Section 1

**Physical-Chemical properties**

# Test Guideline No. 126

## Determination of the Hydrophobicity Index of Nanomaterials Through an Affinity Measurement

4 July 2023

**OECD Guidelines for the Testing of Chemicals**

BETTER POLICIES FOR BETTER LIVES

**OECD/OCDE** **126**

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# OECD GUIDELINE FOR THE TESTING OF CHEMICALS

## Determination of the Hydrophobicity Index of Nanomaterials Through an Affinity Measurement

1. This Test Guideline (TG) describes a method to determine the hydrophobicity index ($Hy$) of nanomaterials (NMs), through an affinity measurement. By measuring their binding rate to different engineered surfaces (collectors), $Hy$ expresses the tendency of the NMs to favour the binding to a non-polar (hydrophobic) surface because of its low affinity for water. The method applies to NMs dispersed in an aqueous medium or to NM powders after their dispersions in aqueous medium, with or without a surfactant, using a recommended protocol.

2. Hydrophobicity is defined as "the association of non-polar groups or molecules in an aqueous environment which arises from the tendency of water to exclude non-polar molecules" (IUPAC Gold Book: https://goldbook.iupac.org/terms/view/HT06964). It then represents the tendency of a substance to repel water. The degree of hydrophobicity of a macroscopic flat surface can be determined by measuring the contact angle (CA) between water droplets at the surface of a solid and the surface of the solid. In general, a hydrophobic flat surface is defined by a CA higher than 90°, a conventional cut-off value used to distinguish hydrophobic from hydrophilic surfaces (1). A few methods are described for characterising the hydrophobicity of NMs, for example dye adsorption assays, contact angle, and hydrophobic interaction chromatography. However, these methods do not enable a quantification of the hydrophobicity of NMs, pose issues of reproducibility and technical difficulties or they require a large amount of material. Extensive reviews of these methods are available in the literature (e.g. 2,3)).

3. Several legislative frameworks, including the EU REACH Regulation 1907/2006, require reporting the octanol/water partitioning coefficient ($K_{ow}$), an indicator of the fate and transport of a chemical in the aquatic compartment and a key parameter of environmental exposure models or for human risk assessment. However, As the behaviour of NMs suspended in fluids may be governed by kinetic rather than thermodynamic factors (4), both the OECD Working Party on Manufactured Nanomaterials (WPMN) (e.g. (5,6, 7)) and the research community (e.g. NANoREG 2015 (8)) concluded that the existing TGs for $K_{ow}$ measurement (TG 107, 117 and 123) are not applicable to nanomaterials. In addition, the European REACH guidance states that $K_{ow}$ is relevant for the dissolved fraction for nanomaterials with water solubility <100 mg/L, but not for the particulate fraction. The OECD WPMN pointed out that it might be necessary to develop other procedures to acquire information on the behaviour of NMs in fluids (9). Hydrophobicity is currently listed as one physicochemical parameter in the "OECD Physical-Chemical Decision Framework for Manufactured Nanomaterials” (10). Interaction of nanomaterials with aquatic and terrestrial organisms (incl. bioaccumulation and persistence) and to human cell surfaces (incl. transport into the human body through skin and lungs, and consequent accumulation in tissues/organs or clearance from blood circulation) is a complex process depending on many factors, involving the NM’s properties as well as its microenvironment. Among other parameters, hydrophobicity could provide information on the affinity of NMs to those matrices and organisms (10). However, development of a quantitative property-property relationship between e.g., hydrophobicity and fate model input parameters such as the attachment affinity

would require more data on both aspects. The use of this TG will facilitate the building of the necessary knowledge base.

4. The method described in this TG aims to determine the hydrophobicity index ($Hy$) of the NMs as dispersed in an aqueous sample (taking into account all treatments that would influence their properties, including coatings) or after dispersion of the powder following a recommended protocol. $Hy$ represents the tendency of NMs to bind to hydrophobic surfaces, providing information on the affinity of NMs to natural matrices and organisms.

5. The original proposal for this TG was based on two articles published by Desmet et al. (11) and Valsesia et al. (12) and validated in an inter-laboratory study between 2019 and 2021 (13).

## INITIAL CONSIDERATIONS AND LIMITATIONS

6. The TG was developed for the determination of the hydrophobicity index of NMs as dispersed in an aqueous medium. If a NM has undergone a surface treatment, the new surface properties are considered the intrinsic property of the tested material. The determination of $Hy$ applies to the material as a whole, with or without surface modification, and not only to its core formulation. However, a surfactant can be added to permit the dispersion of highly hydrophobic NMs, following a recommended protocol. The use of such an additive would partially modify the NMs' surface properties as compared to the pristine material. To take the influence of that modification into account, decreasing concentrations of surfactant (i.e. different surfactant/NMs surface coverage) should be used in a test series, and the measured $Hy$ will be extrapolated to the theoretical index without surfactant ([surfactant] = 0). In the case of NMs in powder form, the dispersion protocol depends on the material to be tested and should guarantee the stability of the resulting dispersion, without modifying the surface properties of the NM (including coating if present on the powder). A decision tree for the treatment of the NMs' sample is presented in **Figure 1**. The prerequisite of dispersion stability is met if the concentration of the NMs in the dispersion is equal or higher to 90% of the initial concentration after a period of 6 hours from completion of the dispersion procedure used. This value of dispersion stability is in accordance with OECD TG 318 (Dispersion Stability of Nanomaterials in Simulated Environmental Media). For determination of the hydrophobicity index, an indicative concentration of $10^6$ to $10^9$ particles/mL is advised. This range generally permits the stability of the colloid and avoids the saturation of the scattering signal in dark-field microscopy while maintaining a countable number of particles to obtain statistically relevant results.

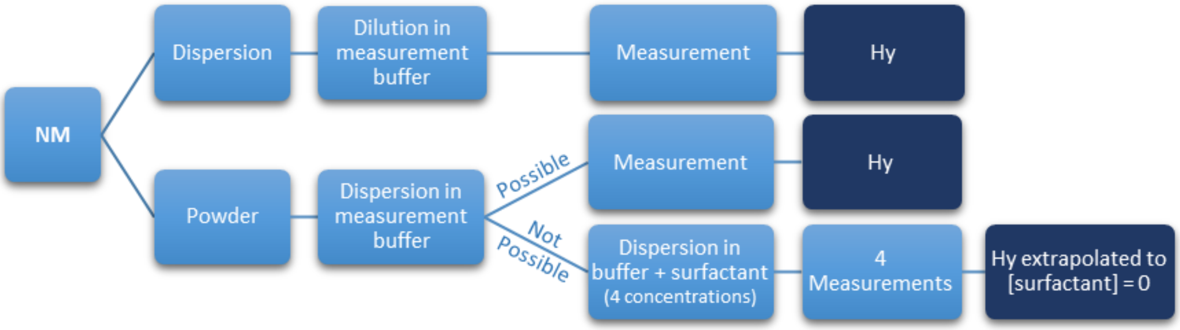

**Figure 1.** Decision tree for the application of the method of this TG.

7. The method applies to NMs that can be detected with a dark-field microscope, without restriction on the composition. A limitation depending on the relation between the NM's light scattering intensity and

size has to be considered: a lower size limit is estimated around 10 nm for metallic NMs and 50 nm for NMs having a low scattering efficiency, with the use of an enhanced dark-field microscope (14,15). The method is not limited to spherical particles. However, the method has not been validated with fibers, and an aspect ratio of 1/3 would be a recommended limit. For soluble NMs, the reduction in size during the measurement time should not be critical, meaning that the NM should still be detectable by dark field microscopy at the end of the measurement. Information on the dissolution behaviour of the NMs should be available and reported. A confirmation step for the applicability of the method for the tested nanomaterial (considering parameters such as size, shape, and solubility) is to detect and measure by dark field microscopy the effective adsorption of the NMs at a concentration of $10^9$ particles/mL on the hydrophilic positively charged collector (or on the hydrophilic negatively charged collector for positively charged NMs) after a contact time of the sample of 12 min. The determined hydrophobicity index $Hy$ of soluble materials will be a consequence of its surface properties as a result to its interaction with the dispersion medium. The following materials were considered in the inter-laboratory exercise: naked polystyrene particles, gold nanoparticles stabilised with citrate, carboxylate polystyrene particles, hydrophobic $TiO_2$ nanoparticles stabilised with natural organic matter and the commercial food additive $TiO_2$ (E171).

## PRINCIPLE OF THE TEST

8. The test enables the quantification of the hydrophobicity index ($Hy$) of the NMs by measuring the binding rate of the dispersed NMs to different collectors. The collectors are surfaces that are engineered to display different properties. The parameter $Hy$ is a direct measurement of the tendency of the NM to bind to a hydrophobic surface instead of remaining in the water phase. The test uses three different collectors. The hydrophobic collector is the one that measures the hydrophobicity of the NM, i.e., involving hydrophobic interactions between the NMs and the collector. The two other collectors are hydrophilic and positively and negatively charged and play the role of reference and negative control. For NMs that are negatively charged, the hydrophilic positively charged collector is the reference, to which the binding rate is maximised by favouring electrostatic interactions. For positively charged NMs the hydrophilic negatively charged collector plays this role. The binding rate to the reference collector is only limited by the NM's transport to the surface. The other collector (of the same surface charge as the NM) is the negative control. The hydrophobicity index is defined as the logarithm of the ratio between the NM's binding rate on the hydrophobic collector and the NM's binding rate on the reference collector, the hydrophilic one on which the binding rate is maximised.

9. The collectors are engineered to display different hydrophobicity and surface charges. They present the following properties:

- a very low surface roughness (<< the NM size)
- a certain value of the polar component of the surface free energy as described below
- a certain ζ-potential in the measurement medium as described below

10. According to the Owens–Fowkes–Wendt theory (16,17) the total surface energy of a solid is the sum of the dispersive component (taking into account the non-polar interactions), called $\gamma_{LW}$ (Lifschitz–van der Waals component), and of a polar component, called $\gamma_{AB}$ (acid–base component). Solid materials with low $\gamma_{AB}$ are considered to be "hydrophobic". The increase of the $\gamma_{AB}$ of a solid corresponds to an increase of its hydrophilicity. The hydrophobic collector can be based on a fluorocarbon coating as in the proficiency test, providing a very low $\gamma_{AB}$. The $\gamma_{AB}$ can then be increased by the addition of layers of hydrophilic material.

11. The NMs already dispersed in an aqueous medium are subsequently diluted in Phosphate Buffer (PB) before performing the measurements. In the case of powder form, the NM is dispersed in PB. A surfactant can be added to favour the dispersion of highly hydrophobic NM. In this case, a series of 4 measurements using decreasing concentrations of surfactant (with the minimum concentration that enables to obtain a stable dispersion) should be performed. The $Hy$ value measured for each concentration permit to extrapolate the $Hy$ value of the NM without surfactant ($Hy_x$ at [surfactant] = 0). The dispersion is deaerated and then injected on the collectors by means of a liquid cell where it will be transported to the surface. The number of particles binding to the collector as a function of time (binding rate) determines their affinity to the collector. The imaging of bound particles as a function of time is done by means of dark-field microscopy and a camera. An image analysis software is used to automatically detect, count the particles and build the binding curves. The bound particles are distinguished from the ones not bound by tracking their position on the image sequence: the ones moving from one frame to another are not counted. The test returns the number of particles binding to the different collectors as a function of time.

12. These experimental data can initially be used to qualitatively assess the NM hydrophobicity ((11,12)). In principle, the closer the binding rate to a hydrophobic collector is to the reference collector the higher the NM hydrophobicity is, as a result of hydrophobic interactions.

13. The binding rate of the NMs to the different collectors is regulated by the XDLVO forces (Extended Derjaguin, Landau, Verwey and Overbeek forces, where Hydrophobic forces are added to the DLVO model) and kinetics limitations imposed by the diffusion of the NMs (18). The XDLVO interaction energy between NMs and surface determines the formation of an energy barrier inhibiting the binding. The XDLVO theory applies to stable NM dispersions in aqueous samples.

14. When the energy barrier is comparable with the thermal energy, the NM is able to bind to the surface in a stable thermodynamic state. This happens when Van der Waals or hydrophobic forces can counterbalance the electrostatic repulsion. When the electrostatic forces are attractive, the only factor limiting the binding rate of the particles to the surface is the transport of the particles. The reaction balance can be written as:

$$[NM] + [S] = [NM - S]$$

Where $[S]$ is the binding site on the surface and $[NM - S]$ is the complex nanomaterial-binding site. The equilibrium constant of the reaction is the proportion of the products and the reactants:

$$K_{eq} = [NM - S]/([NM] + [S])$$

15. The equilibrium constant $Keq$ cannot be determined since the reaction occurs only in one direction (4). Thus, it is impossible to determine the interfacial Gibbs free energy of adhesion using a single surface. The thermodynamic relation between the Gibbs free energy and the equilibrium constant is expressed by the equation:

$$\Delta G0 = -kTlnK_{eq}$$

Where $k$ is the Boltzmann constant and $T$ the temperature. On the other hand, $\Delta G0$ is the limiting parameter that inhibits the binding of NMs to the surface. According to the Boltzmann distribution the binding rate on a surface is:

$$v = v_{max}e^{-(\Delta G0/kT)}$$

where $vmax$ is the maximum binding rate (for a spontaneous reaction limited only by the transport of the particles to the surface). Thus, $\Delta G0/kT$ is:

$$-\Delta G0/kT = ln(v/vmax)$$

16. A Hydrophobicity index ($Hy$) is defined, based on the affinity of the material for a hydrophobic solid phase (the hydrophobic collector) as compared to a hydrophilic one which favours the electrostatic attraction forces (the reference collector, negatively or positively charged according to the measured NM’s surface charge, on which the binding velocity is maximum,) as described in paragraph 8. $Hy$ can be expressed as:

$$Hy = log(v_{Hy}/vmax)$$

$Hy$ is a direct measurement of the tendency of the NM to bind to the hydrophobic collector, rather than staying in the aqueous phase, which is directly related to the NMs hydrophobicity character.

17. In the method, $v_{Hy}$ is the binding rate on the hydrophobic collector, which is directly related to the hydrophobicity of the NM, i.e., where binding is driven by hydrophobic interactions between the NMs and the collector. $vmax$ is measured on the collector on which the binding rate is maximised by favouring electrostatic interactions. NMs being in most cases negatively charged in the measurement conditions, the binding rate to the hydrophilic positively charged collector is only limited by the NMs transport to the surface. For positively charged NMs the binding rate on the hydrophilic negatively charged collector is used instead.

18. Since $vHy/vmax$ is always < 1, the $Hy$ values are always < 0. $Hy$ values close to zero, indicate a high affinity to the hydrophobic collector (as show in figure 2), i.e., particles behaving as hydrophobic chemicals. As an indication, a NM is considered hydrophobic when the value of this index is between 0 and -1, while a hydrophilic one shows values lower than -1. The closer to 0 the $Hy$ is, the more hydrophobic the NM is.

19. As described in paragraph 11, in the case of a hydrophobic NM for which a surfactant is used to perform the dispersion, a value of $Hy$ is calculated for each concentration. Each $Hy$ that is measured corresponds to the hydrophobicity index of the NM whose properties are modified by the surfactant. These values are then plotted to extrapolate the value of $Hy$ without surfactant by linear regression ($Hy_x$ at [surfactant] = 0). This extrapolation is done with a concentration range in which $Hy$ is linearly proportional to the surfactant concentration, starting from the minimum concentration allowing to obtain a stable dispersion. If it is not the case and the plot is not linear, the surfactant concentrations should be decreased to avoid saturation.

20. The principle of the method is illustrated in **Figure 2**.

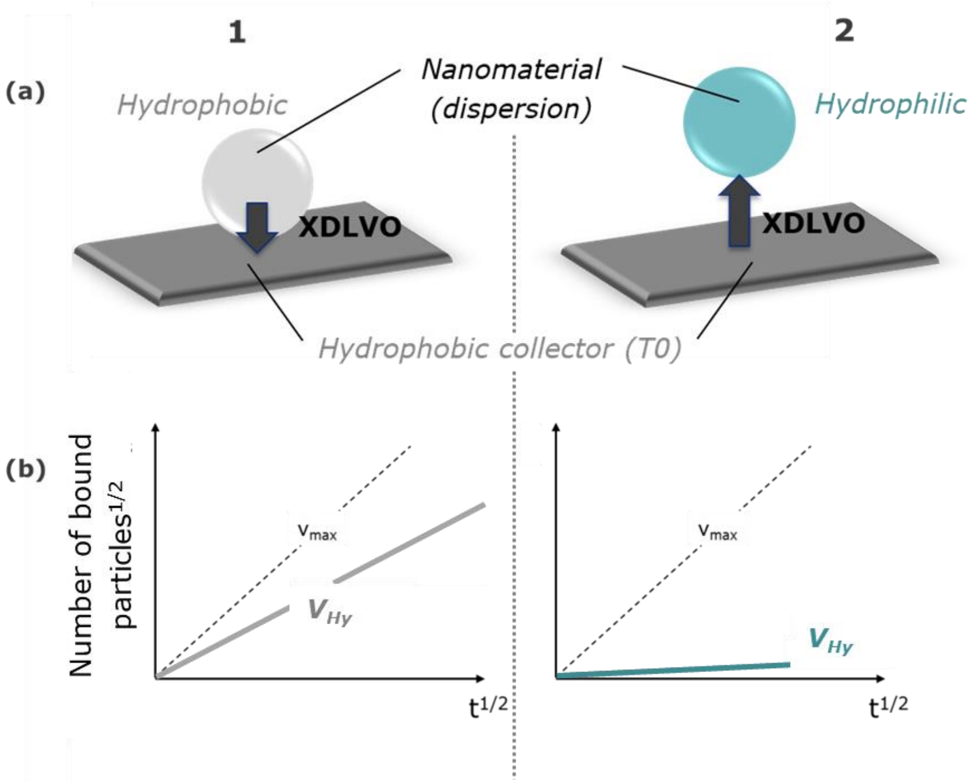

**Figure 2.** Principle of the method for the quantification of the hydrophobicity index of NMs. The NMs in stable dispersion are allowed to approach the collector by diffusion. (a). The energy balance described by the XDLVO theory drives the stable binding of the hydrophobic NM to the hydrophobic collector (situation in column 1), while the hydrophilic NM is repelled (situation in column 2) (b) The square root of the number of bound particles is presented as a function of the square root of time. The binding rate on the hydrophobic collector $vHy$ is plotted for the NMs with respect to the maximum binding rate $vmax$ occurring when the electrostatic forces are dominating. The closer the slope for $vHy$ is to the slope for $vmax$, the more hydrophobic a particle is.

## DEMONSTRATION OF PROFICIENCY

21. As a demonstration of proficiency, a commercially available standard material should be used on the collectors as a control. A hydrophobic noble metal NM such as gold nanoparticles (AuNP) of 70 nm can be used. The NM should be dispersed in phosphate buffer (PB) 10 mM, pH 7 at a concentration of $10^9$ particles/mL and tested according to the test procedure described below (paragraph 29). A binding of around 1 particle per 1 $\mu m^2$ should be obtained after 12 minutes of incubation on the hydrophobic collector. This test defines the parameters of the setup (microscope and camera).

22. In these conditions the hydrophobicity index of the material stated in the previous paragraph should be > -1.

## VALIDITY OF THE TEST

23. For the test to be valid, the following criteria apply:

The physico-chemical properties of the dispersion of the NMs to be measured meet the following criteria:

The NMs are in a stable dispersion in phosphate buffer at 10 mM at pH 7 (with or without the use of surfactant). The prerequisite of stable dispersion is met if the concentration of the particles in the dispersion is equal or higher than 90% of the initial concentration after a period of 6 hours from completion of the dispersion procedure used, the value of dispersion stability is in accordance with OECD TG 318. Mass concentration of the dispersion should be measured in the top 0.5-1 cm of the test vial. A concentration of $10^6$ to $10^9$ particles per mL is advised. The physico-chemical properties of the surface of the collectors meet the following criteria:

- Root Mean Square roughness < 2 nm
- Surface free energy components (polar and dispersive) as described in **table 1**
- Stability in time (no change in the binding rate over 12 min).

**Table 1**

| Surface | Example of coating | Roughness (RMS) | Contact Angle in water (deg) | $\gamma_{AB}$ (mJ/m$^2$) |
|---|---|---|---|---|
| Hydrophobic | Fluorocarbon or alkyl-silane (18) | < 2nm | > 100 | < 1 |
| Positive | 5 polyelectrolyte layers, ending with PDDA | | < 70 | > 10 |
| Negative | 4 polyelectrolyte layers, ending with PSS | | < 70 | > 10 |

## DESCRIPTION OF THE METHOD

### *Test preparation*

#### *Apparatus and chemical reagents*

24. Standard laboratory equipment, including but not limited to:

    - Calibrated pipets for sample preparation and dispensing (5 mL, 1 mL, 0.1 mL, 0.02 mL volume)
    - Microscope glass slides used as collectors
    - Commercial bottomless channel slide with a self-adhesive underside to which own substrates can be mounted, or homemade microfluidic channels
    - Optical microscope equipped with a camera for dark-field observations

25. Materials:

    - Water ($H_2O$) – ultrapure de-ionized water (18 MΩ resistivity) is used for all preparations and dilutions
    - Phosphate Buffer (PB) 10 mM, pH = 7
    - NM in the form of dry powder or aqueous dispersion
    - Surfactant (e.g. NOM), diluted in PB, used only for hydrophobic NMs non dispersible alone

#### *General conditions*

26. All experiments should be performed in triplicate at 20 +/- 1°C.

### *Preparation of nanomaterial stock dispersion*

27. The material needs to be dispersed in an aqueous medium and deaerated. It is recommended to use a dispersion protocol that guarantees the dispersion to meet the conditions of stability without modification of the surface properties and chemical nature of the NMs. For instance, some very high-power sonication protocol could remove the coating from the NM's surface, and this should be avoided since it would alter the properties of the NM. Surface treatments are not considered here a modification, but an intrinsic property of the tested material, the method is applicable to these surface treated NMs. The NMs already dispersed in an aqueous medium are diluted in PB. In the case of powder form, the NM is dispersed in PB. A surfactant can be added to favour the dispersion of highly hydrophobic NMs that are not dispersible otherwise. In this case, a series of 4 increasing concentrations of surfactant should be prepared, starting from the minimum concentration necessary to obtain a stable dispersion. Each will be measured following the same protocol.

28. Three different surfaces or collectors (hydrophobic, positive, and negative) characterised by the parameters specified in **Table 1** should be prepared on a transparent substrate or purchased from functionalised slide providers. An example of preparation protocol is given in the **Annex**.

### *Mounting of the microfluidic cell*

29. For easier setting of the focus on the surface in dark-field microscopy, the collector needs to be cleanly scratched in a horizontal line from side to side lengthwise on the middle of the slide (across the width) as shown in figure 3. Each substrate is then mounted with microfluidic cells of controlled volume, surface, and height. Commercially available multi-channel sticky slide (e.g., Ibidi, 6-channel or Chipshop 16-channel sticky slides) or homemade microfluidic cells can be used for the measurement according to the conditions presented in the test procedure.[1]

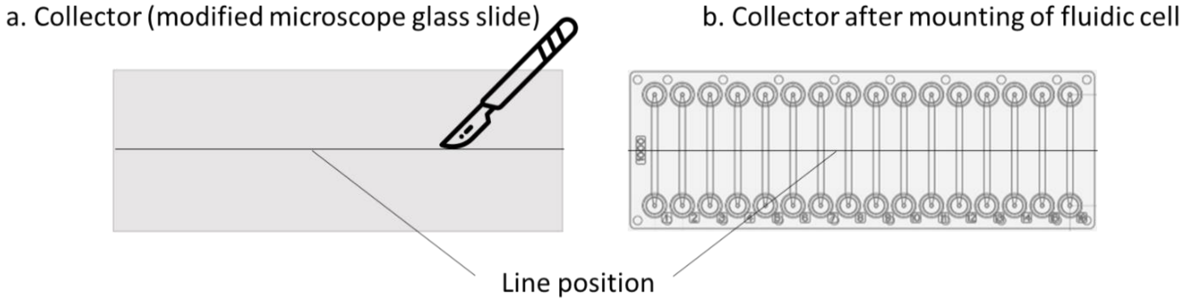

**Figure 3.** Practical design for collectors' preparation. **a**. the microscope glass slide providing the hydrophobicity and charge properties is cleanly scratched in a horizontal line on the middle of the slide, **b**. a slide of fluidic cells is mounted on the top of the collector, the presence of the line in the middle of fluidic channels will permit an easier setting of the focus of the microscope.

### *Test procedure: Measurement of nanomaterial binding to collectors by dark-field microscopy*

30. In order to measure the NMs binding rates on the different collectors, dark-field microscopy videos (image sequences) are recorded. It is also possible to use phase contrast and differential interference contrast microscopy, as well as any technique capable of single particle tracking analysis. The imaging is done with the following parameters: 10x magnification, Numerical Aperture, NA = 0.15. The field of view

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[1] The development of the test was performed with Ibidi 6-channels and Chipshop 16-channels sticky slides but any commercially available multi-channel slide or homemade microfluidic system can be used.

of the microscope should be minimum of 0.4 mm. The channel on which the measurement is done should be centred to avoid any effect of the border. The focus, illumination and contrast parameters should be adjusted based on the line made on the collector (see **figure 3**). The image recording software should be configured to acquire at least one image every 30 seconds for 12 min after the start of sample injection.

31. A volume of sample equivalent to the total volume of the channel and its reservoirs (e.g., 20 µL to be injected in a 16-channel slide as the ones provided by Chipshop) should be injected with a pipette in order to completely fill the channel. The image acquisition should start immediately after injection and last for 12 min.

32. In principle, the analysis can also be done with techniques providing a global signal of the NMs binding on the collector as long as it allows a kinetics analysis (e.g. Quartz Crystal Microbalance or Surface Plasmon Resonance (19, 21, 22). As for the microscopic method, the comparison of the kinetics of binding to the different collectors will permit to determine the hydrophobicity index of the nanomaterial. The comparison of methods has however not been assessed in inter-laboratory tests yet.

## DATA AND REPORTING

### *Data treatment*

33. The measurement of the number of particles binding to the collectors per frame is done using the free software ImageJ and its Trackmate plugin[2]. In dark-field microscopy, particles are brighter than the background. Particle detection is performed by thresholding of the pixel intensity distribution. The analysis then consists in an automatic detection of the particles for each frame, and the tracking of their positions within the sequence of frames. The objects not moving for more than two frames in a row counting back from the last frame are counted. This automatic calculation quantifies the number of bound particles per frame. The time is calculated by multiplying the frame number by the frame delay (30 seconds), to reach the number of bound particles per unit of time, corresponding to the velocity of binding of the material on the collector. The resulting text file can be imported in a data treatment software for fitting.

34. The number of bound particles on each collector can be plotted as a function of the square root of time to obtain a linear trendline as shown in **figure 4**. This function is calculated for each collector. It can be fitted with a linear function using the fitting wizard available in Microsoft Excel® or in any kind of data treatment software. The fit returns the slope of the line, called v, representing the velocity at which the NMs bind to the different collectors.

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[2] It can be freely downloaded from *https://imagej.net/download.html*

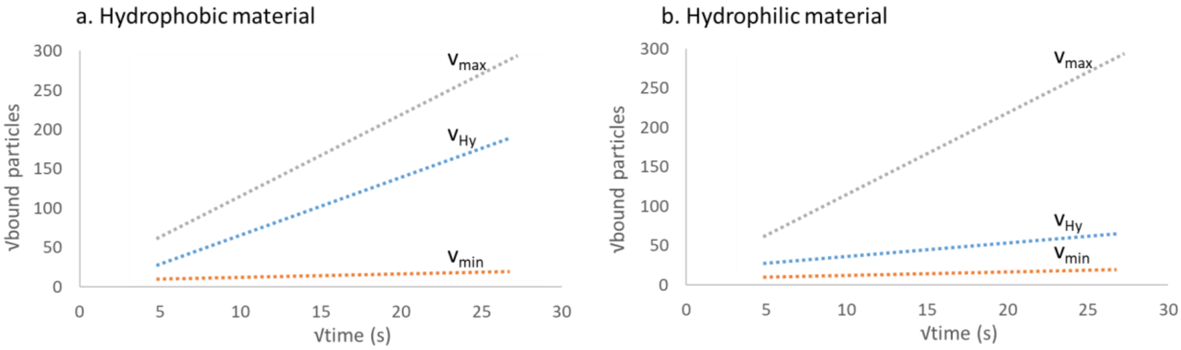

**Figure 4.** Graphical example of the result of the image analysis for (a) a hydrophobic and (b) a hydrophilic material. The linear trendlines are obtained from the plot of the square root of the number of bound particles as a function of the square root of time in seconds. The slope of the trendlines obtained for the different collectors. $v_{Hy}$ represents the binding rate on the hydrophobic collector, $v_{max}$ is the maximum binding rate occurring on the hydrophilic collector with a surface charge opposite to the one of the NM (when the electrostatic attractive forces are dominating) and $v_{min}$ is the binding rate on the hydrophilic collector with the same surface charge as the NM.

35. The binding velocities are used for calculation of the hydrophobicity index $Hy$, defined as the logarithm of the ratio between the values of the binding rate on the hydrophobic collector $v_{Hy}$, and on the hydrophilic collector presenting dominant electrostatic attraction $v_{max}$ (hydrophilic positively charged collector for negatively charged material as it is generally the case in the medium of measurement and hydrophilic negatively charged collector for positively charged material), as detailed in the equations in paragraphs 16-18. Indicatively, a NM is considered hydrophobic when the value of this index is between 0 and -1, while a hydrophilic one shows values lower than -1. The closer to 0 the more hydrophobic a NM is.

36. In the case of a hydrophobic NM for which a surfactant is used to perform the dispersion, a value of $Hy$ is calculated for each concentration of surfactant. Each $Hy$ corresponds to the hydrophobicity index of the NM whose properties are modified by the surfactant. These values are then plotted to extrapolate the value of $Hy$ without surfactant by linear regression (**figure 5**). This extrapolation is done with a concentration range in which $Hy$ is linearly proportional to the surfactant concentration. If it is not the case and the plot is not linear, the surfactant concentrations should be decreased to avoid saturation.

### *Analysis of data / Evaluation of test results*

37. The first qualitative assessment comes from the dispersibility of the NM into the test medium. If this condition cannot be achieved, the NM is considered highly hydrophobic and $Hy$ is assumed to be close to 0. To quantify $Hy$ in that case, a surfactant is added to favour the dispersion of highly hydrophobic NMs, as described in paragraphs 27 and 36.

38. The calculation of $Hy$ using the logarithm of the ratio of $v_{Hy}/v_{max}$ enables a quantitative assessment of the hydrophobicity index of the measured NM. The more negative the $Hy$, the less hydrophobic is the NM. An indicative scale of measured materials is given in Figure 5.b.

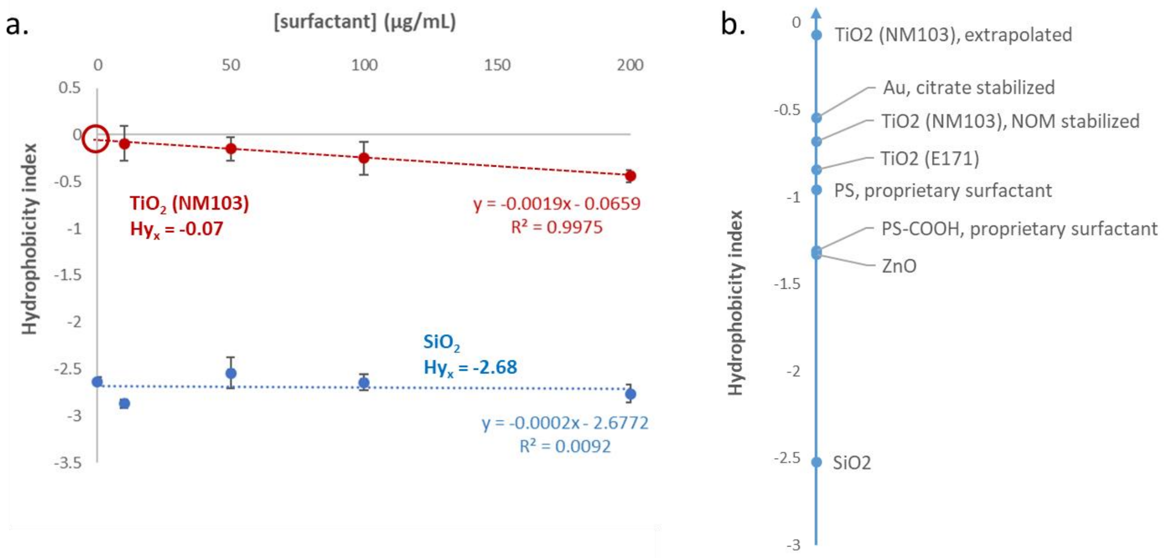

**Figure 5.** a. Example of extrapolation of $Hy$ for two NMs: a hydrophobic NM non-dispersible alone in an aqueous medium (NM103, hydrophobic coated $TiO_2$), and a hydrophilic NM dispersible without surfactant ($SiO_2$), based on measurements performed using decreasing concentrations of surfactant. $Hy_x$ is the extrapolated value to [surfactant] = 0. b. Indicative scale of the Hydrophobicity index ($Hy$) for materials tested with the method. The closer to 0, the more hydrophobic is the material.

## Test report

The test report should describe, but not be limited to, the following elements.

### *Test conditions*

*For the collectors:*

- source, lot number, limit date for use
- values of the contact angle
- values of the other parameters (optional when following the proposed preparation protocol)

*For the microfluidic cell:*

- source, lot number, limit date for use

*For the dispersant medium*

- limit date for use
- measurement of pH, and salt concentration
- type and concentration of surfactant (if used)

*For the test particles and the standard reference particles:*

- source, lot number, CAS number, limit date for use
- concentration in number of particles per ml
- size distribution (recommended)
- dispersion protocol
- dissolution behaviour

*For the dark-field microscope:*

- objective
- numerical aperture

### *Results*

### *Results of the standard reference*

- binding rates for the hydrophobic collector $v_{Hy}$, and for the two hydrophilic collectors $v_{min}$, $v_{max}$
- qualitative hydrophobicity
- hydrophobicity index

### *Results of the tested nanomaterial*

- graph of the square root of the number of bound particles vs. square root of time
- binding rates for the hydrophobic collector $v_{Hy}$, and for the two hydrophilic collectors $v_{min}$, $v_{max}$
- qualitative hydrophobicity
- hydrophobicity index

### *Discussion of results*

### *Conclusion*

## ABBREVIATIONS

| | |
|---|---|
| **CA** | contact angle |
| $\Delta G^{max}$ | energy barrier |
| $\gamma_N^{AB}$ | polar component of the surface free energy of the particle |
| $Hy$ | Hydrophobicity index |
| **JRC** | Joint research centre |
| $K_{ow}$ | octanol/water partitioning coefficient |
| **kT** | product of Boltzmann constant and temperature |
| **NM** | nanomaterial |
| **nm** | nanometre |
| **PDDA** | poly(diallyldimethylammonium chloride) |
| **PE** | polyelectrolyte |
| **PSS** | poly(sodium 4-styrenesulfonate) |
| **RMS** | root mean square |
| **SOP** | Standard Operating Procedure |
| **TG** | Test Guideline |
| $v$ | velocity of adsorption |
| $v_{Hy}$ | velocity of adsorption on the hydrophobic collector |
| $v_{max}$ | maximum velocity of adsorption |
| **XDLVO** | eXtended Derjaguin Landau Van Overbeek |

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## ANNEX. EXAMPLE OF PREPARATION AND CHARACTERISATION OF THE COLLECTORS

### Apparatus and chemical reagents

- Classical microscope glass slides or fluorocarbon coated glass slides for collectors preparation
- Optical tensiometer to verify the contact angle of the prepared collectors
- Commercial bottomless channel slide with a self-adhesive underside to which own substrates can be mounted, or homemade microfluidic channels
- Water ($H_2O$) – ultrapure de-ionized water (18 MΩ resistivity) is used for all preparations and dilutions
- poly(diallyldimethylammonium chloride) (PDDA) 2% solution in water for self-assembly layer-by-layer modification of the substrate
- poly(sodium 4-styrene sulphonate) (PSS) 2% solution in water for self-assembly layer-by-layer modification of the substrate

### Example of preparation of the collectors

39. Three different surfaces or collectors (hydrophobic, positive, and negative) characterised by the parameters specified in Table 1 (paragraph 23) should be prepared on a transparent substrate. Alternatively, the collectors could be purchased from functionalised slide providers.

40. The other parameters of the collectors as obtained in the conditions described below should be close to the following ones. The refractive index should be around 1.3 (not for the optical properties of the layer but as an indication of an appropriate chemical composition) and thickness approximately 100 nm, both parameters can be measured by ellipsometry for the three collectors. The ζ-potential of the collectors at pH = 7 should be negative for the "hydrophobic" surface (fluorocarbon coated) as well as for the hydrophilic "negative" one. The hydrophilic "positive" surface should have a ζ -potential close to 0. The condition of stability of the surface over time (its hydrophobicity degree) can be evaluated by measuring the droplet contact angle of the different surfaces.

41. The following protocol is given as an example for producing collectors in house according to the parameters in Table 1. It is also possible to use commercially available hydrophobic microscope slides or to prepare them using a different method (e.g. using octadecyltrichlorosilane as in J. X. H. Wong and H-Z Yu, 2013) as long as the obtained surfaces have the properties described in Table 1.

42. Classical microscope glass slides should be modified by different deposition processes in order to fabricate the collectors with different surface properties. Three glass slides are first thoroughly washed with ethanol and ultrapure water, and dried under nitrogen flow. A fluorocarbon coating is deposited to generate a hydrophobic surface (another type of hydrophobic coating based on non-fluorinated precursor can also be used, such as Alkyl-silanes). Plasma polymerisation can be performed using pure octofluorocyclobutane ($C_4F_8$) as the gas precursor at a pressure of 3.5 Pa, applying a power of 142 W for 5 min. One slide is kept unmodified after this fluorocarbon deposition step to act as the purely hydrophobic collector.

43. In order to tune the surface hydrophobicity of the fluorocarbon coated slides and to give a more hydrophilic character, a layer-by-layer deposition of two polyelectrolytes (PE) is then performed on the two other slides. The fluorocarbon coated modified substrates are dipped for 2 min in poly(diallyldimethylammonium chloride) (PDDA) 2% solution in water and in poly(sodium 4-styrene sulphonate) (PSS) 2% in water in a petri dish placed on a rocker for gentle agitation. The self-assembly deposition of each PE layer starts from PDDA (positively charged) and alternates with PSS (negatively

charged). After each step, the substrate is rinsed with ultrapure water and dried under nitrogen flow. One of the substrates is modified with 4 layers (PDDA-PSS-PDDA-PSS) and the other one with 5 (adding a last layer of PDDA). The adhesion of PE layers permits the modification of the surface free energy components of the collectors.

### *Characterization of the collectors*

44. The collectors characterization should be fully performed when a protocol different from the one proposed as an example is used for the preparation, in order to ensure that the correct parameters are obtained. When the example protocol is followed, the contact angle as shown in Table 1 is the only parameter to check.

45. The surface free energy components of the three collectors can be determined by measuring the contact angle of the solid surfaces with a polar (water) and a non-polar solution (bromonaphtalene). In brief, 2 μL of the probe liquid is dropped from a calibrated micro-syringe over each substrate (taken in triplicate) at three different locations, the nine measurements should then be averaged. The contact angle is measured after each step of the surface modification procedure.

46. ζ-potential measurements of the collectors can be performed at pH 7 to verify the surface charge in test conditions (using for example the streaming potential method, as described in Desmet et al., 2017). In the case of collectors produced in house from classical glass slides, the evaluation of the following parameters can also be performed: the thickness and refractive index of each deposited layer, which can be evaluated by Ellipsometry, and the roughness, which should be assessed by atomic force microscopy.