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Surface characterization of gold nanoparticles for nanomaterial specific toxicity screening: FT-IR method

Caractérisation de surface des nanoparticules d'or pour criblage de toxicité spécifique de nanomatériau: méthode FT-IR

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Contents

Page

Foreword

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ISO/TS 14101 was prepared by Technical Committee ISO/TC 229, *Nanotechnologies*.

Introduction

Gold nanoparticles (AuNPs) can be controlled with regard to size, shape and surface ligands, making them ideal for the study of relationships between their physicochemical properties and cytotoxicity on living bodies[1][2][3]. Among the various properties of AuNPs, surface ligand characteristics, such as the chemical composition, molecular structure and quantity of bound molecules, were found to play an important role in determining the behaviour of AuNPs, e.g. the degree of aggregation or agglomeration in solution, binding with biomolecules in cell culture media and cytotoxicity to living cells[4][5][6][7][8][9][10][11][12]. On the other hand, surface ligand modification is not always successful in the synthesis step, and the degree of ligand exchange should be identified prior to the property specific cytotoxicity test of AuNPs in order to obtain reliable and consistent results.

FT-IR (Fourier transform infrared) absorption spectroscopy is one of the most useful tools of NP surface ligand identification and quantification. By using the FT-IR method, the structures and relative quantities of ligand molecules bound to NP surfaces can be analysed[13][14][15][16][17][18][19][20]. However, the low concentrations and aqueous environment of synthesized AuNPs will complicate the interpretation of measurement results. Low concentrations of AuNPs result in small absorbance values, which can easily be influenced by background noise or the absorbance of trace impurities. Since cytotoxicity tests are performed in aqueous environments, we should analyse what is on the surface of AuNP in aqueous solutions if we want to study the effect of the surface characteristics on cytotoxicity of AuNPs. However, water molecules strongly absorb IR light over a wide frequency range, disabling IR absorption analysis on the solutes in very low concentrations. It is necessary to develop measurement guidelines by which the above issues can be minimized. In this project, we seek to develop a Technical Specification (TS) for the observation of chemical moieties bound to the synthetic AuNP in the form of dehydrated films, which can deliver the information about the molecular species bound to AuNPs when they were in aqueous solutions. Although the standardization of FT-IR measurement procedures will be the basis for this Technical Specification, a great deal of weight will also be given to the sample preparation procedure for correct FT-IR analysis.

Surface characterization of gold nanoparticles for nanomaterial specific toxicity screening: FT-IR method

1 Scope

This Technical Specification provides guidelines for the identification of the surface bound molecules using FT-IR of dehydrated gold nanoparticle (AuNPs) films both before and after nanomaterial (NM) cytotoxicity testing.

NOTE 1 AuNPs may have surface bound ligands prior to testing and may be additionally covered (or coated) with organic- or bio-molecules during the cytotoxicity test.

NOTE 2 Nucleic acids, amino acids, lipids or membrane components binding to AuNPs can be observed by FT-IR spectroscopy by detection of absorption bands corresponding to phosphodiester, amine or lipid, respectively, although the type of nucleic acids, proteins or lipid cannot be identified in detail based on IR spectra.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendment) applies.

ISO/TS 27687, *Nanotechnologies — Terminology and definitions for nano-objects — Nanoparticle, nanofibre and nanoplate*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/TS 27687 and the following apply.

3.1
attenuated total reflection mode
ATR Mode
instrumental mode of operation in which the incident angle of IR light on the crystal is adjusted to be higher than the critical angle

NOTE The light is completely reflected by the upper surface of the crystal, and the intensity of the light is attenuated through absorption by materials covering the upper surface of the crystal. The frequency of IR light absorbed is used to identify the absorbed chemical moiety, and the fraction of light that is absorbed is used to quantitate the amount of that moiety present.

3.2
dialysis
process by which small molecules or ions diffuse through a membrane, thus causing their separation from larger molecules in solution and from suspended matter

[ISO 6107-2:2006, definition 38]

3.3
Fourier transform infrared spectroscopy
FT-IR
analytical chemical technique based on absorption of infrared radiation by chemical moieties in the specimen, used to identify and quantitate the absorbing chemical moieties

3.4
limit of detection
LOD
measured quantity value, obtained by a given measurement procedure, for which the probability of falsely claiming the absence of a component in a material is β, given a probability α of falsely claiming its presence

NOTE 1 Adapted from ISO/IEC Guide 99:2007, definition 4.18.

NOTE 2 LOD may be determined as 2,776 times the standard deviation of the measurements of 5 replicate blanks under conditions of repeatability with IUPAC recommended values of 0,05 for both α and β.

NOTE 3 See also ISO 17191.

3.5
limit of quantification
LOQ
lowest value of an analyte that can be determined with an acceptable level of accuracy and precision

NOTE 1 LOQ may be determined as 10 times the standard deviation of the photometric noise, which will give relative precision $\sigma_A/A \leq 10$ % for the minimum signal level A.

NOTE 2 See Reference [24].

3.6
molecular weight cut-off value
MWCO
molecular weight of solute that is retained by more than 90 % after 16 h dialysis

NOTE See References [25] and [26].

3.7
nano-object
material with one, two or three external dimensions in the nanoscale

[ISO/TS 27687:2008, definition 2.2]

NOTE Generic term for all discrete nanoscale objects.

3.8
nanoparticle
NP
nano-object with all three external dimensions in the nanoscale

[ISO/TS 27687:2008, definition 4.1]

NOTE If the lengths of the longest to the shortest axes of the nano-object differ significantly (typically by more than three times), the terms nanorods or nanoplate are intended to be used instead of the term nanoparticle.

3.9
nanoscale
size range from approximately 1 nm to 100 nm

[ISO/TS 27687:2008, definition 2.1]

NOTE 1 Properties that are not extrapolations from a larger size will typically, but not exclusively, be exhibited in this size range. For such properties the size limits are considered approximate.

NOTE 2 The lower limit in this definition (approximately 1 nm) is introduced to avoid single and small groups of atoms from being designated as nano-objects or elements of nanostructures, which might be implied by the absence of a lower limit.

3.10
pre-tested distilled water
DW
distilled water validated to be free from IR absorbing impurities by FT-IR measurement

3.11
relative centrifugal force
RCF
acceleration force relative to the Earth's gravity

3.12
surface plasmon resonance band
SPR
range of frequencies of absorbed light, where the absorption is the result of the collective oscillation of electrons within the near-surface region of a solid

NOTE SPR occurs in thin metal films or metallic NPs.

4 Symbols and abbreviated terms

AuNP	gold nanoparticle
IR	infrared
MW	molecular weight
SCM	serum containing media
UV/Vis	ultraviolet/visible
× *g*	Earth's gravimetric acceleration as a reference unit for the relative centrifugal force

5 Sample preparation mode

5.1 Removal of unbound molecules

5.1.1 General

Since FT-IR absorption spectroscopy measures total molecular species in the sample film, all unbound molecules that are active IR absorbing species except solvents shall be removed from the solution before preparing the sample film in order to correctly identify the molecules bound to the surface of AuNPs.

5.1.2 Dialysis

Dialysis is an efficient method for separating unbound molecules from AuNPs when membranes with adequate MWCOs are available. If used adequately, dialysis membranes reduce the concentration of unbound molecules according to the volume ratio of the sample and dialysis solutions, generally retaining more than 90 % of NPs. It is recommended that MWCO is lower than half the MW of the species to be retained, and higher than three times the MW of the species intended to pass through. Because the efficiency of dialysis membrane depends on the charge and shape of molecules, the unbound molecular removal efficiency of a dialysis membrane shall be verified before the separation of AuNPs from unbound molecules. Prior to the efficiency test, the membrane shall be tested if it is free from IR absorbing impurities. The procedure for impurity test is as follows:

a) fill the dialysis bag with 0,5 ml to 3 ml of pretested DW;

b) seal the DW containing dialysis bag with a proper clip and dialyse 16 h in a bath containing pretested DW (≥600 ml in volume);

c) clean the surface of ATR crystal or IR window by using a solvent moistened cotton swab;

d) take a required volume (2 or ≥ 200 μ l) of liquid in the dialysis bag;

NOTE Use 2 μl for the ATR method and ≥ 200 μL for the transmission method.

e) drop the sampled liquid on an ATR crystal or IR window and dry it in a dehydration chamber (see 5.2); this procedure is called "drop and dry";

f) measure the FT-IR spectrum of any potential impurities dissolved in the sampled liquid by using the procedure in 6.2 or 6.3;

g) if there is no IR band exceeding LOD in the frequency region of interest, the membrane is considered free from IR active impurities.

The membrane that is free from IR active impurities can be used for the dialysis. The procedure for the test of dialysis efficiency is as follows:

a) fill the dialysis bag with 0,5 ml to 3 ml of solution that contains only molecules to be removed by dialysis;

— molecules to be removed by dialysis correspond to surface ligands before and after exchange, or biomolecules in SCM or cell extracts for binding test;

— the concentration of sample solution is set to the maximal amount of surface ligands that can exist in the NP suspension. This value may be the same as the added amount of ligand molecules for exchange or estimated from the number of gold atoms on the surface, which can be calculated from the lattice constant[27] and the diameter of AuNP. The maximal number of ligand molecules is the number of gold atoms on the surface assuming the monolayer binding without steric hindrance among ligands.

b) seal the sample containing dialysis bag with a proper clip and dialyse 16 h in a bath containing pretested DW (≥600 ml in volume);

c) take a required volume (2 or ≥ 200 μl) of sample in the dialysis bag;

d) "drop and dry" the sample solution on an ATR crystal or IR window;

e) measure the FT-IR spectrum of sample film by using the procedure in 6.2 or 6.3;

f) take a required volume (2 or ≥ 200 μl) of sample in the bath;

g) "drop and dry" the sample solution on an ATR crystal or IR window;

h) measure the FT-IR spectrum of sample film by using the procedure in 6.2 or 6.3;

i) compare the spectra obtained in steps e) and h). If neither spectrum shows bands above LOD, repeat with an increased concentration of the sample solution. Since both samples "in the dialysis bag" and "in the bath" should be the same composition after proper dialysis, the membrane is considered validated if they both have bands higher than LOD and the intensities are the same within measurement uncertainty.

5.1.3 Centrifugation

Centrifuge method can be used for the separation of AuNPs from unbound molecules especially when the MWs of unbound molecules are too high to find a dialysis membrane with adequate MWCO. Insufficient centrifugation speeds will result in the suspension of AuNPs in the supernatant, which can be identified from SPR band absorption observed by using UV/Vis absorption spectroscopy. Excessive speeds will result in sedimentation of unbound molecules. Efficient re-suspension of AuNPs should be guaranteed among the centrifuge method to obtain sufficient concentration of AuNPs in solution for FT-IR analysis.

UV/Vis absorption spectrum can be used to provide information about efficient sedimentation and re-suspension according to the SPR band observation. If the absorption spectrum of unbound molecules is properly excluded in the SPR absorption region, degree of aggregation/agglomeration can also be estimated by the broadening of SPR absorption band of AuNPs. The procedure to determine combination of centrifugal force and time for efficient sedimentation of AuNPs without interference from unbound molecules is as follows:

a) fill a 2 ml micro-centrifuge tube with 1,5 ml of AuNP solution;

b) sediment AuNPs by centrifugation with the force and time recommended in Table 1;

c) drain as large a volume as possible of the supernatant from the tube without disturbing the pellet; measure the UV/Vis absorption spectrum of the supernatant between 400 nm and 750 nm against DW as background;

   — check if the SPR band of AuNP is observed considering the absorption of unbound molecules;

d) if the maximum SPR absorbance is higher than 0,05, which is the lower limit of UV/Vis quantification[28], repeat steps a) to c) with increased centrifugal force or centrifugation time increased by 20 %;

e) if the maximum SPR band absorbance is lower than 0,05, the centrifugal force and time is high enough for sedimentation.

Table 1 — Recommended RCF and time for sedimentation of AuNPs as a function of particle diameter

AuNP size in diameter (nm)	**Sample volume** (ml)	**RCF**	**time** (min)
5	1,5	19 000	60
15	1,5	10 000	20
30	1,5	5 000	20
50	1,5	2 000	20
100	1,5	1 000	20

The procedure to establish the upper limit of centrifugal force and time is as follows:

f) fill a 2 ml micro-centrifuge tube with 1,5 ml of solution that contains all the other components except AuNP such as free ligand solution before and after exchange, SCM or cell extracts;

g) centrifuge the tube with the centrifugal force and time established through steps a) to e);

h) take 1 ml of supernatant without including any potential sediment;

i) take a required volume (2 or ≥ 200 μ l) of the supernatant solution; “drop and dry” the solution on the ATR crystal or IR window and measure an FT-IR spectrum;

j) vortex the remaining solution in the tube and take a required volume (2 or ≥ 200 μ l) of the remaining solution; “drop and dry” the solution on the ATR crystal or IR window and measure an FT-IR spectrum;

k) triplicate steps from f) to j) as independent experiments; average the FT-IR spectra of the supernatant (Spectrum IR-1) and the remaining solutions (Spectrum IR-2), respectively;

l) compare “Spectrum IR-1” with “Spectrum IR-2”; if any absorption band in “Spectrum IR-2” exceeding LOQ is greater than 1,3 times “Spectrum IR-1”, the centrifugal force or time should be reduced to avoid sedimentation of unbound molecules. If all the absorption bands in “Spectrum IR-2” exceeding LOQ are within 0,7 to 1,3 of those in “Spectrum IR-1”, the upper limit of centrifugal force and time is established;

m) if the centrifugal force and time established through steps a) to e) are higher than the upper limit values obtained through steps f) to l), the centrifugal force and time can be reduced until they reach the lower limit where the maximum SPR absorbance of the supernatant solution is as high as 0,05.

After the centrifugal force and time have been determined, AuNPs shall be separated from the unbound molecules through the following procedure:

n) fill a 2 ml micro-centrifuge tube with 1,5 ml of AuNP solution;

o) sediment AuNPs with the centrifugal force and time determined through steps a) to m);

p) drain the supernatant from the tube in as large a volume as possible without disturbing the pellet;

q) add 1,5 ml of DW to the pellet in the tube; vortex the tube for 30 s to establish stable suspension;

r) repeat steps o) to q) three times;

s) the solution after performing step r) shall be used to make a sample film for the FT-IR measurement;

t) if the AuNP pellet does not re-suspend due to agglomeration in step q), a small amount of acid or base can be added for efficient re-suspension;

   — for amine functionalized AuNPs, add 150 μl of 1 N HCl to 1,5 ml AuNP suspension after step q) and vortex again for 30 s;

   — for carboxylate functionalized AuNPs, add 150 μl of 1 N NaOH to 1,5 ml AuNP suspension after step q) and vortex again for 30 s;

   — these additives are washed out in the step r).

5.2 Dehydration

Removal of water in the sample film is necessary, since IR light is strongly absorbed by water. To make a dehydrated AuNP film on an ATR crystal or IR window, contamination should be avoided and it should be validated by the blank test as in the following procedure:

a) measure the time required for dehydration of AuNP solution in a dehydration chamber used for the experiment. The dehydration chamber can be a (vacuum) oven or a (vacuum) desiccator. In case of using the "vacuum" oven or desiccator, the vacuum pump shall be an oil-free type, or a cold trap shall be equipped between the chamber and the vacuum pump;

b) put the blank ATR crystal or IR window in the dehydration chamber during the time duration required for dehydration of AuNP solution;

c) measure the IR intensity spectrum through the blank ATR crystal or IR window;

d) clean the surface of the ATR crystal or IR window with a high purity isopropanol moistened cotton swab. Measure the IR intensity spectrum through the cleaned ATR crystal or IR window;

e) calculate the FT-IR spectrum of the ATR crystal or IR window that was incubated in the dehydration chamber;

f) if there is no IR band exceeding LOD in the frequency region of interest, the dehydration process is free from IR active impurities. Otherwise try to find the source of impurity and reduce the IR absorbance below LOD while repeating steps from b) to e).

5.3 Screening test for impurities in DW from sample tubes

Some conical tubes and micro-centrifuge tubes release hydrocarbon impurities when they contain DW for more than a few minutes. Although the absorbance values due to these impurities are very low ($<10^{-3}$), it can seriously affect the analysis of surface ligands on AuNPs, since the FT-IR absorbance values from AuNPs themselves may also be very low. It is best to avoid sample tubes that release hydrocarbon

impurities. Before testing the impurities from the sample tubes, DW shall be tested if it is free from impurities. The procedure for the impurity test of DW is as follows:

a) take a required volume (2 or ≥ 200 μl) of DW;

b) "drop and dry" DW on the ATR crystal or IR window;

c) measure the IR intensity spectrum through the ATR crystal or IR window (see 6.2 or 6.3);

d) clean the ATR crystal or IR window surface with a high purity isopropanol moistened cotton swab. Measure the IR intensity spectrum through the cleaned ATR crystal or IR window;

e) calculate the FT-IR spectrum of the drop-and-dried DW;

f) if there is no IR band exceeding LOD in the frequency region of interest, then use DW that is free from IR active impurities. This is called "pretested DW". Otherwise try to find the source of impurity and reduce the IR absorbance below LOD by repeating steps a) to e).

Once DW has been validated to be impurity-free, contamination of DW by FT-IR active impurities from sample tubes can be tested by the following procedure:

a) fill the sample tube (micro-centrifuge or conical tube) with pretested DW up to 80 %;

b) close the lid and vortex the tube for 30 s;

c) maintain DW in the sample tube for more than 30 min;

d) take a required volume (2 or ≥ 200 μl) of liquid in the sample tube;

e) "drop and dry" sampled liquid on the ATR crystal or IR window;

f) measure the FT-IR spectrum of potential impurities in DW stored in the sample tube (see 6.2 or 6.3;

g) if there is no IR band exceeding LOD in the frequency region of interest, the sample tube is free from IR active impurities. This is called "pretested sample tube". Otherwise, clean the sample tube with retested DW or high purity isopropanol and repeat steps from a) to f) after drying the sample tube. If there is still IR band exceeding LOD in the frequency region of interest, use a sample tube supplied from the other companies and repeat the test through steps from a) to f).

6 FT-IR measurement procedure

6.1 General

In this clause, measurement procedures for FT-IR analysis are described. The ATR and transmission method using water insoluble IR transmitting materials can be used.

6.2 ATR method

The ATR method is applicable to quantitative analysis of thin films such as AuNP film prepared from solution. An aliquot of the AuNP suspension or solution is deposited on the ATR crystal element. The solvent is evaporated and AuNPs or solvated molecules form a thin film, with thickness proportional to the concentration. Provided that the film remains in intimate contact with the crystal and its thickness is less than the penetration depth of the evanescent wave above the ATR crystal, the measured absorption signal is proportional to the thickness of the film. Since the concentration of AuNPs in solution is generally low (<1 μm), the sample film prepared from the solution is very thin (<1 μm thick). It is recommended to use ZnSe or diamond as the materials for ATR crystals with circular active window region of approximately 2 mm in diameter. Those materials are good to be used as IR windows because they are waterproof and exhibit high transparency in IR wavelength region. One of the main advantages of using the ATR mode is that the sample volume needed for FT-IR measurements is very small (approximately 2 μl) due to the small active area of the ATR crystal. IR absorption profile in the

FT-IR spectrum obtained by using the ATR method cannot be directly compared with those obtained by transmission method because the reflected IR intensities are distorted along with the frequency at the surface of the ATR crystal. Nevertheless, the FT-IR spectrum obtained by using ATR method provides quantitative information if they are compared with the spectra obtained by the same method, and if the linear range of the method is not exceeded. The procedure for the measurement of FT-IR spectrum by using the ATR method with minimized background distortion is as follows:

a) turn on the FT-IR spectrophotometer and start flowing dry nitrogen (N_2) to purge IR optics along the beam path. Then warm-up and purge instrument for more than 60 min. The time required for purge completion shall be tested according to the purge test procedure in 6.4. It is generally recommended to leave the instrument switched on permanently to avoid the stabilization period;

b) cool the IR detector by using liquid nitrogen (N_2) or other cooling systems supplied with the instrument, if required. It is recommended to use detector that provides LOD at least $3{,}0 \times 10^{-4}$ in absorbance unit, within the time duration validated in the procedure according to 6.4;

c) verify instrument stability;

d) clean crystal with a high purity isopropanol moistened cotton swab;

e) "drop and dry" sample onto crystal;

f) measure sample single-beam spectrum; also record resolution and maximum integration time;

g) clean crystal *in situ* by using a solvent moistened cotton swab;

h) measure background single-beam spectrum;

i) calculate sample absorbance spectrum from background and sample single-beam spectra.

Measuring the background after the sample is better here because there is a smaller time interval and the crystal does not need to be removed.

6.3 Transmission method

High pressure pressed pellets are frequently used in the transmission method for the FT-IR spectrum measurement, however, the small IR absorption of AuNPs can be highly perturbed by the background distortion according to the morphologies of pellets. It is recommend making a dehydrated sample film on a water insoluble, transparent IR window in preparing the sample for the application of transmission method, which will provide more consistent results than using pellets in view of background stability. The procedure for the measurement of FT-IR spectrum by using the transmission method is as follows:

a) turn on the FT-IR spectrophotometer and start flowing dry N_2 to purge IR optics along the beam path. Then warm-up and purge instrument for more than 60 min. The time required for purge completion shall be tested according to the purge test procedure in 6.4. It is generally recommended to leave the instrument switched on permanently to avoid the stabilization period;

b) cool the IR detector by using liquid N_2 or the other cooling system supplied with the instrument, if required. It is recommended to use a detector that provides LOD at least $3{,}0 \times 10^{-4}$ in absorbance unit, within the time duration validated in the procedure according to 6.4;

c) verify instrument stability;

d) clean IR window with a high purity isopropanol moistened cotton swab;

e) "drop and dry" sample onto IR window;

f) measure sample single-beam spectrum; also record resolution and maximum integration time;

g) take off the IR window and remove sample film by using a solvent moistened cotton swab;

h) measure background single-beam spectrum;

i) calculate sample absorbance spectrum from background and sample single-beam spectra;

j) instead of steps g) and h), it is desirable to use a sample window shuttle that can be equipped with two windows for sample and background measurement, respectively;

   — with the instrument equipped with the sample window shuttle, purged atmosphere in the IR beam direction can be maintained during the intensity spectrum measurements of sample and background;

   — in this case, the background intensity spectrum shall be measured by using a blank window that is clean and composed of the same material and shape as the window used for sample film preparation;

   — the absorption difference spectrum between two blank IR windows for sample and background, respectively, shall be measured and subtracted from the sample absorption spectrum.

6.4 Determination of time required for complete purge

6.4.1 General

Since the FT-IR method provides very sensitive detection of molecular compounds even in gas phase, purging the atmosphere in the direction of IR light shall be performed to avoid the interference from the absorption by water vapour, carbon dioxide and the other possible impurities in the air. Purge can be performed by flowing dry N_2 gas along the whole region where IR light passes through. Insufficient purge usually results in IR bands in the region of water vapour and carbon dioxide due to the concentration difference of those molecules between sample and background measurement. Therefore, the time required for complete purge shall be determined and applied before the measurement of IR intensity spectrum. The following procedure shall be performed to determine the time required for complete purge.

6.4.2 ATR method

The ATR method is as follows:

a) install a clean ATR crystal at the crystal position in the instrument while supplying dry N_2 through purge input in the instrument;

b) record the IR intensity spectrum reflected from the ATR crystal as background;

c) wait for predetermined time duration, e.g. 10 min, for purge. The predetermined time can be preferably set as the time difference between the sample and background measurement in the daily analysis;

d) record the IR intensity spectrum reflected from the ATR crystal as the signal; calculate FT-IR absorption spectra of the clean ATR crystal;

e) wait for predetermined additional time duration, e.g. 10 min, c) for further purge;

f) record the IR intensity spectrum reflected from the ATR crystal as the signal; calculate FT-IR absorption spectra of the clean ATR crystal;

g) compare the spectra obtained by the last two measurements;

   — if the two IR absorption bands agree within LOD at the entire wavelength, the time required for complete purge is determined as the purge time until the former measurement;

   — otherwise, repeat steps e) to g) until IR absorption bands from the last two measurements agree within LOD at the entire spectrum.

6.4.3 Transmission method

The transmission method is as follows:

a) install a clean IR window at the window position in the instrument while supplying dry N_2 through purge input in the instrument;

b) record the IR intensity spectrum transmitted through the IR window as background;

c) wait for predetermined time duration, e.g. 10 min, for purge. The predetermined time can be preferably set as the time difference between the sample and background measurement in the daily analysis;

d) record the IR intensity spectrum transmitted through the IR window as the signal; calculate FT-IR absorption spectra of the clean IR window;

e) wait for predetermined additional time duration, e.g. 10 min, c) for further purge;

f) record the IR intensity spectrum transmitted through the IR window as the signal; calculate FT-IR absorption spectra of the clean IR window;

g) compare the spectra obtained by the last two measurements;

   — if the two IR absorption bands agree within LOD at the entire wavelength, the time required for complete purge is determined as the purge time until the former measurement;

   — otherwise, repeat steps e) to g) until IR absorption bands from the last two measurements agree within LOD at the entire spectrum.

6.5 Linear range of IR band intensity versus concentration

For the purpose of quantitative analysis, it is required to determine the linear range of IR band absorbance versus concentration of AuNPs for specific vibrational modes. Because the IR absorptivity of molecular vibrational band may change after binding with AuNPs, the quantification is not for the amount of ligands bound on AuNP but only for the band intensity here. When the linearity of IR band intensity versus concentration is established, it is possible to identify the ligand exchange by observing the relative band intensities (see 7.1). This can be done by observing the IR band intensities of sample films made with serially diluted AuNP solutions, respectively. The experimental procedure to determine the linear concentration range in respect to the IR band intensity is as follows:

a) take a required volume (2 or ≥ 200 μl) of sample; "drop and dry" the solution on the ATR crystal or IR window;

b) measure the FT-IR spectrum according to the procedure in 6.2 or 6.3;

c) serially dilute sample solution to the halves in concentration (e.g. 1/2, 1/4, 1/8, 1/16); prepare at least four serially diluted solutions;

d) measure the FT-IR absorption spectrum of each diluted solution;

e) set the inverse of dilution factor as "*x*" (e.g. 1/2, 1/4, 1/8, 1/16) and the absorbance of highest IR band as "y"; plot those values in linear scale;

f) determine the concentration range where the absorbance is linearly dependent on the inverse of dilution factor covering at least three points;

g) calculate the relative intensity ratios of the IR bands to the highest band at each spectrum obtained with serial dilution;

h) plot the relative intensity ratios of the IR bands versus the inverse of dilution factor; determine the concentration range where the relative intensity ratios are independent of dilution factor covering at least two points;

i) the concentration range for the valid FT-IR spectrum analysis shall satisfy both the conditions in steps f) and h).

6.6 LOD and LOQ determination

6.6.1 General

For the purpose of quantitative analysis, it is necessary to determine the LOD and LOQ for each vibration frequency. According to ISO 17191, $\sigma_A/A \leq 10$ %, calculated as 10 times the standard deviation of the photometric noise. Therefore, LOD and LOQ can be calculated as 2,776 and 10 times the standard deviation of the photometric noise, respectively ($2.776\sigma_A$ for LOD and $10\sigma_A$ for LOQ). The procedure for the analysis of photometric noise for LOD and LOQ estimation at each frequency is as follows (it is also important to set up and stabilize the instrument as described in 6.2 or 6.3).

6.6.2 ATR method

The ATR method is as follows:

a) install a clean ATR crystal at the crystal position in the instrument;

b) record the IR intensity spectrum reflected from the ATR crystal;

c) perform a surface cleaning procedure of ATR crystal by using a solvent moistened cotton swab;

d) record the IR intensity spectrum reflected from the ATR crystal as the background;

e) calculate FT-IR absorption spectrum of the clean ATR crystal;

f) measure at least five independent IR spectra by repeating from steps b) to e);

g) calculate standard deviations of FT-IR absorption spectra obtained in the steps from a) to f) at each frequency;

h) calculate LOD at each frequency by multiplying the standard deviations by 2,776;

i) calculate LOQ at each frequency by multiplying the standard deviations by 10.

6.6.3 Transmission method

The transmission method is as follows:

a) install a clean IR window at the sample position in the instrument;

b) record the IR intensity spectrum transmitted through the IR window;

c) take-off and perform a surface cleaning procedure of IR window by using a solvent moistened cotton swab;

d) re-install the cleaned window and record the IR intensity spectrum transmitted through the IR window as the background;

e) calculate FT-IR absorption spectrum of the clean IR window;

f) measure at least five independent IR spectra by repeating from a) to e);

g) calculate standard deviations of FT-IR absorption spectra obtained in the steps from a) to f) at each frequency;

h) calculate LOD at each frequency by multiplying the standard deviations by 2,776;

i) calculate LOQ at each frequency by multiplying the standard deviations by 10.

6.7 Repeatability determination

For the purpose of quantitative analysis, it is necessary to determine the measurement repeatability of the IR absorption bands. Repeatability can be assessed as 95 % confidence interval obtained from the repetitive absorption measurement[29]. The procedure for the analysis of IR absorption band repeatability is as follows:

a) take a required volume of sample solution and measure FT-IR spectrum of dried sample according to 6.2 or 6.3;

   — the concentration of sample solution shall be adjusted within linear range according to 6.5;

b) analyse the IR band intensities that exceed LOQ intensities;

c) obtain at least three independent IR spectra of the sample films made of the same solution by repeating the sampling, measurement and spectrum calculation procedure;

d) calculate the standard deviations of IR bands from the observed IR spectra;

e) calculate 95 % confidence interval of relative repeatability ($CI_{95\,\%,rRpt}$) from the standard deviation and the number of independent observation by using the following equation;

$$CI_{95\%,rRpt} = \left[k \times s(A_v) / \sqrt{N} \right] / A_{v,m} \quad (1)$$

where

$CI_{95\,\%,rRpt}$ is 95 % confidence interval of relative repeatability;

k is coverage factor for a given confidence level and the number of date (values are summarized in Reference [30]);

$s(A_v)$ is the standard deviation of the absorbance from N data;

N is the number of independent observations;

$A_{v,m}$ is the mean value of measured absorbance.

7 Application examples

7.1 Degrees of ligand exchange

The degree of ligand exchange from one compound to the other can be assessed by comparing the relative intensities. In this assessment, all absorption bands that exhibit intensities higher than the LOQ can be used. In addition, the band intensity should be linearly dependent on the concentration of AuNP solution as described in 6.5. Exemplified procedure to validate the ligand exchange is as follows:

a) take 1 ml of AuNPs solution before ligand exchange. Separate AuNPs from unbound ligands according to 5.1.2 or 5.1.3 and provide AuNP suspension in DW;

b) “drop and dry” the solution prepared in step a) on the ATR crystal or IR window and measure the FT-IR spectrum. Obtain the IR spectrum of AuNPs before ligand exchange from this measurement;

c) take a required volume of free ligand solution that will be exchanged on AuNP surface. “Drop and dry” this solution on the ATR crystal or IR window and measure the FT-IR spectrum. Obtain the IR spectrum of free ligand molecules from this measurement;

d) take 1 ml of AuNPs solution after ligand exchange. Separate AuNPs from unbound ligands according to 5.1.2 or 5.1.3 and provide AuNP suspension in DW;

e) "drop and dry" the solution prepared in step d) on the ATR crystal or IR window and measure the FT-IR spectrum. Obtain the IR spectrum of AuNPs after ligand exchange from this measurement;

f) list the absorption bands exceeding LOQ from highest frequency band to the lowest one in the IR spectrum of AuNP before exchange. Assign serial numbers to the absorption bands starting from "1" for the highest frequency band;

g) calculate the relative absorbance of each IR band in three spectra obtained in steps b), c) and e) normalized with -CH_2 stretching band intensities, respectively;

h) if -CH_2 stretching band intensities are smaller than LOQ in any spectrum, use the other absorption band, whose intensities exceed LOQ in all the FT-IR spectra obtained in steps b), c) and e), for normalizing;

i) plot the relative intensities of IR bands versus assigned numbers;

j) compare the relative intensity plots of AuNP before exchange and free ligand. Check if there is any IR band exhibiting difference in the relative intensity ratios between two spectra;

   — if there is no IR band differing relative intensity, this method cannot be used;

   — if there is any IR band differing relative intensity, mark the IR band for comparison;

k) compare the relative ratios of marked IR bands of ligand exchanged AuNP with the other two. Estimate the degree of exchange qualitatively from the ratio of distinct IR bands;

l) a case study result of ligand exchange validation on the surface of AuNPs is presented in Annex A.

7.2 Qualitative measurement of biomolecular binding

Binding of major biochemical moieties to the surface of AuNP can be measured through the identification of amine, phosphodiester or lipid vibration modes. This observation can be made before and after the cytotoxicity test of AuNPs. The results may be used to infer the binding of biochemical moieties AuNP surfaces. Procedure of these moieties on AuNP surfaces using FT-IR during the cytotoxicity test is as follows:

a) measure the FT-IR spectrum of AuNP to be used for cytotoxicity test;

b) add a fixed volume of AuNP solution to the sample tube that contains live cells and cell culture media;

c) maintain the mixture in an incubator for fixed time duration;

d) at predetermined time intervals, take upper solution in the tube without drawing cells;

e) fill the sampled solution into approximately 2 ml micro-centrifuge tube and sediment cells or cell fragments drawn with the upper solution by centrifuging the tube with 250 × g;

f) take the supernatant that may contain AuNPs, unbound biomolecules and salts. Remove salts and unbound molecules from the solution by using dialysis or centrifuge method;

g) measure the FT-IR spectrum of separated AuNPs. Compare this spectrum with the spectrum obtained in step a). Check if new IR bands are detected in the FT-IR spectrum obtained in the last spectrum;

   — binding of biochemical moieties characteristic of proteins to AuNPs can be qualitatively measured by simultaneous detection of 3 510 $cm^{-1}$to 3 310 cm^{-1} (aliphatic/aromatic and primary/ secondary amine stretch) band and 1 650 cm^{-1} 1 550 cm^{-1} (aliphatic/ aromatic and primary/ secondary amine bend) band[31][32][33];

   — binding of biochemical moieties characteristic of lipid or membrane components to AuNPs can be qualitatively measured by detection of increased band at 2 935 cm^{-1} to 2 915 cm^{-1} (methylene C-H asym. stretch), 2 865 cm^{-1} to 2 845 cm^{-1} (methylene C-H sym. stretch) and 1 485 cm^{-1} to 1 445 cm^{-1} (methylene C-H bend) band[34][35][36];

— binding of biochemical moieties characteristic of nucleic acids to AuNPs can be qualitatively measured by simultaneous detection of 1 240 cm^{-1} to 1 220 cm^{-1}/1 130 cm^{-1} to 1 080 cm^{-1} (phosphodiester backbone sym./asym. stretch) band and 1 500 cm^{-1} to 1 300 cm^{-1} (base sugar vibration) band[37][38][39];

h) information about IR band assignment for functional groups can be found in Reference [40];

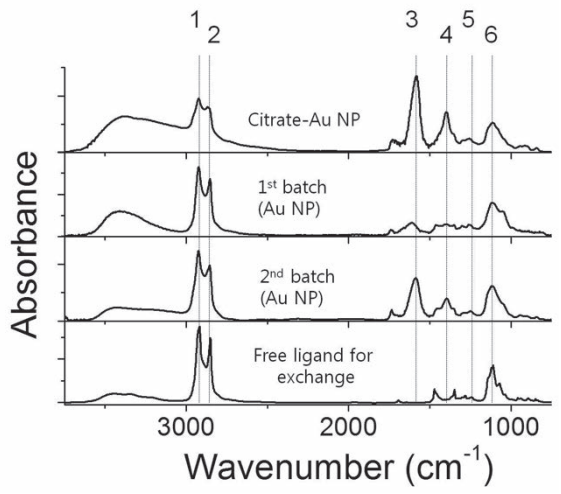
i) an example of qualitative measurement of biochemical moieties characteristic of proteins on the surface of AuNPs in SCM is presented in Annex B.

Annex A
(informative)

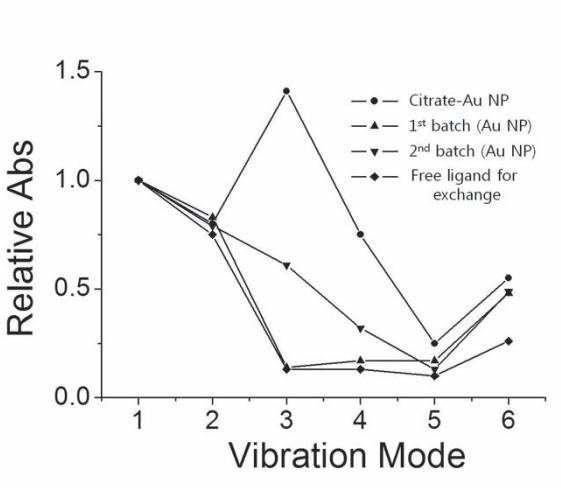
Case study for validation of ligand exchange

In this annex, a validation example of the AuNP surface ligand exchange by using FT-IR analysis has been presented. The surface of AuNPs has been changed from citrate to HS-$(CH_2)_{11}$-$(OCH_2CH_2)_3$-OH (triethylene glycol mono-11-mercaptoundecyl ether; TEMUE) and FT-IR spectra before and after the ligand exchange were monitored. For this case study, ligand exchange reactions were performed in two separate experiments delivering two independent batches of AuNP solutions (1st and 2nd batches in Figure A.1).

Since there are two moieties of methylene groups and three moieties of carboxylate groups in citrate, the absorbance values of -CH_2 vibration modes (mode 1 and 2) are relatively smaller than those of C = O vibration modes (mode 3 and 4) in the FT-IR spectra. On the other hand, there are 17 moieties of methylene groups and no carboxylate group in TEMUE, the absorbance values of -CH_2 vibration modes (mode 1 and 2) become larger than those at the frequency region of C = O vibration modes (mode 3 and 4) in the FT-IR spectrum. Such difference can be observed in the comparison of FT-IR spectra (Figure A.1, A) and the relative absorbance pattern of vibration modes to the mode 1 changes according to the degree of ligand exchange. [Figure A.1, b)] Comparison of the relative intensities for vibration modes showed that the exchange in the 1st batch was more effective than that in the 2nd batch. Such qualitative interpretation could not be deduced by simple band detection.



a)



b)

Key

The numbers on each line represent different vibration modes. Vibration frequencies and related functional groups are as follows:

1 2 924 cm^{-1} CH_2 asym. stretch

2 2 854 cm^{-1} CH_2 sym. stretch

3 1 581 cm^{-1} C = O stretch

4 1 396 cm^{-1} carboxylate C-O-H bend

5 1 257 cm^{-1} carboxylate C-O stretch

6 1 111 cm^{-1} alcoholic C-O stretch

Figure A.1 — a) FT-IR spectra of free ligand molecules and AuNPs of which the surface ligands were exchanged from citrate to TEMUE; b) relative IR absorption band intensities of free and AuNP bound ligands. The relative ratio was normalized to mode 1

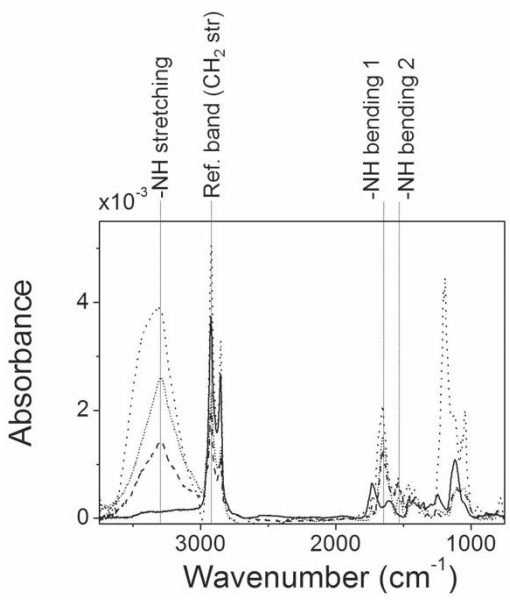
Annex B
(informative)

Case study for qualitative measurement of biochemical moieties binding to the AuNP surface

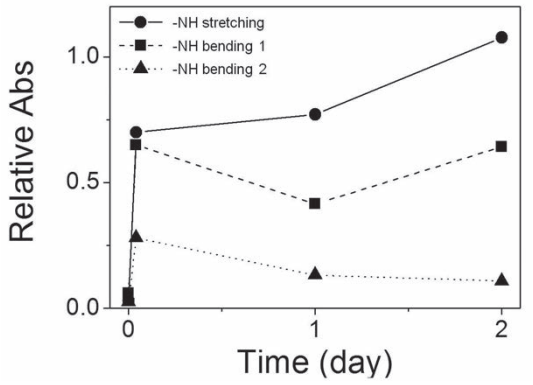
In this annex, evidence of biochemical moieties binding to alkyl chain carboxylate bound AuNP by using FT-IR spectroscopy has been presented. The binding of biomolecules to the surface of AuNP can be observed through the assignment of amine, phosphodiester or lipid vibration modes.

Figure B.1 shows that biomolecular adsorption on the surface of AuNPs can be monitored by observing FT-IR spectra in view of relative intensities. Solution of 30 nm AuNPs having 11-(Carboxymethoxy triethylene glycol) undecane thiol (CMTEUT) on their surface were mixed with 20 % FBS containing cell culture media in 1:1 volume ratio. A fixed portion of the mixture solution was sampled at predetermined time interval. Then AuNPs were separated from unbound molecules and FT-IR spectra were recorded.

As shown in Figure B.1, N-H bond related vibrational modes appear after maintaining AuNPs in 10 % FBS containing cell culture media. When the absorption intensities at 3 311 cm^{-1} assigned to N-H str., 1 653 cm^{-1} and 1 550 cm^{-1} for N-H bending were normalized by the band intensity at 2 924 cm^{-1} assigned to CH_2 asym. str., the resultant values from the SCM mixture were much higher than the values obtained from the solution before mixing.



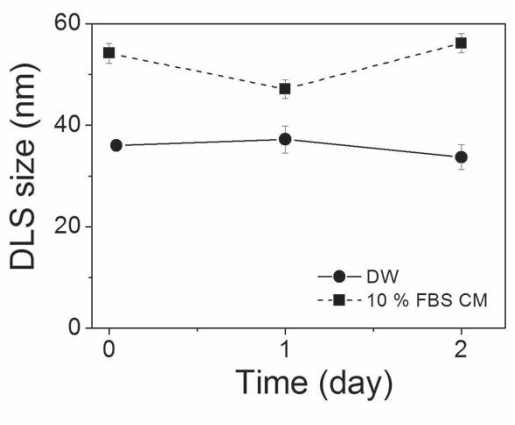
a)



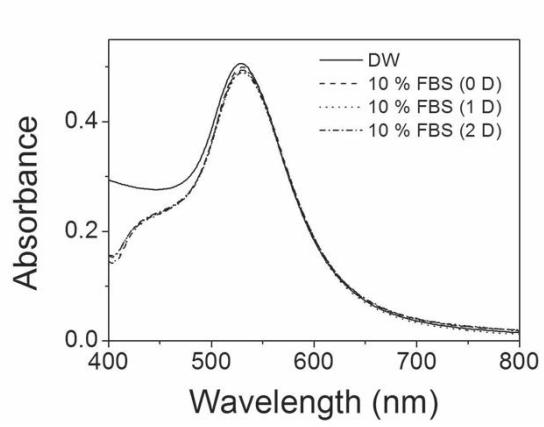
b)

Figure B.1 — a) FT-IR spectra of AuNP films obtained by using the ATR mode. Sample films were made of AuNPs with CMTEUT ligand in distilled water or 10 % FBS cell culture media after removal of unbound molecules by centrifuge; b) relative intensities of IR absorption bands assigned to amides on the surface of AuNPs. The relative ratio was normalized to the -CH_2 asym. str. mode intensities

As shown in Figure B.2, DLS sizes of AuNPs increased by approximately 50 % while there was very little change among the UV/Vis absorption spectra of AuNPs in solution after mix with SCM. These results indicated that hydrodynamic radius of AuNPs increased due to binding of additional molecules and the increase was not due to agglomeration of AuNPs regarding almost invariant the SPR band absorption spectrum in the longer wavelength region.



a)



b)

Figure B.2 — a) DLS size of AuNPs with CMTEUT ligand in distilled water (DW) or 10 % SCM after time elapsed. For DW, 0 day means just after the free ligand removal by centrifuge method. For 10 % SCM, 0 day means 1 h after mixing of AuNPs with 20 % SCM in 1:1 volume ratio; b) UV/Vis absorption spectra of AuNPs in DW or 10 % SCM after time elapsed

The above combined results from three different techniques suggest protein binding to AuNPs in 10 % SCM to increase hydrodynamic radius of AuNPs and IR absorption intensities at the frequencies assigned to primary or secondary amine vibrations (3 311 cm^{-1} for N-H str. and 1 653 and 1 550 cm^{-1} for N-H bend). It is possible to detect and identify biomolecule type, binding to AuNPs during cytotoxicity test according to the procedure described in 7.2.

Annex C
(informative)

Selection guide for window materials

IR windows for transmission mode should be highly transparent and exhibit few absorption band in the IR frequency region. They are also required to be water stable since most of the AuNPs are provided in aqueous solution. Since the transmittance curve of IR windows varies according to their width, it is recommended to refer the transmittance curves posted in the supplier's website or product catalogues.

As an example, ZnSe, Si and diamond are suitable window materials for the application of IR absorption spectroscopy. They are waterproof and have longer cut-off wavelengths than most of IR window materials.

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