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(54) Title: PLANT NANOBIONICS FOR ENHANCED PHYTOREMEDIATION

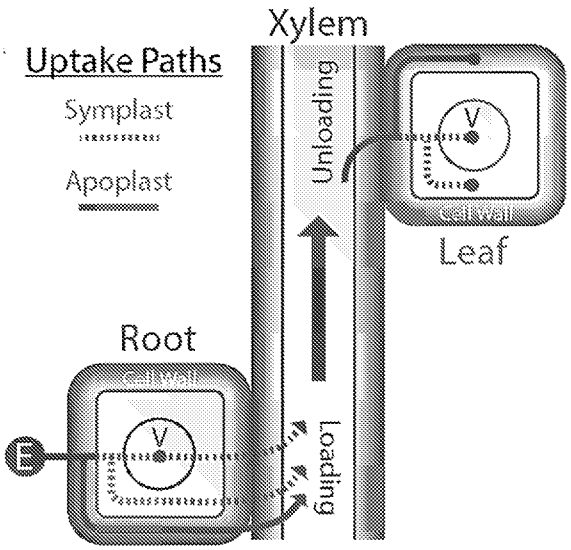


FIG. 1A

(57) Abstract: The present disclosure provides methods of conducting phytoremediation of soil containing one or more contaminants comprising growing plants in soil treated with nanoparticles, wherein the plants uptake the nanoparticles.

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PLANT NANOBIONICS FOR ENHANCED PHYTOREMEDIATION

CROSS REERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 63/509,506, filed June 21, 2023, the contents of which are herein incorporated by reference in their entirety.

FEDERAL FUNDING STATEMENT

[0002] This invention was made with government support under DE-SC0019112 awarded by U.S. Department of Energy. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION

[0003] Widespread environmental contamination by heavy metals, perfluorinated compounds, and a host of other toxic organics—paired with the rapidly progressing climate crisis—necessitates sustainable soil and groundwater remediation schemes. As the world progresses toward a net-zero carbon future, there exist over five million brownfield sites globally that require remediation and redevelopment to mitigate urban sprawl. Phytoremediation and redevelopment provide increased economic growth, reduced human health risk, reduced environmental injustice, and mitigate urban sprawl. Forms of phytoremediation include removal and transformation. Removal involves contaminant uptake from the environment and sequestration in the plant, whereas transformation involves degradation and volatilization of the contaminant.

[0004] The use of plants for phytoremediation is a carbon-negative, self-sustaining, and inexpensive approach to remove and transform nonpoint sources of environmental contamination. Phytoremediation exploits the way plants naturally interact with the environment to remove or transform contaminants from soil, water, or air matrices where they no longer pose a direct threat to human health. Despite these attractive features, phytoremediation is exceedingly time consuming due to slow kinetics or low levels of biomass production, limiting their practical application to low-value lands. This largely stems from the limited number of plants that can be used for remediation of contaminants of interest, resulting in a less-than-ideal library of plant properties to choose from.

[0005] Certain plants function in phytoremediation as hyperaccumulators. Naturally occurring hyperaccumulators of arsenic (As) can accumulate several orders of magnitude more As in their tissues than most other plant species. Hyperaccumulators primarily store As in their shoots, easing the necessary harvesting and disposal processes. However, the known

hyperaccumulating species lack the biomass production to be highly effective phytoremediators, have limited growing conditions, and there only exist a few known contaminants that can be hyperaccumulated.

[0006] Accordingly, there exists a need in the art for improved methods of phytoremediation that ideally mimic hyperaccumulators.

SUMMARY OF THE INVENTION

[0007] The present disclosure provides a method of conducting phytoremediation of soil containing one or more contaminants comprising growing plants in soil treated with nanoparticles, wherein the nanoparticles sorb the one or more contaminants, and wherein the plants uptake the nanoparticles.

[0008] In certain embodiments, the nanoparticles are engineered to sorb the one or more contaminants. In certain embodiments, the nanoparticles accumulate in the protoplast, and in other embodiments, the nanoparticles do not accumulate in vacuoles.

[0009] In certain embodiments, the plants uptake the nanoparticles through symplastic transport, and in certain embodiments, the nanoparticles translocate from the roots to the shoots of the plants.

[0010] In certain embodiments, the shoot uptake coefficient is about 100 mL kg^{-1} d^{-1}.

[0011] In certain embodiments, the one or more contaminants are heavy metals and/or organic pollutants, in certain embodiments the heavy metals are arsenic, cadmium, chromium, lead, nickel, and zinc, and in certain embodiments the heavy metal is arsenic.

[0012] In certain embodiments the plants comprise *Zea mays*.

[0013] In certain embodiments, the soil is treated to comprise nanoparticles at 200, 400, 600, 800 or 1000 mg per kilogram of soil.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] **FIG. 1** provides a summary of solute fate and transport in plant systems. **FIG. 1A** shows a schematic illustration of symplastic (solid line) and apoplastic (dashed line) transport pathways for uptake from the environment to plant leaf cellular compartments. Red circles indicate solute sequestration. **FIG. 1B** is an illustration of plant transport as a resistors-in-series model.

[0015] **FIG. 2** provides nanoparticle (NP) design considerations for enhancing contaminant uptake *in planta*. **FIG. 2A** is an illustration of NP carrying a molecular species (e.g., arsenic, As) across the lipid bilayer of a plant cell through adsorption, membrane softening, and

internalization. **FIG. 2B** shows accumulation rate of NPs in in the shoots versus roots of various plant species. **FIG. 2C** shows the fate of NPs *in planta* with respect to their size and surface charge. Pointed symbols depict NP localization in the root exudate (RE), cell wall or intercellular space (CW / ICS), cytoplasm (CY), protoplast (P), or vacuole (V). Circles represent data where the precise localization area was not provided (N/A). Lines represent the threshold for cross-over of nanoparticles into the protoplast (solid) and vacuole (dashed) as predicted by the lipid exchange envelope penetration (LEEP) model. **FIG. 2D** shows normalized shoot uptake rate (α, mL kg^{-1} d^{-1}) with respect to NP size and surface charge. **FIG. 2E** shows mass flow rate of As into plant shoots ($Q_{s,As}$) with respect to NP concentration in soil and its As adsorption capacity (K_{As}) for α of 10 ml kg^{-1} d^{-1} (left) and 100 mL kg^{-1} d^{-1}.

DETAILED DESCRIPTION OF THE INVENTION

[0016] The inventors have used plant nanobionics, which uses nanomaterials to confer non-native functionalities to plants, to develop the next generation of phytoremediation. This approach has many advantages in that it is species-independent and much faster to implement than omics-based approaches. In particular, the inventors have developed nanobionic phytoremediation methods with fast, targeted removal of contaminants at high capacities, and have found that the use of nanoparticles can mimic the performance of naturally occurring hyperaccumulating plant species, but engineered for plants possessing more beneficial phytoremediation properties (e.g., high biomass production).

[0017] In particular, the inventors have discovered that nanoparticle adsorbents enhance the uptake and translocation of contaminants such as arsenic (As) from the environment to the plant shoots. Without wishing to be bound to any particular mechanism, the nanoparticles act as a "nanocarrier" that can be engineered to favorably interface with the plant cellular space for greater uptake. Historically, nanoparticles studied in the literature have largely remained in the roots rather than translocate to the shoots. In contrast, the inventors have developed methods associated with and enabling the development and engineering of nanoparticles with enhanced translocation from the root to shoots for engineering more effective phytoremediators.

[0018] The methods of the invention comprise conducting phytoremediation of soil containing one or more contaminants comprising growing plants in soil treated with nanoparticles, wherein the nanoparticles sorb the one or more contaminants, and wherein the plants uptake the nanoparticles.

[0019] The soil may be contaminated with one or more contaminants for which phytoremediation is appropriate, i.e. for which phytoremediation can remove all or part of the contaminant. In certain embodiments, the one or more contaminants may be inorganic and/or organic pollutants, including heavy metals. Heavy metals include, but are not limited to, arsenic, cadmium, chromium, lead, nickel, and zinc. Organic pollutants include, but are not limited to, e.g., halogenated hydrocarbons, polycyclic aromatic hydrocarbons, polychlorinated biphenyl, organochlorine pesticides, total petroleum hydrocarbons, and per- and polyfluoroalkyl substances including perfluorosulfonic acids and perfluorocarboxylic acids.

[0020] The plants may be any plant suitable for uptake of nanoparticles comprising the one or more contaminants. As used herein, "plants" refers to a single species or a combination of species of plants. In certain embodiments, the plants are high biomass production plants. In certain embodiments, the plants are non-hyperaccumulating plants. In certain embodiments, the plants comprise *Zea mays*.

[0021] As used herein, the term "nanoparticle" refers to articles having at least one cross-sectional dimension of less than about 1 micron. A nanoparticle can also be referred to as a "nanostructure" or "nanocarrier." A nanoparticle can have at least one cross-sectional dimension of less than about 500 nm, less than about 250 nm, less than about 100 nm, less than about 75 nm, less than about 50 nm, less than about 25 nm, less than about 10 nm, or, in some cases, less than about 1 nm. As used herein "about" means ± 10% which may include any amount less than that even if not specifically enumerated herein, e.g. ±9%, ±8%, ±7%, ±6%, ±5%, ±4%, ±3%, ±2%, or ±1%. Reference to "±" herein means plus, minus, or plus and minus.

[0022] Any biocompatible nanoparticle comprising any materials may be used, provided the nanoparticles are not phytotoxic. Examples of nanoparticles include nanotubes (e.g., carbon nanotubes), nanowires (e.g., carbon nanowires), graphene, and quantum dots, among others. It would be within the purview of one of skill in the art, in view of the disclosure herein, to engineer suitable nanoparticles with appropriate parameters, including, but not limited to size, charge, and chemistry to optimize NP-lipid interactions for increased uptake, for use with the methods disclosed herein.

[0023] The nanoparticles for use with the methods disclosed herein sorb the one or more contaminants in the soil, wherein the plants uptake the nanoparticles carrying the contaminants. In certain embodiments, the nanoparticles are engineered to adsorb the one or more contaminants. The nanoparticles may completely remove a given contaminant or

contaminants, or they may partially remove them, e.g. reach about 50-100% removal. As used herein, when a range is recited, that range is meant to refer to any particular value or sub-range within the recited range, even if not specifically enumerated herein. Thus, the nanoparticles may remove, e.g. 50%, 60%, 70%, 80%, or 90% of a given contaminant, or they may remove, e.g. 50-75%, 60-80%, 70-90%, or 80-90% of a given contaminant.

[0024] In the methods disclosed herein, the soil is treated with sufficient nanoparticles to effectively remove a given contaminant. In certain embodiments, the soil is treated to comprise nanoparticles at 200, 400, 600, 800 or 1000 mg per kilogram of soil.

[0025] The nanoparticles may accumulate in the protoplast, and further, they may not accumulate in vacuoles. In particular, in certain embodiments, the nanoparticles enter the root protoplast and do not accumulate in root vacuoles. Uptake of the nanoparticles may be through symplastic transport.

[0026] The nanoparticles may translocate from the roots to the shoot of the plants. In certain embodiments, the shoot uptake coefficient (α) is may be about 75 mL kg^{-1} d^{-1} to about 125 mL kg^{-1} d^{-1}. The shoot uptake coefficient may be about 80 mL kg^{-1} d^{-1} to about 120 mL kg^{-1} d^{-1}, or about 85 mL kg^{-1} d^{-1} to about 115 mL kg^{-1} d-1, or about 90 mL kg^{-1} d^{-1} to about 110 mL kg^{-1} d-1. In certain embodiments, the shoot uptake coefficient is about 100 mL kg^{-1} d^{-1}.

EXAMPLES

Example 1: Solute Uptake

[0027] There are two primary pathways that solutes, including nanoparticles, can transport from the roots to the shoots of the plant: the apoplast and symplast. Apoplastic transport describes solute movement outside of the plant cell membrane, such as in the cell wall or intercellular spaces. Complete transport from the roots to the shoots via the apoplast, although generally considered a low resistance pathway, does not exist without the presence of defects in the Casparian band, an evolutionary development by plants to prevent the uptake of unwanted solutes. Therefore, solutes and nanoparticles alike are generally expected to transport along the symplast to reach the plant shoots. The symplast requires internalization of these species through the cell membrane, where solutes and nanoparticles can then move cell to cell across the plasmodesmata on their way to the plant vasculature.

[0028] Solute uptake from the environment and translocation to the shoots was modeled as resistors-in-series, comparing common, hypertolerant, and hyperaccumulating plants to elucidate the bottleneck for rapid sequestration. In this case, the solute transport driving force is transpiration, which can be defined by the negative water potential across the plant. This

approach was employed to quantitatively evaluate the uptake efficiency of various plant species to arsenic (As), a common environmental contaminant of major concern, as proof of concept. 36 independent studies of plants interacting with As or nanoparticles were modeled.

[0029] The quantitative form of the model was $Q = \Delta\Psi / R_i$, where Q is the mass flow rate of the contaminant or nanoparticle, $\Delta\Psi$ is the negative water potential across the plant, R_i is the areal resistance of the contaminant or nanoparticle to transport. The series of resistances included resistance to uptake of the contaminant or nanoparticle from the environment into the root (R_U), loading into the xylem (e.g., vasculature) from the root (R_X), translocation from the vasculature into the leaves (R_T), and sequestration from the leaves into a cell compartment (R_S).

[0030] Based on literature data of typical plant species, the model assumed $\Delta\Psi$ of 0.7 MPa.

[0031] Given the practical relevance of transporting the contaminant or nanoparticle from the root to shoot, regardless of end destination within the shoot, the model ignored sequestration into a cell compartment (i.e., the model neglected R_S). Additionally, the model assumed that R_T is negligible relative to the R_X and R_U due to the large microporous environment that comprises the xylem, in stark contrast to the pathways found within the symplast.

Example 2: Nanoparticle Uptake and Translocation in Plants

[0032] There is substantial controversy in the literature regarding the transport of nanoparticles *in planta*, with several contradicting studies on the ability of nanoparticles to transport via symplastic pathways. A thermodynamic lipid-nanoparticle interaction model was employed that uses nanoparticle surface charge and size to determine plant cell internalization for elucidating the fate of nanoparticles *in planta* for the first time. This model, termed the Lipid Exchange Envelope Penetration (LEEP) model, was previously developed for isolated protoplast lacking cell walls.

[0033] The quantitative form of the model was as follows:

$$\zeta = \pm\left(\frac{\varepsilon_M + \varepsilon_W}{\varepsilon_W}\right)\left(\frac{d}{a}\right)e^{\kappa(d-a)}\sqrt{\frac{(\Gamma - a\gamma_0 + 4a\Delta\Delta H\rho_n)L}{2a\varepsilon_0\varepsilon_M\left(1-\frac{\varepsilon_M}{\varepsilon_W}\right)}}$$

[0034] Where ζ, is the threshold zeta potential for nanoparticle entry, ε_M and ε_W are relative permittivity of the plant membrane and medium respectively, d is the effective charge radius, a is the nanoparticle radius, κ^{-1} is the Debye screening length, Γ is the pore line tension, γ_0 is the resting membrane tension, $\Delta\Delta H$ is the change in free energy due to lipid binding on

nanoparticle, ρ_n is the lipid density on nanoparticle, and L is the approximate thickness of the membrane dielectric.

[0035] Key assumptions used in this model were a $\Delta\Delta H$ of $0.04k_BT$ and that the pore line tension of the protoplast membrane was less than that of the membrane surrounding the vacuole.

[0036] Results indicated that nanoparticles predicted to internalize within the protoplast, but not in the vacuole, have a substantially higher likelihood of finding their way to the plant shoots rather than being sequestered in the root system.

[0037] Uptake kinetics of nanoparticles into the plant shoots were described by a shoot uptake coefficient (α), which described the normalized uptake rate of nanoparticles from the plant root into the shoot with respect to the nanoparticle concentration (C_{NP}) dosed in the soil (i.e., $\alpha = Q/C_{NP}$).

[0038] It was found that nanoparticles that met the criteria (of entering protoplast but not being internalized in the vacuole) tended to translocate to the shoots at ≥10-fold rates. These findings reveal several new and exciting insights. First, the results indicate that efficient nanoparticle transport into the plant shoots does not rely upon defective apoplastic transport, but rather symplastic transport. This development is a significant advancement for understanding nanoparticle transport in plants. Further, the LEEP model, although a thermodynamic model that was only tested for isolated plant protoplasts/chloroplasts, can inform nanoparticle uptake kinetics as well as predict internalization in plant cell vacuoles.

[0039] A mechanistic framework for predicting the performance of plant nanobionic-based phytoremediators was established based on these findings. This framework relies on the nanoparticle adsorption capacity, amount of nanoparticles introduced to the soil, and the α coefficient of the nanoparticle. We found that nanoparticles engineered to have an α coefficient of 100 mL kg^{-1} d^{-1} can readily outcompete the As accumulation rates of common plants (1 mg kg^{-1} d^{-1}) and can compete with the As accumulation rates of hyperaccumulators at sufficiently high nanoparticle concentrations and adsorption capacities. This framework suggests that optimizing the nanoparticle surface to increase the α coefficient (e.g., using hydrophobic coatings to modulate $\Delta\Delta H$) is a critical step to realizing this technological approach to phytoremediation. Optimizing the surface interactions must be done mindfully to maintain the balance between nanoparticle size and surface charge that enables internalization in the protoplast but not the vacuole.

[0040] The design schemes developed herein are independent of the plants' species and can thus be used to substantially improve the phytoremediation ability of high biomass production plants. For *Zea Mays*, we predict an order of magnitude improvement in the As accumulation rate when using engineered nanoparticles. Plant nanobionics thus lends itself as an exciting technique to engineer high biomass, non-hyperaccumulating plants as fit-for-purpose, next-generation phytoremediators.

We claim:

1. A method of conducting phytoremediation of soil containing one or more contaminants comprising growing plants in soil treated with nanoparticles, wherein the nanoparticles sorb the one or more contaminants, and wherein the plants uptake the nanoparticles.

2. The method of claim 1, wherein the nanoparticles are engineered to adsorb the one or more contaminants.

3. The method of any one of claims 1-2, wherein the nanoparticles accumulate in the protoplast.

4. The method of claim 3, wherein the nanoparticles do not accumulate in vacuoles.

5. The method of any one of claims 1-4, wherein the plants uptake the nanoparticles through symplastic transport.

6. The method of any one of claims 1-5, wherein the nanoparticles translocate from the roots to the shoots of the plants.

7. The method of claim 6, wherein the shoot uptake coefficient (α) is about 100 mL kg^{-1} d^{-1}.

8. The method of any one of claims 1-7, wherein one or more contaminants are heavy metals and/or organic pollutants.

9. The method of claim 8 wherein the heavy metals are arsenic, cadmium, chromium, lead, nickel, and zinc.

10. The method of claim 9 wherein the heavy metal is arsenic.

11. The method of any one of claims 1-10, wherein the plants comprise *Zea mays*.

12 The method of any one of claims 1-11, wherein the soil is treated to comprise nanoparticles at 200, 400, 600, 800 or 1000 mg per kilogram of soil.

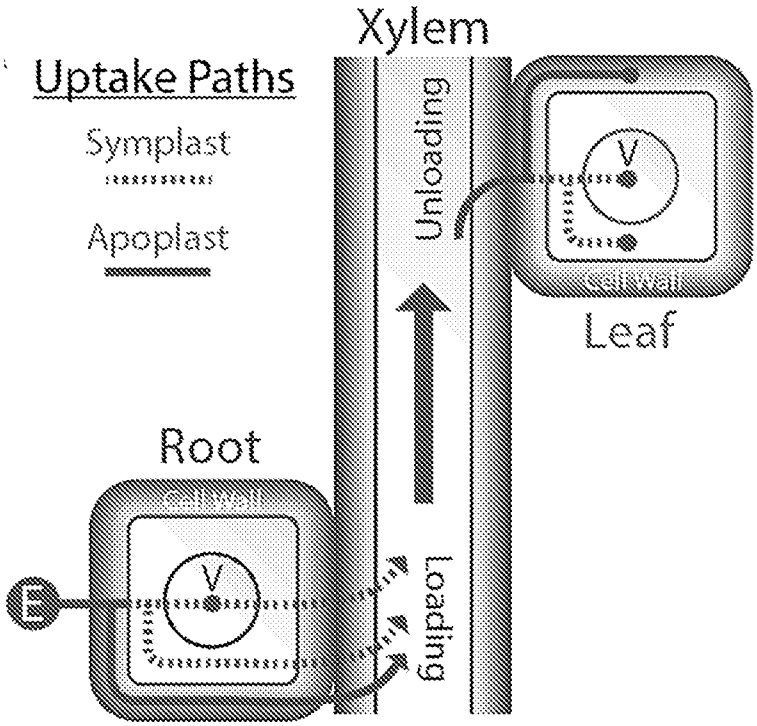


FIG. 1A

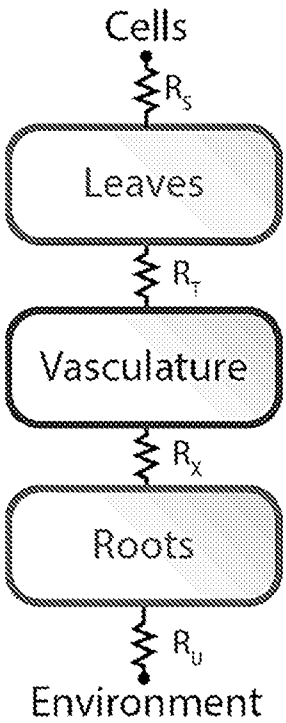


FIG. 1B

FIG. 1

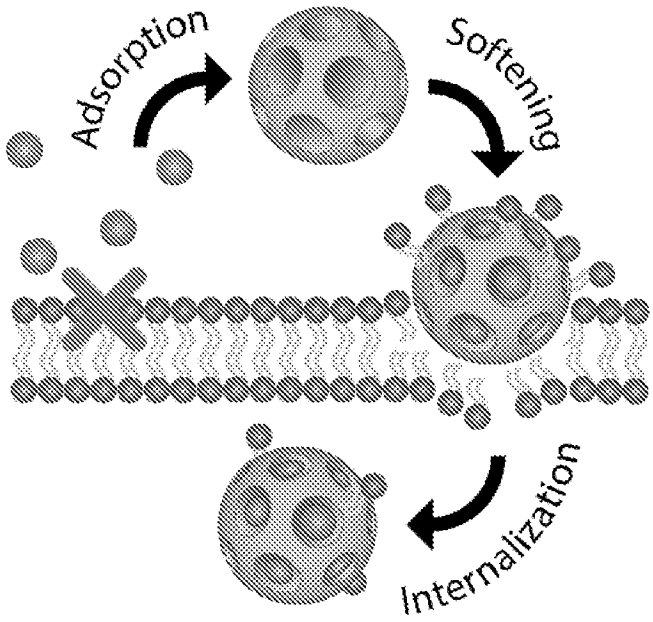


FIG. 2A

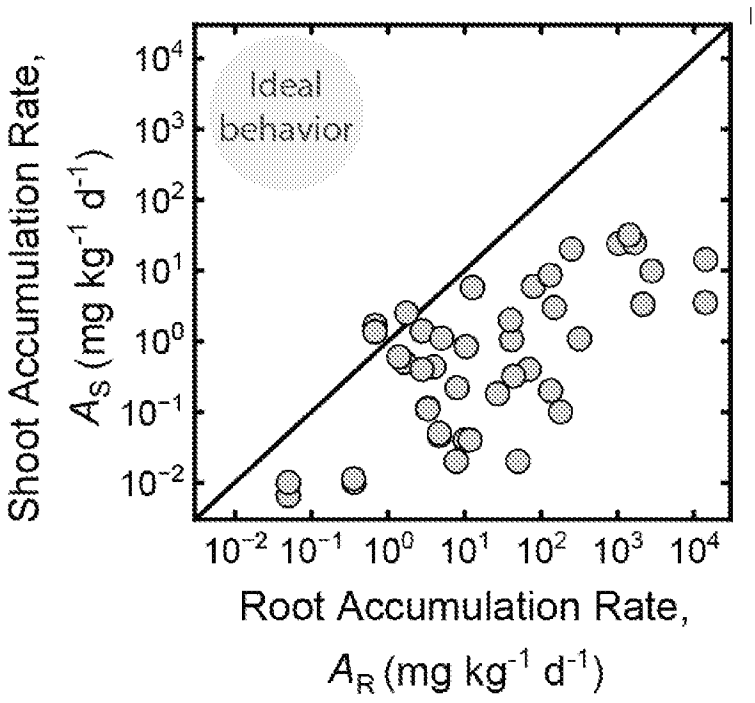


FIG. 2B

FIG. 2

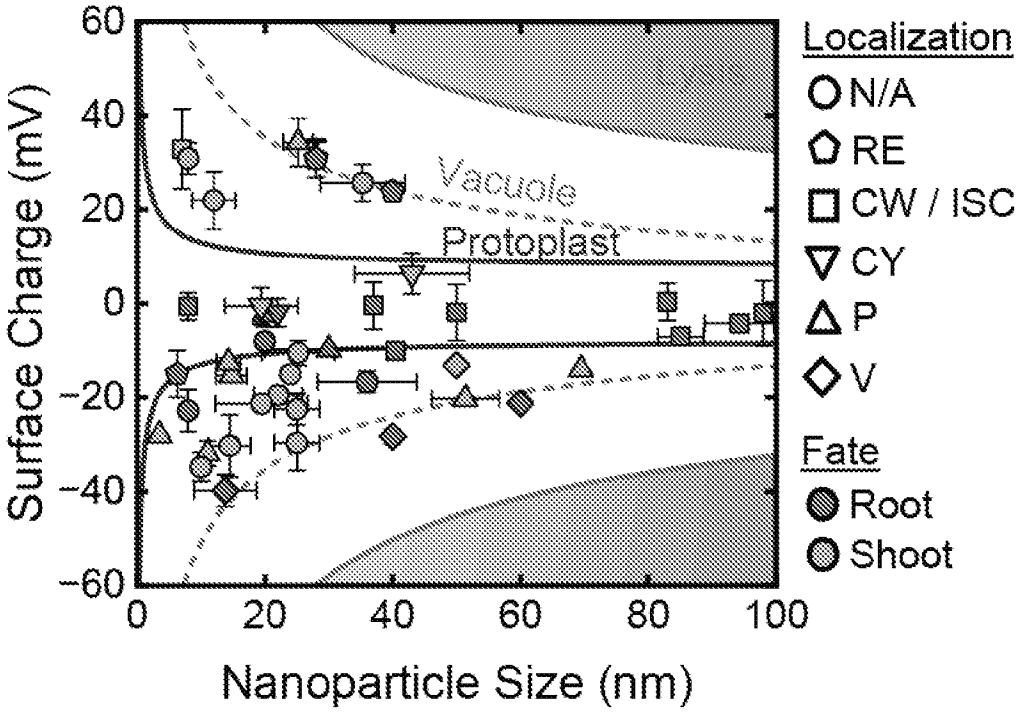


FIG. 2C

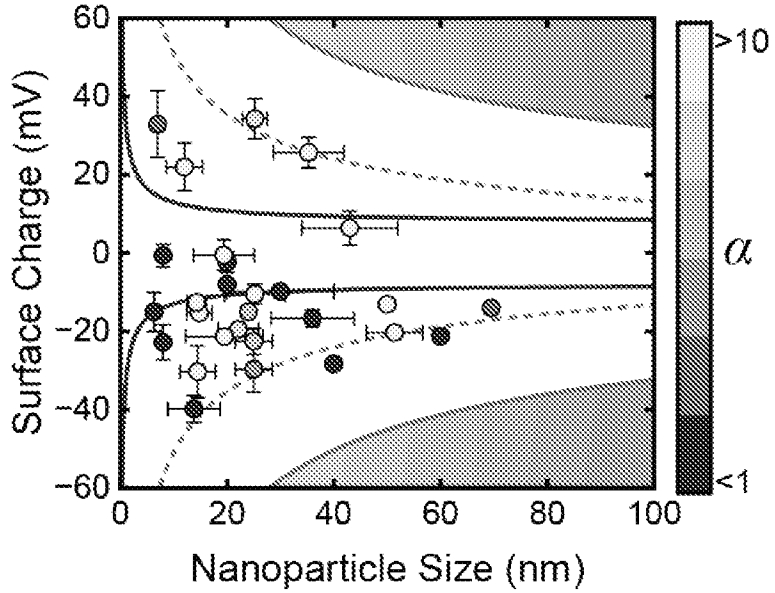


FIG. 2D

FIG. 2 (con't.)

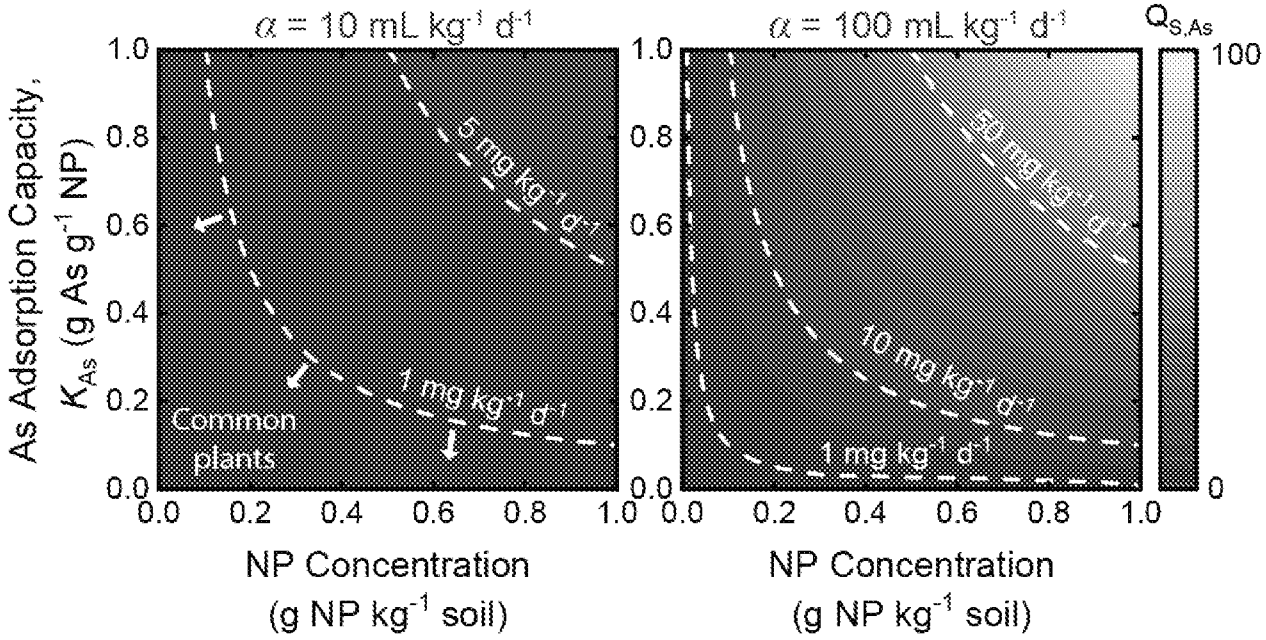


FIG. 2E

FIG. 2 (con't.)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/025618

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***B09C 1/10*** (2024.01); ***B01J 20/28*** (2024.01); *B09C 1/08* (2024.01)

CPC: ***B09C 1/105; B01J 20/28007;*** *B09C 1/08*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 111517408 B (SHANGHAI JIAO TONG UNIVERSITY) 11 May 2021 (11.05.2021) see machine translation	1, 2
A	CN 111545194 B (NANJING INSTITUTE OF SOIL CHINESE ACADEMY OF SCIENCES) 27 April 2021 (27.04.2021) see machine translation	1-4
A	CN 111530919 A (SHANGHAI JIAO TONG UNIVERSITY) 14 August 2020 (14.08.2020) see machine translation	1-4
A	CN 106362699 A (DONGHUA UNIVERSITY) 01 February 2017 (01.02.2017) see machine translation	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 July 2024 (06.07.2024)	**15 July 2024 (15.07.2024)**

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US **Commissioner for Patents** **P.O. Box 1450, Alexandria, VA 22313-1450**	**MATOS** **TAINA**
Facsimile No. **571-273-8300**	Telephone No. **571-272-4300**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/025618

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☑ Claims Nos.: **5-12**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).