Lulek J, Nowak I, Synthesis and characterization of SBA-16 type mesoporous materials containing amine groups, *Microporous Mesoporous Mater.* 220 (2016) 231–238, 10.1016/j.micromeso.2015.09.006.
- [83]. Hori K, Higuchi T, Aoki Y, Miyamoto M, Oumi Y, Yogo K, Uemiya S, Effect of pore size, aminosilane density and aminosilane molecular length on CO₂ adsorption performance in aminosilane modified mesoporous silica, *Microporous Mesoporous Mater.* 246 (2017) 158–165, 10.1016/j.micromeso.2017.03.020.
- [84]. Nordmann J, Buczka S, Voss B, Haase M, Mummenhoff K, *In vivo* analysis of the size- and time-dependent uptake of NaYF₄:Yb,Er upconversion nanocrystals by pumpkin seedlings, *J. Mater. Chem. B* 3 (1) (2015) 144–150, 10.1039/C4TB01515K.
- [85]. Servin AD, Castillo-Michel H, Hernandez-Viezas JA, Diaz BC, Peralta-Videa JR, Gardea-Torresdey JL, Synchrotron micro-XRF and micro-XANES confirmation of the uptake and translocation of TiO₂ nanoparticles in cucumber (*Cucumis sativus*) plants, *Environ. Sci. Technol* 46 (14) (2012) 7637–7643, 10.1021/es300955b. [PubMed: 22715806]
- [86]. Larue C, Laurette J, Herlin-Boime N, Khodja H, Fayard B, Flank A-M, Brisset F, Carriere M, Accumulation, translocation and impact of TiO₂ nanoparticles in wheat (*Triticum aestivum* spp.): influence of diameter and crystal phase, *Sci. Total Environ* 431 (2012) 197–208, 10.1016/j.scitotenv.2012.04.073. [PubMed: 22684121]
- [87]. Mahony D, Cavallaro AS, Mody KT, Xiong L, Mahony TJ, Qiao SZ, Mitter N, In vivo delivery of bovine viral diarrhoea virus, E2 protein using hollow mesoporous silica nanoparticles, *Nanoscale* 6 (12) (2014) 6617–6626, 10.1039/C4NR01202J. [PubMed: 24811899]
- [88]. Stalmans P, Van Aken EH, Melles G, Veckeneer M, Feron EJ, Stalmans I, Trypan blue not toxic for retinal pigment epithelium *in vitro*, *Am J. Ophthalmol* 135 (2) (2003) 234–236, 10.1016/S0002-9394(02)01891-3. [PubMed: 12566035]
- [89]. Corredor E, Testillano PS, Coronado M-J, González-Melendi P, Fernández-Pacheco R, Marquina C, Ibarra MR, de la Fuente JM, Rubiales D, Pérez-deLuque A, Risueño M-C, Nanoparticle penetration and transport in living pumpkin plants: *in situ* subcellular identification, *BMC Plant Biol.* 9 (1) (2009) 45, 10.1186/1471-2229-9-45. [PubMed: 19389253]
- [90]. Indrasekara ASDS, Paladini BJ, Naczynski DJ, Starovoytov V, Moghe PV, Fabris L, Dimeric gold nanoparticle assemblies as tags for SERS-based cancer detection, *Adv. Healthcare Mater* 2 (10) (2013) 1370–1376, 10.1002/adhm.201200370.
- [91]. Asli S, Neumann PM, Colloidal suspensions of clay or titanium dioxide nanoparticles can inhibit leaf growth and transpiration via physical effects on root water transport, *Plant Cell Environ.* 32 (5) (2009) 577–584, 10.1111/j.1365-3040.2009.01952.x. [PubMed: 19210640]

- [92]. Hong F, Yang F, Liu C, Gao Q, Wan Z, Gu F, Wu C, Ma Z, Zhou J, Yang P, Influences of nano-TiO₂ on the chloroplast aging of spinach under light, *Biol. Trace Elem. Res* 104 (3) (2005) 249–260, 10.1385/BTER:104:3:249. [PubMed: 15930594]
- [93]. Saw CLL, Guo Y, Yang AY, Paredes-Gonzalez X, Ramirez C, Pung D, Kong A-NT, The berry constituents quercetin, kaempferol, and pterostilbene synergistically attenuate reactive oxygen species: involvement of the Nrf2-ARE signaling pathway, *Food Chem. Toxicol* 72 (2014) 303–311, 10.1016/j.fct.2014.07.038. [PubMed: 25111660]
- [94]. Mudunkotuwa IA, Grassian VH, Citric acid adsorption on TiO₂ nanoparticles in aqueous suspensions at acidic and circumneutral pH: surface coverage, surface speciation, and its impact on nanoparticle – nanoparticle interactions, *J. Am. Chem. Soc* 132 (42) (2010) 14986–14994, 10.1021/ja106091q. [PubMed: 20919713]
- [95]. Bishop LM, Yeager JC, Chen X, Wheeler JN, Torelli MD, Benson MC, Burke SD, Pedersen JA, Hamers RJ, A citric acid-derived ligand for modular functionalization of metal oxide surfaces via “click” chemistry, *Langmuir* 28 (2) (2012) 1322–1329, 10.1021/la204145t. [PubMed: 22145802]
- [96]. Peer WA, Brown DE, Tague BW, Muday GK, Taiz L, Murphy AS, Flavonoid accumulation patterns of transparent testa mutants of *Arabidopsis*, *Plant Physiol.* 126 (2) (2001) 536–548, 10.1104/pp.126.2.536. [PubMed: 11402185]
- [97]. Yin W, Zhou L, Ma Y, Tian G, Zhao J, Yan L, Zheng X, Zhang P, Yu J, Gu Z, Zhao Y, Phytotoxicity, translocation, and biotransformation of NaYF₄ upconversion nanoparticles in a soybean plant, *Small* 11 (36) (2015) 4774–4784, 10.1002/smll.201500701. [PubMed: 26099115]
- [98]. Serag MF, Kaji N, Gaillard C, Okamoto Y, Terasaka K, Jabasini M, Tokeshi M, Mizukami H, Bianco A, Baba Y, Trafficking and subcellular localization of multiwalled carbon nanotubes in plant cells, *ACS Nano* 5 (1) (2011) 493–499, 10.1021/nn102344t. [PubMed: 21141871]
- [99]. Low PS, Chandra S, Endocytosis in plants, *Annu. Rev. Plant Biol* 45 (1) (1994) 609–631, 10.1146/annurev.pp.45.060194.003141.
- [100]. Gan Q, Dai D, Yuan Y, Qian J, Sha S, Shi J, Liu C, Effect of size on the cellular endocytosis and controlled release of mesoporous silica nanoparticles for intracellular delivery, *Biomed. Microdevices* 14 (2) (2012) 259–270, 10.1007/s10544-011-9604-9. [PubMed: 22124885]
- [101]. Hu L, Mao Z, Zhang Y, Gao C, Influences of size of silica particles on the cellular endocytosis, exocytosis and cell activity of HepG2 cells, *J. Nanosci. Lett* 1 (1) (2011) 1–16.
- [102]. Oh N, Park J-H, Endocytosis and exocytosis of nanoparticles in mammalian cells, *Int. J. Nanomedicine* 9 (Suppl. 1) (2014) 51–63, 10.2147/IJN.S26592. [PubMed: 24872703]
- [103]. Jiang X, Röcker C, Hafner M, Brandholt S, Dörlich RM, Nienhaus GU, Endo- and exocytosis of zwitterionic quantum dot nanoparticles by live HeLa cells, *ACS Nano* 4 (11) (2010) 6787–6797, 10.1021/nn101277w. [PubMed: 21028844]
- [104]. Wang Y, Gu H, Core-shell-type magnetic mesoporous silica nanocomposites for bioimaging and therapeutic agent delivery, *Adv. Mater* 27 (3) (2015) 576–585, 10.1002/adma.201401124. [PubMed: 25238634]

Fig. 1. SEM image of (a) MSNP, (b) MSNPT, (c) MSNPA and (d) MSNPTA (scale bar 500 nm for all images).

Fig. 2.

Bright field (left) and corresponding fluorescence microscopic image (right) of *Solidago nemoralis* hairy roots after RITC-tagged MSNPTA uptake: (a) & (b) without and (c) & (d) with trypan blue addition for 100 μg/mL nanoparticle solution (exposure time 150 ms). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

Fig. 3. Magnified fluorescence image showing intracellular localization of MSNPTAs for 100 $\mu\text{g/mL}$ nanoparticle concentration (a) in absence of trypan blue and (b) in the presence of trypan blue.

Fig. 4. Viability of *Solidago nemoralis* hairy roots culture after exposure to 2.5 mg/mL functionalized MSNPs for 48 h and re-culturing: (a) control after 0 week, (b) only TiO₂ functionalized MSNPs after 0 weeks, (c) TiO₂-amine functionalized MSNPs after 0 weeks, (d) control after 2 week, (e) only TiO₂ functionalized MSNPs after 2 weeks and (f) TiO₂-amine functionalized MSNPs after 2 weeks.

Fig. 5.

Radical scavenging activity (RSA) of MSNPs functionalized with only TiO_2 and TiO_2 -amine group when nanoparticles (25 mg, 2.5 mg/mL in MS media) were exposed to 500 mg hairy roots, leftover solution and control solution (error bars are standard deviation from quadruplicate measurements and statistically significant difference in mean ($p < 0.05$) from unpaired t -test shown by 'asterisk').

Fig. 6. ^3H -methyllycaconitine (^3H -MLA) binding displacement activity of (a) *Solidago nemoralis* hairy root extracts and (b) TiO_2 and amine functionalized MSNPs (25 mg, 2.5 mg/mL) and their eluent in ethanolic citric acid (20% w/v) exposed to 500 mg hairy root compared to control (error bars are standard deviation from quadruplicate measurements and statistically significant difference in mean (p < 0.05) from unpaired t-test shown by 'asterisk').

Fig. 7.

Comparative radical scavenging activity (RSA) of root extracts after exposure to control (no nanoparticles), only TiO₂ functionalized MSNPs and TiO₂-amine functionalized MSNPs (error bars are standard deviation from triplicate measurements and statistical difference in mean ($p < 0.05$) from unpaired t -test represented by 'asterisk').

Table 1

BET specific surface area, BJH pore volume and average pore diameter of TiO₂ functionalized, amine functionalized, and TiO₂-amine functionalized mesoporous silica nanoparticles (MSNPs) with optimum TiO₂ content compared to non-functionalized MSNPs.

Particle types	Average particle diameter	Amount of TiO ₂	Amount of amine	BET surface area	BJH pore volume	Average pore diameter
	nm	mg/g particle	mmol/g particle	m ² /g	cm ³ /g	nm
MSNP	165 ± 19 ^a	–	–	953	1.21	2.76
MSNPT	185 ± 29	425 ± 9.2 ^b	–	629	0.59	2.21
MSNPA	172 ± 26	–	2.00 ± 0.16 ^b	400	0.57	2.50
MSNPTA	174 ± 32	407 ± 13	0.65 ± 0.07	325	0.33	1.97

^a Standard deviation values resulted from measurement of 20 particles selected randomly (using ImageJ Software).

^b Standard deviation values resulted from quadruplicate measurement for chemical analysis.