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TITLE:

Preparation of Nanoparticles for ToF-SIMS and XPS Analysis

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SUMMARY:

A number of different procedures for preparing nanoparticles for surface analysis are presented (drop casting, spin coating, deposition from powders, and cryofixation). We discuss the challenges, opportunities, and possible applications of each method, particularly regarding the changes in the surface properties caused by the different preparation methods.

ABSTRACT:

Nanoparticles have gained increasing attention in recent years due to their potential and application in different fields including medicine, cosmetics, chemistry, and their potential to enable advanced materials. To effectively understand and regulate the physico-chemical properties and potential adverse effects of nanoparticles, validated measurement procedures for the various properties of nanoparticles need to be developed. While procedures for measuring nanoparticle size and size distribution are already established, standardized methods for analysis of their surface chemistry are not yet in place, although the influence of the surface chemistry on nanoparticle properties is undisputed. In particular, storage and preparation of nanoparticles for surface analysis strongly influences the analytical results from various methods, and in order

to obtain consistent results, sample preparation must be both optimized and standardized. In this contribution, we present, in detail, some standard procedures for preparing nanoparticles for surface analytics. In principle, nanoparticles can be deposited on a suitable substrate from suspension or as a powder. Silicon (Si) wafers are commonly used as substrate, however, their cleaning is critical to the process. For sample preparation from suspension, we will discuss drop-casting and spin-coating, where not only the cleanliness of the substrate and purity of the suspension but also its concentration play important roles for the success of the preparation methodology. For nanoparticles with sensitive ligand shells or coatings, deposition as powders is more suitable, although this method requires particular care in fixing the sample.

INTRODUCTION:

Nanomaterials are defined as materials having any external dimension between 1 nm and 100 nm or having an internal or surface structure on this scale[1]. Due to the unique properties arising from their small scale and correspondingly large surface area (among other factors), they find increasing use in a wide variety of fields including agriculture, chemistry, automotive construction, cosmetics, environment, medicine, printing, energy, and textiles. This increased use means that both humans and the environment will be exposed, on a hitherto unknown scale, to these materials whose toxicological properties are not yet fully known, and whose size enables their facile integration into biological or environmental systems[2].

After the fundamental properties of surface area and particle size/size distribution, surface chemistry and coatings were identified as the most crucial property of nanomaterials[3]; smaller particles have a higher surface area per unit mass, and therefore a higher ratio of surface to bulk atoms. Indeed, for nanoparticles of 1 nm size, over 70% of atoms can be found at corners or edges; this strongly influences surface properties such as chemisorption which is highly dependent on the atomic-scale surface morphology[4]. Regulations dealing with nanomaterials require accurate data regarding physicochemical properties and reliable estimates of the toxicological properties of these materials. In order to efficiently estimate toxicological properties from physical and chemical properties of nanomaterials, the nanomaterials community requires reliable, standardized, and verified analytical procedures. Projects such as ACEnano[5] aim to collect and correlate accurate and verifiable physical data from nanoparticles in a framework allowing better regulation and characterization of nanomaterials. This drive towards standardized analytical procedures has also been supported by the editors of ACS Nano, wishing "to consolidate and to agree on methods of characterization and minimum levels of analysis of materials[6]". Furthermore, XPS and ToF-SIMS offers new possibilities for elucidating the particle architecture of core-shell nanoparticles[7,8].

X-ray photoelectron spectroscopy (XPS) and Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS), compared in **Table 1**, are well-established methods for investigation of surface atoms. In XPS, the sample is irradiated with x-rays having an energy between 1 and 2 keV, causing emission of electrons due to the photoelectric effect. These emitted electrons, having a kinetic energy in the same range, correlate to the binding energy of the electrons in the solid; the appearance of photoelectrons at these defined binding energies and measurable intensities therefore allows quantitative analysis of the composition. Since the mean free pathway of these

photoelectrons is below 10 nm, XPS is a highly surface sensitive technique for quantitative analysis. Furthermore, detailed analysis of the binding energies in highly resolved spectra enables the quantitative determination of the valence states of these electrons.

In ToF-SIMS the surface is sputtered with a focused ion beam (primary ions), with the ions ejected from the material (secondary ions) collected and analyzed in a time-of-flight mass spectrometer. The mass/charge pattern obtained allows the determination of the elemental, isotopic, or molecular composition. Due to the mean free pathway of the secondary ions, this technique is also highly surface sensitive and has an information depth of 1–2 nm but is at best semi-quantitative, due to the matrix effect whereby the ionization probability (and therefore yield) of secondary ions is strongly influenced by their surrounding matrix. ToF-SIMS can be operated in either static or dynamic mode; the difference between the two is the primary ion flux impacting the surface. Static SIMS keeps the primary ion flux to a level that impacts (i.e., fragments) a maximum of 1%-10% of the surface; the surface remains relatively undisturbed, which allows analysis of the top atomic layers of material. Since even static SIMS causes some destruction to the surface, it is considered to be less "non-destructive" of the two methods.

These surface-sensitive techniques allow analysis of the first few nanometers of the material, including intentional or unintentional coatings, which, for nanomaterials, can significantly influence material properties. Examples of intentional coatings are capping layers on quantum dots to improve photoluminescence quantum yields and reduce environmental reactivity[9], alumina or silica coatings for the prevention of photocatalytic activity of titania nanoparticles in sun blockers[10], surface functionalization to enable bioconjugation and subsequent biological activity[11], coatings for diagnostic and drug delivery applications[12], and fluorocarbon coatings on magnetic particles for ferrofluids and core-shell metallic systems to enhance catalyst properties[13]. Unintentional coatings, such as oxidation, surface contamination, or protein coronas in biological systems have a similarly strong influence on nanoparticle properties and it is crucial that experimental preparation procedures ensure that the coating and more generally the surface chemistry of the nanomaterial is not destroyed or transformed. It is also crucial to evaluate the properties of the nanoparticles as they are in-situ, as their properties can be drastically altered by the change[2,14,15]. In addition, the concentration of stabilizers in the nanoparticle suspension can dramatically influence the analysis and structural integrity of the nanoparticles; the presence of a stabilizer can result in large unwanted signals (for example, C, H, O, and Na) in the analysis, while its removal can result in damage or agglomeration of the nanoparticles.

Due to their size and surface area, the storage conditions of nanoparticles also affect their behavior, both as stored powders/suspensions and as prepared samples. The effect of sub-optimal storage conditions, particularly room-temperature storage and exposure to light, have been shown in various studies to cause degradation of the nanoparticles which has been shown to alter the particles' physical, chemical, and/or toxicological properties[14–18]. Smaller nanoparticles have been shown to oxidize more rapidly than larger ones with oxidation/degradation rates dependent on storage conditions[15] as well as surface chemistry[14]. The effects of the nanoparticle degradation during storage have been shown to significantly

affect physicochemical properties including toxicity[14], while the oxidative growth can proceed inwards at the expense of the core[15].

The careful storage and preparation of nanomaterials is therefore essential for an accurate surface analysis, and any factors which could influence the sample surface and/or the quality of the measurements should be carefully considered. It should be noted that due to the relatively low spatial resolution of XPS (in the µm range) and ToF-SIMS (a few hundred nm), only a small subset of the nanoparticles can be investigated; these methods average over an area and do not have the ability to image single particles as is possible with techniques such as electron microscopy. For this reason, any analysis requires deposition of the nanoparticles in a continuous layer to ensure no interference from the substrate. Electron microscopy and XPS/ToF-SIMS are therefore often used together as complementary methods for nanomaterial analysis.

Aside from changes in surface chemistry, the main challenges for preparation of nanoparticle samples for XPS and ToF-SIMS analysis are to prepare a layer that is: homogeneous, to increase reproducibility; gapless, to minimize the contribution of the substrate to the spectra; thin enough to avoid charging effects (for non-conductive samples); and securely fixed to the substrate, to avoid free nanoparticles entering and damaging ultrahigh vacuum instruments

Nanoparticles can be deposited onto the substrate from suspension or as a powder. Firstly, we will discuss the different methods for depositing nanoparticles from suspension. Silicon wafers are a commonly used substrate for suspension deposition, because they are relatively cheap, readily available as a highly pure product consisting of pure or doped silicon (doping avoids charging effects), and for most nanoparticles the spectral peaks do not overlap with peaks typical for nanoparticles. This last point is important; before analysis it should be ensured that the substrate peaks are well separated from the peaks expected from the nanoparticles, otherwise interpretation of the spectra is complicated or impossible and the continuous coverage of the substrate by the nanoparticles cannot be verified. Before using silicon wafers, an extensive cleaning procedure (described in this publication) is necessary to remove (organic) contaminants and to increase the surface wettability. Other suitable substrates such as gold films, highly ordered pyrolytic graphite (HOPG), or indium foils have been successfully used, but a discussion about their preparation is beyond the scope of this work[19–22].

Secondly, we present methods for depositing nanoparticle powders on a substrate for XPS and ToF-SIMS analysis and present the advantages and disadvantages of each method, allowing researchers new to the techniques to find the optimal preparation method for their purposes. Thirdly, we discuss cryofixation, which is a suitable preparation method to conserve features such as the agglomeration behavior, organic corona, solid/aqueous interface[23,24] or distribution in biological media[25] of NPs. Cryofixation, typically fast freezing of material in a liquid nitrogen–cooled cryogen and analysis in the frozen-hydrated state, allows the analysis and visualization of nanoparticles directly in complex matrices. This procedure does not cause ice crystal formation but forms amorphous ice that keeps membranes and cellular and tissue structures in their native biological state, avoiding damage caused by water crystallization processes and enabling the exact chemical distribution of all cell metabolites and cell membrane compounds to be

maintained[26–28]. This preparation method may be of particular interest for presenting an exact chemical map of the actual NP agglomerate or heteroagglomerate, visualizing the exact chemical space in close proximity to the nanoparticle directly in suspension, or correlating either cell tissue-specific features or intra-cellular compartments within NP agglomerates or heteroagglomerates.

As shown through the results presented in this work, the most suitable procedure in a particular case is dependent on a variety of parameters such as the nanoparticles' hydrophilicity, stability, conductivity, state (e.g., powder or suspension) and the analytical question at hand (e.g., size, bulk properties, or surface coatings). A variety of methods are presented here that can be used for preparation of NPs for surface analysis, as well as a comparison of their advantages and disadvantages.

PROTOCOL:

CAUTION: The toxicological properties of nanoparticles are still under investigation; due to their size they can present unique hazards in humans as well as in the environment even when they consist of intrinsically non-hazardous materials. Before undertaking any work with nanoparticles, a proper risk assessment should be completed, and appropriate engineering controls, lab procedures, and PPE (personal protective equipment) put in place, depending on the hazard level of the materials to be studied[29–32].

1. Preparation of Si wafers

NOTE: These steps are necessary to remove undesired (organic) contamination and increase the surface wettability. All solvents used should be at least ACS grade. A standard sonication bath (35 kH and 120 Watts) is suitable.

1.1 Wet chemical cleaning of Si wafers

1.1.1 Put the Si wafer in a beaker with isopropanol and ultrasonicate for 5 min.

1.1.2 Transfer the Si wafer to a beaker with an alkali glass cleaning solution and ultrasonicate for 10 min.

1.1.3 Put the wafer in a beaker with ultrapure water. Change the water 10 times by pouring out the water and refilling the beaker; the Si wafers will remain at the bottom due to the capillary effect.

1.1.4 Dry the wafer with clean N_2 gas.

NOTE: Drying with N_2 prevents the formation of "coffee rings" and other artefacts from water drying.

1.1.5 Put the wafer in a second beaker with isopropanol and ultrasonicate for 10 min.

1.1.6 Dry the wafer with clean N_2 gas.

1.1.7 Put the wafer in a beaker with ethanol and ultrasonicate for 10 min.

1.1.8 Dry the wafer with clean N_2 gas. The protocol can be paused here.

1.2 Plasma or UV/ozone cleaning of silicon wafers

1.2.1 Introduce the Si wafer in the plasma or the UV/ozone cleaner and switch on for 30 min.

NOTE: Wafers should be plasma- or UV/ozone-cleaned immediately before use.

2. Nanoparticle deposition from suspension

NOTE: The most common exposure route for nanoparticles is by inhalation. Working with suspensions can minimize exposure hazards.

2.1 Preparation of nanoparticle suspension from powder

NOTE: All quantities described here are examples. The method should be optimized for the particular nanoparticles used in each case.

2.1.1 Accurately weigh 15 mg of nanoparticle powder (± 10%) into a 10 mL tube.

2.1.2 Accurately weigh in approximately 8 mL ultrapure water.

2.1.3 Close the tube, pack in a 50 mL centrifuge tube with paper towel and place in the vortexer at 3,000 rpm for 15 min.

2.2 Drop-casting of electrically conductive nanoparticles from aqueous suspension

2.2.1 Place the wafer in the UV/ozone cleaner for 30 min.

2.2.2 Place the wafer in one half of the wafer holder and place a 3 µL drop of nanoparticle suspension in the center of the ring.

2.2.3 Mount a 6.07 mm diameter Viton O-ring on the wafer around the droplet. Take care that the ring does not touch the droplet.

2.2.4 Place the wafer in a vacuum desiccator under a vacuum of 4 mbar for 15 min to dry the wafer.

2.2.5 Remove the wafer from the desiccator and examine using light microscopy and XPS to determine that the particle layer is homogeneous and closed. Repeat steps 2.2.1 and 2.2.2 until analysis shows a closed and homogeneous layer. The protocol can be paused here.

2.3 Spin-coating of electrically non-conductive nanoparticles from aqueous suspension

2.3.1 Place the wafer in the UV/ozone cleaner for 30 min.

NOTE: By spin-coating suspensions of different concentrations using the same protocol, different levels of surface coverage can be achieved.

2.3.2 Program the spin-coater. A suitable sample program is: step 1: 500 rpm/s ramp to 1,000 rpm (5 s); step 2: 1,000 rpm/s ramp to 2,000 rpm (3 min); step 3: deceleration at 2,000 rpm/s to 0 rpm.

2.3.3 Insert the wafer into the spin-coater and switch on the vacuum for fixation.

2.3.4 Deposit 80 µL of the suspension on the wafer and start the program.

2.3.5 Remove the wafer from the spin-coater.

2.3.6 Store the sample in a new, clean wafertray. The protocol can be paused here.

2.3.7 Analyze the sample using SEM to confirm gapless coverage of the substrate.

3. Nanoparticle deposition from powder

3.1 Nanoparticle deposition on double-sided adhesive tapes (“stick and go”)

3.1.1 Fix the double-sided adhesive to the sample holder and remove the liner.

3.1.2 Take a spatula-tip of the nanoparticle powder and dip it onto the adhesive.

3.1.3 Spread the sample over the adhesive and press into the adhesive with the spatula, until as much of the powder is adhered as possible.

3.1.4 Check that the powder is fixed on the tap by inverting and tapping the sample holder, and by blowing a stream of gas (e.g., nitrogen) across it. The protocol can be paused here.

NOTE: Alternatively, a small amount of powder can be placed on a cleaned surface (Alu foil or glass slide) and pressed from above with the adhesive and double-sided sample holder.

3.1.5 Place a spatula-tip of the powder onto the cleaned surface. Press the sample holder with the adhesive onto the powder from above.

3.1.6 Check that the powder is fixed on the tap by inverting and tapping the sample holder, and by blowing a stream of gas (e.g., nitrogen) across it. The protocol can be paused here.

3.2 Preparation of pressed powder pellets

3.2.1 Thoroughly clean all parts of the pellet die, taking care not to scratch the polished surface.

3.2.2 Invert the pellet die and rest on a small spacer.

3.2.3 Insert the plunger and one stainless steel pellet, with polished side up, and pull the plunger through until there is enough space to fill with the powdered sample.

3.2.4 Fill the die with a small amount of sample (1 large spatula tip), and then insert the second stainless steel pellet with the polished side facing the sample.

3.2.5 Place the base onto the body and carefully invert. If a vacuum is desired and available, attach the vacuum pump to the base of the pellet die.

3.2.6 Place the die into a press, making sure it is centered.

3.2.7 Apply a light load (2 kN) for approximately 20 s and release.

3.2.8 Apply a heavier load (6 kN) for 2 min and release.

3.2.9 After the load is released, release the vacuum pump.

NOTE: Due to the different material properties of various nanoparticles, it may be advantageous to prepare a series of pellets with different loads and load times to determine the optimum pellet pressing conditions.

3.2.10 Invert the die, place the extractor ring into position, and place a light load (up to 1 kN) between the plunger and the extractor ring.

3.2.11 Remove the die parts from the press and carefully extract the sample pellet with tweezers.

3.2.12 Gently mount the sample on a cleaned Si wafer using double-sided adhesive. The protocol can be paused here.

4. Cryofixation of nanoparticle suspensions

4.1.1 Fill the main chamber of the fast-freeze device with liquid nitrogen.

4.1.2 Fill the cooled fast-freeze chamber with the cryogen (propane).

4.1.3 Allow the fast-freeze device to cool to its operating temperature.

NOTE: The fast-freeze device requires some time to reach operating temperature prior to the sample preparation, therefore a reasonable timeframe (a few hours) is required for cryofixing the samples.

4.1.4 Drop-cast 10–20 µL of NP suspension onto a cleaned Si wafer with a pipette.

4.1.5 Holding the Si wafer with fixing tweezers, place it inside the plunge freeze device.

4.1.6 Move the fixing tweezers to the plunge position.

4.1.7 Press the button to drop the sample inside the cryogen.

4.1.8 Wait several seconds until the sample is completely frozen.

4.1.9 Transfer the frozen samples as fast as possible into a cooled environment.

4.1.10 Place the cryofixed sample (Si wafer) into the sample holder and transfer it inside the instrument.

NOTE: For transport, dry ice is recommended and short-term sample storage is possible. The samples can be measured in a frozen state with a cooled instrument or with conventional ToF-SIMS settings after stabilization by freeze-drying the sample.

REPRESENTATIVE RESULTS:

This paper presents a variety of sample preparation methods for surface analysis of nanoparticles. Since the physicochemical properties of a specific NP will define both the optimal method for sample preparation (e.g., drop-casting vs. spin coating) and the best procedure for that method (for example, requiring different substrates or solvents), the suitability of the method used should be validated via alternative analytical methods and optimized if necessary. The results seen in this publication are consistent with previously published literature in showing the need for consistent protocols and procedures for sample preparation as well as the need for quality checks to ensure that the sample preparation and purification methods are appropriate, successful, and do not damage the nanoparticles[22,33–36].

Sampling and storage methods for NPs have not been addressed here, as they are described in detail in various other references[14-18,34,37–39]. Naturally, great care should be taken that the samples analyzed are representative of the overall nanoparticle distribution and suitable sampling methods developed and validated. Storage conditions have also been shown to strongly affect nanoparticle properties over a period of months and should therefore be carefully considered. As an example, we recommend that nanoparticles should be stored in small amounts

in sealed containers away from light, ideally below 4 °C. It is also crucial that storage, sampling and sample preparation is consistently performed according to validated procedures as well as is being documented in detail. This documentation should include the metadata from the NPs themselves, such as provenance information and storage conditions[40]. Tools such as electronic lab notebooks (ELNs) may be useful for consistent documentation of procedures and NP metadata, as well as enabling the production of data according to the FAIR principle (Findable, Accessible, Interoperable, and Reusable).

Accurate and correct surface analysis of NPs firstly requires a suitable choice of substrate. We have used cleaned Si wafers as substrates because they are readily available, durable, easily cleaned, conducting and sufficiently flat, however depending on the goals of the analysis the oxide surface layer can be a drawback, as the adventitious hydrocarbons on the substrate cannot be differentiated from those on the nanoparticles. When necessary, other materials such gold or polymeric coatings on Si wafers, Si_3N_4 wafers, or HOPG (highly oriented pyrolytic graphite) can be used[19–22]. The first step in sample preparation described in this paper is cleaning the Si wafer, shown as a schematic in **Figure 1**. The efficacy of the cleaning process can be verified by a variety of methods including XPS, as shown in **Figure 2**. The main contaminant (adventitious carbon) is typical for samples stored in air and is significantly reduced after the cleaning process. Additionally, hydroxylating the wafer surface via UV or ozone treatment avoids the coffee-ring effect from deposition from aqueous suspension by enhancing wettability and leading, therefore, to a more homogeneous distribution of the nanoparticles as shown in **Figure 3**. Alternative wet chemical cleaning methods for Si wafers may be used as needed; here only a reproducibly clean surface is required rather than the complete removal of all organic contaminants or the oxide layer. If the protocol is paused between the cleaning and suspension deposition steps, the wafer should be treated again under plasma or UV/ozone and the suspension deposited ideally within 15 min of treatment.

The suspension of 60 nm Au-Ag core-shell nanoparticles shown in section 2.2 contained a significant amount of sodium citrate as a stabilizer, which is a common occurrence in nanoparticle suspensions. For accurate analysis of these particles and their surface properties, particularly via XPS, as much stabilizer should be removed as possible, as it attenuates the signal from the nanoparticles and causes charging effects. In order to establish the optimal purification method for these nanoparticles, shown as SEM micrographs in **Figure 4**, they were either dialyzed in ultrapure water or purified using centrifugation and re-dispersion in triplicate. Although dialysis would seem a gentler method and centrifugation and re-dispersion more likely to cause agglomeration and aggregation of the particles, the SEM images show significant deformation and damage of the Au-Ag nanoparticles after dialysis (**Figure 4B**), while the centrifuged/re-dispersed particles are still intact (**Figure 4C**). This is particularly remarkable with metallic nanoparticles; our hypothesis is that there is an optimum amount of sodium citrate that enables some stabilization of the solution while not interfering with the signal for the nanoparticles, and removal of too much stabilizer causes damage to the nanoparticles. A previous report shows that there is an optimum number of centrifugation cycles for removal of most of the sodium citrate; exceeding this number causes some NP aggregation[33]. In this study, nine dialysis cycles (a total of 36 h) were required to obtain similar citrate concentration; however, this method resulted in

a higher amount of aggregation than centrifugation as well as causing a decrease in surface functionalization. These results demonstrate the importance of verifying each step in the preparation procedure for each different type of nanoparticle, particularly with unknown samples.

The 60 nm Au-Ag core-shell nanoparticles used in this example are suitable for drop-casting due to their electrical conductivity, because charging effects are not an issue and a thick spot can be generated by repeated deposition using relatively little equipment. This thicker layer has the advantage of giving more reproducible measurements, and casting from a more concentrated suspension can save time by reducing the number of deposition steps. The deposition can be influenced by the substrate wettability; poor wetting can produce a thick nanoparticle spot which is advantageous for conductive samples, while good wetting can produce a more homogeneous nanoparticle layer, which can be useful for both conductive and insulating samples. As described in the protocol, drop-casting of nanoparticle suspensions usually requires repeated applications to obtain a thick layer with full coverage; this should be verified using XPS, but may also be quickly and easily verified using optical microscopy. **Figure 5** shows the evolution of droplet coverage in a drop-casting of Au-Ag core-shell nanoparticles from aqueous solution; in this case, 13 drop-casting steps are required to achieve full coverage. Drop casting is particularly suitable for conductive particles, or the ones where charging effects can be adequately compensated. As with the other methods described in this publication, drop-casting should be optimized for each sample as different NP materials will have different properties concerning information depth and concentration and film thickness limits. It is important to avoid too thick films which can cause stacking of organics in turn inhibiting the NP signal.

A homogeneous and good quality coating helps to ensure consistent and reproducible results. In addition to the suspension concentration, solvent, and spin-coating parameters, the quality of spin-coated suspensions can also be negatively influenced by the presence of dust or other large macro- or microscopic particles. **Figure 6** shows the improvement in spin-coating quality of a nanoparticle suspension after filtration with an 0.45 µm syringe filter. The filter should be selected to ensure that it does not remove nanoparticles from the suspension. The three different suspension concentrations described in the protocol (90, 9.0 and 0.9 mg/mL of 135 nm PS-PTFE core-shell nanoparticles) were spin-cast under the same conditions and analyzed using SEM and XPS. The top image and spectrum in **Figure 7** show the film cast from the 90 mg/mL suspension, which shows a thick and gapless multilayer coverage in the SEM image as well as a notable absence of Si peaks in the CPS spectra, indicating no contribution of the substrate to the spectrum. This sample is ideal for XPS or ToF-SIMS analysis; additionally, the smaller F1s peaks from the shell of the particles can be clearly seen in the absence of a large signal from the substrate. The second sample cast from the 9.0 mg/mL suspension shows the particles in small single-layer agglomerates, which do not completely cover the surface. This sample is too thin and inhomogeneous for XPS or ToF-SIMS analysis. Furthermore, quantitative analysis can be impaired due to the contribution of adventitious carbon on the substrate even after careful cleaning; at the very least, such an effect must be considered in the uncertainty budget of the measurement. This sample would, however, be ideal for SEM or TEM analysis of particle size distribution using image analysis software, as the particles exist in a single layer and in a sufficient number (within

the image) to provide a statistically significant evaluation. The sample cast from the lowest concentration (0.9 mg/mL) does not provide either continuous coverage or sufficient particle density to make it suitable for analysis of either surface chemistry or particle size distribution. A reliable quantitative analysis is not at all possible due to the dominant influence of the substrate.

Al_2O_3-TiO_2 core-shell NPs with either a PDMS or glycerol outer layer were prepared via drop-casting from suspension as well as from powder using the "stick-and-go" method in order to compare the effects of the different preparation methods on the sensitive outer layer. The samples were analyzed with ToF-SIMS, where in the spectra was analyzed using Principal Components Analysis (PCA). PCA is a statistical technique for reducing the dimensionality of large data sets by creating new uncorrelated variables (the principal components), which maximize the variance in the data[41–45]. The separation of different sample sets on the principal component graph allows the results to be more easily analyzed and grouped. On the PCA scores plot in **Figure 8B**, which shows the discrimination power of each data set in comparison to all other data sets (i.e., between different sample sets) the two samples prepared from powder show very different scores, while the samples prepared from dispersion show very similar scores. The loading plots shown in **Figure 8C** indicate the relationship between variables, i.e., which peaks contribute the most to the respective principal components. All principal components are sorted according to their contribution to the observed difference between the data sets, i.e., PCA1 contributes the most to the observed separation of the different data sets. PC1 is dominated by the presence (PDMS-coated NPs prepared from powder) or absence (all other samples) of PDMS peaks, while PC2, the factor accounting for the second largest variation within the data sets, enables the differentiation of the Al_2O_3 and the organic capping on the NPs. This indicates that the measured spectra of NPs prepared from suspension are very similar and suggests that the PDMS and glycerol layers may have been removed or damaged by preparation from suspension, from either the suspension itself or the drying process, with dominating signals from the Al_2O_3 or TiO_2.

While pressed pills can provide advantages for preparation of powdered samples such as ease of handling and stability in ultrahigh-vacuum instruments (including the ability to sputter without dislodging NPs in the high-vacuum chamber), the high forces involved may also damage sensitive nanoparticles, as has already been seen with other preparation methods. A suitable protocol should be prepared and validated.

In the case of NP dispersions, cryofixation of drop-cast sample suspensions avoids coffee ring effects (because of the instantaneous fixing of the NP suspension and therefore elimination of drying effects) as well as the preservation of larger structures present in the suspension. Additionally, the application of adhesive tape is avoided. This in turn is reflected in reduced signals, which may be attributed to salts, contaminants, or other artifacts of the sample preparation procedure in the respective mass spectra as shown in **Figure 9**. The main advantage of cryofixation is the ability to conserve "as is" the chemical space around the nanoparticles and/or the chemical entity of the particle agglomerates or heteroagglomerates as well as their correlation to biological features within tissues or single cells or even the co-localization to intracellular compartments, without disruption from sample handling steps such as drying, drop-casting, etc[46,47]. We have demonstrated the applicability of the cryofixation technique within the

current paper and have highlighted the advantages of cryofixation for TiO_2 nanoparticles. We stress that cryofixation is particularly suitable for the analysis of biological samples due in their natural state without the dislocation of chemicals due to sample preparation artifacts. For more in-depth information about fixation techniques for biological samples the reader is referred to literature[19,25,27,48,49].

FIGURE AND TABLE LEGENDS:

Table 1: Comparison of various methods for surface analysis.

Table 2: Comparison of different sample preparation methods.

Figure 1: Cleaning process for Si wafers.

Figure 2: XP spectra of Si wafer before and after cleaning. Survey before (gray) and after (red) cleaning, showing the decrease of the carbon amount from 13 at% to 2 at%. The spectra were obtained with a Kratos Supra DLD (Manchester, UK) with a monochromatic Al Kα radiation. The samples were fixed with double-adhesive tape on the sample holder, pass energy was 80 eV, step width 1 eV, dwell time 500 ms. The "hybrid lens mode" was used. The X-ray spot size was 300 x 700 µm². A flood gun was used for charge compensation. For quantitative analysis, the software package UNIFit 2020[50] was used, using the peak areas of the corresponding photoelectron peaks corrected with a Tougaard background and normalized with Scofield factors, inelastic mean free pathways and the transmission function.

Figure 3: Effect of UV/Ozone cleaning on homogeneity of particle dispersion in the drop-casting of PTFE-PMMA core-shell nanoparticles from aqueous suspension. The wafers cleaned with UV/ozone show a significant decrease in coffee-rings, as well as better adhesion of the particles to the surface.

Figure 4: Treatment options for removing impurities (e.g., stabilizers) from nanoparticle suspensions SEM images showing the effect of dialysis (top right) and centrifugation and re-dispersion in triplicate (bottom right) on 60 nm Au-Ag core-shell nanoparticles. The nanoparticles are clearly damaged by the dialysis, while centrifugation has no visible affect. All scale bars are 100 nm.

Figure 5: Optical microscope images from drop-casting of 60 nm diameter Au-Ag core-shell nanoparticles from aqueous suspension onto silicon wafers, showing sufficient coverage after 13 drops.

Figure 6: Spin-coated nanoparticle suspension, before (left) and after (right) filtration with an 0.45 µm syringe filter. The improvement in quality after filtration can clearly be seen.

Figure 7: SEM images and XPS spectra of PMMA-PTFE core-shell nanoparticles spin-cast at various concentrations, showing the effect of substrate peaks (from insufficient coverage) on the XPS spectra.

Figure 8: Principal Component Analysis (PCA) score plot, derived from ToF-SIMS spectra of glycerol- and PDMS-coated Al_2O_3-TiO_2 core-shell NPs (**A**) Schematic of NP structure; (**B**) Scores and (**C**) Loading plots after ToF-SIMS analysis of drop-cast (dispersion) and "stick-and go" (powder) preparation methods. PC1 represents peaks correlating to PDMS fragments; PC2 separates samples with an organic coating (samples prepared from powder) from Al_2O_3 peaks seemingly without surface coating. Spectra were measured in positive mode on an IONTOF ToF-SIMS IV instrument (ION-TOF GmbH, Münster, Germany) in the spectrometry mode (HCBU) with a 25 kV Bi_3^+ ion beam with a maximum dose density of 10^{12} ions/cm^2. A field of view of 150 × 150 µm was scanned in sawtooth mode with 125 × 125 pixels.

Figure 9: Section of ToF-SIMS mass spectra of TiO_2 NPs (**A**) prepared from powder with the "stick and go" method and (**B**) after cryofixation of the NP dispersion. A ToF-SIMS instrument (ION-TOF V; Ion-TOF GmbH, Münster, Germany) was used for mass spectrometry analyses with a pulsed 30 keV Bi_3^+ liquid metal ion gun (LMIG, direct current (dc), 16 nA). Each spectrum was acquired by scanning the ion beam over a sample area of 500 × 500 µm. Positive secondary ions were acquired in the mass range 0–1,200 Da using 10^6 Bi_3^+ pulses.

DISCUSSION:

A number of methods have been presented for the preparation of nanoparticles for surface analysis using XPS and ToF-SIMS. We have summarized the advantages and disadvantages of these methods, as well as possible sources of error and suitability for different materials, in **Table 2**. As shown in the representative results, the preparation of nanoparticles can strongly influence the success of the resulting surface analysis. In addition, not all methods are suitable for all particle types due to factors such as signal interference with the substrate or mounting materials, charging effects in non-conducting thick films, state of the nanoparticles as a powder or suspension, potential damage to sensitive outer layers, destruction of biological structures and information on aggregation and interfaces, or vulnerability of sensitive ultrahigh-vacuum instruments to free nanoparticles.

Because XPS and ToF-SIMS measurements average over an area rather than measuring single particles, it is only possible to obtain reproducible results from homogeneous layers; aggregation or agglomeration of the particles on the substrate should therefore be avoided. Additionally, too-thick layers of non-conductive materials cause charging effects during analysis, which can lead to undesired artefacts in the spectra, especially partial charging which cannot be compensated with a flood gun. On the other hand, incomplete films show strong signals from the substrate or mounting materials (e.g., adhesives), which can interfere with sensitive peaks from the particle surface. The ideal thickness of the film is material dependent and should be determined experimentally by analysis of films of different thicknesses. In particular, samples prepared using spin coating should be analyzed with SEM to ensure completeness of the coating.

Working with NP suspensions presents fewer exposure hazards and safety requirements compared to working with NP powders. Drop-casting is a relatively simple method with low

equipment requirements and is particularly suitable for conductive nanoparticles in suspension where film thickness is not a concern. While the samples can easily be dried under atmospheric conditions, the vacuum desiccator serves to reduce the drying time for the droplets as well as protecting the wafers from contamination. The Viton ring is used to modify the evaporation patterns of the droplet and thereby minimize the formation of coffee-rings. Evaporation patterns can also be influenced by varying the substrate hydrophilicity using cleaning protocols or by application of alternative coatings[51,52], by evaporating in solvent atmospheres[53], or even by heating the substrate[54]. Spin-coating is recommended for suspensions of non-conductive nanoparticles in suspension because it is capable of generating a homogeneous particle layer that is thin enough to avoid charging effects but still thick enough to prevent the Si substrate from contributing to the XPS and ToF-SIMS spectra. For each individual NP system and concentration, both the centrifuge and spin-coating parameters must be optimized but can then very reliably be reproduced even on different instruments. Because the spin-coated drop is always in the middle of the wafer, the radius of rotation is irrelevant and the unit "revolutions per minute" (rpm) can be used. The suspension could alternatively be deposited on the wafer after starting the program; however, this would require different spin-coating parameters and a greater amount of suspension to obtain a thicker coating.

Because of their extremely small size, nanoparticles may detach from the substrate and move freely inside the ultrahigh-vacuum chamber when impacted with an ion or x-ray beam. This is a particular problem for samples prepared with powder. In some cases, the nanoparticles can penetrate into the sensitive components of the instrument requiring expensive and time-consuming maintenance. Due to the applied acceleration voltage, the danger of damaging sensitive parts is larger with ToF-SIMS than with XPS. Powdered samples, particularly those prepared using the "stick and go" method, should be carefully checked to ensure the powders are fixed securely enough, especially for ToF-SIMS analysis. This can be confirmed by, for example, holding the sample upside down and blowing a stream of gas (e.g., N_2) across it. Prior to analysis, the samples can also be left overnight in the airlock or other initial sample entry chamber of the instrument, where a stable vacuum can indicate that there are no loose particles from the sample. Nanoparticles prepared as pellets, however, can even be sputtered (at low acceleration voltages) without damaging the instrument; this method can eliminate contaminants, particularly hydrocarbons, introduced from the pill press and can also enable bulk analysis of the particles.

Preparation of NP powders in the sample holder stub allows for the preparation of samples with defined geometry and a macroscopically flat surface. Critical points are the cleanliness of the tool for pressing the sample, and the use of a low pressure to avoid changes in the nanoparticle surface due to this procedure. It has the disadvantages of needing a relatively high amount of material, and potential problems with loss of material in high-vacuum instruments. We do not recommend this method for ToF-SIMS analysis, as the particles are not compressed or secured in any way.

Regarding the NP material, the first consideration for sample preparation is elimination or minimizing of interference between NPs and substrates of similar material; for example, Si wafers

are an unsuitable substrate for analysis of SiO_2 NPs using XPS and ToF-SIMS, even with sufficient sample coverage. Metallic or inorganic nanoparticles can be readily analyzed as powder on an adhesive (assuming they contain no organic layers or coatings) due to the lack of signal interference between the nanoparticles and the double-sided adhesive, a preparation method which would be unsuitable for polymeric NPs. Metallic nanoparticles have more flexibility in terms of possible film thickness used due to the absence of charging effects, and may be drop-cast with relatively little equipment; however, they are likely to contain large amounts of impurities and stabilizers from their synthesis, which must be carefully removed without damage to the particles. Polymeric nanoparticles may more easily be damaged by die pressing but may also more readily hold together in the pellet, depending on the pressures used. Pressed pills can also damage sensitive or soft organic coatings on the NP surface. Direct deposition from the solution has the potential to damage sensitive coatings either through the suspension or the drying process but is advantageous for analyzing NPs already present in suspension. Cryofixation is a suitable method for the analysis of chemical structures, surfaces or interfaces in suspension which would be damaged or destroyed by various other sample preparation techniques, but requires a specialized cryoequipment for both XPS and ToF-SIMS[46,47].

While this paper describes several exemplary methods that can be used for sample preparation, in every case the method should be optimized and validated using alternative analytical methods. A detailed overview of the influence of different factors was recently published[22]. Besides the development and validation of suitable preparation methods, the documentation of these steps is also of paramount importance[40]. This publication presents some easy-to-handle methods and is a guide to modify or develop new methods according to the requirements of the specific task.

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The authors have no competing interests to disclose.

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Figure 1

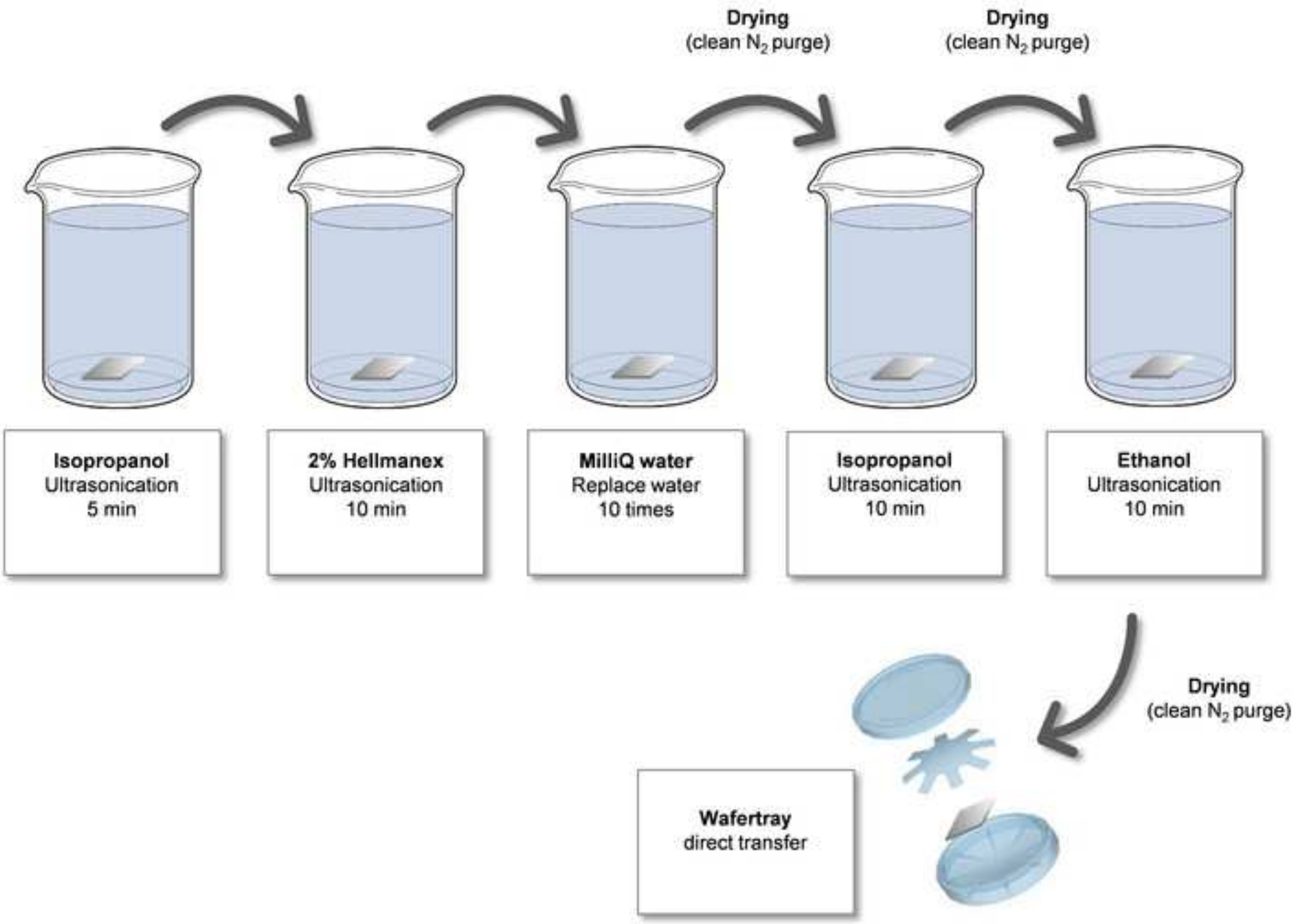

Figure 2

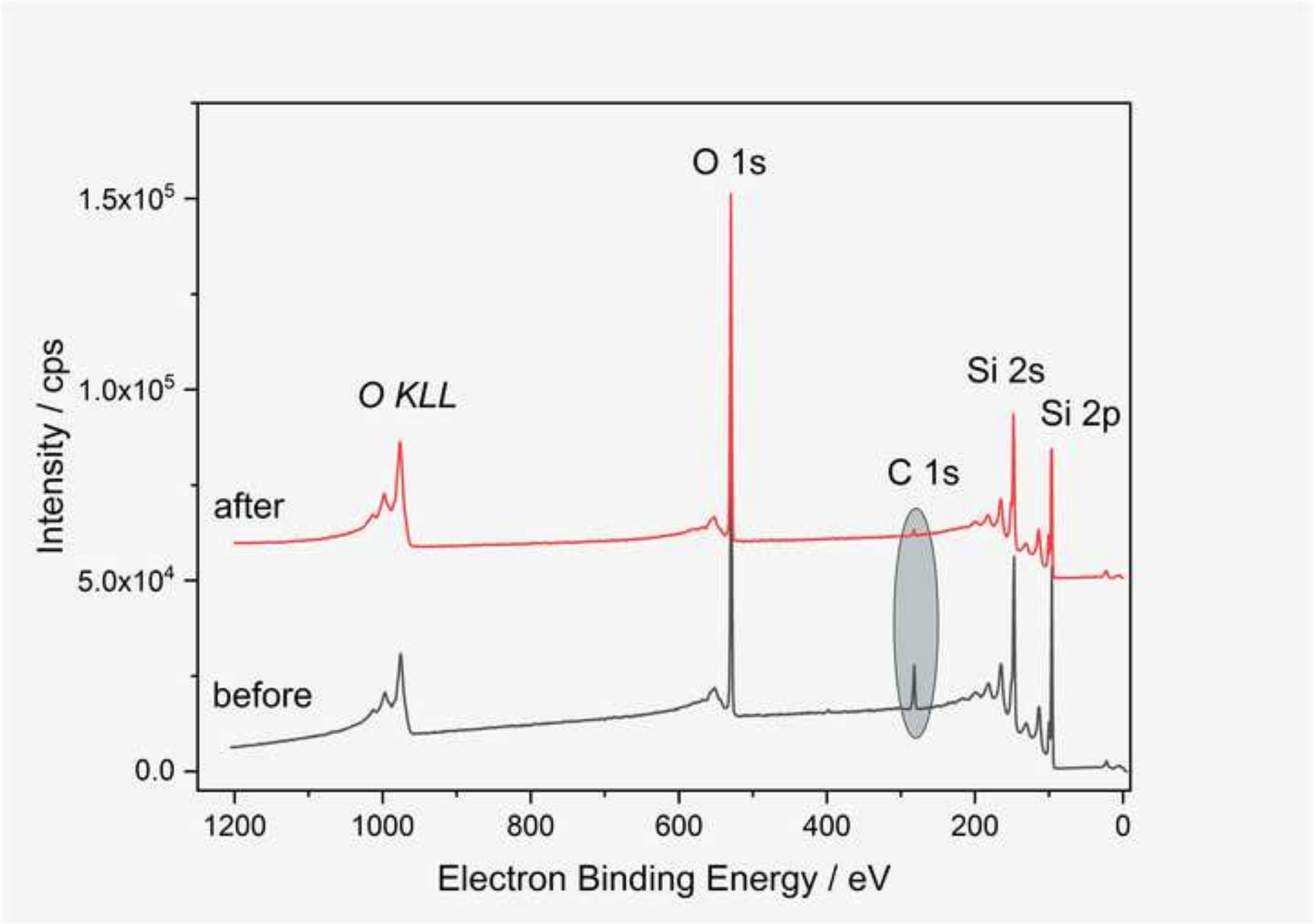

Figure 3

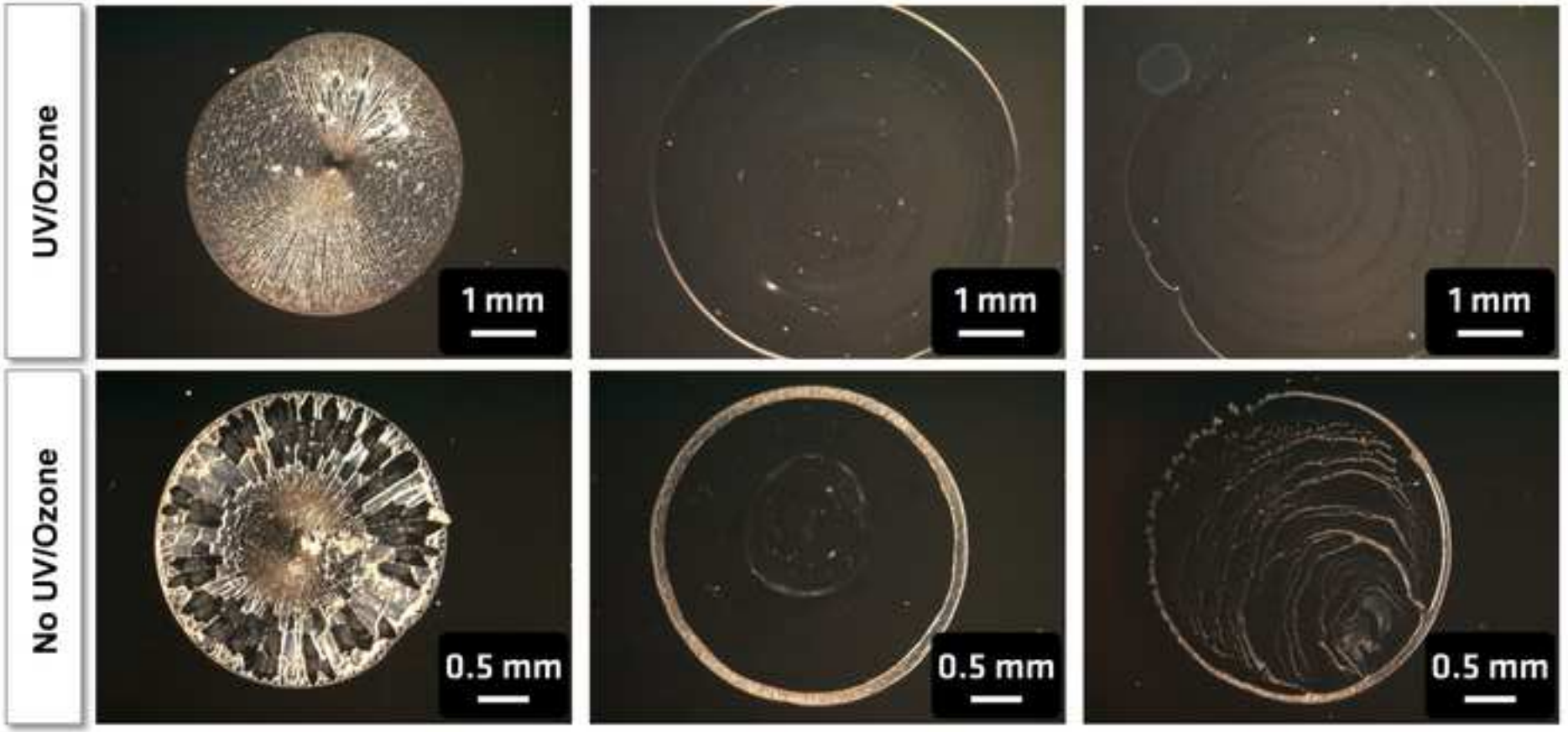

Figure 4

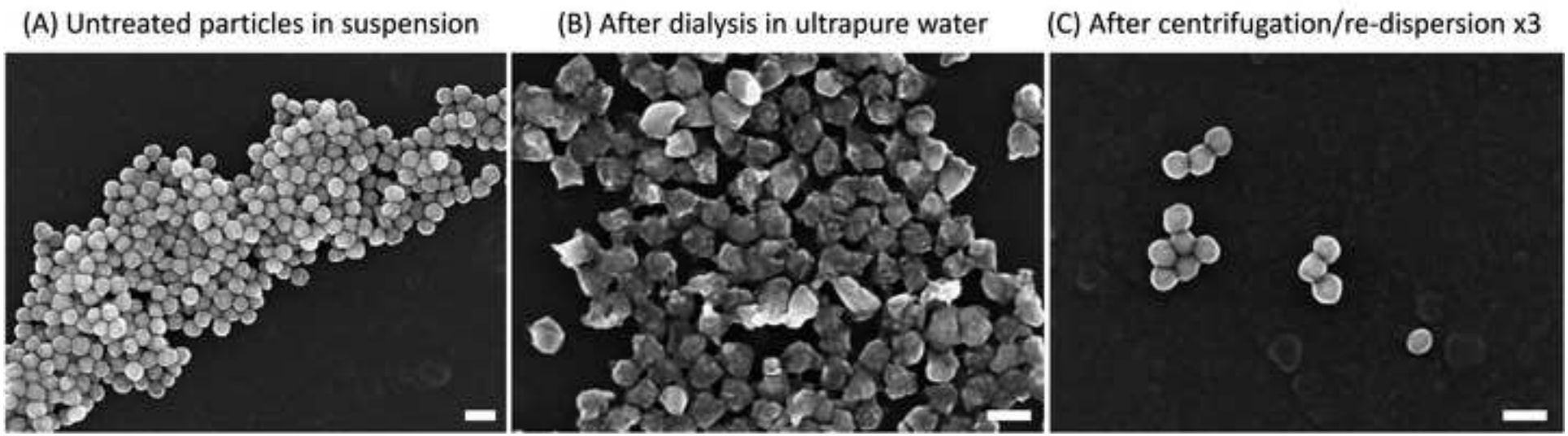

Figure 5

Click here to access/download;Figure;Figure 5 Optical microscope drop casting_REVISED.jpg

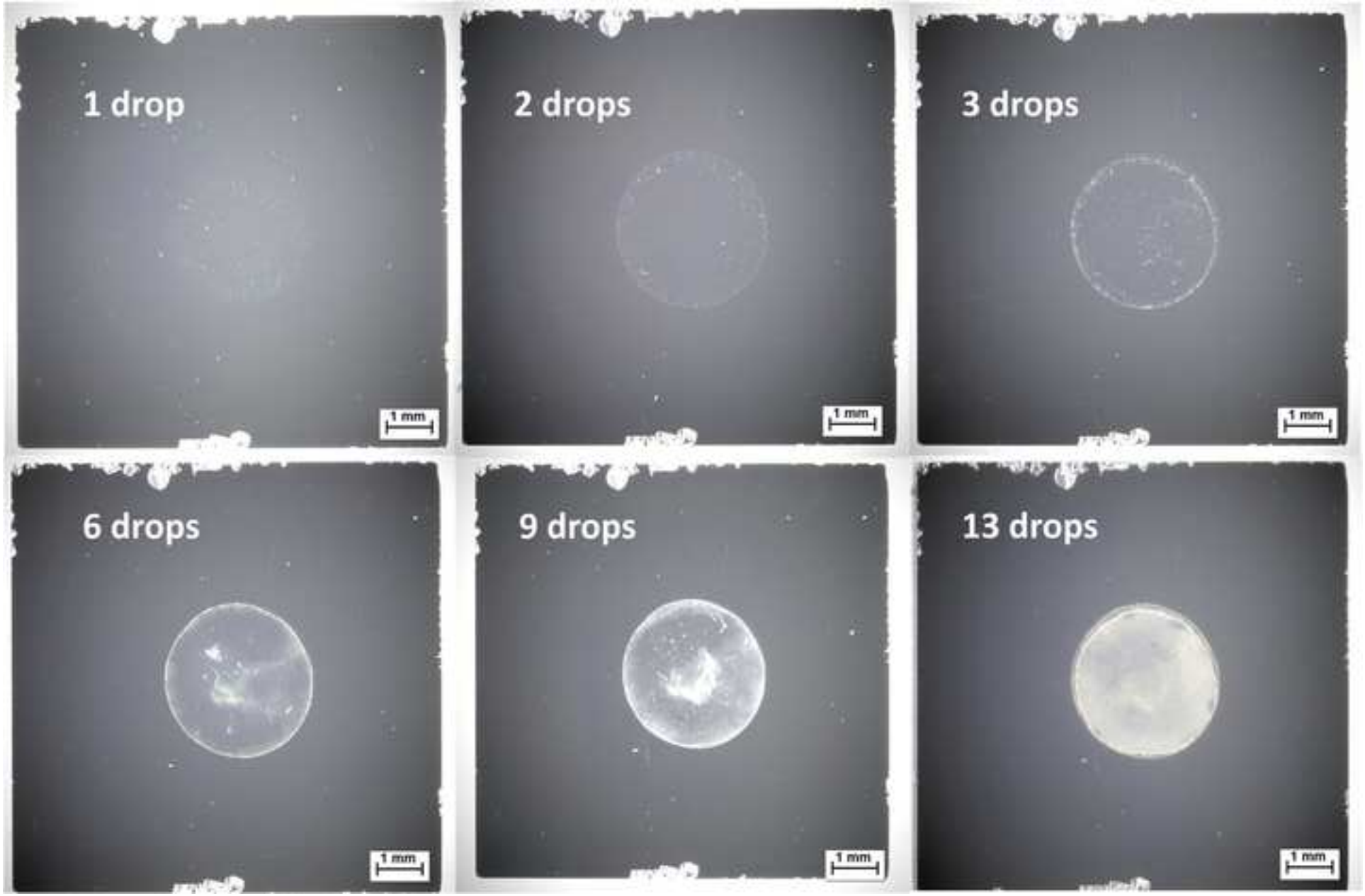

Figure 6

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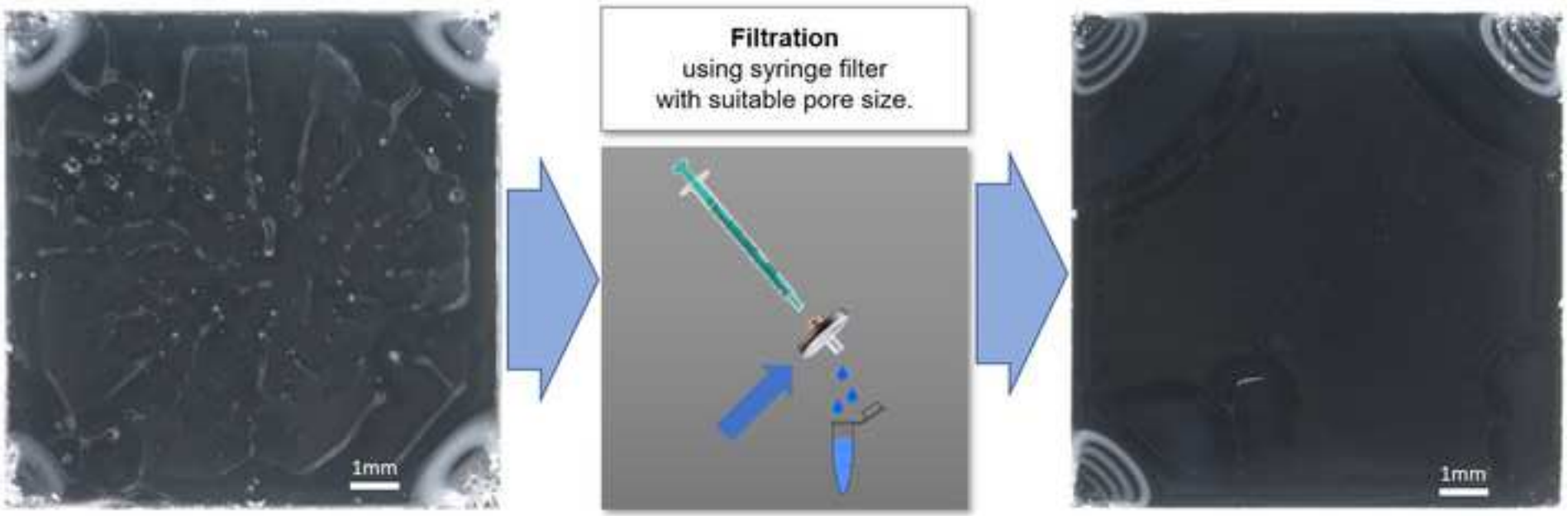

Figure 7

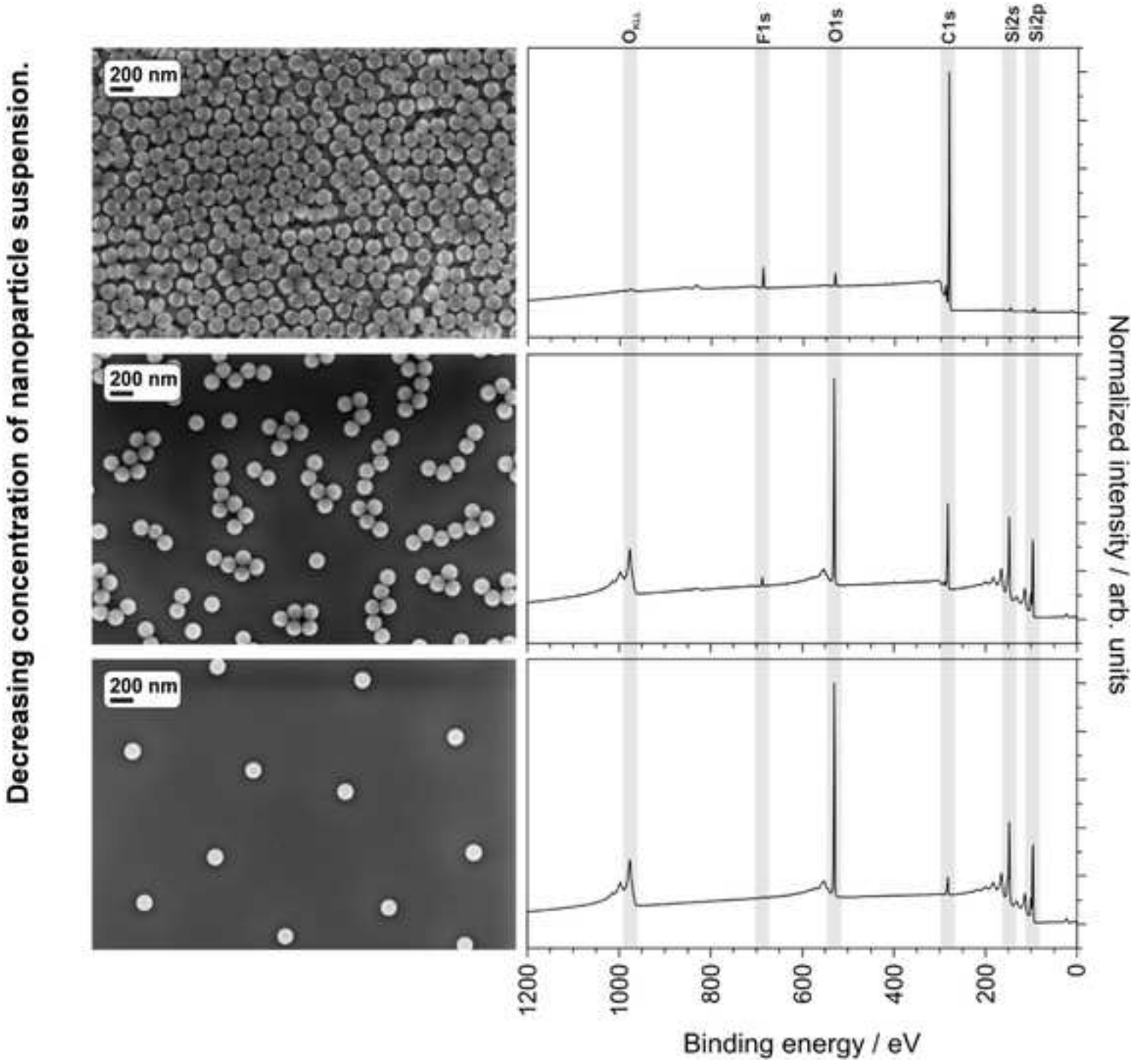

Figure 8

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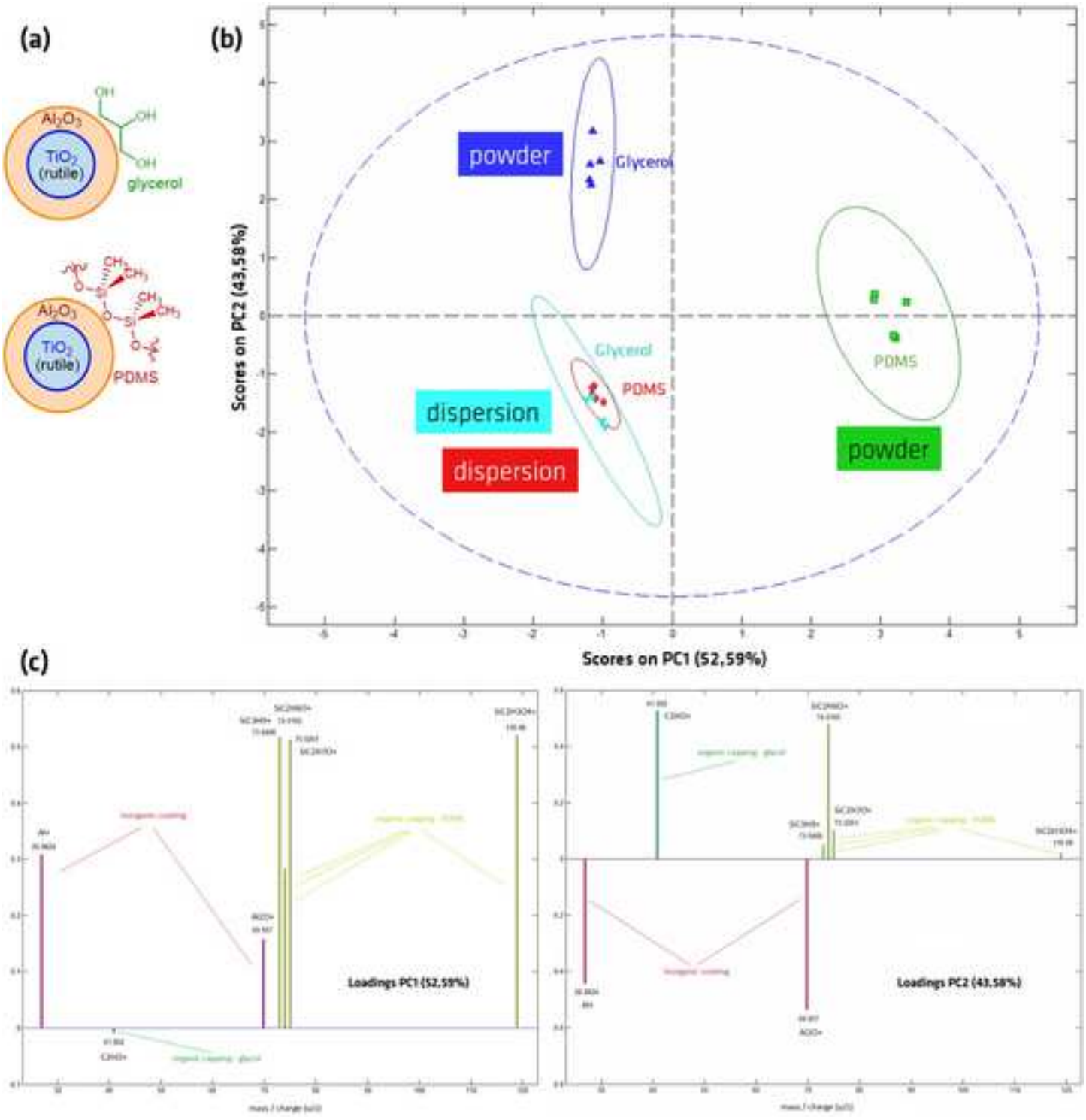

Figure 9

Click here to access/download;Figure;Figure 9 Mass Spec TiO2 NPs powder-cryo fix_REVISED.jpg

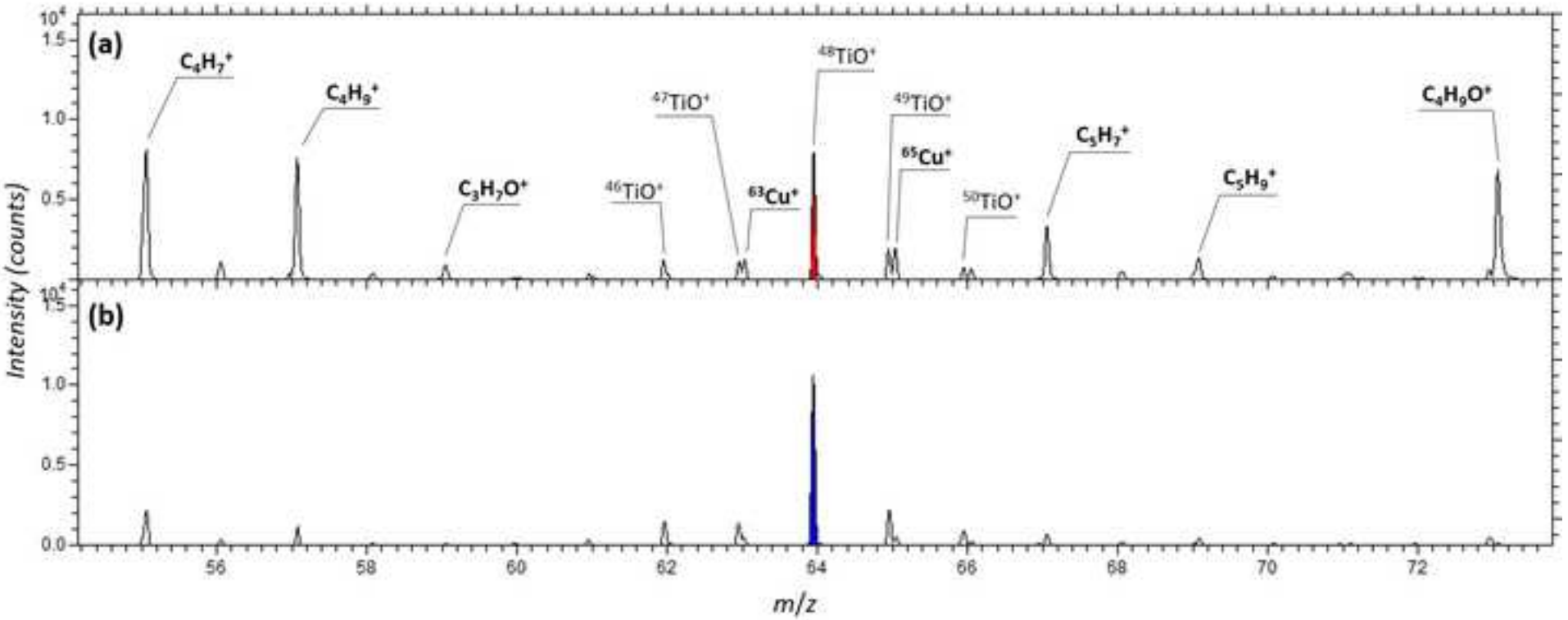

Table 1: Comparison of XPS and ToF-SIMS as methods for surface analysis

	XPS	ToF-SIMS
Probe Beam	Photons	Ions
Analysis Beam	Electrons	Ions
Spatial Resolution*	> 1 µm	0.1 µm
Sampling Depth	0.5 – 7.5 nm	<2 nm
Detection Limit	0.01 -0.1 atom %	ppb
Quantification	Excellent (semi quantitative)	Challenging (matrix effects)
Information Content	Elemental Chemical bonding	Elemental Molecular
Organic Analysis	Excellent	Excellent in static mode

* specified by the manufacturer

Table 2: Comparison of various methods for sample preparation described in this paper

Method	Suitable for	Gives	Advantages
Dialysis	Purification	Removal of stabilisers/ impurities	Simple, low effort, no complicated equipment
Centrifugation/re-dispersion	Purification	Removal of stabilisers/ impurities	More control over process, simultaneous concentration
Drop casting (suspension)	Conductive NPs without sensitive outer layer	Relatively thick coated spot	Simple, no complicated equipment
spin coating (suspension)	Conductive or non-conductive NPs without sensitive outer layer	Thin homogeneous layer, or single particles	Consistent settings
"stick and go" (powder)	Inorganic conductive and non-conductive NPS with sensitive outer layer	Powder spot on adhesive	Simple, low effort, no complicated equipment,
deposition in hole of a stub (powder)	XPS analysis; conductive/non-conductive organic or inorganic particles	Lightly pressed nanoparticle sample	No contact with other materials
Pressed pellets (powder)	Conductive and non-conductive NPS, polymeric NPs	Solid pellet	Enables analysis of polymeric NPs as powder
Cryo-fixation (suspension)	NP suspensions with sensitive ligand layer; biological samples	Solid sample	Conserves morphology, native biological state and corona, reduces coffee ring effect

Disadvantages	Caution	Controls	Check
Lack of control over process	May cause damage to nanoparticles	Time	Damage to nanoparticles (SEM)
Labour-intensive, requires centrifuge	May cause aggregation or agglomeration	Centrifuge rotation speed, quantity of solvent	Agglomeration/ aggregation/ damage to nanoparticles (SEM)
Can give inhomogeneous thickness, time-intensive	Suspension preparation may damage sensitive NP shells	Suspension concentration, solvent (substrate wettability)	Coverage (light microscopy/XPS)
Requires experimental determination of optimal parameters	Filter out dust/impurities, coverage may be inconsistent	Concentration, spin coating parameters, solvent	Pre-filtration, Coverage, layer thickness (SEM/XPS)
Unsuitable for organic or C-containing NPs, Inconsistent film thickness	Danger of NP release into instruments	Fixation of NPs onto adhesive	Stability under high vacuum conditions
No secure fixation of NPs; unsuitable for ToF-SIMS	Dager of NP release into instruments	None	Lightly tilt to the side, to ensure powder is compacted
May damage or contaminate NP surface	Materials should be cleaned thoroughly to avoid surface contamination; may damage surface	Size, pressure, time	Stability under high vacuum conditions
Sophisticated and expensive preparation and sample handling, requires skilled user	high degree of skills required for sample handling and sample storage	Concentration, droplet size, temperature	Preservation of vitrification

Name of Material/Equipment	Company	Catalog Number
4-figure Laboratory balance	Kern & Sohn GmbH	ADB200-4A
5 mm Pellet die	Specac	GS03060
Alkali glass cleaning solution	Sigma-Aldrich	Hellmanex™ III Z805939
Carbon adhesive tabs	Plano	"Leit-Tabs" G3347
Clean laboratory beakers	any	
Cryo-freezer	Electron Microscopy Sciences	EMS-002 Cryo Workstation
Dialysis tube with fasteners	Medicell Membranees Ltd	DTV12000.06.30
Die press	any	
Disposable syringe, 1 mL, Luer-slip	TH Geyer	Labsolute 7657545
Double-sided adhesive	3M	Removable Repositionable Tape 665
Dry ice	Linde AG	ICEBITZZZ®
Eppendorf transfer pipette and tips	Eppendorf	various
Ethanol, ACS grade	Merck KGaA	1009832500
FFP2 or FFP3 mask	various	
Isopropanol, ACS grade	Merck KGaA	1096342500
Lab coat, gloves and goggles	any	
Laboratory centrifuge	Eppendorf	Centrifuge 5430
Laboratory fume hood	any	
Laboratory stirrer & stirrer bar	NeoLab	D-6010
Lint-free wipes	Kimberley Clark Professional	Kimtech Science Precision wipes
Liquid Nitrogen	Linde AG	Stickstoff flüssig 5.0
Microtube/centrifuge tube 1,5 mL	T.H. Geyer GmbH & Co. KG	Labsolute 7696751
Nitrogen 5.0	any	
Pasteur pipette, PE, plastic 3 mL	TH Geyer	Labsolute 7 691 203
Pasteur pipette, PE, plastic 3 mL	TH Geyer	Labsolute 7 691 203
Powder sample holder	BAM workshop	
Propane	Sigma-Aldrich	769037
Sample vial or centrifuge tube 1 mL	Greiner Bio-One GmbH	Cellstar 188 261
Silicon wafers	any	
Spin-coater	SPS Europe	SPIN150i-NPP
Syringe filter 0,45 µm	Th Geyer	Labsolute 7699803

ToF-SIMS	IONTOF GmbH	ToF-SIMS IV or V, equipped with Bi LMIG a
Tweezers for handling Si wafers	any	
ultrapure water	TKA	MicroPure 08.1202
Ultrasonicator	Bandelin	Sonorex Super
UV/Ozone cleaner	NanoBioAnalytics	UVC-1014
Vacuum dessicator	any	
Vacuum pump (membrane/diaphragm)	Vacuubrand GmbH	Type MD-4T
Viton O-ring 6.07 x 1.78 mm	Betech GmbH	2-010, FKM 80
Vortexer	Heathrow Scientific	Vortexer HS120212
Wafer Holder 25mm coin style	Semiconductor Production Systems Europe	eWB0091-ASSY-1
XPS	Kratos	Kratos Axis Ultra DLD

Comments/Description

Special cleaning solution for cuvettes

e.g. 300 mL

Molecular weight cut-off (MWCO) 12-14 kDa
Capable of 2 kN force
Any appropriate volume can be used

For short term storage/cooling
Check correct size for planned pipetting volume

For working with nanoparticles from non-hazardous materials, when not in a fume hood or glove box

necessary for working with nanoparticles

Recommended for working with Si wafers
Only for cooling of the cryogen.

99.999% purity

"Home-made" sample holder
The cryogen should be of highest possible purity.
Should be capable of being fixed in the Vortexer
ideally 1cm^2 pre-cut

For smaller samples; larger versions exist for larger sample volumes

and flood gun

Dear Sir or Madam,

Please find below a list of Editor's and reviewers' comments (blue) along with our responses (black) for the manuscript JoVE61758 - [EMID:aa08e990fdc9af0c]. We would like to thank all the reviewers for their constructive comments which have helped us to greatly improve the paper.
While specific changes according to the wish of the reviewers have been highlighted in the revised text, the large sections that have been re-organised were not highlighted.

Best regards,
Dr. Francesca Bennet & Dr. Jörg Radnik

Editorial Comments:

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

- The protocol has been revised to ensure all instructions are in the imperative voice. Some of the text previously as a "note" has been moved to the results/discussion sections as appropriate.

1) Some examples NOT in the imperative: 2.2.1, 2.2.2, - All instructions have been changed to the imperative

2) Split up long steps (e.g., 2.2.2, 3.3.2, etc) – long steps have been split up

3) 2.4: please keep subheadings short. – The title of the subheading has been changed and is now shorter.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video.**Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

- The protocol has been reviewed and modified to ensure it is consistent with requirements.

1) 1.1.5: Mention sonication frequency in Hz and Amplitude in Watts. – These have been mentioned in the instructions, based on the manufacturers specifications, however they are set for the model of sonicator bath and we cannot change them.

2) 2.1.1: 15 mg of what? -Sentence has been changed to "15 mg of nanoparticle powder"

• **Protocol Numbering:**

1) Line 192: This heading can be deleted. - The heading has been deleted.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

- The protocol has been modified to form a cohesive narrative with all sub-steps clearly described. The highlighted sections remain below 2,75 pages, including sub-headings and spaces and excluding Notes.

• **Discussion:**

1) JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6

paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol. -The discussion has been modified and extended to include these points.
2) Avoid subheadings – subheadings have been removed.

• **Figures:**
Scale bars have been added to Figures 5 and 6. The revised figures are attached.

• **Commercial Language:** Commercial product names have been removed.

• **Table of Materials:** Table of materials has been sorted alphabetically.

• None of the figures used have been previously published.

Comments from Peer-Reviewers:

_____

Reviewers' comments:

Reviewer #1:

Minor Concerns:

* The authors mention substrates other than SiO2, but only in passing. As a matter of fact, my laboratory has never used SiO2. The reason is that the partially oxidized adventitious hydrocarbon layer on the SiO2 is matched by a similar layer on the nanoparticles. Since we are interested in studying the nanoparticle surface, it is necessary for us to distinguish which is which. We tried removing the SiO2 surface layer but found that it reformed over about 4 hours when exposed to air and, while it took a bit longer in the XPS vacuum, the layer reformed even there. We prefer HOPG, where a new, clean layer can be exposed by Scotch tape peeling. If the authors choose to keep their demonstrations on SiO2, I suggest that they spend a moment discussing its pitfalls.

We choose silicon wafer because they are very common, and we have good experience with them. Of course, an extensive cleaning procedure is necessary before using them which is a part of this contribution. We have added this point in the Introduction (ll. 172 – 175) and mentioned other substrates which can be used.

* Line 107: I suggest that the word, "destroys", be replaced by the more appropriate word, "fragments". "Destroys" has been replaced with "fragments".

* Line 563: I suggest that the discussion on Principal Component Analysis be accompanied by a reference or two. References have been added to the text; this section was moved to "Representative Results" in response to other comments and is now at line 472.

* Line 581: I suggest that the word, "destroyed", be replaced bu the more appropriate word, "removed". "Destroyed" has been replaced with "removed"

* Lines 726 to 777 are a repeat of what comes before it. The accidental duplication of the references has been corrected.

* Figure 9: The authors should include units on the axes. The figure has been corrected to include units.

Reviewer #2:

Manuscript Summary:

This manuscript provided useful storage and deposition approach for the surface analysis of ToF-SIMS and XPS. In addition, Table 2 is very useful to summary the preparation methods for various nanoparticle samples. I think the paper can be published as it is.

Reviewer #3:

Manuscript Summary:

Recommendation: Re-evaluate after major revision
Major Concerns:
This article present in detail standard procedures for preparing nanoparticles for ToF-SIMS and XPS Analysis. However, there is not clear conclusion to the question "what is most suitable sample preparation method for XPS and/or ToF-SIMS?" If you present the results of surface analysis according to the optimized sampling method, reader can clearly understand the advantages of the each sampling method. Rearranging the data or providing a clear explanation would be a good study to guide nanoparticle sampling. The answer to this question “what is the most suitable method?” is: it depends. This is the entire basis of this paper; each method has its advantages and drawbacks, as well as varying suitability for different materials, which we have summarized in Table 2. This table is a guide for the reader which indicating method is suitable for their requirements and which not. The goal of this paper is to clearly present the important steps for a number of sample preparation methods, which are then available for other researchers to use depending on their requirements (NP material, cost, equipment available, level of success, sensitivity to surface layers). We discuss in the paper that for the particular materials in question, the sample preparation method should ideally initially be confirmed by other methods (e.g. electron microscopy) to determine that the NPs are not damaged or aggregated/agglomerated and the method is therefore suitable. See in particular Figure 4.

1. The authors should reference other works regarding the analysis of nanoparticles by using XPS and ToF-SIMS. Many studies have focused on the effect of sample preparation method on the results of surface analysis, particularly for nanoparticles. The introduction of this manuscript needs to be improved to discuss about the relevant works and to emphasize the novelty of the authors' work.
The aim of this paper is to present various sample preparation methods, that they can be easily used, reproduced and modified by other researchers; as such, the focus is more on the state of the art in this field. The paper has been modified to include a number of relevant references on the topic.

2. It is rather unclear what type of nanoparticle is used in this study. This should be clearly stated in the abstract and the introduction. Also, many studies state that the intrinsic physicochemical properties of nanoparticles affects their behaviors during the sample preparation steps (i.e., agglomeration, coffee-ring effects, etc.). These detailed information must be provided. The various different types of nanoparticles used in this study have been described in the text and the figure captions. We agree with the reviewer’s comment that the NP properties affect their behaviors during the sample preparation steps; for that reason, we state that the suitability of the preparation methods should be confirmed by other analytical techniques, e.g. electron microscopy. It is also the reason why we present a variety of preparation methods in this paper as well as a comparison table, so that the optimal method can easily be selected and used.

3. The authors need to provide a more detailed rationale for comparing the results of cryo-fixation method with those of other three methods. Just like the authors have stated, cryo-fixation is a commonly used sample preparation method for biological matrices such as tissues. The authors did not mimic biological environment as it is beyond the scope of this manuscript, so the use of cryo-fixation method should be justified. This point has been addressed in the Representative Results section (ll. 578 – 587).

4. The comparison of the sampling method presented in Table 2 looks reasonable, however, is somewhat speculative as the results of XPS and ToF-SIMS analysis are not fully integrated. The influence of the various sampling methods on the results of surface analysis should be discussed. Otherwise, this manuscript will fail to address the interest of many readers. The major question is as follows: What is the most suitable sample preparation method for XPS and/or ToF-SIMS? We understand this interest of the reader very well, but unfortunately, the only one method does not exist. The aim of this study to present some “easy-to-handle” methods and show the advantages, pitfalls and ways to validate the methods.

5. Detailed measurement conditions for XPS and ToF-SIMS are missing. Measurement conditions have been included in the relevant figure captions. (Figure caption 2 and 9)

6. In the introduction, the authors stated that XPS/ToF-SIMS results obtained from the different sample preparation methods (drop casting, spin coating, cryo-fixation etc.). However, each sample preparation method went through different purification steps and these steps were discussed in a very detailed manner, while the impact of the sampling method itself is poorly elaborated. Sampling has been addressed briefly in the text, with references to suitable literature. (line 448) A more detailed explanation is not possible due to the limits of the journal.

Overall, this manuscript requires a significant improvement in terms of scientific writing and organization. The aim of this study (i.e., Preparation of nanoparticles for ToF-SIMS and XPS analysis) is very intriguing and can address the needs of the laboratory personnel in the fields of surface analysis however, the conclusion is not fully supported by the experimental results. The introduction, Representative Results and Discussion have been revised according to editorial comments; we feel that this improves the organization of the manuscript. One aim of this publication is to show suitable methods to check each preparation step which can be easily done.

Minor Concerns:
1) In the case of Figure 5, the suitable number of drop casting times may different for XPS and SIMS (excessive stacking of organics inhibits the NPs signal), so further explanation is needed. This has been addressed in a brief comment in the Representative Results section. (lines. 517-519)
2) In the case of Figure 7, the sampling result according to the concentration was linked to the XPS analysis. Since the subject of the paper is about a sampling method suitable for SIMS and XPS, it needs to be mentioned about suitable for ToF-SIMS analysis condition. This paper addresses preparation methods for XPS and/or ToF-SIMS analysis. In this case we show XPS results to demonstrate the effects of different experimental parameters in the sample preparation. We endeavored to show exemplary spectra of both methods. A clear aim of this work is to encourage the reader to perform suitable checks for their specific problem.
3) In figure 8, it is an over-interpretation of the data in the PCA analysis to conclude that the powder sampling method is more suitable than the drop casting method without any description of the loading value. Figure 8 has been modified to include the loadings plots.
4) In Figure 9, the cryo samples, there was no image of the coffee ring effect mentioned in the text, and it was written without a clear rationale for the low level of contamination in the spectrum. Our phrase was misleading. We have d it and clarify that we do not observe a coffee-ring effect after cryo-fixation.
5) Please show XPS or ToF-SIMS images or data after wet chemical cleaning or alkali glass cleaning. This is shown in Figure 2.
6) Please write down the full name of PPE. This has been corrected in the text.
7) In Figure 2, please show the difference of XPS results before and after cleaning. This is shown clearly in Figure 2 in the grey-coloured ellipse.
8) In Figure 3, please show the difference of XPS or ToF-SIMS results with and without coffee ring. Figure 7 shows the differences in XPS spectra from films of sufficient and insufficient coverage, respectively. This demonstrates that continuous coverage is necessary for analysis of the NPs without interference from the substrate. Because coffee rings form depositions of varying coverage (usually with insufficient coverage in the centre of the ring), we see it as obvious that the more uniform coverage, the better.
9) In Figure 5, please show the difference of ToF-SIMS and XPS results obtained from optical images. See previous point regarding uniform and complete coverage.
10) Please explain the difference of ToF-SIMS or XPS results with and without fliteration. See previous points regarding uniform coverage.
11) In Figure 8, please explain the peak difference for PCA difference with ToF-SIMS spectra (and

peak list). Figure 8 has been modified to include the loadings plots for all the peaks used.
12) In Figure 9, specify the sampling method in a) and b). Please identify salt, contamination or other artifact signals in the spectra of cryo-fixation samples. The figure has been modified to show peak allocations of impurities.

Reviewer #4:

Manuscript Summary:

The preparation of nanoparticles for surface analysis, SIMS, XPS, AES etc. is very important and this paper is welcome. It appears to give appropriate details and is thus likely to be a useful paper and set of procedures. There are bits of information and context that, in my view, are missing from the manuscript and if added would strengthen and usefulness and impact of the paper and related video.

Major Concerns:

1. These procedures are very important to analyzing and sometimes preparing nanoparticles for other uses. There are two important aspects related to this:

a. Recording and reporting what is done is important. ISO standard 20579-4 deals with recording and reporting information about what has happened to nanoparticles (sometimes called provenance information) that is important for validating meaning of some analyses and potentially other uses of nanoparticles. For referencing purposes I assume that users might reference this paper and call out procedure numbers (e.g. Drop casting as per Bennet et al. procedure 2.4). Labeling appropriate for such referencing should be kept in mind as the paper moves forward. This comment has been addressed in the Discussion and the reference to the ISO norm added.

b. In some cases, XPS and other analyses is related to immediate use of nanoparticles. Thus, cleaning for surface analysis is not the only object of the cleaning process. Noting, as others have observed, La Spina et al. for example, that there are impacts of "over cleaning" and even functionality after different cleaning processes should be at least noted in the paper. The effects of over-cleaning has been addressed in the Representative Results section with the reference.

Minor Concerns:

2. In discussing uses of XPS referencing papers related to coating thicknesses seem relevant, the comparison paper by Powell et al on two methods may be relevant. Thank you for this comment. We add this important study.

3. There are several references to Cryo XPS at least one of which might be used. A quick google search found a very new one, I am familiar with one by Andrey Shchukarev in surface and interface analysis. The references have been included.

4. In section 2, nanoparticle distribution from suspension, not everything works well in ultrapure water. Should some qualifications be noted in that section? In 2.2.3 the repeat as necessary may need some qualification. After a few rounds cleaning changes XPS little, but particle agglomerate. The “repeat as necessary” in Section 2.2.3 has been modified. The possible need for alternative substrates and solvents and the need for validation and optimization of these methods in individual cases has been briefly addressed in the Representative Results.

5. In the representative results the deformation and damage to nanoparticles is called out. This is important and something more about the type of damage should be noted. It was not obvious to me in the figure. We think the damage to the shape of the nanoparticles is obvious in the figure, and it is beyond the scope of the paper to analyse this in-depth. Representative Results and Discussion have been modified to include other references on nanoparticle preparation which also discuss damages.

6. Below are several references I might have expected to see. There are others that might be relevant, but I agree that too many is less relevant here, but some of the nuances and impact of cleaning and preparation are important to note. Belsey et. al. highlights the challenge of following procedures for sample prep and the need for some type of quality check. The book chapter by Baer

et. al. (you already reference a different chapter in the same book) has some relevant procedures. References called out above or some I might have expected to see:

The references have been included at appropriate points in the text and we thank the reviewer for the helpful suggestions which have improved the quality of the manuscript.

N.A. Belsey, D.J.H. Cant, C. Minelli, J.R. Araujo, B. Bock, P. Br€uner, D.G. Castner, G. Ceccone, J.D.P. Counsell, P.M. Dietrich, M.H. Engelhard, S. Fearn, C.E. Galhardo, H. Kalbe, J.W. Kim, L. Lartundo-Rojas, H.S. Luftman, T.S. Nunney, J. Pseiner, E.F. Smith, V. Spampinato, J.M. Sturm, A.G. Thomas, J.P.W. Treacy, L. Veith, M. Wagstaffe, H. Wang, M. Wang, Y.-C. Wang, W. Werner, L. Yang, A.G. Shard, VAMAS inter-laboratory study on measuring the thickness and chemistry of nanoparticle coatings using XPS and LEIS, J. Phys. Chem. C 120 (2016) 24070-24079.

Donald R Baer, David JH Cant, David G Castner, Giacomo Ceccone, Mark H. Engelhard, Ajay S Karakoti (2019) Preparation of nanoparticles for surface analysis Chapter 4.2 in Characterization of Nanoparticles: Measurement Processes for Nanoparticles by Elsevier, Elsevier (2019)

ISO 20579-4:2018 Surface chemical analysis — Guidelines to sample handling, preparation and mounting — Part 4: Reporting information related to the history, preparation, handling and mounting of nano-objects prior to surface analysis

Determining Thickness and Completeness of the Shell of Polymer Core-Shell Nanoparticles by X-Ray Photoelectron Spectroscopy, Secondary Ion Mass Spectrometry and Transmission Scanning Electron Microscopy The Journal of Physical Chemistry CAnja Müller,†Thomas Heinrich,†Sven Tougaard,‡Wolfgang S. M. Werner,§Martin Hronek,§Valentin Kunz,†Jörg Radnik,†Jörg M. Stockmann,†Vasile-Dan Hodoroaba,†Sigrid Benemann,†Nithiya Nirmalananthan-Budau,‖Daniel Geißler,‖Katia Sparnacci,⊥and Wolfgang E. S. Unger*,†

R. La Spina, V. Spampinato, D. Gilliland, I. Ojea-Jimenez, G. Ceccone, Influence of different cleaning processes on the surface chemistry of gold nanoparticles, Biointerphases 12 (2017) 031003.

C. J. Powell,*† W. S. M. Werner,‡ A. G. Shard,§ and D. G. Castner Evaluation of Two Methods for Determining Shell Thicknesses of Core-Shell Nanoparticles by X-ray Photoelectron Spectroscopy, J Phys Chem C Nanomater Interfaces. 2016 Oct 6; 120(39): 22730-22738.
. doi: 10.1021/acs.jpcc.6b07588

Andrey Shchukarev Madeleine Ramstedt, Cryo-XPS: probing intact interfaces in nature and life First published: 18 April 2016 Surface and interface analysis https://doi.org/10.1002/sia.6025

JiříŠkvarlaaMáriaKaňuchováaAndreyShchukarevbAnnaGirováaIvanBrezánia Cryo-XPS - A new technique for the quantitative analysis of the structure of electric double layer at colloidal particles? Colloids and Surfaces A: Physicochemical and Engineering Aspects Volume 586, 5 February 2020, 124234

Reviewer #5:

Manuscript Summary:

Dear authors

The paper deals with a very important and complex problem of nanoparticles preparation for XPS and ToF-SIMS analysis. The paper is quite detailed and the different protocols presented well described and discussed. However, in my opinion, some major points have to be addressed before

publications

Major Concerns:
The major concern is related to the peculiar properties of the nanomaterials and the detailed description of the used protocols. In particular, many studies have shown that each type of nanoparticle could have a different behavior depending upon synthesis, handling, storage and transport [see for instance D. R. Baer, et al., Preparation of nanoparticles for surface analysis, In " CHARACTERIZATION OF NANOPARTICLES - Measurement Processes for Nanoparticles" V.-DAN HODOROABA W. E.S. UNGER A. G. SHARD (eds), Elsevier inc, ISBN: 978-0-12-814182-3, p. 295, (2020)]. For this information about provenance, synthesis protocol, eventual ageing issues and storage conditions are of a paramount importance to select the preparation protocols.The authors mention this in the introduction, but not in the discussion giving the impression that the presented protocols can be applied to all nanoparticles only depending upon their status.
In fact, many studies have shown that a detailed standard protocol for surface analysis valid for all nanomaterials/nanoparticles is not easy to achieve and adaptations of each step are most of the time unavoidable. For instance in ch 2.1 page 6 it seems that the detailed value gives (e.g. 15 mg, 3000rpm...) should be used in all cases. This is misleading. We agree completely with this remark of the reviewer. To avoid any misunderstanding we add an appropriate remark at the end of the manuscript and in the representative results and protocol section.
Similarly the preparation of the nanoparticles in powder form by pressing or the substrate cleaning. The proposed steps are suitable but not mandatory. In some cases cleaning Si wafer with ultrapure water, hexane, acetone and ethanol (5 min in ultrasound bath) can be enough to obtain a suitable substrate. The same applies to UV/ozone or plasma cleaning steps: they may help in some cases such as that illustrated but also be neutral (no help) or even deleterious by rendering the substrate too hydrophilic. This point has been addressed briefly in the Representative Results.

Another example of possible misunderstanding is the sample preparation by pressing in case of nano-powders. Some nanoparticles can be strongly affected by pressing them too strongly (e.g. Ceria TiO2). Could the authors address this point? This point has been addressed in the Discussion.
In general, I would suggest the authors to indicate which steps they consider mandatory and which ones only recommended or suggested given the particular cases illustrated. This has been addressed briefly in the introduction and representative results.

Although the example reported are well described and illustrated in the case of fig 7 some concern related to the substrate contribution remains. Although the O 1s peak is likely related only to the Si signal (that is quite low at high coverage), the quantitative analysis can lead to wrong values because of the contribution of hydrocarbon present onto the substrate even after the careful cleaning protocol (fig1). Can the author discuss more in detail this result? We have added a discussion about the influence of adventitious carbon in ll. 540 – 543 and 548 – 549.

Minor Concerns:
suspension purification (2.2). The authors indicate that dialysis can be more dangerous than centrifugation and resuspension. However, in the discussion (page 15) they suggest to avoid agglomeration. This is unclear because in many cases of nanoparticles stabilized by additives and surfactants it is difficult to avoid agglomeration or aggregation. Moreover, the drop casting procedure shown in fig 3 clearly show the nanoparticles agglomeration/aggregation.
In the case of dialysis it is important to know the membrane composition and surface treatment to avoid sample contamination. Which dialysis membrane were used? How they were cleaned?
avoiding agglomeration/aggregation and producing monolayer samples will limit te use of XPS to nanoparticles with a diameter larger than 10nm (upper limit of analysis dept).. Can the authors discuss this point? The experimental section has been modified to include preparation of the dialysis tube. The details of the membranes used are described in the Table of Materials. As discussed previously, the experimental values used in this paper are exemplary and the method should be

modified and validated for different nanoparticle types. We are not aiming to produce monolayers of nanoparticles, but rather a layer of uniform coverage, sufficient thickness to avoid substrate interference but sufficient thinness to avoid charging effects.

Table 1: how the analysis depth and lateral resolution of XPS were determined? Why the XPS depth profiling is considered excellent for insulating materials? in fact it depends upon the configuration and the erosion gun used (single Ar beam is definitely not good for profiling a polymer nano film
Thank you for this valuable comment. We have modified table 1.

Fig 9: It seems that the reduction signal is related to other peaks (contaminants or additives) rather than TiO fragment. Please explain. This has been described in the text; the other peaks have been reduced compared to the TiO peaks.

Thank you for the attention
Regards