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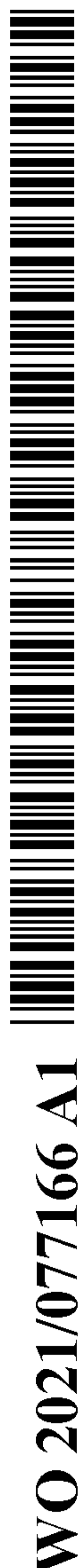
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(54) **Title:** CONTRAST AGENTS

(57) **Abstract:** The present disclosure generally relates to contrast agents for use in imaging and/or as biosensors. The present disclosure also relates to processes for preparing the contrast agents and methods of imaging using the contrast agents. The contrast agents can be provided as solid composite particles for use in various compositions. The solid composite particles may comprise a solid core encapsulated by a cross-linked polymer that is further encapsulated by a porous outer shell.



## CONTRAST AGENTS

### Field

The present disclosure generally relates to contrast agents for use in imaging and/or as biosensors. The present disclosure also relates to processes for preparing the contrast agents and methods of imaging using the contrast agents. The contrast agents can be provided as solid composite particles for use in various compositions.

### Background

*In vivo* biosensing is an emerging area in which dynamic fluctuations in biomarkers of health and disease are monitored in a continuous and real-time manner. Despite the benefits that *in vivo* biosensing offers this area is still developing. Current examples use a variety of detection platforms and include electrochemical sensing of therapeutic drug levels, optical and electrochemical sensing of glucose, and magnetic resonance imaging of neurotransmitters (A. A. Lubin, K. W. Plaxco, *Acc. Chem. Res.* 2010, 43, 496; L. R. Bornhoeft, A. Biswas, M. J. McShane, *Biosensors (Basel)* 2017, 7; Y. Luo, E. H. Kim, C. A. Flask, H. A. Clark, *ACS Nano* 2018, 12, 5761). However, optical systems suffer from poor depth penetration, electrochemical systems require “wiring” and are ensemble methods, and MRI is not suitable for routine, real-time applications. Accordingly, there is a need for new or alternative biosensors, particular those that can be used *in vivo* for continuous and real-time monitoring.

Ultrasound (also referred to as US) is one of the most commonly used medical imaging techniques as it is portable, non-invasive and allows for tuneable tissue penetration. Ultrasound can be enhanced with the use of contrast agents (USCA). Most commercially available USCA have a phospholipid shell and a perfluoro carbon gas core, which are administered intravenously and used to image blood perfusion in organs and blood flow rate in the heart. USCAs can also be utilized to diagnose diseased tissues, this is done by targeting ligands conjugated to the phospholipid shell. The highly echogenic gas provides high imaging contrast, however the imaging window is short (15-20 minutes) because the gas diffuses out of the shell or the microbubbles burst in response to ultrasound waves. Accordingly, these USCAs are not suitable for long-term imaging or biomarker monitoring studies.

There is also need for alternative or improved biosensors and/or contrast agents that enable detection and identification of various diseases, disorders and/or conditions in subjects.

## Summary

The present disclosure relates to solid composite particles and compositions thereof, which can be used as contrast agents for imaging and/or as biosensors. The solid composite particles can comprise a solid core encapsulated by a cross-linked polymer that is further encapsulated by a porous outer shell.

In one aspect, there is provided a solid composite particle comprising: a solid core; one or more polymeric layers encapsulating the solid core, wherein at least one polymeric layer comprises or consists of a cross-linked polymer containing a plurality of functional groups; and a porous outer shell encapsulating the one or more polymeric layers. In some embodiments, each polymeric layer comprises or consists of a cross-linked polymer containing a plurality of functional groups.

In one embodiment, the solid composite particle is an ultrasound contrast agent. In another embodiment, the solid composite particle can be used as a biosensor in combination with imaging techniques, such as a pH sensor in combination with ultrasound imaging. In another embodiment, the cross-linked polymer is a pH sensitive polymer. In another embodiment, the functional groups of the pH sensitive polymer are acid or base groups having a predetermined pKa or pKb. In one example, the pH sensitive polymer is a poly(methacrylic acid) or a copolymer thereof.

In another embodiment, the solid composite particle can be used as a redox sensor in combination with ultrasound imaging. In another embodiment, the one or more polymeric layers comprise polyionic layers. In another embodiment, the polyionic layers comprise alternating layers of a polycation and a polyanion. In another embodiment, the polyionic layers are cross-linked with a redox sensitive cross-linking agent, for example a phenol or polyphenol.

In one embodiment, the outer shell comprises an organosilica.

In one embodiment, the solid core is solid silica core.

In another aspect, there is provided a composition comprising the solid composite particles according to any embodiments or examples thereof as described herein, and an acceptable excipient.

In an embodiment, the composition can be an imaging composition and/or a pharmaceutical composition comprising a pharmaceutically acceptable excipient. The imaging composition may be an ultrasound imaging composition.

In another aspect, there is provided a method for using the solid composite particles according to any embodiments or examples thereof as described herein, as a contrast agent and/or biosensor. In one embodiment, the use is for ultrasound imaging and/or a pH sensor. In one embodiment, the use is for ultrasound imaging and/or a redox sensor.

In another aspect, there is provided a method of enhancing the contrast in an ultrasound image generated for use in medical diagnosis by administering to a region of a subject the solid composite particles or composition thereof according to any embodiments or examples thereof as described herein, and obtaining an ultrasound image of the region.

In another aspect, there is provided a process for preparing a composition comprising a plurality of solid composite particles comprising:

contacting a plurality of individual solid cores with one or more polymers comprising functional groups to provide a plurality of individual solid cores each encapsulated by one or more polymeric layers;

cross-linking at least some of the one or more polymeric layers to form, on each of the solid cores, a cross-linked polymer containing a plurality of functional groups; and

applying a porous coating to the one or more polymeric layers to form a porous outer shell encapsulating the one or more polymeric layers present on each of the solid cores to form the plurality of solid composite particles.

In one embodiment, the process comprises a layer-by-layer polymerisation process for encapsulating the one or more polymeric layers on the solid cores.

Any aspect, embodiment or example, as herein described, shall be taken to apply *mutatis mutandis* to any other aspect, embodiment or example, as herein described, unless specifically stated otherwise.

The present disclosure is not to be limited in scope by the specific aspects, embodiments or examples, as described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the disclosure, as described herein.

Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter (e.g. solid particles), group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or groups of compositions of matter.

### **Brief Description of Drawings**

**Figure 1.** (a) Synthesis of exemplified  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particles. Starting with a silica core ( $\text{SiO}_2$ ), alternating layers of  $\text{PMA}_{\text{SH}}$  and PVPON (6 bilayers) were deposited using a layer-by-layer process.  $\text{PMA}_{\text{SH}}$  layers were cross-linked through the thiol functionality and the PVPON removed ( $\text{SiO}_2@\text{PMA}_{\text{SH}}$ ) by washing with PBS (pH 7.4). The  $\text{SiO}_2@\text{PMA}_{\text{SH}}$  particles were then encapsulated in a porous MPS shell to form the exemplified ultrasound contrast agent ( $\text{SiO}_2@\text{PMA}_{\text{SH}} @\text{MPS}$ ). (b) Synthesis of exemplified  $\text{SiO}_2@\text{PSS}/\text{PAH}/\text{TA}@\text{MPS}$  particles. Starting with a silica core ( $\text{SiO}_2$ ), a layer of PSS is deposited onto the silica core (1). A layer of PAH is then deposited into the PSS coated silica core (2). This is repeated using a layer-by-layer process to create 6 bilayers of PSS/PAH (3). The polymer layers were cross linked with tannic acid (4) The  $\text{SiO}_2@\text{PSS}/\text{PAH}/\text{TA}$  particles were then encapsulated in a porous MPS shell (5) to form the exemplified ultrasound contrast agent ( $\text{SiO}_2@ \text{PSS}/\text{PAH}/\text{TA}@\text{MPS}$ ).

**Figure 2.** TEM image of (a)  $\text{SiO}_2$  core starting material, (b)  $\text{SiO}_2@\text{PMA}_{\text{SH}}$  particles showing a polymer film deposited on the surface of the  $\text{SiO}_2$  cores, and (c)  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  showing a silica layer on top of the  $\text{PMA}_{\text{SH}}$  film with the surface

becoming rough. (d) Size distribution of  $\text{SiO}_2$ ,  $\text{SiO}_2@\text{PMA}_{\text{SH}}$  and  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particles, measured on TEM micrographs (\*\*\*\* $P < 0.0001$ ).

Figure 3. Representative TEM images of microtomed  $\text{SiO}_2@\text{PMASH}@\text{MPS}$  particles.

**Figure 4.** pH sensitivity of  $\text{SiO}_2@\text{PMA}_{\text{SH}}$  coated particles. Confocal microscopy of Cy5-labelled  $\text{SiO}_2@\text{PMA}_{\text{SH}}$  in 40 mM HEPES buffer at (a) pH 4 and (b) pH 7. (c) Plotted values of confocal images of  $\text{SiO}_2@\text{PMA}_{\text{SH}}$  at pH 4 and 7 to obtain the average diameter of the  $\text{SiO}_2@\text{PMA}_{\text{SH}}$  particle at the respective pH.

**Figure 5.** (A) 3D printed mould designed in Rhino 5. The 3D printed mould was used to create B. (B) PDMS poured and degassed in mould in A and cured overnight, this mould was designed to hold a 20  $\mu\text{L}$  agar sample with insets allowing for both depth alignment during ultrasound measurement also vertical and horizontal alignment prior to imaging. (C) Phantom ultrasound measurements of sample showing section view and plane view alignment.

**Figure 6.** (a) Normalized calibration curve of  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particle (referred to in the figures as sUN) suspended in agar (1 % in 40 mM HEPES buffer, pH 7) imaged over 72 hrs at 24 hr time points. (b) Normalized calibration curve of  $\text{SiO}_2$  cores (negative control) and  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particles, suspended in agar (1 % in 40 mM HEPES buffer, pH 7) with imaging every 24 hrs at 40 MHz, showing the relationship between pH and US backscatter (\*\*\*\* $P < 0.0001$ ). (c) Ultrasound images of  $\text{SiO}_2$  cores and  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particles. Shown are Z-projections of the phantom image stacks. The circles highlight the cross-section of the sample in comparison to the mould surfaces used for image setup.

**Figure 7.** Ultrasound imaging of control and exemplified contrast agent in tissue. (a) The corresponding grey values of the  $\text{SiO}_2$  cores (control), taken from region of interest (ROI) measured at 15 min time points as integrated density over the image stacks. The measured ROI for the control showed no statistical difference in US backscatter with change of pH. (b) *Ex-vivo* US images of  $\text{SiO}_2$  cores (control) following the same protocol as a. (c) The corresponding grey values of the  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particles, taken from region of interest (ROI) measured at 15 min time points as integrated density over the image stacks \*\*\*\* $P < 0.0001$ , ns  $P < 0.9421$ . (d) *Ex vivo* US images of

SiO<sub>2</sub>@PMA<sub>SH</sub>@MPS particle injection into a pocket of HEPES buffer at pH 4. After 15 minutes a further 100 µL of HEPES pH 4 was injected, After another 15 minutes, 100 µL of HEPES at pH 7 was injected.

**Figure 8.** *Ex vivo* ultrasound images with SiO<sub>2</sub>@PMA<sub>SH</sub>@MPS subcutaneous IV injection into a pocket of HEPES buffer at pH 4. Time 0 is 1 hr post rattle injection. (a) at time 0 ‘rattles’ had been injected into subcutaneous into a pocket of HEPES buffer (40 mM, pH 4). (b) After 250 µL injection of HEPES buffer (40 mM, pH 4). Monitored for 40 minutes. (c) After 250 µL injection of HEPES buffer (40 mM, pH 7). Monitored for 40 min. (d) The corresponding grey values measured at 10 min time points as integrated density over the image stacks \*\*\*\*P < 0.0001, ns P < 0.9421.

**Figure 9.** Ultrasound imaging of exemplified contrast agent *in vivo*. (a) The corresponding grey values of SiO<sub>2</sub>@PMA<sub>SH</sub>@MPS, taken from region of interest (ROI) measured at 5 min time points as integrated density over the image stacks \*\*\*\*P < 0.0001, ns P < 0.9421. (b) The corresponding US images for time points in graph a.

**Figure 10.** Harmonic data collected with pulsed ultrasound for the exemplified SiO<sub>2</sub>@PMA<sub>SH</sub>@MPS particles.

**Figure 11:** Redox sensitivity of an exemplified contrast agent. The exemplified contrast agent is a SiO<sub>2</sub>@PSS/PAH/TA@MPS particle cross-linked with tannic acid on a TEOS core with an MPS shell). The particles are suspended in 1 % agar, then incubated overnight with Fe(II) or Fe(III) in solution.

**Figure 12:** Proposed reaction mechanism of tannic acid with (a) Fe(III) and (b) Fe(II).

**Figure 13:** Concentration dependence of ultrasound intensity (a) Normalized calibration curve of 800 nm TEOS silica cores suspended in 1 % agar with increasing concentration

of TEOS core material from 0- 40 w/v %. (b) Ultrasound images of phantoms with 0 w/v% (control), 5 w/v%, 20 w/v% and 40 w/v% TEOS silica cores.

**Figure 14:** The effect of core particle composition on ultrasound backscatter.

Ultrasound backscatter was measure for particles comprising tetraethyl orthosilicate (TEOS) and (3-mercaptopropyl)trimethoxysilane (MPS).

### **Description of Embodiments**

The present disclosure relates to solid composite particles and compositions thereof, which can be used as contrast agents and/or as biosensors. The solid composite particles can comprise a solid core encapsulated by a cross-linked polymer further encapsulated by a porous outer shell. The cross-linked polymers can comprise functional groups, such as acid groups, and for example have a predetermined pKa. At least according to some embodiments or examples as described herein, the solid composite particles have been shown effective for use as biosensors in combination with imaging techniques, such as a pH sensor with ultrasound imaging.

### *Terms*

In the following description, reference is made to the accompanying drawings which form a part hereof, and which is shown, by way of illustration, several embodiments. It is understood that other embodiments may be utilized and structural changes may be made without departing from the scope of the present disclosure.

With regards to the definitions provided herein, unless stated otherwise, or implicit from context, the defined terms and phrases include the provided meanings. Unless explicitly stated otherwise, or apparent from context, the terms and phrases below do not exclude the meaning that the term or phrase has acquired by a person skilled in the relevant art. The definitions are provided to aid in describing particular embodiments, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Furthermore, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

All publications discussed and/or referenced herein are incorporated herein in their entirety.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present disclosure. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

Throughout this disclosure, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e., one or more) of those steps, compositions of matter, groups of steps or groups of compositions of matter. Thus, as used herein, the singular forms “a”, “an” and “the” include plural aspects unless the context clearly dictates otherwise. For example, reference to “a” includes a single as well as two or more; reference to “an” includes a single as well as two or more; reference to “the” includes a single as well as two or more and so forth.

Those skilled in the art will appreciate that the disclosure herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the disclosure includes all such variations and modifications. The disclosure also includes all of the examples, steps, features, methods, compositions, coatings, processes, and coated substrates, referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

The term “and/or”, e.g., “X and/or Y” shall be understood to mean either “X and Y” or “X or Y” and shall be taken to provide explicit support for both meanings or for either meaning.

Unless otherwise indicated, the terms “first,” “second,” etc. are used herein merely as labels, and are not intended to impose ordinal, positional, or hierarchical requirements on the items to which these terms refer. Moreover, reference to a “second” item does not require or preclude the existence of lower-numbered item (e.g., a “first” item) and/or a higher-numbered item (e.g., a “third” item).

As used herein, the phrase “at least one of”, when used with a list of items, means different combinations of one or more of the listed items may be used and only one of the

items in the list may be needed. The item may be a particular object, thing, or category. In other words, “at least one of” means any combination of items or number of items may be used from the list, but not all of the items in the list may be required. For example, “at least one of item A, item B, and item C” may mean item A; item A and item B; item B; item A, item B, and item C; or item B and item C. In some cases, “at least one of item A, item B, and item C” may mean, for example and without limitation, two of item A, one of item B, and ten of item C; four of item B and seven of item C; or some other suitable combination.

As used herein, the term “about”, unless stated to the contrary, typically refers to +/- 10%, for example +/- 5%, of the designated value.

It is to be appreciated that certain features that are, for clarity, described herein in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features that are, for brevity, described in the context of a single embodiment, may also be provided separately or in any sub-combination.

Throughout the present specification, various aspects and components of the invention can be presented in a range format. The range format is included for convenience and should not be interpreted as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range, unless specifically indicated. For example, description of a range such as from 1 to 5 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 5, from 3 to 5 etc., as well as individual and partial numbers within the recited range, for example, 1, 2, 3, 4, 5, 5.5 and 6, unless where integers are required or implicit from context. This applies regardless of the breadth of the disclosed range. Where specific values are required, these will be indicated in the specification.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The reference to “substantially free” generally refers to the absence of that compound or component in the composition other than any trace amounts or impurities

that may be present, for example this may be an amount by weight % in the total composition of less than about 1%, 0.1%, 0.01%, 0.001%, or 0.0001%. The compositions as described herein may also include, for example, impurities in an amount by weight % in the total composition of less than about 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.01%, 0.001%, or 0.0001%. An example is the amount of water that may be present in an organic solvent.

As used herein, the term “biosensor” is defined broadly and is a sensor (e.g. a chemical-based sensor) that is used in a biological environment.

### SOLID PARTICLES

The solid composite particles comprise a solid core encapsulated by a cross-linked polymer that is further encapsulated by a porous outer shell. In other words, the cross linked polymer provides an intervening layer between the solid core and the porous outer shell.

Any reference to “solid” in relation to the “solid composite particles” refers to the particle being substantially solid, for example to essentially exclude any microbubbles, liposomes, or other particles having gaseous or liquid core type configurations, for example. It will be appreciated that a “solid” composite particle may contain a degree of porosity, such as in the solid core and/or outer shell, for example a mesoporous organosilica solid core or a solid core prepared in the presence of a porogen, which is further described herein. It will also be appreciated that any reference to “composite” relates to the particle being comprised of different layers or components such as by comprising a solid core, porous outer shell, and intervening polymeric layer located between the solid core and porous outer shell, which may vary according to any embodiments or examples as described herein.

In one aspect, there is provided a solid composite particle comprising:

a solid core;

one or more polymeric layers encapsulating the solid core, wherein at least one polymeric layer comprises or consists of a cross-linked polymer containing a plurality of functional groups; and

a porous outer shell encapsulating the one or more polymeric layers.

In some embodiments, there is provided a solid composite particle comprising:

a solid core;

one or more polymeric layers encapsulating the solid core, wherein each polymeric layer comprises or consists of a cross-linked polymer containing a plurality of functional groups; and

a porous outer shell encapsulating the one or more polymeric layers.

In some embodiments, the functional groups are responsive to an analyte (e.g. biomarker) such that a property of the solid composite particle is responsive to the analyte. In some embodiments, the functional groups are responsive to an analyte (e.g. biomarker) such that the ultrasound contrast property of the solid composite particle is responsive to the analyte. In some embodiments, the functional groups bind to an analyte such that the ultrasound contrast properties of the solid composite particle are responsive to the analyte. In some embodiments, the functional groups bind to an analyte such that the ultrasound contrast of the solid composite particle increases or decreases on binding of the analyte. In some embodiments, the functional groups react with an analyte such that the ultrasound contrast properties of the solid composite particle are responsive to the analyte. In some embodiments, the functional groups react with an analyte such that the ultrasound contrast of the solid composite particle increases or decreases on reaction with the analyte. In some embodiments, the functional groups are pH sensitive such that the ultrasound contrast of the solid composite particle increases or decreases depending on the local pH. In some embodiments, the functional groups are redox sensitive such that the ultrasound contrast of the solid composite particle increases or decreases depending on the redox state of an environment and/or biomarker.

The solid composite particle can be provided or used as a contrast agent, for example a contrast agent for imaging, and/or as a biosensor. In one example, the solid composite particles are used as a redox sensor, pH sensor, ultrasound contrast agent, or combination thereof. In one example, the solid composite particles are used as a pH sensor, ultrasound contrast agent, or combination thereof. In one example, the solid

composite particles are used as a redox sensor, ultrasound contrast agent, or combination thereof.

The solid composite particle may be provided in various polygonal shapes, such as spherical, elliptical, or cylindrical. In one example, the solid composite particle is spherical. The solid composite particle may be a solid nanoparticle. The solid composite particle may be a solid silica nanoparticle comprising a polymeric layer as an intervening layer between a solid silica core and a porous organosilica outer shell.

The solid composite particle may, for example, have a diameter (in nm) between about 10 to 15000, 50 to 12000, 100 to 10000, 200 to 8000, 300 to 6000, 400 to 4000, 500 to 2000, or 750 to 1500. The solid composite particle may have a diameter (in nm) of at least about 50, 100, 250, 500, 750, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, or 10000. The solid composite particle may have a diameter (in nm) of less than about 15000, 12000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 750, 500, 250, or 100. In some embodiments, the solid composite particle has a diameter (in nm) between 100 to 10000. The solid composite particle may have a diameter (in nm) in a range provided by any two of these upper and/or lower values.

The solid composite particle may have a measurable backscatter property determined by, for example, ultrasound, or indirectly by atomic force microscopy.

The solid composite particle may be stable in an aqueous or biological fluid for at least about 12 hours, 1 day, 1 week, 1 month, 6 months, or 12 months etc. For example, the particle may be stable in an aqueous or biological fluid at 37°C for at least about 12 hours, 1 day, 1 week, 1 month, 6 months, or 12 months etc. In some embodiments, the particle may be stable between a pH of 1 to 9, for example under acidic conditions or under basic conditions. In some embodiments, the particle may be stable at physiologically relevant pH. In some embodiments, the solid composite particle is stable after administration to a subject for at least about 12 hours, 1 day, 1 week, 1 month, 6 months, or 12 months.

### Solid Core

The solid core may be formed from any suitable material. For example, the solid core is selected from a suitable material which may provide support (e.g. a scaffold) for the polymeric layer or layers and/or may provide back scattering properties.

The solid core may comprise or consist of any suitable inorganic material (e.g. silica, metal and/or metal oxide) or organic material (e.g. polymeric), or a combination thereof.

For example, the suitable inorganic material may be any pharmaceutically acceptable and/or non-toxic inorganic material (e.g. silica or calcium carbonate). The inorganic material may be selected from any suitable metal, non-metal (e.g. carbon) or semiconductor material (e.g. silicon) and suitable derivatives thereof (e.g. oxides such as silica or carbonates such as calcium carbonate).

The solid core may comprise or consist of an inorganic oxide or carbonate (e.g. silica or calcium carbonate). In one embodiment, the solid core is silica. The silica core may be a mesoporous silica core or a solid silica core (e.g. non-porous silica). The silica core may also comprise one or more internal cavities (e.g. has a degree of porosity). The one or more internal cavities may be naturally occurring (i.e. form during the process of preparing the silica core without the addition of a porogen) or be formed by including one or more porogens (e.g. tannic acid) when preparing the solid core. As would be appreciated by the person skilled in the art, pore size may be tuned, for example, by controlling the stirring rate, surfactant type (if used) and template size. The porosity of the solid core may be measured using techniques known to the person skilled in the art, for example, TEM, gas absorption (BET method), mercury porosimetry or liquid phase absorption of a fluorescent dye (e.g. methylene blue or evans blue). In some embodiments, the solid core may be microporous (e.g. pore size less than 2 nm) or mesoporous (e.g. pore size between about 2 nm and 50 nm). For example, the solid core may comprise one or more pores having a pore size (in nm) of less than about 50, 45, 40, 35, 30, 25, 20, 15, 10, 5, 4, 3, 2, 1, 0.5 or 0.1. For example, the one or more pores may have a pore size (in nm) of greater than about 0.1, 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40 or 45. For example, the one or more pores may typically have a pore size (in nm)

between 0.1 to 50, 0.5 to 50, 1 to 50, 2 to 50, 2 to 40, 2 to 30, 2 to 20, 2 to 10, 10 to 50, 20 to 50, 30 to 50, 40 to 40, 10 to 40, 20 to 30, or 10 to 20 or 30 to 40. The one or more pores may have a pore size (in nm) in a range provided by any two of these upper and/or lower values.

The silica cores may be commercially available or alternatively may be synthesised using techniques known to the person skilled in the art, for example via the Stober method or other methods as described under the heading “PROCESSES FOR PREPARING PARTICLES” below.

The solid core may comprise or consist of an inorganic compound, such as silica (i.e. SiO<sub>2</sub>) and/or calcium carbonate, which may optionally be blended with an organosilane compound (e.g. MPS, TEOS or TSPA). In some embodiments, the solid core is solid silica core comprising an inorganic silica, and optionally one or more compounds selected from an organosilane and organosilica.

In some embodiments, the solid core may further comprise an organosilane or organosilica compound. For example, the solid core may comprise or consist of various inorganic silicas including any blends thereof with organosilanes or organosilicas compounds (e.g. MPS, TEOS or TSPA). The solid core may be provided by a mesoporous organosilica, for example prepared from a bridged organosilane precursors. The contents of the following reference are herein incorporated in its entirety by reference thereof: Inagaki, Shinji; Norihiro Mizoshita; Takao Taniab (2011), "Synthesis, properties, and applications of periodic mesoporous organosilicas prepared from bridged organosilane precursors", *Chem. Soc. Rev.* 40 (2): 789–800. In one example, 3-mercaptopropyl trimethoxysilane (MPS) may be used to prepare solid core silica particles, which may comprise silanol (Si-OH) groups and thiol groups on the surface thereof. The thiol groups for example may be used to further functionalise with polymers, polymerization initiators, etc., for facilitating application of the polymeric layer thereto.

In one embodiment, blending TEOS silica cores with organosilanes and/or organosilicas provides one option in which to tune the density of the solid core. For

example, pure TEOS can provide a highest density and pure organosilane can provide a lowest density, and blends thereof can provide particular densities therebetween.

In some embodiments, the solid cores may optionally further comprise one or more inorganic or polymeric particles. For example, one or more particles may optionally be incorporated (e.g. interspersed or embedded) within the core (e.g. within the silica core) or provided as a layer on the surface of the solid core. The particles may be selected from any one of the materials defined herein in relation to the solid cores, e.g. calcium carbonate particles. In one embodiment, the solid core may further comprise (e.g. incorporates) one or more additional additives to increase ultrasound backscatter.

In one embodiment, the solid core may comprise or consist of a metal and/or a metal oxide (e.g. a metal core or a cluster of metals). The metal may be gold or silver, or a combination thereof including an alloy thereof. Other metals or metal oxides may also be suitable as understood by the person skilled in the art.

Suitable solid cores may be synthesised via techniques known to the person skilled in the art, for example via “bottom-up” processes such as thermal decomposition or hydrothermal processes.

In another embodiment, the solid core may comprise or consist of one or more organic materials such as a synthetic polymer and/or a natural polymer. For example, the suitable organic material may be any pharmaceutically acceptable and/or non-toxic organic material. Examples of synthetic polymers include polylactic acid, polyglycolic acid and copolymers thereof. Examples of natural polymers include hyaluronic acid, chitosan, and collagen. Other polymers may also be suitable as understood by the person skilled in the art.

In one embodiment, the solid core may comprise a polymeric material (e.g. a polymer matrix) wherein one or more particles may be incorporated (e.g. interspersed or embedded) within the polymeric material or provided as a layer on the surface of the polymeric material. For example, the solid core may comprise a polymer matrix wherein one or more silica particles and/or calcium carbonate particles are incorporated (e.g. interspersed or embedded) within the polymeric material or provided as a layer on the surface of the polymeric material. Other solid particles may also be incorporated or

coated within or on the polymer matrix, for example any one of the solid materials defined herein in relation to the solid cores, e.g. calcium carbonate particles.

In one embodiment, the surface of the solid core may be non-functionalised. In another embodiment, the surface of the solid core may be functionalised with one or more moieties to aid attachment of the solid core to the polymer layer shell. In some embodiments, the surface of the solid core may be functionalised to provide terminal functional groups selected from—CHO, —COOH, —SO<sub>3</sub>H, —CN, —NH<sub>2</sub>, —SH, —COSH, and —COOR. Where applicable, these moieties may be protonated or deprotonated to form a positively or negatively charged moiety which can attach to the molecule. In one embodiment, the surface of the solid core is functionalised with a carboxylic acid moiety (-COOH) and/or a thiol moiety (-SH). In one embodiment, the solid core comprises silica wherein the surface that is not functionalised (i.e. comprises a bare surface which is terminated by Si—OH moieties). In another embodiment, the solid core comprises silica wherein the surface is functionalised with one or more moieties selected from —CHO, —COOH, —SO<sub>3</sub>H, —CN, —NH<sub>2</sub>, —SH, —COSH, and —COOR. For example, the silica surface may be functionalised with carboxylic acid and/or thiol moieties. The surface functionalisation, for example the carboxylic acid and thiol functionalities, can be incorporated onto the surface of the solid core by any means known to the person skilled in the art.

In some embodiments, the solid core may be selected to provide a certain mechanical property, such as stiffness, density, or hardness. The stiffness may be measured by Young's modulus, and in one example the Young's modulus of the solid core is less than that of the shell.

In some embodiments, the solid core may be selected to provide a certain backscatter (i.e. reflectance) property. As would be appreciated by the person skilled in the art, for sensing and/or imaging purposes there should be a density mismatch (i.e. a difference in density) between the tissue where the imaging and/or sensing is being performed and the solid composite particle. In some embodiments, the material used for the solid core is selected to provide a suitable density mismatch. For example, the solid core may comprise mesoporous silica if the solid composite particle is to be used for imaging bone.

The backscatter (i.e. reflectance) properties of the solid particles may be measured, for example by a clinical or pre-clinical ultrasound machine, using techniques known to the person skilled in the art. In some embodiments, the solid composite particles are suspended in liquid (e.g. aqueous liquid) and placed in a container such as a tube, beaker balloon and the like and placed under an ultrasound transducer. In some embodiments, the solid composite particles are encapsulated into a hydrogel (e.g. agar, poly(acrylamide) and the like) and placed under an ultrasound transducer. In some embodiments, the solid composite particles are embedded or coated on solid object (e.g. bone, medical implant/device and the like) and placed under an ultrasound transducer. The backscatter (i.e. reflectance) properties of the solid particles are then measured and/or determined. In some embodiments, the ultrasound frequency used is between 0.1 to 50 MHz. In some examples, the ultrasound frequency used is 40 MHz. In some embodiments, the ultrasound mechanical index is used between 0-4. Alternatively, the backscatter (i.e. reflectance) properties may be determined by indirect measurements, for example, AFM using the samples prepared as above. In some embodiments, the backscatter (i.e. reflectance) properties of the solid particles may be measured using the conditions and methods described in Example 3 (Figure 6).

The solid core may typically have a diameter (in nm) between about 5 to 15000, 10 to 100, 100 to 1000, 50 to 12000, 100 to 10000, 200 to 8000, 300 to 6000, 400 to 4000, 500 to 2000, or 750 to 1500. The solid core may have a diameter (in nm) of at least about 5, 10, 25, 50, 100, 250, 500, 750, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, or 10000. The solid core may have a diameter (in nm) of less than about 15000, 12000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 750, 500, 250, or 100. In some embodiments, the solid core has a diameter (in nm) between about 10 to 10000. The solid core may have a diameter (in nm) in a range provided by any two of these upper and/or lower values.

The solid core may for example comprise of a plurality of smaller particles agglomerated or clustered together (e.g. a plurality of nanoparticles or microparticles forming a cluster). Alternatively, the solid core may be a single particle (e.g. a spherical silica particle).

The solid core may have any suitable shape. For example, the core may be substantially spherical (e.g. a nucleated sphere), non-spherical, oval, elliptical, icosahedral, dodecahedral, or cylindrical, rod-shaped, pyramidal, cube-like, disk-shaped, wire-like, or irregularly shaped. In one example, the solid core is spherical. In another embodiment, the solid core may be isotropic (e.g. spherical) in shape or anisotropic (e.g. non-spherical) in shape.

The solid core may typically have an aspect ratio (i.e. the ratio of a length to a width, where the length and width are measured perpendicular to one another, and the length refers to the longest linearly measured dimension) of 1.0 to 10.0, 1.0 to 5.0, or 1.0 to 2.0. In one embodiment, the solid core may have an aspect ratio of about 1.0 to 2.0, for example about 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2.0. In one embodiment, the solid core may have an aspect ratio of about 1.0 (e.g. the solid core has a spherical morphology).

The weight % of the solid core in the particle (as a total weight % of the particle) may typically be provided between 0.001 to 60, 0.01 to 50, 0.1 to 40, or 1 to 30. The weight % of the solid core in the particle (as a total weight % of the particle) may be at least about 0.001, 0.01, 0.1, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 55. The weight % of the solid core in the particle (as a total weight % of the particle) may be less than about 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10, 5, 1, 0.1, or 0.001. In some embodiments, the weight % of the solid core in the particle (as a total weight % of the particle) is between 1 to 30. The weight % of the solid core in the particle (as a total weight % of the particle) may be provided in a range between any two of these upper and/or lower values.

#### Polymeric Layer

One or more polymeric layers are provided to encapsulate the solid core, which in turn are encapsulated by the porous outer shell. At least one polymeric layer can comprise or consist of a cross-linked polymer containing a plurality of functional groups. In some embodiments, each polymeric layer can comprise or consist of a cross-linked polymer containing a plurality of functional groups. In some embodiments, at least one polymeric layer that is not cross-linked may be present. The number of polymeric layers provided as intervening layers between the solid core and porous outer shell may be 1, 2,

3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15. In one example there is provided a single polymeric layer as an intervening layer between the solid core and porous outer shell. When the one or more polymer layers comprises alternate layers of two different polymers, the number of bilayers may typically be 1, 2, 3, 4, 5, 6, or 7. In one example there is provided six bilayers as an intervening layer between the solid core and porous outer shell.

In another example, each of the polymeric layers consist of the same or different cross-linked polymer.

In another example, the functional groups of the cross-linked polymer are capable of reactivity with a biomarker. The functional groups of the cross-linked polymer may be capable of reactivity with a biomarker for reversible conversion of the cross-linked polymer between a first configuration and a second configuration effective for altering an ultrasound contrast property of the particle. The property being altered may be a mechanical property, for example a property selected from stiffness and/or hardness. It will be appreciated that stiffness may be measured by a Young's Modulus value, and alteration of this property has shown to impact image contrast through modification of backscatter (i.e. reflectance) in ultrasound transmission. Hardness may also be measured or determined by a packing density and/or hydrogen bonding property, and again may impact image contrast. In some embodiments, the crosslinks comprise functional groups capable of reactivity with a biomarker. In some embodiments, the polymer comprises functional groups capable of reactivity with a biomarker. In some embodiments, both the crosslinks and the polymer comprise functional groups capable of reactivity with a biomarker.

In another example, the functional groups of the cross-linked polymer can be capable of reactivity with a biomarker for reversible conversion of the cross-linked polymer between a first higher contrast configuration and a second lower contrast configuration.

In another example, the biomarker is a hydrogen ion.

In another example, the biomarker is iron, e.g. Fe(II) and/or Fe(III).

In another example, the biomarker is a biomarker of oxidative stress, for example, a reactive oxygen species (ROS), a reactive nitrogen species (RON) or  $O^{\cdot-}$ .

In another example, the cross-linked polymer is a pH sensitive polymer. As used herein, the term “pH sensitive polymer” refers to polymers that include in their structure a functional group that is an acid or a base (e.g. weak acid or weak base) and, depending on the functional group, can accept or release protons in response to changes in the pH. It will be appreciated that the pH sensitive polymer can be selected to provide a predetermined pKa or pKb. For example, the functional groups of the pH sensitive polymer can be acid or base groups to provide a predetermined pKa or pKb. The acid or base groups can provide reversible protonation and deprotonation at a predetermined pH for reversible conversion of the cross-linked polymer between a first higher contrast configuration and a second lower contrast configuration, for example.

At least according to some embodiments or examples as described herein, a pH sensitive polymer has been shown to increase ultrasound backscatter at a certain pH. For example, where the pH sensitive polymer comprises acid functional groups, a lower pH environment (for example, an environment having a pH of less than the pKa) can provide for a higher contrast (improved backscatter/reflectance of signal) than compared with an environment having a higher pH (for example, an environment having a pH of greater than the pKa). Without wishing to be bound by theory, it is understood that when the functional groups on the pH sensitive polymer are acid groups, in a lower pH environment (e.g. a pH less than about 5), the acid groups are protonated and it is thought that the polymer is more densely packed with higher stiffness leading to improved backscatter/reflectance. The improved backscatter/reflectance is understood to result in an increase in ultrasound contrast. The acid groups in a higher pH environment (i.e. less acidic and more alkaline or basic, e.g. a pH greater than about 5) dissociate to form its conjugate base counter anion species, such as a carboxylate for carboxylic acids, and it is thought that the anionic groups repel each other leading to a polymer that is more expanded, less densely packed, lower stiffness, that leads to poorer backscatter/reflectance. The poorer backscatter/reflectance is understood to result in a decrease in ultrasound contrast.

In one example, the functional groups of the pH sensitive polymer are acid groups selected from one or more carboxylic acids, sulphur containing acids, and/or phosphorus containing acids. In some embodiments, the polymer is a carboxylated polymer or a derivative thereof, for example, poly(acrylic acid), poly(meth)acrylic acid and poly(glutamic acid) or derivatives thereof. In some embodiments, the polymer is a sulfonated polymer or a derivative thereof, for example, poly(styrene sulfonate) (PSS) or derivatives thereof.

In another example, the functional groups of the pH sensitive polymer are base groups selected from an amine groups. Amine groups include, but are not limited to, amino groups ( $\text{NH}_2$ ), alkylamine groups, and/or dialkylamine groups. The amine groups may be an alkyl amine or dialkylamine. In other examples, there can be provided aminated polymers and derivatives, e.g. poly(ethylene imine), poly(2-(diisopropylamino)ethyl methacrylate), poly(2-(diethylamino)ethyl methacrylate), poly(2-(dimethylamino)ethyl methacrylate) or poly(allylamine). In some examples, the polymer is poly(allylamine hydrochloride) (PAH).

In other examples, the cross-linked polymer comprising functional groups may be provided by biopolymers and derivatives: e.g. hyaluronic acid, tannic acid, or chitosan or derivatives or analogs thereof.

In another example, the pH sensitive polymer is selected from the group consisting of polyacrylic acid, polymethacrylic acid, copolymers of acrylic acid and acrylates or methacrylates, and copolymers of methacrylic acid and acrylates or methacrylates. In one example, the pH sensitive polymer is selected from a poly(methacrylic acid) or a copolymer thereof. In another example, the pH sensitive polymer is selected from poly(styrene sulfonate) or a copolymer thereof. In another example, the pH sensitive polymer is selected from poly(allylamine hydrochloride) or a copolymer thereof. In some examples, the one or more polymeric layers comprise alternate layers of poly(styrene sulfonate) or a copolymer thereof and poly(allylamine hydrochloride) or a copolymer thereof.

In another example, the one or more polymeric layers comprise polyionic layers. It will be appreciated the polyionic layers can be pH sensitive. In some embodiments, the

functional groups of the cross-linked polymer are cationic groups or anionic groups or a combination of anionic and cationic groups. In some embodiments, the polyionic layers comprise alternating layers of a polycation (e.g. poly(allylamine hydrochloride)) and a polyanion (e.g. poly(styrene sulfonate)). Polycationic polymers include polymers containing amine groups (e.g. tertiary amine groups), polymers containing morpholino, pyrrolidine and piperazine groups and polymers containing pyridine and imidazole groups. The polycationic polymers may be selected from poly(allylamine hydrochloride), poly(N, N -dimethylaminoethyl methacrylate), poly(L-lysine), poly(L-arginine), poly(ethylene imine) (PEI), poly-diallyldimethylammonium chloride, chitosan, poly[(2-dimethylamino)ethyl methacrylate), poly[(2-diethylamino)ethyl methacrylate), poly(2-diisopropylamino)ethyl methacrylate), poly(acryloylmorpholine), poly(N-ethylpyrrolidine methacrylate), poly(N-acryloyl-N'-alkenyl piperazine), poly(4-vinylpyridine) poly(2-vinylpyridine), poly(N-vinylimidazole), poly[6-(1H-imidazol-1-yl)hexyl-methacrylate], poly(propylenimine)dendrimer, poly(ethylenimine) dendrimer (PEI) or poly(amidoamine) dendrimer. Polyanionic polymers include poly(carboxylic acids), poly(phosphoric acids), poly(sulfonic acids), poly(amino acids) and poly(boronic acids). The polyanionic polymers may be selected from poly(acrylic acid), poly(methacrylic acid), poly(glutamic acid), poly(ethylacrylic acid), poly(propylacrylic acid), poly(4-vinylbenzoic acid), poly(itanoic acid), hyaluronic acid, poly(vinylphosphonic acid), poly(4-vinyl-benzyl phosphonic acid), poly (ethylene glycol methacrylate phosphate), poly(vinylsulfonic acid), poly(styrene sulfonic acid), poly(4-styrenesulfonic acid), poly(3-sulfopropyl methacrylate potassium salt), poly(2-acrylamido-2-methylpropane sulfonic acid), poly(aspartic acid), poly(L-glutamic acid), poly(histidine), poly(vinylphenyl boronic acid) or poly (3-acylamidophenyl boronic acid).

In one example, the polyanion is poly(styrene sulfonate) and the polycation is poly(allylamine hydrochloride). In another example, the polyionic layers can be cross-linked with a redox sensitive cross-linking agent, such as a polyphenol (e.g. tannic acid) or other cross-linking agent as described below. As used herein, the term "redox-sensitive" is intended to refer to a compound (e.g. cross-linking agent or cross-link) comprising a moiety that is capable of undergoing electron transfer including loss of electrons (oxidation) or gain of electrons (reduction). In some embodiments, the redox

sensitive cross-linking agent can be effective to alter localised pH for recognition by the pH sensitive polymeric layer.

While in some embodiments, the change in property of the solid composite particle (e.g. change in ultrasound contrast) is associated with a polymer containing pH sensitive functional groups, it will be appreciated that a polymer containing functional groups that are sensitive to other biomarkers are also within the scope of this disclosure. In some embodiments, the polymer may be an ion responsive polymer (e.g. poly(styrene sulfonic acid) and derivatives thereof). In some embodiments, the polymer may be a redox responsive polymer. For example, the polymer may provide a ROS-responsive system based on, e.g. poly(thioketal)s, poly(oxalate)s, selenium-containing polymers or polymers containing nitroxyl, phenoxyl, quinones, viologens, carbazol or hydrazyl groups. For example, the polymer may provide a reducing condition responsive systems based on, e.g. disulphide and trisulphide containing polymers.

The cross-linking of the polymer layer has been found to be effective for preventing dissolution of the cross-linked polymer in a biological fluid on ionisation thereof (e.g. protonation or deprotonation). The cross-linking groups of the cross-linked polymer may be selected from various hardeners and cross-linking groups, such as from diamines, thiols, or click chemistry type groups (e.g. alkyne-azide conjugation chemistry). In some examples, the cross-linking groups of the cross-linked polymer are selected from diamines. In some examples, the cross-linking groups of the cross-linked polymer are selected from disulphide, in other words the cross-linked polymer is cross-linked by disulphide bonds.

In some embodiments, the cross-linked polymer is prepared with a cross-linking agent. Various suitable cross-linking agents may be used. In some embodiments, the cross-linker agent is an organic compound containing at least two functional groups capable of at least partially reacting with one or more of the functional groups of the polymer in the polymeric layer. In some embodiments, the cross-linking agent may contain at least two functional groups which may be the same or different and selected from epoxy, aziridine, isocyanate, amine, imine, anhydride, NHS ester, imidioester, isothiocyanate, sulfonyl chloride, aldehyde, carbodiimide, anhydride, carbonate, thiol, hydroxyl, and phenol groups. In some embodiments, the crosslinking agent comprises

thiol groups or thiol reactive groups. In some embodiments, the crosslinking agent comprises hydroxyl or phenol groups.

In some examples, the cross-linking agent is a phenol or polyphenol (i.e. the cross-links are formed by a phenol or polyphenol). In some examples, the cross-linking agent is a polyphenol. Polyphenols include both naturally occurring polyphenols (e.g. tannic acid) and synthetic polyphenols (e.g. polyvinyl gallol). In some examples, the cross-linking agent is a phenol. In some embodiments, the cross-linking agent may be tannic acid, gallic acid, syringic acid, pyrogallol, hydroxytyrosol, resorcinol, caffeic acid, resveratrol, glucoside piceid, or a derivative or analog thereof. In some examples, the cross-linking agent may be a tannic acid or a derivative or analog thereof. For example, the cross-linking agent may be tannic acid, gallic acid, syringic acid, pyrogallol, ellagic acid, quercetin, methyl gallate, ethyl gallate, propyl gallate, gallic acid, pyrogallol, polyvinyl gallol, PEG-gallol, chitosan-gallol, gallotanin or gallocatechnin. In some examples, the cross-linking agent is tannic acid. In some embodiments, the polyphenol is a plant polyphenol, for example, hydroxytyrosol, resorcinol, caffeic acid, resveratrol, glucoside piceid, or a derivative or analog thereof.

Further non-limiting examples of cross-linking agents include polydopamines (poly-catechol), coumarins or ascorbic acid.

In some embodiments, the cross-linking groups of the cross-linked polymer may be capable of reactivity with a biomarker for reversible conversion of the cross-linked polymer between a first higher contrast configuration and a second lower contrast configuration. In some embodiments, the cross-linking groups may comprise ion responsive functional groups. In some embodiments, the cross-linking groups may comprise redox sensitive functional groups. For example, the cross-linking groups may provide a redox-sensitive system based on, e.g. polyphenols. By way of non-limiting example, a cross-link formed by a polyphenol such as tannic acid or a derivative or an analog thereof may provide a redox-sensitive system for example, when used to cross-link PSS/PAH. In some embodiments, the cross links are redox sensitive to effect changes in local pH. By way of example, a redox-sensitive cross-linking agent may undergo electron transfer with a biomarker (such as metal ion e.g. Fe(II) or Fe(III)). In some embodiments, the reaction with the biomarker produces hydrogen ions effecting a

change in localised pH. It is thought the change in localised pH converts the cross-linked polymer between a first higher contrast configuration and a second lower contrast configuration or between a first lower contrast configuration and a second higher contrast configuration. By way of illustration, for the PAH/PSS system exemplified herein a reduction in localised pH is thought to lead to polymer expansion, resulting in a drop in US backscatter intensity and an increase in localised pH is thought to lead to a more densely packed polymer resulting in an increase in US backscatter intensity.

It will be appreciated by the person skilled in the art that the degree of cross-linking may be used to alter a chemical or physical property of the cross-linked polymer. For example, the degree of cross-linking can alter the pKa of the cross-linked polymer. By way of illustration, the PAH/PSS system exemplified herein displays a pKa ~8, which can be reduced to a pKa~6 with the addition of tannic acid cross-links. In some embodiments, the cross-linking in the cross-linked polymer provides a ratio of free functional groups to covalently cross-linked functional groups of greater than about 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, or 9:1. In some embodiments, the cross-linking in the cross-linked polymer provides a ratio of free functional groups to covalently cross-linked functional groups of greater than about 2:1. It will be appreciated that the higher the ratio the more free functional groups are available. In the case of a pH sensitive polymer comprising carboxylic acid groups, the higher the ratio the more free carboxylic acid groups that are available for hydrogen bonding than those that have been covalently cross-linked.

In another example, the thickness of the one or more polymer layers (in nm) is between about 0.5 to 200, 2 to 10, 10 to 50, or 50 to 100. The thickness of the one or more polymer layers (in nm) may be at least about 0.1, 0.5, 1, 5, 10, 25, 50, 75, 100, 150, 200, 250, or 300. The thickness of the one or more polymer layers (in nm) may be less than about 300, 250, 200, 150, 100, 75, 50, 25, 10, 5, 1, or 0.5. The thickness of the one or more polymer layers (in nm) may be provide in a range between any two of these upper and/or lower values. In one example, the thickness of the one or more polymer layers (in nm) is about 1 to 25, such as about 10 nm. The thickness can be measured using TEM (dry state, under vacuum).

In another example, the weight % of the combined one or more polymer layers in the particle (as a total weight % of the particle) is between about 5 to 80, 10 to 70, or 20 to 60. The weight % of the combined one or more polymer layers in the particle (as a total weight % of the particle) may be at least about , 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, or 75. The weight % of the combined one or more polymer layers in the particle (as a total weight % of the particle) may be less than about 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10, or 5. The weight % of the combined one or more polymer layers in the particle (as a total weight % of the particle) may be provided in a range between any two of these upper and/or lower values.

In another example, each of the one or more polymeric layers may consist of the same or different cross-linked polymer. For example, the polymeric layers would not comprise any additional components or additives, other than the cross-linked polymer.

In some embodiments, the one or more polymeric layers may be formed by two or more different polymers. For example, the one or more polymeric layers may comprise alternate layers of different polymers. In some embodiments, the one or more polymeric layers may comprise alternate layers of a polymer having acid functional groups and a polymer having base functional groups. Suitable polymers having acid functional groups or polymers having base functional groups include the polymers described herein. In another example, the one or more polymeric layers may comprise alternate layers of polycations (e.g. polycationic polymers) and polyanions (e.g. polyanionic polymers) according to any embodiments or examples thereof as described herein, such as poly(styrene sulfonate) and poly(allylamine hydrochloride).

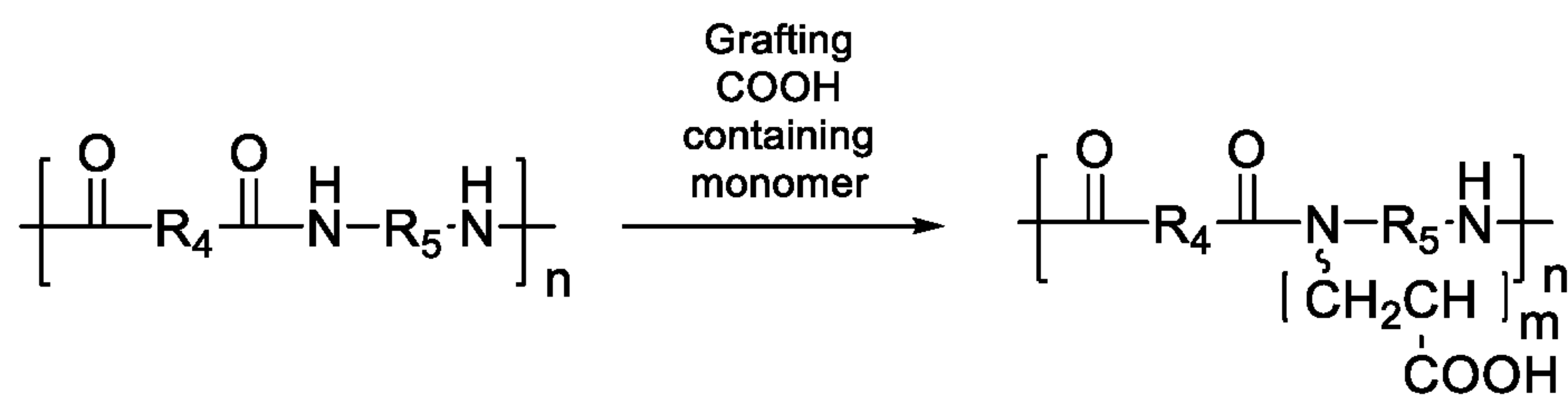
In one embodiment, the plurality of functional groups on the cross-linked polymer can comprise or consist of carboxylic acid groups. In another example, when the functional groups are selected to be acid groups, then the acid groups comprise or consist of carboxylic acid groups. As used herein, “carboxylic acid groups” refers to any chemical moiety that is capable of functioning as a carboxylic acid or can form a carboxylic acid group *in situ*. The carboxylic acid groups may include carboxylic acid groups (e.g. –COOH) or may be formed *in situ* from a precursor hydrolysable to form carboxylic acid groups, such as an anhydride precursor. In one embodiment, the acid group comprises or consists of a carboxylic acid group. A cross-linked polymer may be provided by one or

more organic polymers comprising a mixture of two or more polymers or copolymers (e.g. blend of two or more different polymers). It will be appreciated that the term “polymer” can include “copolymers”. The cross-linked polymer comprising acid groups may be a copolymer, for example wherein at least one comonomer comprises acid functionalities.

The one or more organic polymers comprising carboxylic acid functionalities may be selected from the group consisting of polyacrylic, polyurethane, polyamide, polyether, polyester, polyolefin, co-polymers thereof, and blends thereof. It will be appreciated that each of these polymers contain carboxylic acid functionalities, for example prepared or modified to contain carboxylic acid functionalities, such as by using particular comonomers or by grafting on carboxylic acid functionalities. The one or more organic polymers comprising carboxylic acid functionalities may be independently selected from a copolymer of polyurethane, polyamide, polyether, polyester, and polyolefin. In one example, the one or more organic polymers comprising carboxylic acid functionalities is independently selected from a carboxylic acid copolymer or blend of a polyvinylpyrrolidone and poly(methacrylic acid).

The carboxylic acid functionality can be incorporated into the organic polymer by any means known to the person skilled in the art. For example, the carboxylic acid functionality can be incorporated by copolymerisation, grafting copolymerisation with monomers containing acid functionalities or through post-synthesis modification of the polymer by incorporating acid functionalities into the polymer back-bone. The carboxylic acid functionalities may be provided as pendant groups linked to the polymer chain by alkyl groups, for example straight chain or branched C<sub>1-10</sub>alkyl groups substituted with a carboxylic acid group, or directly attached to the polymer chain as a carboxylic acid moiety.

In some embodiments, the organic polymer may be an acrylic grafted copolymer comprising grafted nylon, polyether or polyester. Typically grafted polymers can be prepared by reacting an activated polymer backbone with a monomer and thermal initiator at an elevated temperature, for example below Scheme 1:

**Scheme 1**

In the above example  $R_4$  and  $R_5$  can each independently be  $C_{1-X}$ alkyl,  $C_{1-X}$  alkylaryl or aryl, and where  $X$  is a positive integer.  $X$  may for example be 22, 20, 18, 16, 14, 12, 10, 8, 6, 4 or 2. It will be appreciated that the grafted polymers can also be prepared using other techniques known to the person skilled in the art.

Non-limiting examples of comonomers containing carboxylic acid functionalities include acrylates such as  $C_{1-4}$  alkyl acrylate, crotonic acid, vinyl benzoic acid, 2-bromoacrylic acid, 2-bromomethacrylic acid, fumaric acid, maleic acid, itaconic acid and muconic acid. In some embodiments, the comonomer containing carboxylic acid functionality is selected from the group consisting of acrylic acid and methacrylic acid. In some examples, the comonomer containing carboxylic acid functionality is acrylic acid. In some examples, the organic polymer comprising carboxylic acid functionalities is a reactant product of an acrylic acid comonomer and olefin comonomer.

Chain extenders may be used and may include low molecular weight polyols and amines. Low-molecular-weight polyols include, but are not limited to, ethylene glycol, propylene glycol, 1,4-butanediol, 1,5-pentanediol, 3-methyl-1,5-pentanediol, 1,6-hexanediol, neopentyl glycol, diethylene glycol, triethylene glycol, tetraethylene glycol, dipropylene glycol, tripropylene glycol, 1,4-cyclohexane diol and 1,4-cyclohexane dimethanol. Amines include, but are not limited to, ethylenediamine, butanediamine, hexanediamine, 1,3-diaminopentane, isophorone diamine, piperazine, Jeffamine polyetheramines.

The carboxylic acid content of the polymer can be measured by determining the acid value of the polymer. The acid value of a polymer can be determined by any method known to a person skilled in the art. For example, the acid value may be calculated by determining the amount of potassium hydroxide (in milligrams) required to neutralize one gram of polymer. In some examples, the acid value of the organic polymer is from 10 to

400, from 10 to 300, from 20 to 250, or from 30 to 200. In some examples, the acid value of the organic polymer is at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, or 200. In some examples, the acid value of the cross-linked polymer is less than about 400, 375, 350, 325, 300, 275, 250, 225, 200, 175, or 150, 125, 100, 75, 50, or 25. The acid value of the organic polymer may be in a range provided by any two of these upper and/or lower values. It will be appreciated that these acid values apply for the various acid groups for the various polymers as described herein.

In some embodiments, the molecular weight of the polymer can be selected by the person skilled in the art to maintain solubility for ease of application or improve in-service adhesion for particular substrates, for example coated composites. In some examples, the polymer has a number average molecular weight of at least about 5000, 7500, 10000, 12500, 15000, 17500, 20000, 25000, or 50000. In some examples, the polymer has a number average molecular weight of less than about 500000, 400000, 300000, 200000, 100000, 75000, or 50000. The polymer may have a number average molecular weight in a range provided by any two of these upper and/or lower values, for example a number average molecular weight in a range of 5,000 to 100,000, 7500 to 200000, or 10000 to 100000.

In some examples, the polymers comprising carboxylic acid functionalities may be provided by a blend of two or more polymers. The polymer blend may comprise or consist of one or more organic polymers comprising carboxylic acid functionalities selected from the group consisting of polyurethane, polyamide, polyether, polyester, and polyolefin, and one or more organic polymers selected from the group consisting of polyurethane, polyamide, polyether, polyester, and polyolefin. The polymer blend may comprise or consist of one or more polymers comprising carboxylic acid functionalities.

### Outer Shell

The porous outer shell typically contains a porosity effective for permitting transport of a biomarker through the outer shell. The porous outer shell may contain a porosity effective for permitting transport of a hydrogen ion through the outer shell. The porous outer shell may contain a porosity effective for permitting transport of a redox

biomarker through the outer shell. The porous outer shell may contain a porosity effective for permitting transport of a metal ion (e.g. Fe(II) or Fe(III) through the outer shell.

In one example, the outer shell is a porous inorganic silica outer shell and/or a porous organosilica outer shell. For example, the outer shell may be prepared from one or more organosilane compounds. Examples of various suitable compounds include MPS or an organosilane blended with inorganic counterparts e.g. TEOS, TSPA, OTES, APS.

In some embodiments, the outer shell is an organosilica outer shell. In one example, the outer shell comprises MPS, TSPA, OTES or APS. In one example, the outer shell comprises MPS.

The outer shell may be porous and be formed using or comprise a porogen agent, for example tannic acid. Other porogens include surfactants such as Tween, cetyltrimethylammonium bromide, triblock copolymers and fluorocarbon surfactants and non-surfactant templates such as tartaric acid, glucose, maltose, kanemite, and boron oxide.

The outer shell may be selected to provide a mechanical property, such as a certain level of stiffness, density, and/or hardness. In one example, the stiffness measured by Young's modulus for the outer shell may be less than a TEOS shell of the same thickness. For example, the outer shell may be provided to be less brittle.

The thickness of the outer shell (in nm) may be between 1 to 200, 1 to 10, 10 to 20, or 50 to 100. The thickness of the outer shell (in nm) may be at least about 0.1, 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, or 200. The thickness of the outer shell (in nm) may be less than about 200, 175, 150, 125, 100, 75, 50, 25, 20, 15, 10, 5, 4, 3, 2, or 1. In some embodiments, the thickness of the outer shell (in nm) is between 1 to 50. The thickness of the outer shell (in nm) may be provided in a range between any two of these upper and/or lower values.

The weight % of the outer shell in the particle (as a total weight % of the particle) may be between 0.00001 to 20, 0.0001 to 15, 0.001 to 10, or 0.1 to 5. The weight % of the outer shell in the particle (as a total weight % of the particle) may be at least about 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. The weight % of the outer

shell in the particle (as a total weight % of the particle) may be less than about 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.1, 0.01, or 0.001. In some embodiments, the weight % of the outer shell in the particle (as a total weight % of the particle) is between 0.1 to 5. The weight % of the outer shell in the particle (as a total weight % of the particle) may be provided in a range between any two of these upper and/or lower values.

The porous outer shell of the particle may be further surface functionalised with a biotargeting molecule, for example an antibody of any kind (IgG, IgM, IgE, etc). These antibodies could be directed against molecules on the surface of cells and/or organs and could be used as a marker.

In another example, an anti-fouling molecule may be incorporated or coated on the exterior surface of the solid composite particle for improved biocompatibility. Examples include, zwitterionic peptides (e.g. EK polypeptides), PEG and/or other suitable hydrophilic, anti-fouling polymer chains.

### COMPOSITIONS

The present disclosure also provides a composition comprising a plurality of solid composite particles as described herein. The plurality of solid composite particles may, for example, comprise nanoparticles and/or microparticles. In some embodiments, the plurality of solid composite particles may, for example, have an average diameter (in nm) between about 10 to 15000, 50 to 12000, 100 to 10000, 200 to 8000, 300 to 6000, 400 to 4000, 500 to 2000, or 750 to 1500. In some examples, the plurality of solid composite particles may have an average diameter (in nm) of at least about 50, 100, 250, 500, 750, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, or 10000. In some examples, the plurality of solid composite particles may have an average diameter (in nm) of less than about 15000, 12000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 750, 500, 250, or 100. In some examples, the plurality of solid composite particles may have an average diameter (in nm) in a range provided by any two of these upper and/or lower values.

The present disclosure also provides a composition comprising the solid composite particles or composition thereof according to any embodiments or examples thereof as described herein, and an acceptable excipient.

The composition may be provides as an ultrasound imaging composition.

The composition may be provided as a pharmaceutical composition comprising a pharmaceutically acceptable excipient.

The pharmaceutically acceptable excipient is pharmaceutically acceptable in the sense of being compatible with the other ingredients of the formulation and not unduly deleterious to the recipient thereof. Generally, suitable pharmaceutically acceptable excipients are known in the art and are selected based on the end use application. The pharmaceutically acceptable carrier may act as a diluent, dispersant or carrier for the solid composite particles and other optional components of the composition. The pharmaceutically acceptable excipient may also contain materials commonly used in pharmaceutically products and can be in a wide variety of forms. For example, the excipient may be water, liquid or solid emollients, silicone oils, emulsifiers, surfactants, solvents, humectants, thickeners, powders, propellants and the like.

The composition may for example contain a solvent, such as water (e.g. water for injection) or a pharmaceutically acceptable organic solvent.

The compositions may further include diluents, buffers, citrate, trehalose, binders, disintegrants, thickeners, lubricants, preservatives (including antioxidants), inorganic salts (e.g., sodium chloride), antimicrobial agents (e.g., benzalkonium chloride), sweeteners, antistatic agents, sorbitan esters, lipids (e.g., phospholipids such as lecithin and other phosphatidylcholines, phosphatidylethanolamines, fatty acids and fatty esters, steroids (e.g., cholesterol)), and chelating agents (e.g., EDTA, zinc and other such suitable cations).

The compositions of the present disclosure may also include polymeric excipients/additives or carriers, e.g., polyvinylpyrrolidones, derivatised celluloses such as hydroxymethylcellulose, hydroxyethylcellulose, and hydroxypropylmethylcellulose, Ficolls (a polymeric sugar), hydroxyethylstarch (HES), dextrates (e.g., cyclodextrins, such as 2-hydroxypropyl- $\beta$ -cyclodextrin and sulfobutylether- $\beta$ -cyclodextrin), polyethylene glycols, and pectin.

Other pharmaceutical carriers, excipients, optional ingredients and/or additives suitable for use in the compositions according to the present disclosure are listed in

"Remington: The Science & Practice of Pharmacy", 19<sup>th</sup> ed., Williams & Williams, (1995), and in the "Physician's Desk Reference", 52<sup>nd</sup> ed., Medical Economics, Montvale, N.J. (1998), and in "Handbook of Pharmaceutical Excipients", Third Ed., Ed. A. H. Kibbe, Pharmaceutical Press, 2000.

The compositions may be formulated for inhalation to the lung, by aerosol, parenteral (including intraperitoneal, intravenous, subcutaneous, or intramuscular injection) or oral administration.

The compositions may be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy.

In some embodiments, the composition is formulated for parenteral delivery. For example, in one embodiment, the composition may be a sterile composition that is suitable for reconstitution in an aqueous vehicle prior to injection.

In one embodiment, a composition suitable for parenteral administration conveniently comprises a sterile aqueous preparation of the solid composite particles, which may for example be formulated to be isotonic with the blood of the recipient.

In some embodiments, the composition comprises the solid composite particles, one or more pharmaceutically acceptable carriers, and one or more additional agents (e.g. contrast agents).

Generally, the composition comprises the solid composite particles in an amount that is effective as a biosensor and/or imaging agent. In some embodiments, the effective amount is provided by a single dose. In some embodiments, the effective amount is provided by one or more doses administered as part of a course of treatment, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 or greater than 27 doses.

The person skilled in the art would understand that the amount of the solid composite particles present in the composition will vary depending on the application and other optional ingredients present in the composition, the desired effect and the like. In some embodiments, the composition comprises the solid composite particles in a concentration between about 1 to 99 wt%, 1 to 90wt%, 1 to 85 wt%, 1 to 80 wt%, 1 to 75

wt%, 1 to 70 wt%, 1 to 65 wt%, 1 to 60 wt%, 1 to 55 wt%, 1 to 50 wt%, 1 to 45 wt%, 1 to 40 wt%, 1 to 35 wt%, 1 to 30 wt%, 5 to 99 wt%, 10 to 99 wt%, 15 to 99 wt%, 20 to 99 wt%, 25 to 99 wt%, 30 to 99 wt%, 35 to 99 wt%, 40 to 99 wt%, 45 to 99 wt%, 50 to 99 wt%, 54 to 99 wt%, 60 to 99 wt%, 65 to 99 wt%, 70 to 99 wt%, 75 to 99 wt%, 80 to 99 wt%, 85 to 99 wt%, 90 to 99 wt%, 5 to 90 wt%, 20 to 80 wt%, 30 to 70 wt%, or 40 to 60 wt%. In some embodiments, the composition comprises the solid composite particles in a concentration between about 1 to 40 wt%, 1 to 35 wt%, 1 to 30 wt%, 1 to 25 wt%, 1 to 20 wt%, 1 to 15 wt%, 1 to 10 wt%, 1 to 5 wt%, 5 to 40 wt%, 10 to 40 wt%, 15 to 40 wt%, 20 to 40 wt%, 25 to 40 wt%, 30 to 40 wt%, or 35 to 40 wt%.

In some embodiments, the solid composite particles may be administered in combination with a further therapeutic, biosensor and/or imaging agent. In some embodiments, the methods and uses described herein also relate to co-administering one or more substances in addition to the solid composite particles to a subject. The term "co-administer" indicates that each of at least two components are administered during a time frame wherein the respective periods of biological activity or effects overlap. Thus, the term includes sequential as well as coextensive administration of compounds. Similar to administering compounds, co-administration of more than one substance can be for therapeutic, biosensor and/or imaging purposes. If more than one substance or compound is co-administered, the routes of administration of the two or more substances need not be the same. The scope of the methods and uses described herein are not limited by the identity of the substance or substances which may be co-administered with the solid composite particles. For example, fluids that may be co-administered include but are not limited to, electrolytes and/or water, salt solutions, such as sodium chloride and sodium bicarbonate, as well as whole blood, plasma, serum, serum albumin and colloid solutions.

### IMAGING AND BIOSENSING

The solid composite particles or composition thereof according to any embodiments or examples thereof as described herein can be used in a variety of ways, including as a biosensor and/or an ultrasound contrast agent. In some examples, the particles or composition thereof according to any embodiments or examples thereof as described herein can be used for the detection of an analyte, such as a biomarker, in a mixture. In some examples, the particles or composition thereof according to any

embodiments or examples thereof as described herein can be used to detect an analyte, such as a biomarker, within a subject such as a human. In some examples, the particles or composition thereof according to any embodiments or examples thereof as described herein can be used as an ultrasound contrast agent. In some embodiments, the solid composite particles or composition thereof may be used in methods of imaging a subject or part of a subject, e.g. as contrast agents useful in ultrasound imaging, methods for detecting the presence of abnormal tissue such as a tumour, and/or for monitoring the size of tumour and/or tumour progression.

The present disclosure provides a method for using the particles or composition thereof according to any embodiments or examples thereof as described herein, as an ultrasound contrast agent, in ultrasound imaging, as a pH sensor and/or redox sensor. The present disclosure also provides use of the particles or composition thereof according to any embodiments or examples thereof as described herein, as an ultrasound contrast agent, in ultrasound imaging, as a pH sensor and/or redox sensor.

The present disclosure also provides the particles or composition thereof according to any embodiments or examples thereof as described herein for use as an ultrasound contrast agent, in ultrasound imaging, as a pH sensor and/or redox sensor.

The present disclosure also provides use of the particles or composition thereof according to any embodiments or examples thereof as described herein in the manufacture of a medicament for use as ultrasound contrast agent, in ultrasound imaging, as a pH sensor and/or redox sensor.

In some embodiments, the particles or composition according to any embodiments or examples thereof as described herein can be used as an iron sensor.

The present disclosure also provides a method of enhancing the contrast in an ultrasound image generated for use in medical diagnosis by administering to a region of a subject the particles or composition thereof according to any embodiments or examples thereof as described herein, and obtaining an ultrasound image of the region.

The present disclosure also provides a method for detecting an analyte in a sample. In some embodiments, the method comprises (i) contacting a sample with the particles or composition thereof according to any embodiments or examples thereof as

described herein; and (ii) detecting a change in the particle, wherein said change corresponds to the presence of the analyte in the sample. In some embodiments, there is provided a method for monitoring pH comprising, (i) contacting a sample with the solid composite particles or composition thereof according to any embodiments or examples thereof as described herein; and (ii) detecting a change in the particle, wherein said change corresponds to the pH. In some embodiments, there is provided a method for monitoring a redox state of a sample, (i) contacting a sample with the solid composite particles or composition thereof according to any embodiments or examples thereof as described herein; and (ii) detecting a change in the particle, wherein said change corresponds to the redox state of the sample. In some embodiments, there is provided a method for monitoring Fe(III) levels, (i) contacting a sample with the solid composite particles or composition thereof according to any embodiments or examples thereof as described herein; and (ii) detecting a change in the particle, wherein said change corresponds to a change in Fe(III) levels. In some embodiments, there is provided a method for monitoring Fe(II) levels, (i) contacting a sample with the solid composite particles or composition thereof according to any embodiments or examples thereof as described herein; and (ii) detecting a change in the particle, wherein said change corresponds to a change in Fe(II) levels. In some embodiments, there is provided a method for monitoring conversion between Fe(III) and Fe(II), (i) contacting a sample with the solid composite particles or composition thereof according to any embodiments or examples thereof as described herein; and (ii) detecting a change in the particle, wherein said change corresponds to conversion between Fe(II) and Fe(III) (e.g. a change in ratio between Fe(II) and Fe(III)).

As used herein, the term "contacting" refers to bringing the sample into physical contact with the solid composite particle under appropriate conditions or *in vivo*. The step of contacting the sample with the solid composite particles described herein may be carried out in any convenient or desired way. Thus, if the contacting step is to be carried out *in vitro*, the sample may optionally be maintained in an appropriate buffer or media and at the appropriate time point the particle or a composition containing the particle can be added to the sample under appropriate conditions, for example at an appropriate concentration and for an appropriate length of time before detection.

If the contacting step is to be carried out *in vivo*, the solid composite particle is administered to a subject in an effective amount. The amount of solid composite particle that will be effective for analyte detection will depend on a number of factors, such as the composition of the particle, the analyte and the location of the sample, and can be determined by standard techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. Such techniques are known to the person skilled in the art. The precise amount to be employed will also depend on the route of administration, and should be decided according to the judgment of the practitioner and each subject's circumstances.

If the contacting step is to be carried out *in vivo*, (i.e. when used in an animal or human body) the solid composite particles described herein or compositions thereof can be administered by methods that are known to the person skilled in the art such as aerosol inhalation, injection and ingestion. In some embodiments, the solid composite particles described herein or compositions thereof are administered to a subject by subcutaneous (s.c.), intraperitoneal (i.p.), intra-arterial (i.a.), or intravenous (i.v.) injection. In some embodiments, the solid composite particles are administered using a pharmaceutically acceptable carrier which can be sterilized by techniques known to those skilled in the art. Pharmaceutically acceptable carriers are known to those skilled in the art and may include saline solutions, buffered solutions and the like. Non-limiting examples of pharmaceutically acceptable carriers are described hereinabove.

In some embodiments, the change in the solid composite particle is selected from the group consisting of a change in the Young's Modulus of the polymer layer, a change in the architecture, a change in density of the polymer layer, a change in the flexibility of the polymer layer, a change in density of the shell, a change in the flexibility of the shell and formation of a partial shell. In some embodiments, the change in the solid composite particle is a change in the Young's Modulus of the polymer layer. For example, the Young's Modulus of the polymer layer may increase on contact with the analyte. In another example, the young's modulus of the polymer layer may decrease on contact with the analyte. In some embodiments, the change in the solid composite particle is a change in the density of the polymer layer. For example, the density of the polymer layer may increase on contact with the analyte. In another example, the density

of the polymer layer may decrease on contact with the analyte. In some embodiments, the change in the solid composite particle is a change in the flexibility of the polymer layer. For example, the flexibility of the polymer layer may increase on contact with the analyte. In another example, the flexibility of the polymer layer may decrease on contact with the analyte.

In some embodiments, the change in the solid composite particle is a change in architecture. The effect of particle architecture on ultrasound backscatter was first described in K. Zhang, H. R. Chen, X. S. Guo, D. Zhang, Y. Y. Zheng, H. R. Zheng, J. L. Shi, *Sci. Rep.* 2015, 5. In some embodiments, the solid composite particle may adopt a rattle architecture on contact with the analyte. In another example, the particle may adopt a solid architecture on contact with the analyte. In one example, the number of interfaces increases on contact with the analyte. In another example, the number of interfaces decreases on contact with the analyte. As used herein, the term “interface” refers to a scattering/reflection surface. Without wishing to be bound by theory, it is thought that a solid architecture has one scattering/reflection surface (i.e. the outer surface), while a “rattle” architecture has at least two scattering/reflection surfaces (the outer surface and the surface of the core) resulting in an increase in ultrasound contrast. In some embodiments, contact with an analyte may cause the particle to switch between a “rattle” architecture and a “solid” architecture.

Any suitable technique known to the person skilled in the art can be used to detect the change in the solid composite particle. In some embodiments, the change in the particle is detected using a technique selected from ultrasound, microscopy, photo acoustics, dynamic light scattering and zeta potential. In some embodiments, the change in the solid composite particle is detected by ultrasound. For example, in some embodiments the change in the solid composite particle causes an increase in ultrasound backscatter or contrast. In alternative embodiments the change in the solid composite particle causes a decrease in ultrasound backscatter or contrast. In some embodiments, the change in the solid composite particle is detected by microscopy, for example TEM, confocal microscopy, or AFM. For example, in some embodiments, the change in the particle is an increase in particle size or a decrease in particle size. These can be detected by microscopy.

The solid composite particle described herein may be used to detect an analyte. In some embodiments, the analyte is a biomarker. In some embodiments, the analyte is a proton (i.e. hydrogen ion). In some embodiments, the particles can be used to detect pH. In one example, the particles can be used to detect if the pH is above or below a predetermined value. In one example, the predetermined value is the pKa of the cross-linked polymer containing a plurality of functional groups. By way of example, the pKa of PMA<sub>SH</sub> as used in the Examples is 4.9. Accordingly, in one example, the solid composite particles can be used to detect if the pH is above or below 4.9. In one example, the solid composite particles can be used to detect if the pH is 4.0 or below. In one example, the particles can be used to detect if the pH is 7.0 or above.

In some embodiments, the sample is a clinical sample. In some embodiments, the clinical sample includes but is not limited to blood, organ, heart, lung, tumour, digestive tract, urinary tract, serum, sputum, mucus, pus, peritoneal fluid and other bodily fluids. In some embodiments, the sample has been removed from a subject. In some embodiments, the sample is in a subject. As used herein, the term “subject” refers to any organism that contains or may contain an analyte and/or requires imaging. In some embodiments, the subject is a mammal, reptile, bird, insect or fish. In some embodiments, however, the subject is a mammal, particularly a primate, domestic animal, livestock or laboratory animal. In one embodiment, the subject is human. In one embodiment, the subject is a non-human mammal.

In another embodiment there is provided a method for detecting an analyte, comprising: contacting the analyte with the solid composite particles or composition thereof according to any embodiments or examples thereof as described herein, the particle having analyte-bound and analyte-unbound states wherein binding of the one or more analyte to the ultrasound molecular sensor causes a modulation in ultrasound signal; and obtaining an ultrasound image.

In some embodiments, the solid composite particles described herein can be imaged using an ultrasound device. Detection of the ultrasound signal from the particles described herein can be performed using techniques known to the person skilled in the art. Ultrasounds can be detected in the transmission or the reflection configuration. Choice of the configuration can be made based on the nature of the sample to be

analyzed. Liquid solutions are amenable to transmission detection but detection within an individual for example may require the use of reflection configuration. In one embodiment transmission measurements are performed using a pulsed ultrasound generated at a transducer. Reflected/transmitted ultrasounds are detected using a second transducer. In some embodiments, the ultrasound frequency used is between 2 to 50 MHz, 2 to 12 MHz and 12 to 50 MHz, In some examples, the ultrasound frequency used is 40 MHz. But it will be appreciated that the actual frequency of excitation depends on the type of particle and the depth at which the particle is located relative to the transducer.

The ultrasound mechanical index (MI) is an ultrasound metric that is a measure of the power of an ultrasound beam. It is a unitless number that can be used as an index of cavitation bio-effects; a higher MI value indicates greater exposure. Levels below 0.3 are generally considered to have no detectable effects. The maximum threshold for diagnostic imaging as an MI value of 1.9. In some embodiments, the ultrasound mechanical index is used between 0-4. In some embodiments, the ultrasound mechanical index is less than 1.9. In some embodiments, the ultrasound mechanical index is between 0.3 and 1.9, for example, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3, 1.5, 1.7, or 1.9. In some embodiments, the ultrasound mechanical index is less than about 0.3, for example 0.1, 0.2 or 0.3.

In some embodiments, the methods and uses described herein are *in vivo* methods and uses. In alternative embodiments, the methods and uses described herein are *in vivo* methods and uses. In some embodiments, samples may be obtained from individuals and analytes measured directly in the sample.

In another embodiment, the solid composite particles or a composition thereof according to any embodiments or examples thereof as described herein can be used to image organs or tumors by accumulation after intra-venous injection. In some embodiments, the solid composite particles can be coated with a targeting agent having an affinity for a target. Suitable targeting agents include antibodies, antibody like molecules and other target binding molecules. In an embodiment, the particles can be injected intravenously and then accumulate specifically at the diseased area for ultrasound imaging.

In another embodiment, there is also provided a method of imaging. The method comprises administering an effective amount of the solid composite particle or a composition thereof to a subject and imaging the subject. In an embodiment, the solid composite particles or a composition thereof according to any embodiments or examples thereof as described herein can be introduced (e.g., administered) to a subject where the particle may include a targeting agent having an affinity for a target. Passive targeting may also be used. After an appropriate amount of time, the subject is exposed to one or more imaging devices so that a signal(s) (e.g., ultrasonic signal) can be detected. The location of the target can be correlated with the location of the detected signal(s).

Accordingly, the present disclosure provides a method of imaging a subject or part of a subject, comprising administering a particle as defined herein, or composition comprising particles as defined herein, to the subject, and carrying out ultrasound imaging of the subject or part thereof.

There is also provided a method of detecting the presence and/or level of a biomarker in a subject or part of a subject, comprising administering a particle as defined herein, or composition comprising particles as defined herein, to the subject, and carrying out ultrasound imaging of the subject or part thereof.

The methods, solid composite particles and compositions described herein can be used to image, detect, study, monitor, and/or evaluate, a condition or disease such pre-cancerous tissue, cancer, or a tumour. For example, the tumour can be more acidic than other tissue, and thus particles/compositions which are ultrasound contrast agents that are responsive to changes in pH may find utility in detecting and monitoring cancers.

Accordingly, there is provided a method of determining whether a subject has a cancer, comprising:

comprising administering a particle as defined herein, or composition comprising particles as defined herein, to the subject;

carrying out ultrasound imaging on the subject's body or a part thereof; and

determining whether the subject has a cancer based on the ultrasound imaging results.

There is also provided a method of imaging a cancer in a subject, comprising:

comprising administering a particle as defined herein, or composition comprising particles as defined herein, to the subject;

carrying out ultrasound imaging on the subject's body or a part thereof.

There is also provided a method of determining the progression of a cancer in a subject, comprising:

administering to a subject having a cancer a first amount of a particle as defined herein or a composition comprising particles as defined herein;

carrying out a first ultrasound imaging step on the subject's body or a part thereof;

subsequently administering to the subject a second amount of a particle as defined herein or a composition comprising particles as defined herein;

carrying out a second ultrasound imaging step on the subject's body or a part thereof; and

determining whether the cancer has progressed based on the first and second ultrasound imaging results.

There is also provided a method of determining the effectiveness of a cancer therapy administered to a subject having a cancer, comprising:

administering to a subject having a cancer a first amount of a particle as defined herein or a composition comprising particles as defined herein;

carrying out a first ultrasound imaging step on the subject's body or a part thereof;

administering to the subject a cancer therapy;

subsequently administering to the subject a second amount of a particle as defined herein or a composition comprising particles as defined herein;

carrying out a second ultrasound imaging step on the subject's body or a part thereof; and

determining the effectiveness of the cancer therapy based on the first and second ultrasound imaging results.

In an embodiment, the method includes imaging pre-cancerous tissue, cancer, or a tumour. In an embodiment, the method can comprise exposing a subject to an imaging device (e.g., ultrasound detection system and/or ultrasound source system). The solid composite particles are administered to the subject prior to exposure and/or during exposure to the imaging device and after a period of time the particle enters the tumour. Subsequently, the particles are detected and the location of the solid composite particles can be determined. The location of the solid composite particles can be correlated with the location of the tumour and/or the presence of the tumour and/or the pH of the tumour microenvironment.

Also provided herein is a particle as defined herein, or a composition comprising particles as defined herein, for use as a biomarker (e.g. pH) sensor, as an imaging agent (e.g. in ultrasound imaging) or as a contrast agent (e.g. an ultrasound contrast agent), for imaging a subject, for determining whether a subject has a cancer, for imaging a cancer in a subject, for determining the progression of a cancer in a subject, or for determining the effectiveness of a cancer therapy administered to a subject.

Also provided herein is the use of a particle as defined herein, for the manufacture of a composition comprising the particles, for use for use as a biomarker (e.g. pH) sensor, as an imaging agent (e.g. in ultrasound imaging) or as a contrast agent (e.g. an ultrasound contrast agent), for imaging a subject, for determining whether a subject has a cancer, for imaging a cancer in a subject, for determining the progression of a cancer in a subject, or for determining the effectiveness of a cancer therapy administered to a subject.

The present disclosure provides a method for using the particles or composition thereof according to any embodiments or examples thereof as described herein, as an ultrasound contrast agent, in ultrasound imaging and/or as a pH sensor.

#### PROCESSES FOR PREPARING PARTICLES

The present disclosure provides a process for preparing a composition comprising a plurality of solid composite particles.

The process can comprise the following steps:

contacting a plurality of individual solid cores with one or more polymers comprising functional groups to provide a plurality of individual solid cores each encapsulated by one or more polymeric layers;

cross-linking at least some of the one or more polymeric layers to form, on each of the solid cores, a cross-linked polymer containing a plurality of functional groups; and

applying a porous coating to the one or more polymeric layers to form a porous outer shell encapsulating the one or more polymeric layers present on each of the solid cores to form the plurality of coated particles.

While the individual solid cores are available commercially, they may also be synthesized using techniques known to the person skilled in the art. In some embodiments, the solid core is a silica nanoparticle that can be made using a synthesis (e.g., a modified Stober approach, for example see *Langmuir* 2005, 21, 4277-4280 and *Langmuir* 2005, 21, 7524-7527) involving water, ethanol, strong base, and a tetraethylorthosilicate precursor. In some embodiments, the solid core is an organosilica nanoparticle that can be made using a synthesis according to the method of Miller, C. R.; Vogel, R.; Surawski, P. P. T.; Jack, K. S.; Corrie, S. R.; Trau, M., Functionalized organosilica microspheres via a novel emulsion-based route. *Langmuir* 2005, 21 (21), 9733-9740. In some embodiments, the solid core is an organosilica nanoparticle that can be made using a synthesis according to the method of A. P. R. Johnston, B. J. Battersby, G. A. Lawrie, L.e K. Lambert, and M. Trau, A Mechanism for Forming Large Fluorescent Organo-Silica Particles: Potential Supports for Combinatorial Synthesis. *Chem. Mater.*, Vol. 18, No. 26, 2006, 6163. In some embodiments, the solid core is a large pore mesoporous silica nanoparticle that may be prepared using the method of Gao, Z and I. Ilya, Zharov Large Pore Mesoporous Silica Nanoparticles by Templating with a Nonsurfactant Molecule, Tannic Acid. *Chemistry of Materials*, 2014, 26:2030-2037.

The diameter or the effective diameter (e.g., if the nanoparticle is rotated about a center of rotation (e.g., to form an imaginary sphere) and the outer most distance from the center of rotation reached (the edges of the imaginary sphere) defines the effective diameter) can be about 20 nm to 5000 nm or a radius of about 10 to 2500 nm. In

addition, the diameter or the effective diameter can be tuned based on concentrations of starting materials.

Any suitable technique can be used to encapsulate the one or more polymeric layers on the solid core. In some embodiments, the process comprises a layer-by-layer process encapsulating the one or more polymeric layers on the solid cores. Typically, this technique involves the layer-wise assembly of polyelectrolytes of opposite charges on the surface of interest, building up a layered system. In some embodiments, the layer-by-layer process comprises assembling alternate layers of a sacrificial polymer with an analyte sensitive polymer (e.g. a pH sensitive polymer). In some embodiments, the layer-by-layer process exploits hydrogen binding interactions (although other types of interactions may be exploited) to form multilayered films by the alternate deposition of a polymer comprising a hydrogen bonding donor and a polymer comprising a hydrogen bonding acceptor. In some embodiments, the sacrificial polymer is a hydrogen bonding acceptor and an analyte sensitive polymer is a hydrogen bonding donor. In alternative embodiments, the sacrificial polymer is a hydrogen bonding donor and an analyte sensitive polymer is a hydrogen bonding acceptor. In some embodiments, the sacrificial polymer is as described herein, for example poly(*N*-vinylpyrrolidone). In some embodiments, the analyte sensitive polymer is as described herein, for example poly(methylacrylic acid) or a derivative thereof. Derivatives include but are not limited to poly(methylacrylic acid) functionalised with one or more crosslinking agents, such as protected thiol functionalities or alkyne functionalities. In some embodiment, assembling a polymer layer comprises contacting the particle with a solution of the desired polymer for a period of time such that the polymer adsorbs to the surface of the particle. The concentration of the polymer solution and contact time may be varied to ensure that the particle is encapsulated. Once a polymer layer has been assembled, the coated particle is washed to remove excess polymer and the process repeating with the other polymer. This process is repeated until the desired number of polymer bilayers is obtained. Any number of polymer bilayers can generated. In another embodiment, the polymeric layer encapsulating the solid core may be assembled by surface initiated polymerisation (“grafting from”). In yet another embodiment, the polymeric layer encapsulating the solid core may be assembled by pre-synthesised polymer conjugation (“grafting to”).

The process comprises cross-linking at least some of the one or more polymeric layers to form a cross-linked polymer containing a plurality of functional groups. In some embodiments, cross-linking comprises treating the one or more polymeric layers with an oxidant, for example to promote formation of disulphide bonds. In some embodiments, the oxidant is chloramine-T. In some embodiments, cross-linking comprises treating the one or more polymeric layers with a triazide linker which reacts with alkyne functional groups through click chemistry. In some embodiments, cross-linking comprises forming disulphides between neighbouring thiols; using amide-crosslinking chemistry (e.g. EDC/NHS or non-aqueous carbodiimides (DCC, DIC)), to link primary amines with carboxylic acids, reductive amination (glutaraldehyde), biotin conjugation and the like. In some embodiments, cross-linking comprises treating the one or more polymeric layers with a phenol or a polyphenol. In some embodiments, cross-linking comprises treating the one or more polymeric layers with a polyphenol. In some embodiments, cross-linking comprises treating the one or more polymeric layers with tannic acid or a derivative or an analog thereof.

In some embodiments, the sacrificial polymer layer is removed before or after cross-linking. In some embodiments, the sacrificial polymer layer is removed after cross-linking. In some embodiments, the sacrificial polymer layer is removed by washing, for example with buffer at pH 6.0 or above. In some embodiments, the sacrificial polymer layer is removed by washing at pH 7.0.

After crosslinking, a porous coating is applied to the one or more polymeric layers to form a porous outer shell encapsulating the one or more polymeric layers present on each of the solid cores to form the plurality of coated particles. In some embodiments, a porous coating is applied to the polymer coated particle by incubating the coating particle in a solution comprising an encapsulating agent. In some embodiments, the solution comprising an encapsulating agent and a porogen. Non-limited examples of porogens are defined herein, e.g. tannic acid. In some embodiments, the solution further comprises a catalyst (e.g. TEA). In one embodiment, the encapsulating agent is an organosilica, for example 3-mercaptopropyltrimethoxysilane, for a period of time. The contact time may be varied to vary the thickness of the porous outer shell. In some embodiments, the contact time is between 10 minutes to 24 hours,

between 20 minutes and 1 hour, or between 20 and 40 minutes. In some embodiments, the contact time is 30 minutes.

The porous coating should be porous to enable the analyte to contact the polymer layer. In some embodiments, a porogen is used to generate the porous coating. In some embodiments, the porogen is tannic acid. The concentration of the porogen can be varied to vary the number of pores formed in the outer layer.

In some embodiments, the polymer coating is pH sensitive and is in the expanded form (e.g. negatively or positively charged form) when the porous layer is applied. In one example, the porous layer is applied at pH 7. In one example, the porous layer is applied in ultra-pure water (e.g. Milli-Q water) at pH 7.

While the composition comprising a plurality of coated particles may be used as is, in some embodiments the coated particles require further processing. The further processed may depend on the desired application. Further processing can include, but is not limited to, buffer exchange, concentrating or the addition of a targeting molecule, anti-fouling coating or fluorescent dye. It will be appreciated that the further processing should have minimum impact on the function of the solid composite particle, e.g. for detecting an analyte or pH. In some embodiments, the process further comprises removing at least a portion of the particles from the composition. For example, the particles may be separated from the suspension using any one of a number of devices and techniques known to those of ordinary skill in the art. Non-limiting examples of removal methods include settling, filtration, and centrifugation. In some embodiments, a targeting molecule may be conjugated to outer surface of the particle. Any suitable conjugation technique may be used for conjugating a targeting molecule. Non-limiting examples, include absorption, maleimide coupling and other bio-conjugation chemistries. In some embodiments, an antifouling molecule may be applied to the surface of the particle. The antifouling molecule is suitable to stop non-specific protein absorption on to the surface of the particle. Any suitable antifouling molecule may be used, for example a polypeptide (e.g. an EK polypeptide) or polyethylene glycol. In some embodiments, the antifouling molecule may encapsulate the particle forming an antifouling layer. However, as would be understood by the person skilled in the art, the antifouling layer should be sufficiently porous to allow the analyte of interest to contact the one or more polymeric layers

encapsulating the solid core, wherein each polymeric layer comprises a cross-linked polymer containing a plurality of functional groups. In some embodiments, the antifouling molecule is not a PEG-silane. In some embodiments, a fluorescent dye may be conjugated to the surface of the particle.

In some embodiments, an a dye, e.g. a fluorescent dye is included during or after formation of the polymer layer. In some embodiments, a fluorescent dye, such as Cy-5, can be added after formation and crosslinking of the polymer layer but before formation of the porous outer shell.

## Examples

### General Materials and Methods

#### *Materials*

Silica particles of 829 nm ( $\text{SiO}_2\text{-R}$ , non-functionalized) were purchased from MicroParticles GmbH (Germany) as a 5 wt% suspension and were used as provided. (3-Mercaptopropyl)trimethoxysilane (MPS,  $M_w$  196.34 g mol<sup>-1</sup>), triethylamine (TEA,  $M_w$  101.19 g mol<sup>-1</sup>), tannic acid (TA,  $M_w$  1701.20 g mol<sup>-1</sup>), poly(methacrylic acid) (PMMA, 30 wt%,  $M_w$  10 kDa), poly(N-vinylpyrrolidone) (PVPON,  $M_w$  40 kDa), poly(sodium 4-styrenesulfonate) (PSS,  $M_w$  70 kDa), poly(allylamine hydrochloride) (PAH,  $M_w$  50 kDa), sodium hydroxide (NaOH), dithiothreitol (DTT), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM), N-chloro-p-toluenesulfonamide sodium salt hydrate (chloramine-T hydrate), 2,2'-dithiodipyridine (DTDP), phosphate buffered saline (PBS) tablets, 2-morpholinoethanesulfonic acid buffer (MES), sodium acetate (NaOAc), 3-(N-morpholino)propanesulfonic acid (MOPS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and Tetrahydrofuran (THF) were obtained from Sigma Aldrich. Cyanine5 maleimide (Cy5) were purchased from Lumiprobe. Polydimethylsiloxane (PDMS) and crosslinking agent SYLGARD™ 184 Silicone Elastomer Curing Agent 0.1 were purchased from Dow chemical.

#### *Imaging*

Imaging of the particles were performed with an Olympus BX 51 microscope equipped with a 40 x objective lens (Olympus UPlanFL N 40 x/1.3, oil, Ph1). A CCD

camera (Olympus XM10) was mounted on the top port of the microscope. Fluorescence images were illuminated with an Hg arc lamp (Olympus U-RFL-T). The images were acquired using Cell Sens [Ver.1.18] and analysed using Image J (version 2.35).

For fluorescence microscopy, the  $\text{SiO}_2\text{@PMA}_{\text{SH}}$  particles were suspended in 40 mM HEPES buffer (pH 4 or pH 7) and then imaged with confocal laser scanning microscopy (CLSM). The images were analysed using Image J (version 2.35) giving the distribution of the  $\text{PMA}_{\text{SH}}$  polymer expansion.

For electron microscopy, transmission electron microscopy (TEM) images of the particles were taken using the FEI Tecnai T20 with a LaB6 emitter, twin lens using acceleration voltage of 200 kV. The samples were prepared by drying an aqueous particle suspension onto a holey carbon grid mounted onto a single tilting holder. The images were acquired using an Orius SCD200D wide-angle CCD camera and analysed using Image J (version 2.35).

## EXAMPLE 1 – PREPARATION OF CONTRAST AGENT

$\text{PMA}_{\text{SH}}$  was synthesised as previously reported (K. Kempe, S. Leen Ng, T. T. Gunawan, K. F. Noi, F. Caruso, *Adv. Funct. Mater.* **2014**, 24, 30, 6187). Briefly,  $\text{PMA}_{\text{SH}}$  was prepared by introducing the protected thiol (2-(pyridylthio)-ethylamine hydrochloride; PDA) functionality. To an aqueous solution of PMA (30 w% in PBS), DMTMM ( $20 \text{ g L}^{-1}$ , 2 fold excess) was added and stirred for 15 min at room temperature. PDA ( $20 \text{ g L}^{-1}$ , 1.5 fold excess) was then added and the reaction mixture stirred at room temperature overnight. The resulting reaction mixture was dialysed against Milli-Q water (3-4 days, with minimum 2 water changes per day) and the  $\text{PMA}_{\text{SH}}$  polymer recovered by freeze drying. The resulting polymer contained 10 % thiol functionality as assessed by  $^1\text{H}$ -NMR and the % signal increase of thiol signals relative to the carboxylic acid signals.

The pH sensitive polymer layer was assembled on a silica core using layer-by-layer assembly as described in Kempe et al 2014 using  $\text{PMA}_{\text{SH}}$  and PVPON in alternating layers (K. Kempe, K. F. Noi, S. L. Ng, M. Mullner, F. Caruso, *Polymer* 2014, 55, 6451). Prior to use,  $\text{PMA}_{\text{SH}}$  (3 mg) was dissolved with DTT (0.5 M) in MOPS buffer (20 mM, pH 8) and stirred for 30 min at room temperature to remove the protecting groups. The solution was added to sodium acetate buffer (40 mM, pH 4) at a

concentration of  $100 \text{ g L}^{-1}$  and adjusted to pH 4. PVPON was dissolved at the concentration of  $100 \text{ g L}^{-1}$  in sodium acetate buffer (40 mM, pH 4) and pH adjusted to pH 4. The silica particles ( $100 \mu\text{L}$ , 5w %) were washed three times with sodium acetate buffer (40 mM, pH 4) prior to use.

The washed  $\text{SiO}_2$  particles were incubated with  $200 \mu\text{L}$  of PVPON solution for 10 min at room temperature with stirring. After 10 min, the particles were washed three times with sodium acetate buffer (40 mM, pH 4). The washed particles were then incubated with  $200 \mu\text{L}$  of  $\text{PMA}_{\text{SH}}$  for 10 min with stirring. This process was repeated to form 6 bilayers, where each bilayer comprises a layer of  $\text{PMA}_{\text{SH}}$  and a layer of PVPON. The  $\text{PMA}_{\text{SH}}$  layers were crosslinked by the addition of chloramine T in MES (20 mM, pH 6). The crosslinked particles were washed three times in MES buffer to remove the PVPON layers, and then washed three times with sodium acetate buffer (40 mM, pH 4).

To coat the MPS shell onto the  $\text{SiO}_2@\text{PMASH}$  particles, the particles were washed and resuspended into Milli-Q water (pH 7). Hydrolysed MPS ( $45 \mu\text{L}$ , 1.62 mM), TEA ( $1 \mu\text{L}$ , 3.19 mM) and TA ( $8.18 \times 10^{-4} \text{ mM}$ ) were added and the solution left to stir for 30 min. The coated ( $\text{SiO}_2@\text{PMASH}@\text{MPS}$ ) particles were washed with Milli-Q. The MPS shell was coated at pH 7 so that the pH sensitive polymer was swollen.

Particle concentration was measured using a haemocytometer. The samples were run in triplicate with the average taken and standard deviation calculated.

A schematic showing preparation of the contrast agent is shown in Figure 1.

## EXAMPLE 2 – CHARACTERISATION OF CONTRAST AGENT

A combination of confocal fluorescence and transmission electron microscopy was employed to characterise the different stages of the contrast agent fabrication. To study the layer-by-layer modified  $\text{SiO}_2$ , the polymer film applied to the silica core was labelled with Cy5 prior to addition of the MPS coat. Briefly, the  $\text{SiO}_2@\text{PMASH}$  particles were incubated with Cy5 maleimide ( $0.5 \mu\text{L}$ ,  $1 \text{ g L}^{-1}$ ) in sodium acetate buffer (40 mM, pH 4) overnight. The Cy-5-maleimide dye reacted with the non-cross-linked thiols present in the polymer. The labelled particles were washed three times with sodium acetate buffer to remove any unreacted dye. If required, the outer MPS shell was applied to the labelled particle as described in Example 1.

To confirm the presence of the polymer layer on the surface of the particles, the SiO<sub>2</sub> cores and the SiO<sub>2</sub>@PMA<sub>SH</sub>-Cy5 particles were observed by TEM and SEM (Figure 2). The SiO<sub>2</sub> cores had a clean surface however the polymer film was observed on the SiO<sub>2</sub>@PMA<sub>SH</sub>-Cy5 particles (Figure 2). The presence of the polymer film after crosslinking is visible as a thin film coating the core (Figure 2). The MPS shell coating was also confirmed by TEM, revealing a rougher layer at the surface of the particles (Figure 2) along with a change in size from silica cores of mean diameter of 840 nm to 1.2 µm. The change in size observed in TEM is not consistent with that observed using confocal microscopy due to material shrinkage under the high vacuum required for TEM. Moreover, TEM images of microtomed materials confirmed the distinct material layers on the surface of the SiO<sub>2</sub> cores (Figure 3).

The response of the polymer layer to changes in pH was then characterized. The SiO<sub>2</sub>@PMA<sub>SH</sub>-Cy5 particles were suspended in sodium acetate buffer at pH 4 and imaged with confocal microscopy. The same particles were then washed into PBS buffer at pH 7 and again imaged with confocal microscopy. The diameter of the particle was found to expand from 1.1 µm to 2.1 µm when the pH changed from pH 4 to pH 7, respectively (Figure 4). Overall, the particles kept their spherical dimensions. The pK<sub>a</sub> of the acid functionality in the PMA<sub>SH</sub> polymer is approximately 4.9 (K. Kempe, K. F. Noi, S. L. Ng, M. Mullner, F. Caruso, *Polymer* **2014**, 55, 6451). Without wishing to be bound by theory, it is thought that at pH 7 the acid groups in the PMA<sub>SH</sub> film are mainly deprotonated resulting in repulsive forces which lead to an expansion of the film as evident from the ~2-fold size increase in particle size observed by confocal fluorescence microscopy (Figure 4).

### EXAMPLE 3 - ECHOGENIC PROPERTIES OF THE CONTRAST AGENT.

To evaluate changes in contrast intensity under different pH conditions, agarose gel phantoms were prepared and imaged with a pre-clinical US. A custom-designed, US-neutral PDMS device was cast as the sample holder (Figure 5a and b), allowing for 3D alignment of the sample to maintain a constant distance from the transducer. Briefly, a phantom mould was designed in Rhinoceros 5 and converted to .stl file format. The phantom mould was 3D printed on a FLASHFORGE NEW Creator Pro Dual Extrusion 3D Printer using acrylonitrile butadiene styrene (Figure 5). 10% crosslinking agent was

added to PDMS and a total volume of 7.5 mL was poured into the 3D printed mould in a desiccator. The 3D printed mould containing PDMS was left under vacuum for 24 hours and was then placed in an oven at 60°C to ensure that the PDMS had crosslinked (Figure 5b).

Gel phantoms were prepared containing either the contrast agent (SiO<sub>2</sub>@PMA<sub>SH</sub>@MPS particles) or SiO<sub>2</sub> particles as a negative control. Briefly, HEPES buffer (40 mM, pH 7) was degassed three times. 50 mL of the degassed buffer was heated to 90 °C with constant stirring. Once at 90 °C, agarose (2 mg) was added and dissolved in the HEPES buffer. After the agarose dissolved, the agarose solution was incubated at 80°C with stirring to make a buffered agar (2 %). The SiO<sub>2</sub>@6PMA<sub>SH</sub>-Cy5@MPS particles were washed with degassed HEPES buffer (40 mM, at desired pH). 10 µL of the particles were pipetted into a microcentrifuge tube and 10 µL of buffered agar was added. The resulting solution was gently mixed with care taken to avoid the incorporation of gas. The resulting solution was then pipetted into a tube mould to make a (1 %) agar suspension. After 10 min, the set agar suspension was pushed out into Eppendorf tube containing HEPES buffer (40 mM, pH 7). Each sample was prepared in triplicate.

Prior to imaging, a bed of ultrasound gel was prepared, and the phantom removed from Eppendorf tube and placed on bed. Agar gels containing particles were initially imaged at pH 7. After imaging at pH 7, the sample was retrieved and placed into 40 mM HEPES buffer (pH 6), washed with 40 mM HEPES buffer (pH 6) to remove residual US gel and incubated in 40 mM HEPES buffer (pH 6) for 16 hours. After 16 hours, the sample was again washed with HEPES buffer and the sample imaged 24 hrs after the first image was collected. This was then repeated for using 40 mM HEPES buffer (pH 5) in the place of 40 mM HEPES buffer (pH 6) for the measurements taken at pH 5. The control sample contained SiO<sub>2</sub> cores in agar.

For ultrasound imaging, the agar phantoms were placed in the PDMS sample holder and covered in US gel. The depth of the phantom was kept constant at 6 mm from transducer. The images were acquired using the 3D scan mode, the scan length was set at 10.526 mm with image capture at 0.038 mm recording 277 frames. All ultrasound images were acquired by FIJIFILM VisualSonics Vevo 2100 Ultrasound, using the MS-550D

transducer at 40 MHz, 10 % dB power and 2D gain at 15. Images were analysed using ImageJ. Each sample was recorded in a .avi stack frames that contained no artefacts (air bubbles or mould reflections) and had the sample in vision where analysed. Regions of interest were drawn on these frames for pH 4, 5, 6 and 7 and the background (BG) and control. The average integrated densities were taken with ImageJ, with the average taken and BG and control BG subtracted. For ex-vevo images frames were selected from frames 1-51 to avoid artefacts from the IV needle, regions of interest were drawn in image J and the average of the integrated density was calculated.

To ensure that the  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particles were not changing due to the time period or incubation setting, the  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particles kept at pH 7 and imaged every 24 hrs for 3 days. No change in backscatter was observed over the 3 day period (Figure 6a). The  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particles or  $\text{SiO}_2$  negative control phantoms were imaged after incubation in HEPES buffer at pH 5, 6, or 7. A two-fold increase in ultra sound contrast was observed with each decreasing step in pH, while the corresponding signal of the  $\text{SiO}_2$  control samples did not change significantly with pH (Figure 6b).

It is thought that the mechanism in which the  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  contrast agent produces a change in contrast is different from traditional USCAs as no gas is encapsulated or generated. Without wishing to be bound by theory, there are a number of possible explanations for the change in ultrasound contrast observed on change in pH. One possible explanation is that the change in pH changes the number of interfaces present in the contrast agent. With the present approach, the number of interfaces in the contrast agent is thought to vary based on local pH. At pH 7, the pH polymer is expanded filling the gap between the solid  $\text{SiO}_2$  core and the organosilica shell. Thus, in the presence of an US beam the contrast agent appears as a solid silica nanoparticle, *i.e.* lower in intensity. In contrast, at pH 4 the polymer film is contracted generating a gap between the two silica components. Consequently, the contrast agent adopts an architecture with increased number of interfaces and corresponding increased ultrasound backscatter intensity.

Density and/or material rigidity may also affect ultrasound backscatter intensity. Previously (in studies on hollow capsules) an increase in polymer flexibility has been

found to lead to a decrease in ultrasound backscatter intensity. In other words, the more rigid the material (i.e. the pH sensitive polymer) the higher the US backscatter. The young's modulus of a polymer is indicative of the flexibility of the polymer. PMAA has a pronounced change in young's modulus over the pH range of 6.5 to 5.4 (N. Elsnér, V. Kozlovskaya, S. A. Sukhishvili, and A. Ferry. *Soft. Mat.* **2006**, 2, 966). Accordingly, it is thought that the thiol functionalized PMAA present in the pH sensitive layer will become more rigid at pH 4 leading to increase in backscatter, while at pH 7 the pH sensitive layer will become more flexible leading to a decrease in ultrasound backscatter. Either of these proposed mechanism would support the changed in observed contrast.

Traditional USCAs are sensitive to the US frequency and intensity due to their reliance on gas. It is thought that the presence of this gas, may for example, lead to inertial cavitation at high frequency and intensity causing rupture of the particle and destruction of the image. In addition, the gas may diffuse out of the USCAs. Consequently, gas filled USCA can only be used for imaging within 5-10 minutes of injection. Hollow capsules composed of silica, poly electrolytes or composite have been shown to suffer from perforation or rupture at low intensity US, leading to signal decrease or image destruction. This has not been observed in the sUN's we attribute this to the silica core, as this shows no sign of destruction in the US beam (sup info fig xx). This has allowed for longitudinal studies of sUN's at high frequency and intensity with no destruction to the image. The phantoms were monitored over the course of 72 hrs at 40 MHz and 10 % power exceeding any current contrast agent at this frequency and intensity, with increase in US backscatter observed only in relation to decrease in pH.

#### **EXAMPLE 5 - ANIMAL TISSUE IMAGING.**

To investigate the performance of the  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particles as an ultrasound contrast agent, ultrasound contrast imaging in animal tissue was performed. Ultrasound imaging was carried out on the flank tissue of freshly culled BALB/c mice, before and after injection of  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particles and  $\text{SiO}_2$  particles as a negative control.

The mice were exsanguinated using heparinised saline (10  $\mu$  per ml, 8 ml total infusion volume) to remove the blood volume. Twin intravenous lines were aligned onto

the flank of the mouse cadaver. First 200  $\mu\text{L}$  of degassed HEPES buffer (40 mM, pH 4) was injected into the cadaver. Following this 25  $\mu\text{L}$  of  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  was injected. The site was monitored for 1 hr with a 3D scan acquired every 10 mins. A further subcutaneous injection of 200  $\mu\text{L}$  HEPES buffer (40 mM, pH 4) was administered into the same injection port and a 3D scan was acquired every 10 mins. A subcutaneous injection of 200  $\mu\text{L}$  HEPES buffer (40 mM, pH 7) was administered, and a 3D scan was acquired every 10 mins. A significant increase in ultrasound contrast was observed upon injection of HEPES pH 4. This decreased upon injection of HEPES pH 7 (Figure 7). The  $\text{SiO}_2$  particle negative control was not affected by the change in pH (Figure 7). It is evident from this study that the signal is stable over at least 60-minutes (Figure 8) which vastly exceeds that of common gas-filled USCAs at high frequency. For figure 7, the cadavers were imaged over a period of 45 minutes with 15 minutes between injection. For figure 8, the cadavers were imaged over a period of 90 minutes with a 10 minute wait for the first injection pH 4 following a 45 minute wait for the pH 7 injection.

#### EXAMPLE 6 – *IN VIVO* IMAGING

The cytotoxicity of the  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particles on NIH/3T3 cells was evaluated using an Alamar blue assay with unlabelled particles using standard procedures. The sUN particles showed negligible toxicity on the cells, with cells having above 80 % viability (data not shown).

To probe the *in vivo* dynamics of the  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particles, the contrast agent or a negative control ( $\text{SiO}_2$  particles) were injected into the flank of a mouse and the particles were monitored for 15-minutes at 5-minute intervals using ultrasound. The ultrasound images were acquired using the same setting as the *in vitro* phantoms and tissue phantoms. A 15-minute time period was required for the particles to equilibrate. These images were collected from one site on the flank of the mouse, in an area which showed minimal US contrast upon injection. In agreement with the *in vitro* phantom results, a 2 fold increase in the backscatter intensity was observed at 5 minute intervals (Figure 9).

To further investigate the stability of the  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particles in the ultrasound beam, harmonic data was also collected with pulsed ultrasound. There was no

change in harmonics post pulse indicating that the  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particle is stable under pulsed conditions (Figure 10), as well as at high frequency and power previously demonstrated by the *in vitro* and *ex vivo*. Accordingly, no component of the  $\text{SiO}_2@\text{PMA}_{\text{SH}}@\text{MPS}$  particles is destroyed under these ultrasound conditions.

#### **EXAMPLE 7 – PREPARATION OF CONTRAST AGENT**

The redox sensitive polymer layer was assembled on a silica core using layer-by-layer assembly as described in Pechenkin et al 2012 using PAH and PSS in alternating layers (Mikhail A. Pechenkin, Helmuth Möhwald and Dmitry V. Volodkin, *Soft Matter*, 2012, 8, 8659). Prior to use, PAH and PSS were dissolved at a concentration of 100 g/L in sodium carbonate buffer (5 mM, pH 8) and the pH adjusted to pH 8. The silica particles (100  $\mu\text{L}$ , 5w %) were washed three times with sodium carbonate buffer (5 mM, pH 8) prior to use. The washed  $\text{SiO}_2$  particles were incubated with 200  $\mu\text{L}$  of PAH solution for 10 min at room temperature with stirring. After 10 min, the particles were washed three times with sodium carbonate buffer (5 mM, pH 8). The washed particles were then incubated with 200  $\mu\text{L}$  of PSS for 10 min with stirring. This process was repeated to form 6 bilayers, where each bilayer comprises a layer of PAH and a layer of PSS. The PAH/PSS bilayers were crosslinked by the addition of tannic acid in MES (20 mM, pH 6). The crosslinked particles were washed three times in MES buffer to remove the residual polymer, and then washed three times with sodium carbonate buffer (5 mM, pH 8).

To coat the MPS shell onto the  $\text{SiO}_2@\text{PAH/PSS/TA}$  particles, the particles were washed and resuspended into Milli-Q water (pH 7). Hydrolysed MPS (45  $\mu\text{L}$ , 1.62 mM), TEA (1  $\mu\text{L}$ , 3.19 mM) and TA ( $8.18 \times 10^{-4}$  mM) were added and the solution left to stir for 30 min. The coated ( $\text{SiO}_2@\text{PAH/PSS/TA}@\text{MPS}$ ) particles were washed with Milli-Q. The MPS shell was coated at pH 6 so that the redox sensitive polymer was swollen. Particle concentration was measured using a haemocytometer. The samples were run in triplicate with the average taken and standard deviation calculated.

#### **EXAMPLE 8 – CHARACTERISATION OF CONTRAST AGENT**

To evaluate changes in contrast intensity under different redox conditions, agarose gel phantoms were prepared and imaged with a pre-clinical US. A custom

designed, US-neutral PDMS device was used as the sample holder. Gel phantoms were prepared containing the contrast agent  $\text{SiO}_2\text{@PAH/PSS/TA@MPS}$  particles. Briefly,  $\text{NaHCO}_3$  buffer (20 mM, pH 8) was degassed three times. 50 mL of the degassed buffer was heated to 90 °C with constant stirring. Once at 90 °C, agarose (2 mg) was added and dissolved in the  $\text{NaHCO}_3$  buffer. After the agarose dissolved, the agarose solution was incubated at 80 °C with stirring to make a buffered agar (2 %). The  $\text{SiO}_2\text{@PAH/PSS/TA@MPS}$  particles were washed with degassed  $\text{NaHCO}_3$  buffer (5 mM, pH 8). 10  $\mu\text{L}$  of the particles were pipetted into a microcentrifuge tube and 10  $\mu\text{L}$  of buffered agar was added. The resulting solution was gently mixed with care taken to avoid the incorporation of gas. The resulting solution was then pipetted into a tube mould to make a (1 %) agar suspension. After 10 min, the set agar suspension was pushed out into Eppendorf tube containing  $\text{NaHCO}_3$  buffer (20 mM, pH 8). Each sample was prepared in triplicate.

Prior to imaging, a bed of ultrasound gel was prepared, and the phantom removed from Eppendorf tube and placed on the bed. Agar gels containing particles were initially imaged after incubation with 1 mM solution of Fe(III) in  $\text{NaHCO}_3$  buffer (5 mM, pH 8). Next, the sample was retrieved and placed into 1mM Fe(II) in the same buffer, washed thoroughly and incubated for 16 hours. The sample was again washed with the same buffer and the sample imaged. The depth of the phantom was kept constant at 6 mm from transducer. The images were acquired using the 3D scan mode, the scan length was set at 10.526 mm with image capture at 0.038 mm recording 277 frames. All ultrasound images were acquired by FIJIFILM VisualSonics Vevo 2100 Ultrasound, using the MS-550D transducer at 40 MHz, 10 % dB power and 2D gain at 15. Images were analysed using ImageJ. Each sample was recorded in a .avi stack frames that contained no artefacts (air bubbles or mould reflections) and had the sample in vision where analysed. The average integrated densities were taken with ImageJ, with the average taken and BG and control BG subtracted.

Figure 11 shows the observed change in ultrasound backscatter (grey scale intensity, y-axis) caused by a change in redox potential. A significant increase in ultrasound backscatter intensity was observed when the  $\text{SiO}_2\text{@PAH/PSS/TA@MPS}$  particles were exposed to Fe (II) species in comparison to Fe (III) species.

Tannic acid has a large amount of phenol groups, which are redox-active. Without wishing to be bound by theory, it is thought that this increase in backscatter is due to the polymer layer expanding in the presence of Fe (III) while being constrained by the outer silica layer, leading to a lower density state. In contrast, it is thought that the polymer layer is in a more compact polymer confirmation in the presence of Fe (II) species. Mechanistically, it is thought that Fe(III) can coordinate with functional groups on the tannic acid and polymers, leading to the oxidation of tannic acid and production of HCl, reducing the local pH (Figure 12a). The reduction in pH is thought to lead to polymer expansion, resulting in the observed drop in US backscatter intensity. Furthermore, as the pH drops, more Fe(III) can enter the expanded polymer system, potentially increase the acidification rate. In contrast, while Fe(II) can also coordinate with tannic acid, it thought to coordinate to a lesser degree, and produces less HCl per coordinated Fe(II) (Figure 12b). Hence, it is thought the decrease in local pH is less, leaving the polymer layer contrasted against the core particle, resulting in a higher US backscatter relative to the Fe(III) state.

#### **EXAMPLE 9 – CONCENTRATION OF SILICA CORE**

Phantom agar moulds composed of different concentrations of TEOS silica cores were prepared using the same approach as described for Example 8. 800 nm TEOS cores were suspended in 1% agar, in concentrations ranging from 0-40% w/v. Ultrasound analysis on phantoms and data analysis was performed as described above for Example 8. Figure 13 shows the observed change in ultrasound intensity caused by increasing the concentrations of the TEOS silica cores in the phantom agar moulds from 0-40% w/v. A non-linear trend between ultrasound intensity and TEOS silica core particle concentration in the gel phantom was observed. This suggests that increasing particle concentration in the imaging field leads to increased intensity.

#### **EXAMPLE 10 – VARIATION OF CORE MATERIAL**

To investigate the effect the core particle composition has on ultrasound intensity, particles were prepared having a silica core (TEOS) and an organosilica core (3-mercaptopropyl trimethoxysilane). The organosilica particles were prepared as described in Miller et al, *Langmuir*, 2005, 21, 21, 9733–9740. Particles were prepared in

1% agar phantom moulds and imaged as described in Example 8. Figure 14 shows the observed ultrasound backscatter intensity for each core particle composition. No difference in ultrasound backscatter intensity was observed for TEOS silica cores and organosilica cores of the same size and concentration.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the above-described embodiments, without departing from the broad general scope of the present disclosure. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

The present disclosure may also provide any and all combinations of various steps, features, embodiments, examples, integers, particles, compositions and/or compounds as disclosed herein or indicated in the specification of this application individually or collectively.

**CLAIMS:**

1. A solid composite particle comprising  
a solid core;  
  
one or more polymeric layers encapsulating the solid core, wherein each polymeric layer comprises a cross-linked polymer containing a plurality of functional groups; and  
  
a porous outer shell encapsulating the one or more polymeric layers.
2. The solid composite particle of claim 1, wherein the solid composite particle is an ultrasound contrast agent and/or pH biosensor.
3. The solid composite particle of claim 1 or claim 2, wherein the solid composite particle has a diameter (in nm) between 100 to 10000.
4. The solid composite particle of claim 1 or claim 2, wherein the solid composite particle is a solid composite nanoparticle.
5. The solid composite particle of any one of claims 1 to 4, wherein the solid core is solid silica core comprising an inorganic silica, and optionally one or more compounds selected from an organosilane and organosilica.
6. The solid composite particle of any one of claims 1 to 5, wherein the solid core has a stiffness or Young's modulus that is less than that of the porous outer shell.
7. The solid composite particle of any one of claims 1 to 6, wherein the solid core has a diameter (in nm) between about 10 to 10000.
8. The solid composite particle of any one of claims 1 to 7, wherein the weight % of the solid core in the particle (as a total weight % of the particle) is between 1 to 30.
9. The solid composite particle of any one of claims 1 to 8, wherein the functional groups of the cross-linked polymer are capable of reactivity with a biomarker for reversible conversion of the cross-linked polymer between a first configuration and a second configuration effective for altering an ultrasound contrast property of the particle.
10. The solid composite particle of claim 9, wherein the biomarker is a hydrogen ion.

- 11 The solid composite particle of any one of claims 1 to 10, wherein the cross-linked polymer is a pH sensitive polymer.
12. The solid composite particle of claim 11, wherein the functional groups of the pH sensitive polymer are acid or base groups having a predetermined pKa or pKb.
- 13 The solid composite particle of claim 12, wherein the functional groups of the pH sensitive polymer are acid groups selected from one or more carboxylic acids.
14. The solid composite particle of any one of claims 1 to 13, wherein the cross-linked polymer is a pH sensitive polymer selected from the group consisting of polyacrylic acid, polymethacrylic acid, copolymers of acrylic acid and acrylates or methacrylates, and copolymers of methacrylic acid and acrylates or methacrylates.
15. The solid composite particle of any one of claims 1 to 14, wherein the cross-linked polymer is a pH sensitive polymer selected from a poly(methacrylic acid) or a copolymer thereof.
- 16 The solid composite particle of claim 12, wherein the functional groups of the pH sensitive polymer are base groups selected from amines.
- 17 The solid composite particle of any one of claims 1 to 11, wherein the functional groups of the cross-linked polymer are cationic groups or anionic groups or a combination of anionic and cationic groups.
18. The solid composite particle of any one of claims 1 to 11, wherein the one or more polymeric layers comprise alternate layers of a polycation and a polyanion.
19. The solid composite particle of claim 18, wherein the polyanion is poly(styrene sulfonate) and the polycation is poly(allylamine hydrochloride).
- 20 The solid composite particle of any one of claims 1 to 19, wherein the cross-linking in the cross-linked polymer is effective for preventing dissolution of the cross-linked polymer in a biological fluid on ionisation thereof.
- 21 The solid composite particle of any one of claims 1 to 20, wherein the cross-linking groups of the cross-linked polymer are selected from disulfides.

22. The solid composite particle of any one of claims 1 to 21, wherein the cross links are redox sensitive to effect changes in local pH.
23. The solid composite particle of any one of claims 1 to 21, wherein the cross links are formed by a phenol or a polyphenol.
24. The solid composite particle of any one of claims 1 to 21, wherein the phenol or polyphenol is selected from the group consisting of tannic acid, gallic acid, syringic acid, pyrogallol, ellagic acid, quercetin, methyl gallate, ethyl gallate, propyl gallate, gallic acid, pyrogallol, polyvinyl gallol, PEG-gallol, chitosan-gallol, gallotannin or galloocatechin.
25. The solid composite particle of claim 24, wherein the phenol or polyphenol is tannic acid.
26. The solid composite particle of any one of claims 1 to 25, wherein the cross-linking in the cross-linked polymer provides a ratio of free functional groups to covalently cross-linked functional groups of greater than about 2:1.
27. The solid composite particle of any one of claims 1 to 26, wherein the thickness of the one or more polymer layers (in nm) is between 1 to 25.
28. The solid composite particle of any one of claims 1 to 27, wherein the weight % of the combined one or more polymer layers in the particle (as a total weight % of the particle) is between 10 to 70.
29. The solid composite particle of any one of claims 1 to 28, wherein each of the polymeric layers consist of the same or different cross-linked polymer.
30. The solid composite particle of any one of claims 1 to 29, wherein the porous outer shell has a porosity effective for permitting transport of a hydrogen ion through the outer shell.
31. The solid composite particle of any one of claims 1 to 30, wherein the outer shell is an organosilica outer shell.
32. The solid composite particle of any one of claims 1 to 31, wherein the outer shell has stiffness or Young's modulus that is higher than that of the solid core or polymeric layers.

33. The solid composite particle of any one of claims 1 to 32, wherein the thickness of the outer shell (in nm) is between 1 to 50.
34. The solid composite particle of any one of claims 1 to 33, wherein the weight % of the outer shell in the particle (as total weight % of the particle) is between 0.1 to 5.
35. The solid composite particle of any one of claims 1 to 34, wherein the porous outer shell of the particle is surface functionalised with a biotargeting molecule.
36. A composition comprising a solid composite particle as defined in any one of claims 1 to 35 and an acceptable excipient.
37. The composition of claim 36, wherein the composition is an ultrasound imaging composition.
38. The composition of claim 36, wherein the composition is a pharmaceutical composition comprising a pharmaceutically acceptable excipient.
39. Use of a solid composite particle as defined in any one of claims 1 to 35 as an ultrasound contrast agent, in ultrasound imaging, as a pH sensor and/or as a redox sensor.
40. A method of enhancing the contrast in an ultrasound image generated for use in medical diagnosis by administering to a region of a subject a particle as defined in any one of claims 1 to 35 or composition as defined in any one of claims 36 to 38, and obtaining an ultrasound image of the region.
41. A process for preparing a composition comprising a plurality of solid composite particles comprising:
- contacting a plurality of individual solid cores with one or more polymers comprising functional groups to provide a plurality of individual solid cores each encapsulated by one or more polymeric layers;
  - cross-linking at least some of the one or more polymeric layers to form, on each of the solid cores, a cross-linked polymer containing a plurality of functional groups;
  - applying a porous coating to the one or more polymeric layers to form a porous outer shell encapsulating the one or more polymeric layers present on each of the solid cores to form the plurality of solid composite particles.

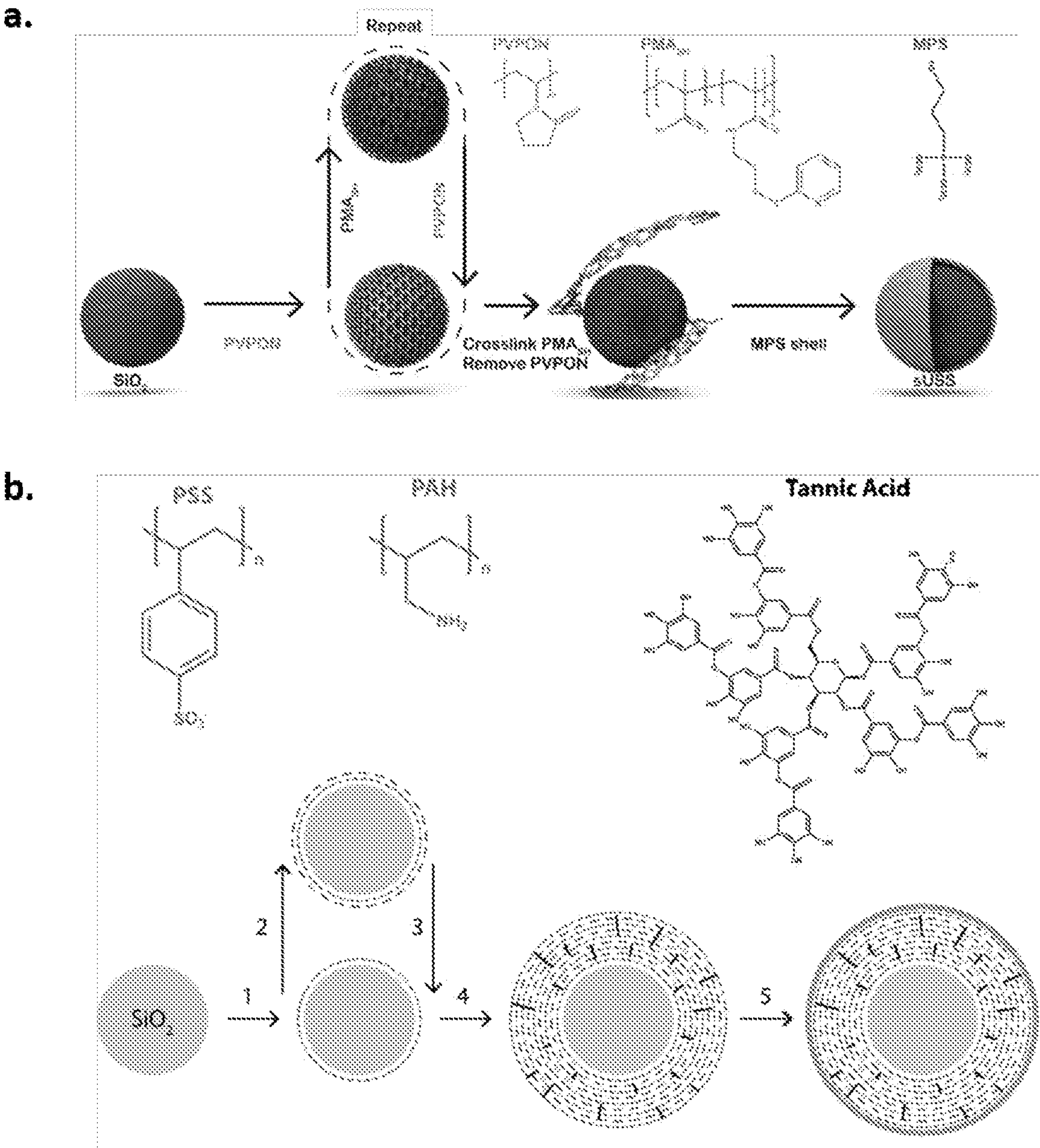


FIGURE 1

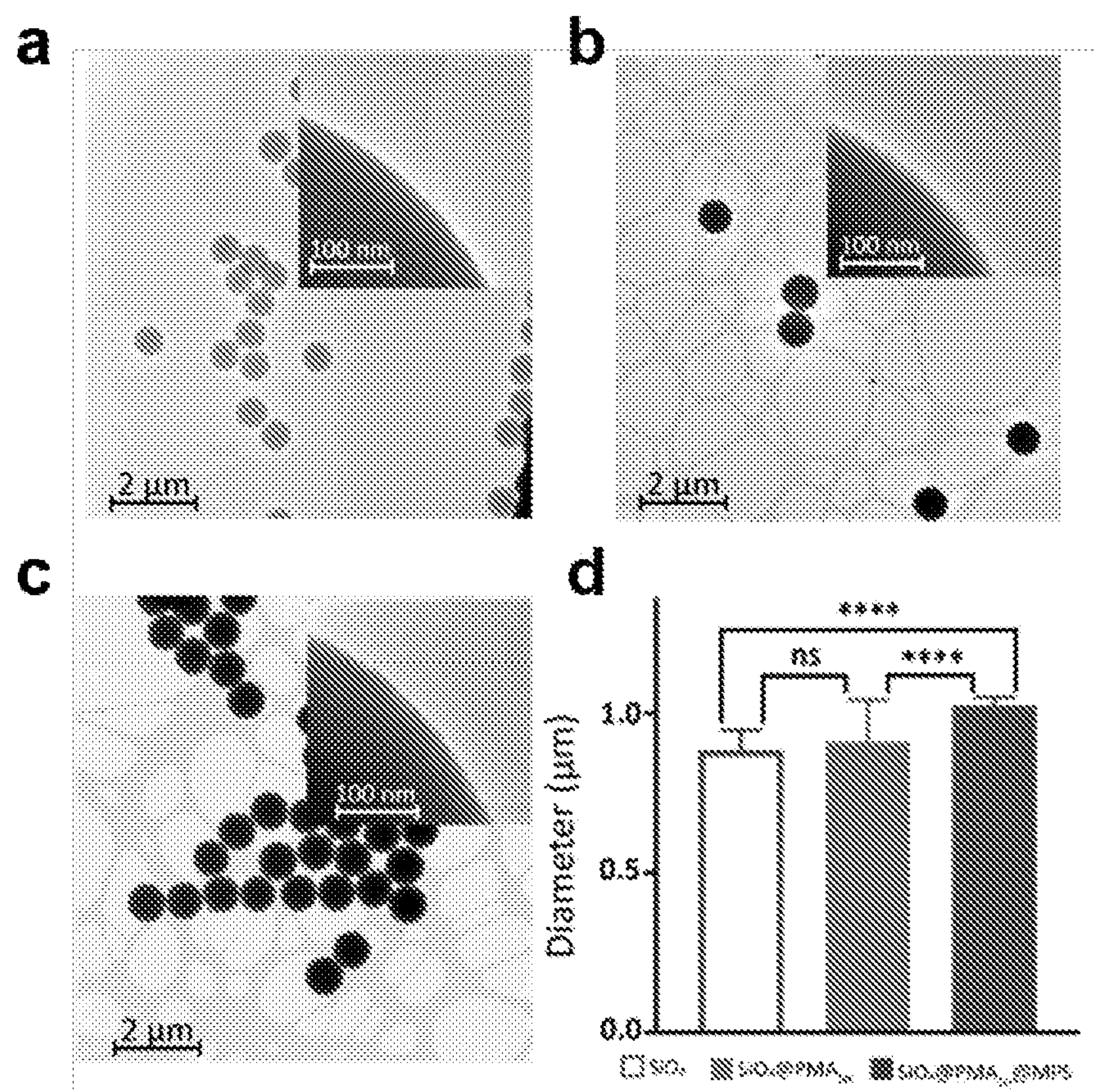


FIGURE 2

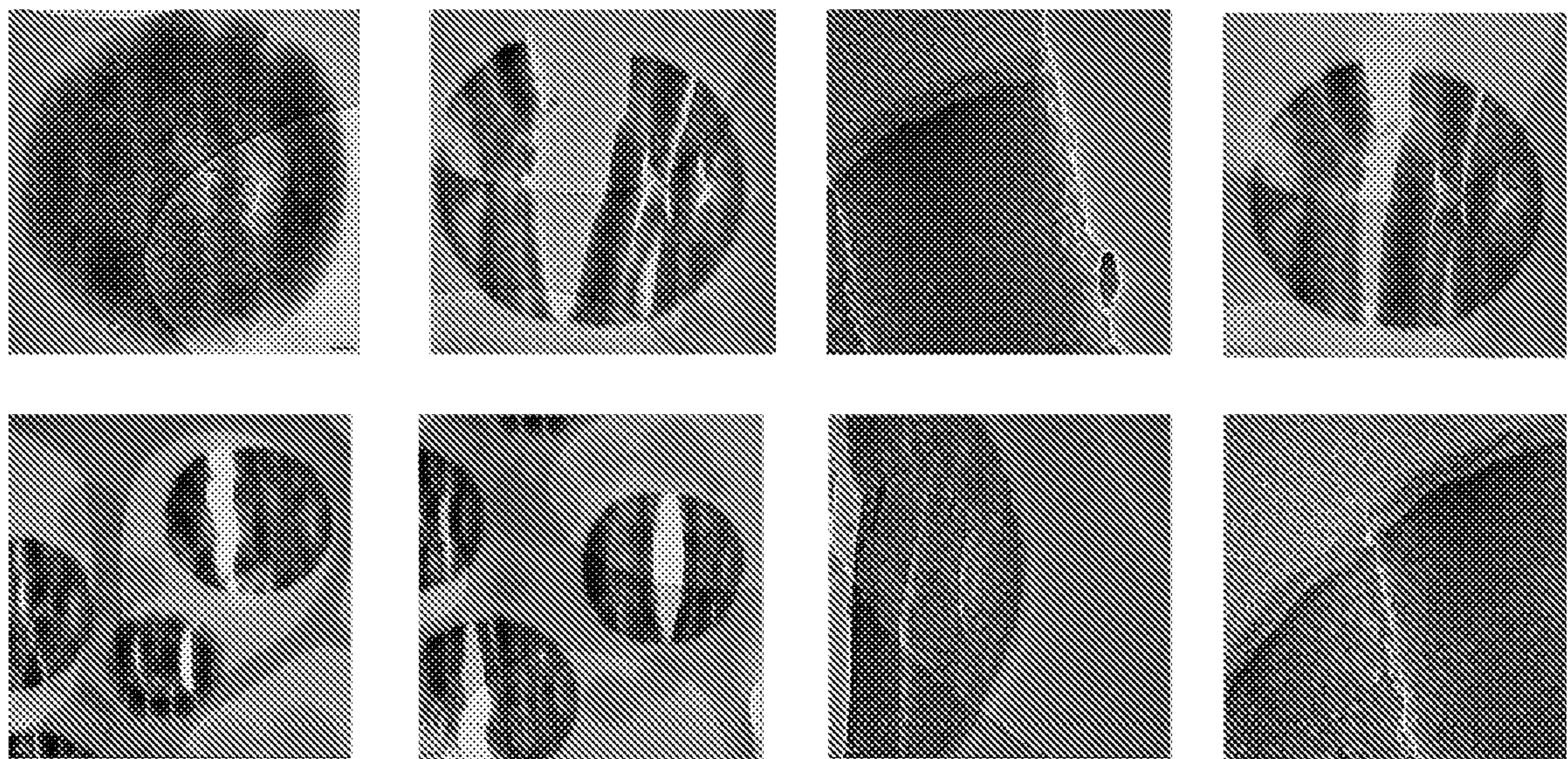


FIGURE 3

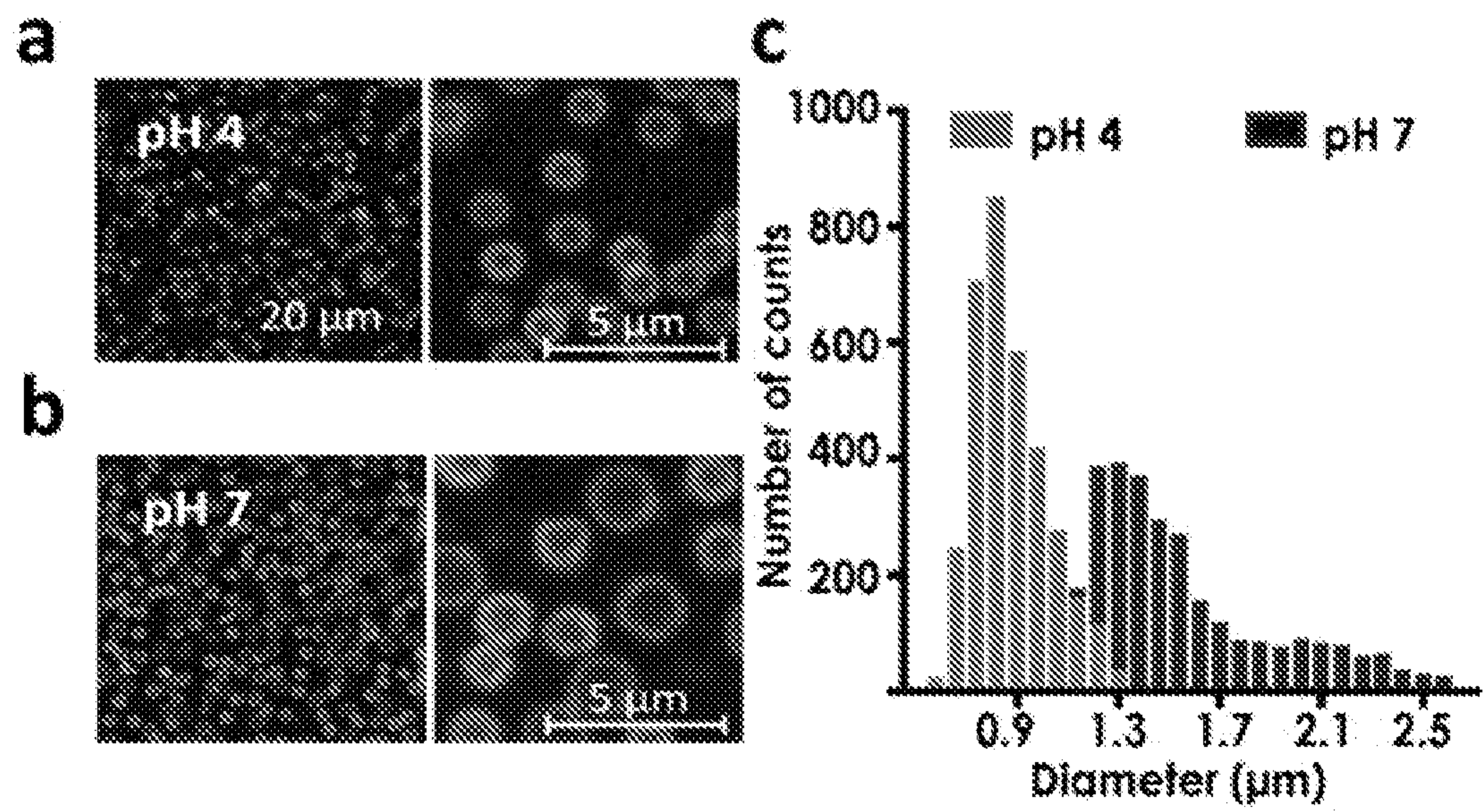


FIGURE 4

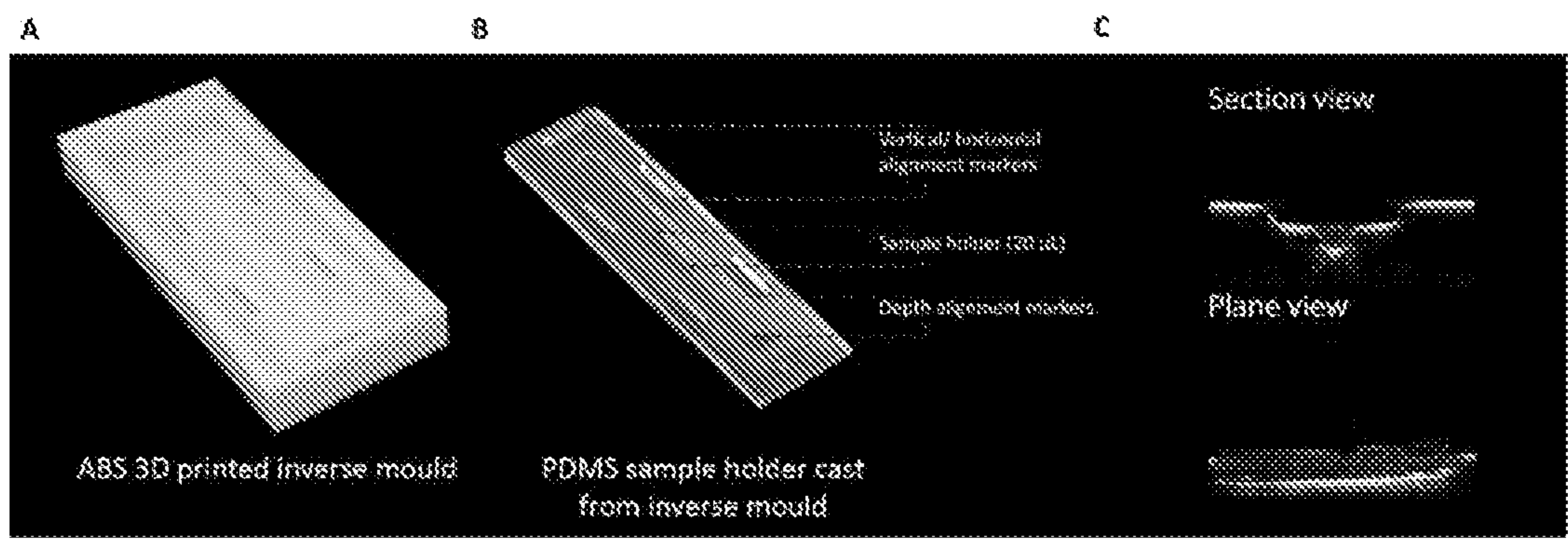


FIGURE 5

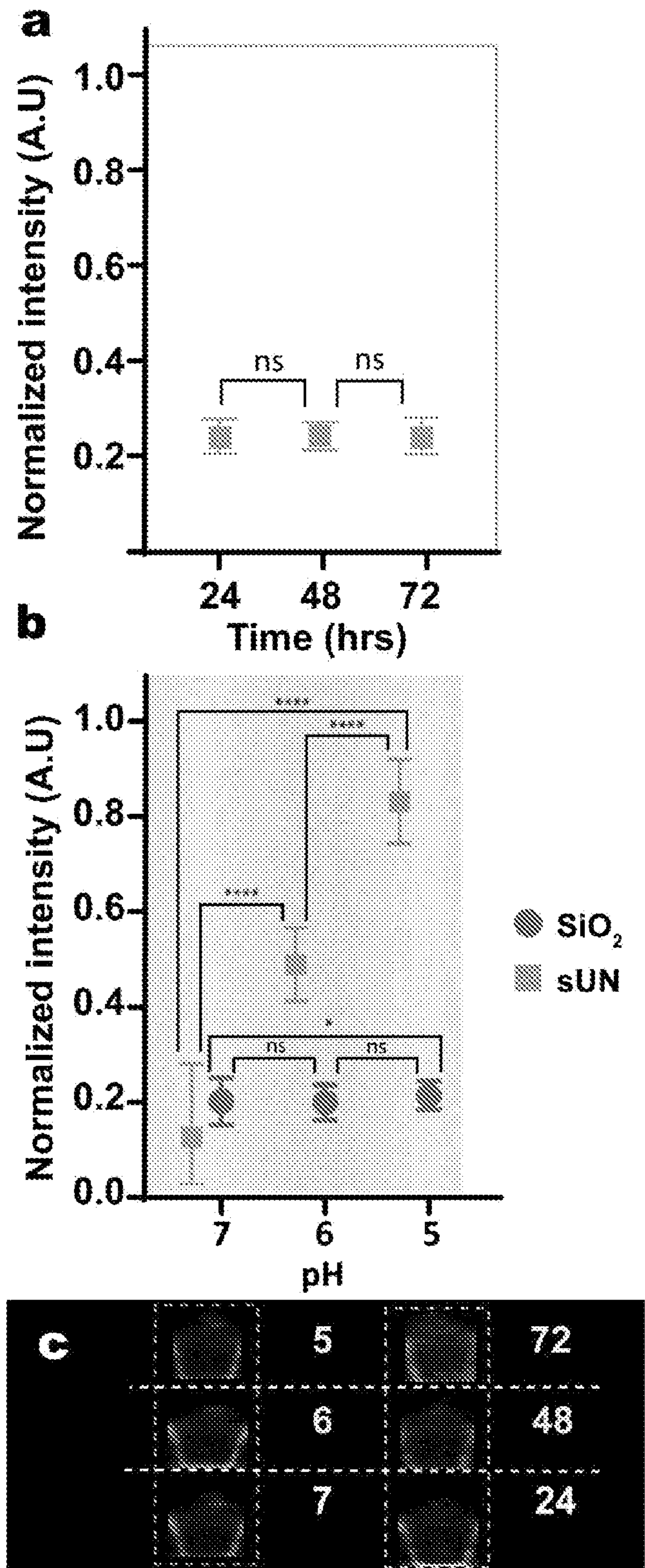


FIGURE 6

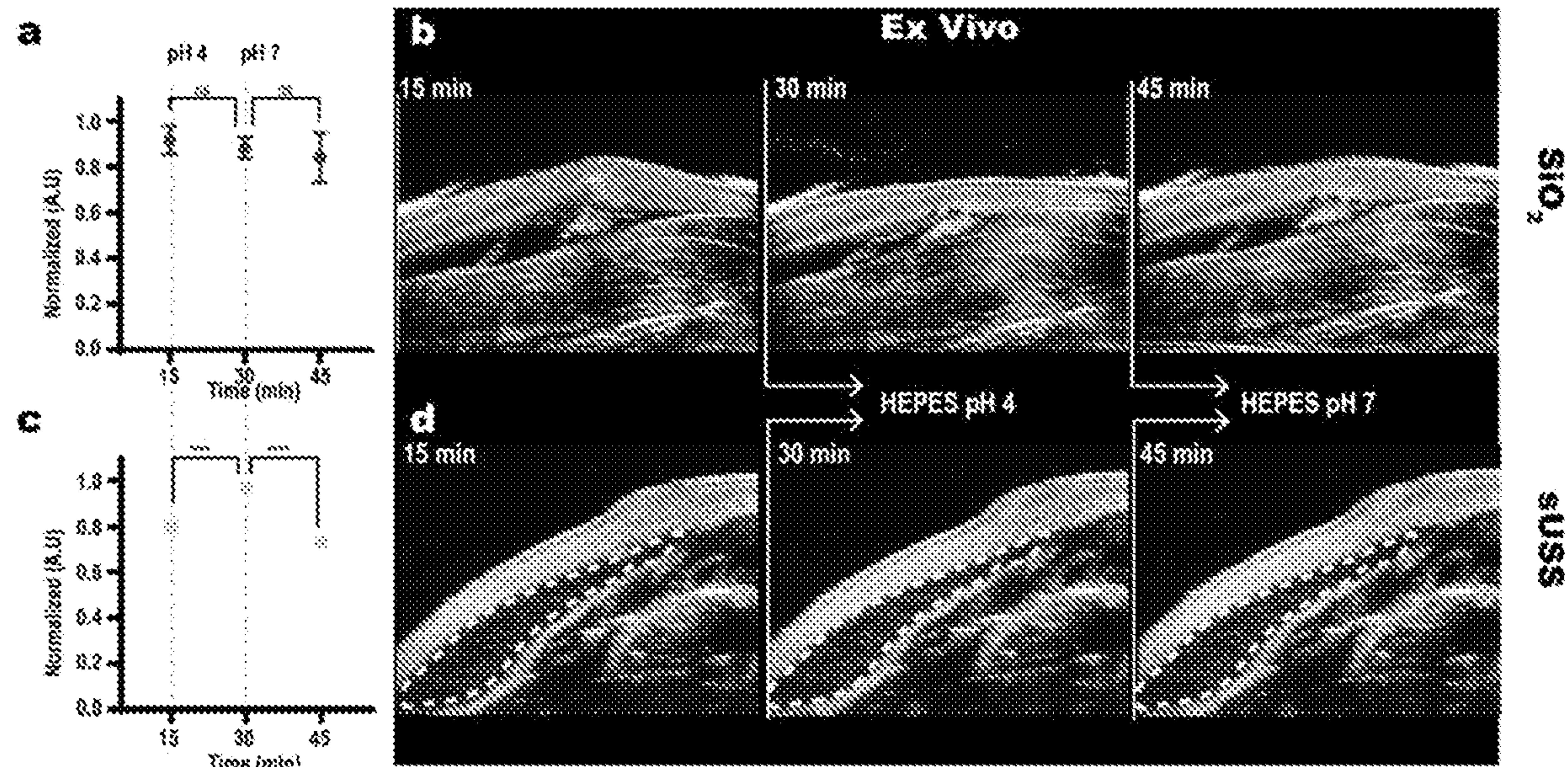


FIGURE 7

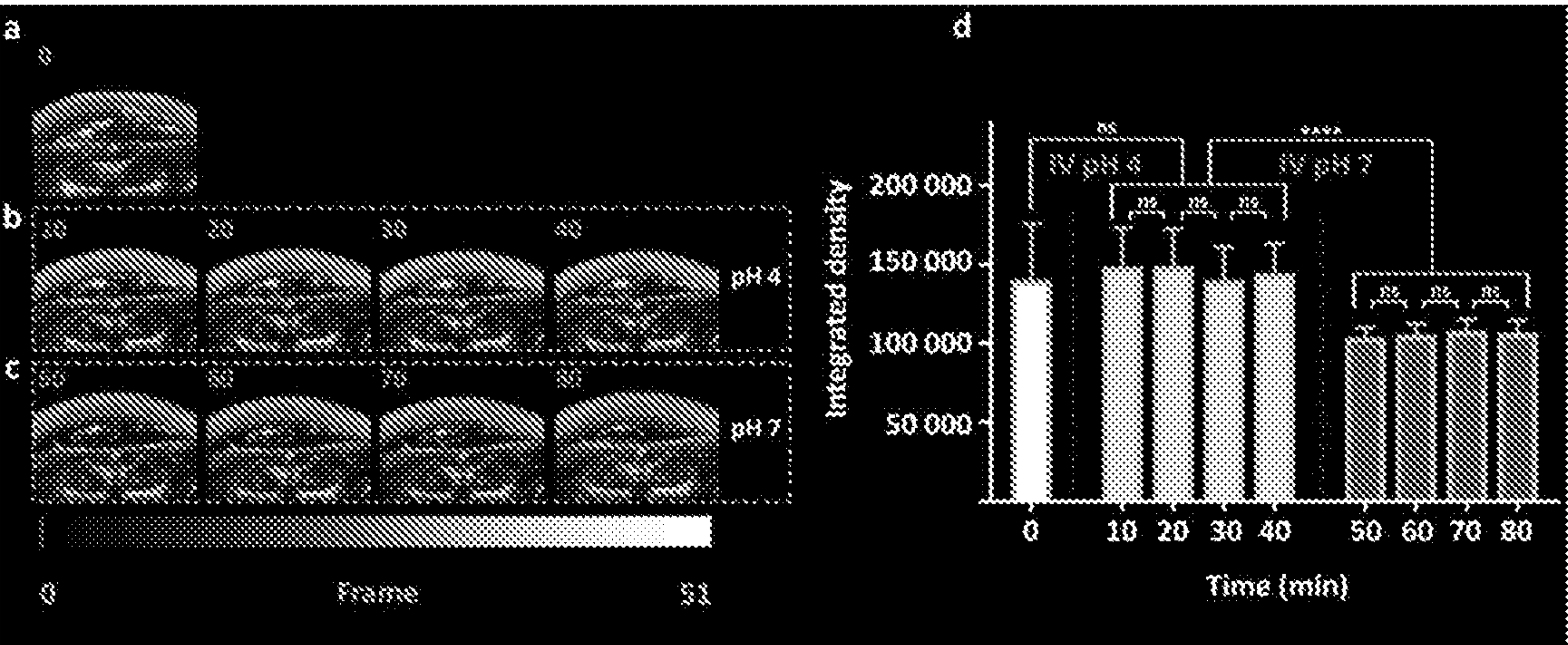


FIGURE 8

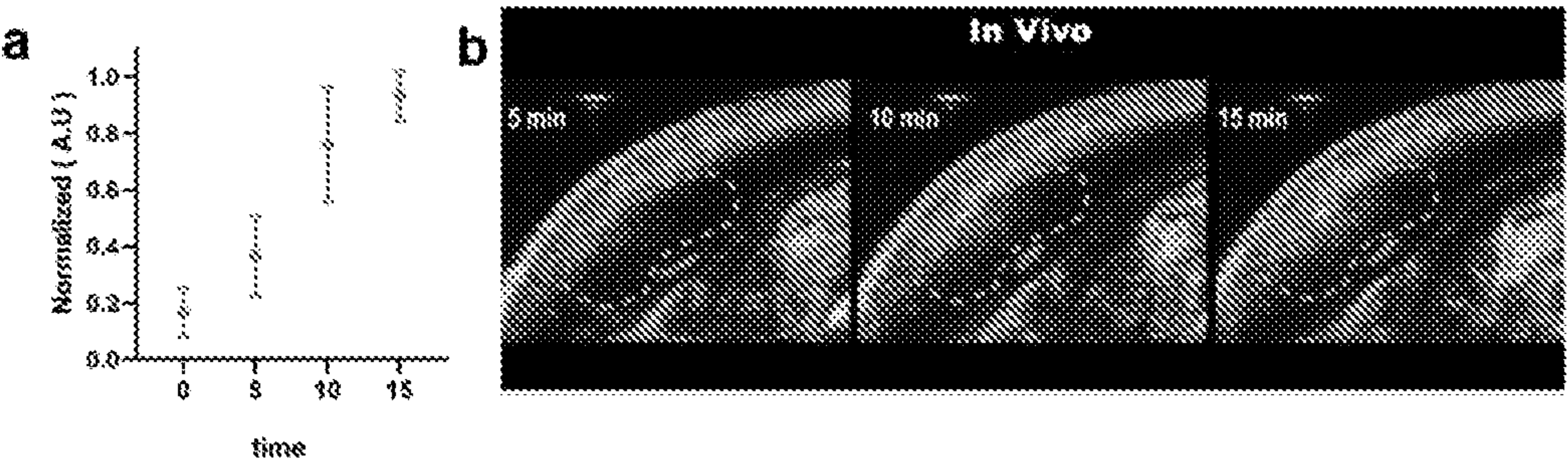


FIGURE 9

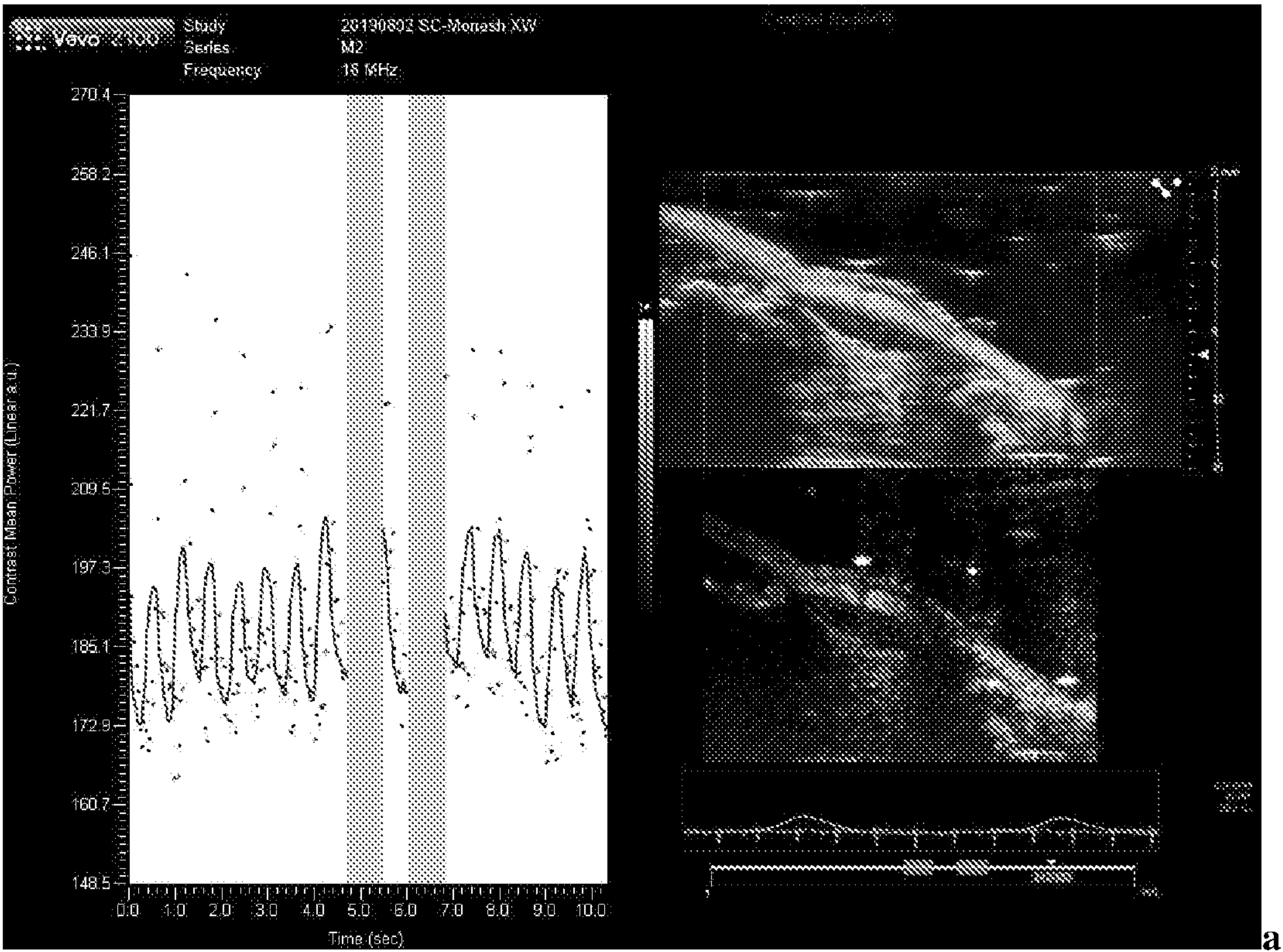


FIGURE 10

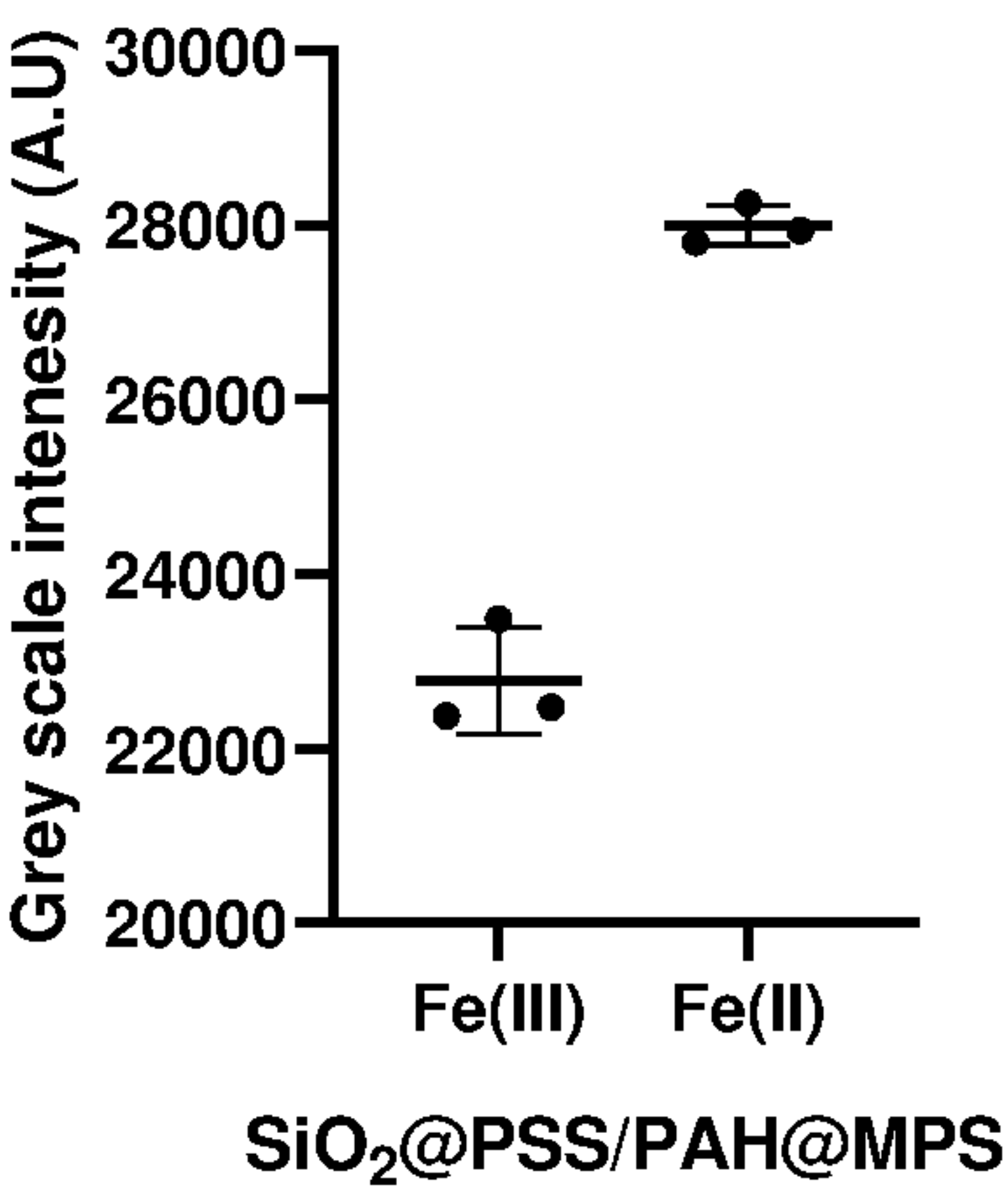


FIGURE 11

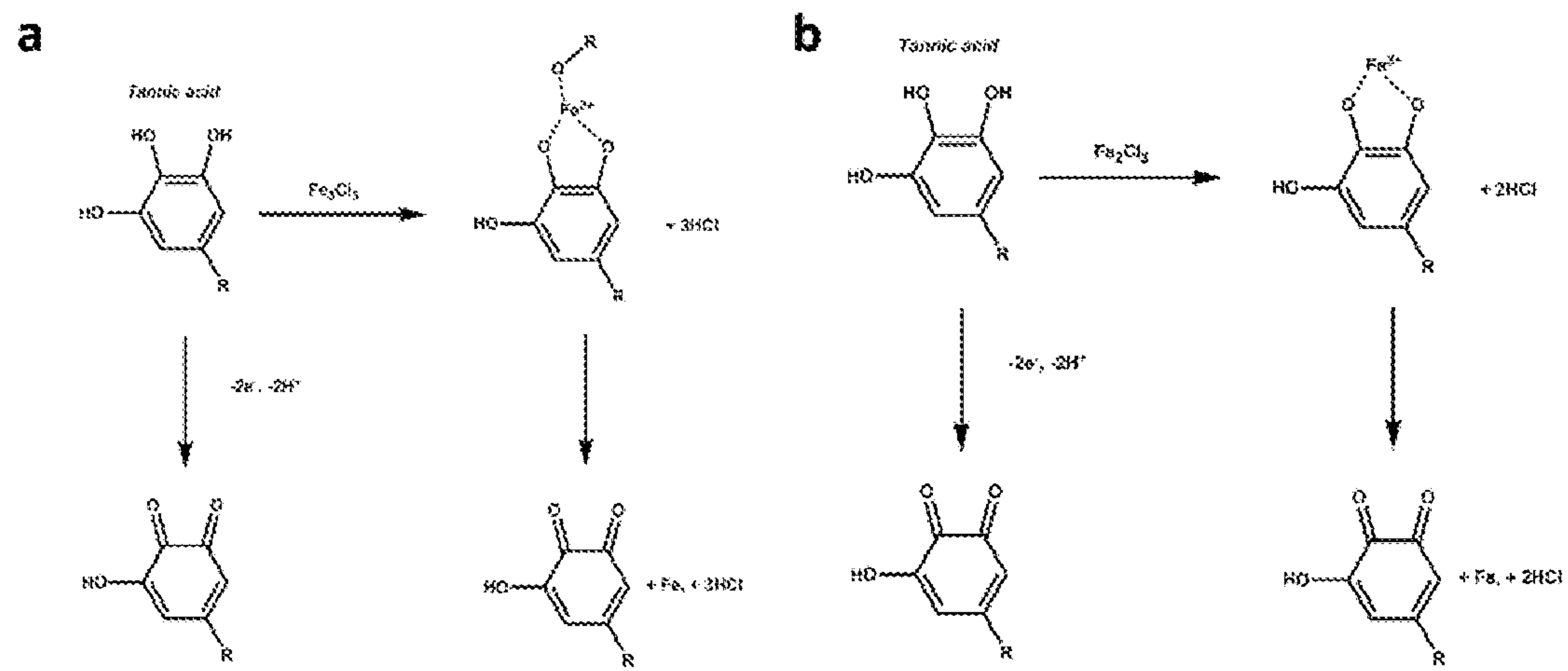


FIGURE 12

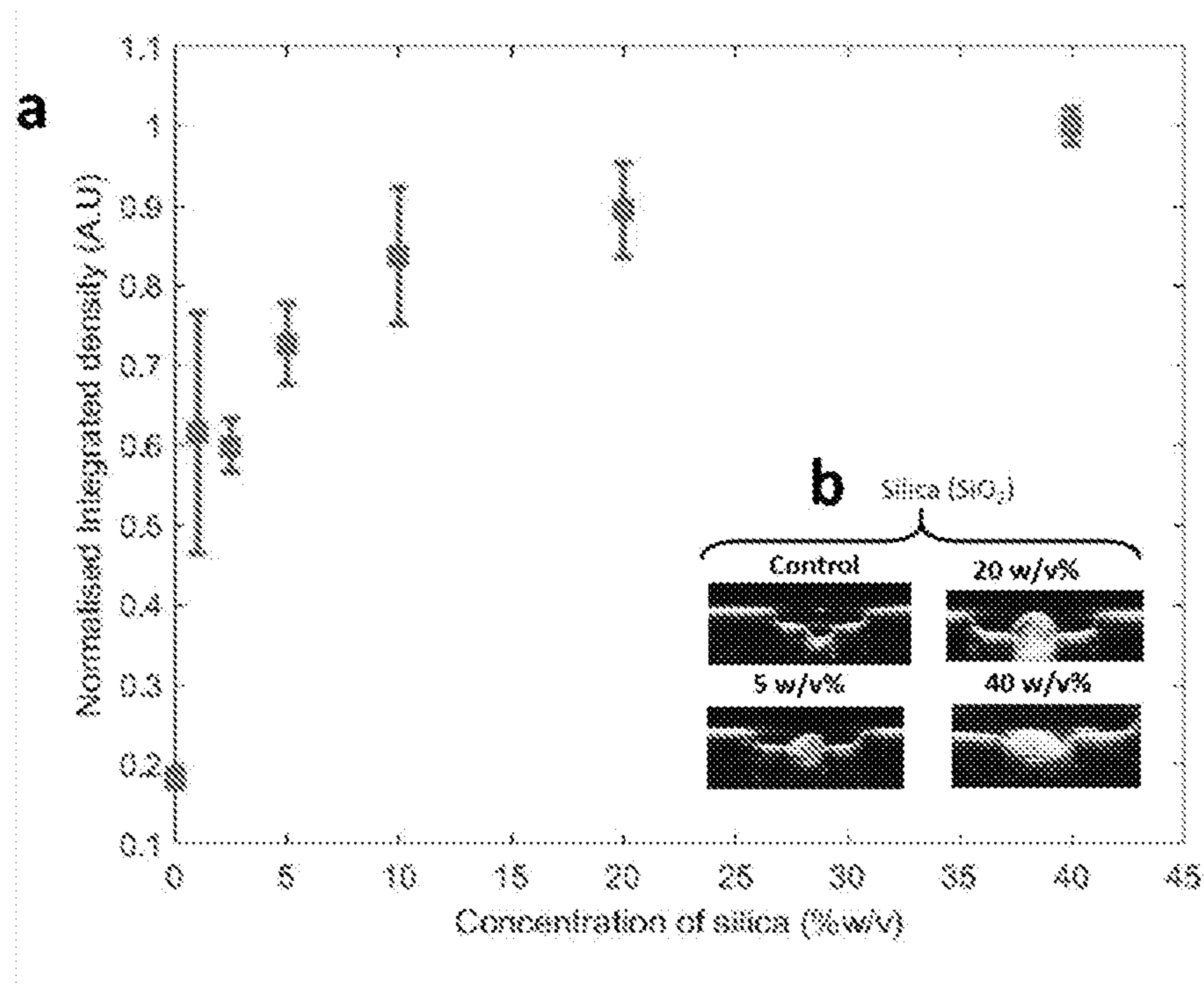


FIGURE 13

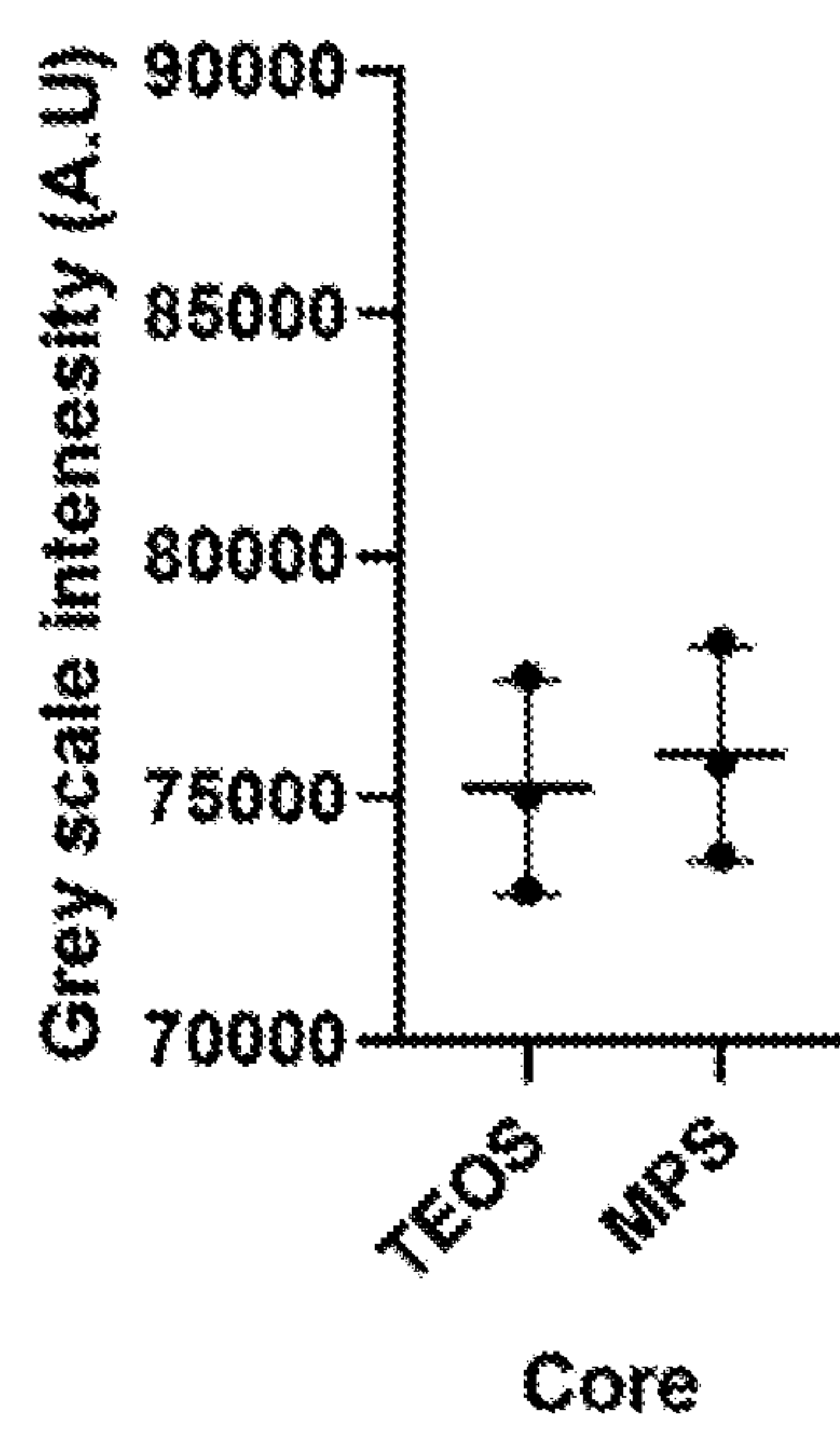


FIGURE 14

## INTERNATIONAL SEARCH REPORT

International application No.  
**PCT/AU2020/051137**

## A. CLASSIFICATION OF SUBJECT MATTER

**A61K 49/22 (2006.01) A61K 49/00 (2006.01) C01B 33/149 (2006.01) B82Y 30/00 (2011.01) C07F 7/02 (2006.01)**  
**A61K 47/69 (2017.01) C08L 33/10 (2006.01) C08L 39/06 (2006.01) C08L 83/02 (2006.01)**

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)

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

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

Database: CAPLUS, MEDLINE, PATENW, ESPACENET, ADISCTI, ADISINSIGHT, ADISNEWS, BIOSIS, BIOTECHNO, DDFB, DDFU, DGENE, DRUGB, EMBASE, ESBIOBASE, IFIALL, IPA, KOSMET, NAPRALERT, PCTGEN, PQSCITECH, SCISEARCH, TOXCENTER, USGENE, USPATFULL, USPATOLD, USPAT2, Google Scholar Keywords: radiographic contrast agents, nanoparticle, composite particle, core shell, silica solid, core, pH response, layer by layer, polymethacrylic, encapsulate, coat, cover, porous, inorganic, metal, superficially and similar. IPC/CPC: A61K49/22, C01B33/00, C07F7/02 and lower marks. Applicant/Inventor search in Espacenet & IPA internal database.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------|-----------------------|
|           | Documents are listed in the continuation of Box C                                  |                       |

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

|                                                                                                                                                                         |                                                                                                                                                                                                                                                  |
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| "P" document published prior to the international filing date but later than the priority date claimed                                                                  |                                                                                                                                                                                                                                                  |

Date of the actual completion of the international search  
8 December 2020Date of mailing of the international search report  
08 December 2020

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| INTERNATIONAL SEARCH REPORT                           |                                                                                                                                                                                                                                                                                                                                                                                                 | International application No. |
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| Information on patent family members                                                                                                                                                                                                                                  |                  | PCT/AU2020/051137             |                  |
| This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information. |                  |                               |                  |
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| End of Annex                                                                                                                                                                                                                                                          |                  |                               |                  |
| Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.                                                                                                                                             |                  |                               |                  |