
**Nanotechnologies — Assessment of
protein secondary structure during an
interaction with nanomaterials using
ultraviolet circular dichroism**

*Nanotechnologies — Évaluation de la structure secondaire des
protéines durant une interaction avec des nanomatériaux à l'aide du
dichroïsme circulaire ultraviolet*

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PROOF / ÉPREUVE



Reference number
ISO/TS 23459:2020(E)

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ISO/PRE TS 23459

<https://standards.iteh.ai/catalog/standards/sist/591f96e4-05e0-42f1-8178-330df9c28e1d/iso-prf-ts-23459>



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Published in Switzerland

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Nano-objects and their aggregates and agglomerates (NOAA) are currently produced in large mass quantities globally and used in a variety of applications. However, there is concern about their interaction with biological systems, including proteins, which could lead to reversible or irreversible alterations in their secondary structure. The latter could affect the functionality and conformation of protein, which in turn might affect the overall bio-reactivity of the proteins. The monitoring of the occurrence of such alterations could thus provide important information on the interaction of NOAAs with biological systems.

The process of folding of polypeptides in biological media produces the secondary structure of proteins which determines their bioactivity. The important features of this structure include hydrogen bonds between the amine hydrogen and carbonyl oxygen atoms in the peptide backbone and disulfide bonds between two cysteine residues.

The protein secondary structure could be affected by exposing it to certain metallic ions and bioactive compounds. Furthermore, it is also influenced by different buffer ionic strength, pH values, and temperature^[1]. Alterations in the functionality and conformation of proteins can be attributed to reorganization (so-called misfolding) and changes of the overall molecular dimension that accompany the folding process. Some diseases, such as amyotrophic lateral sclerosis (ALS), Alzheimer's and Parkinson's, are a consequence of misfolded proteins^[2].

There are several standard techniques for determining the molecular structures/conformations and folding process of proteins and upon their interaction with NOAAs. These include high-field nuclear magnetic resonance (NMR) Fourier-transform infrared (FT-IR), Raman spectroscopy and ultraviolet circular dichroism (UV-CD) spectroscopies^{[3][4][5][6]}. In addition, a novel technique synchrotron radiation circular dichroism (SRCD) spectroscopy is a sensitive method to provide information on protein secondary structures and folding^[7].

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Nanotechnologies — Assessment of protein secondary structure during an interaction with nanomaterials using ultraviolet circular dichroism

1 Scope

This document specifies measurement protocols and test conditions to determine alterations to protein secondary structure induced by their interaction with nanomaterials using ultraviolet circular dichroism (UV-CD) spectroscopy.

This document does not apply to the characterization of conformational changes of disordered proteins.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/TS 80004-1, *Nanotechnologies — Vocabulary — Part 1: Core terms*

ISO/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

ISO/TS 80004-4, *Nanotechnologies — Vocabulary — Part 4: Nanostructured materials*

ISO/TS 80004-6, *Nanotechnologies — Vocabulary — Part 6: Nano-object characterization*

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3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/TS 80004-1, ISO/TS 80004-2, ISO/TS 80004-4, ISO/TS 80004-6 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1

nanoparticle

NP

nano-object with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly

Note 1 to entry: If the dimensions differ significantly (typically by more than three times), terms such as “nanofibre” or “nanoplate” may be preferred to the term “nanoparticle”.

[SOURCE: ISO/TS 80004-2:2015, 4.4]

3.2

nanomaterial

material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale

Note 1 to entry: This generic term is inclusive of nano-object and nanostructured material.

Note 2 to entry: See also “engineered nanomaterial”, “manufactured nanomaterial” and “incidental nanomaterial”.

[SOURCE: ISO/TS 80004-1:2015, 2.4]

3.3

circular dichroism

optical effect of the differential absorption of left- and right-handed circularly polarized light

Note 1 to entry: Ultraviolet circular dichroism spectroscopy is used to investigate the secondary structure of proteins.

4 Abbreviated terms

Ag-NP silver nanoparticle

Au-NP gold nanoparticle

AU absorbance unit

BSA bovine serum albumin

CD circular dichroism

DLS dynamic light scattering

HSA human serum albumin

MRE mean residue ellipticity

MWCNT multiwall carbon nanotubes

NOAA nano-objects and their aggregates and agglomerates

PAA-GNP poly (acrylic acid)-coated gold nanoparticles

SRCD synchrotron radiation circular dichroism

SWCNT single-wall carbon nanotubes

UV-CD ultraviolet circular dichroism

UV-Vis ultraviolet-visible

5 Nanomaterial protein interactions

In a biological environment, NOAA can easily interact with proteins such as apolipoproteins, fibronectin, human serum albumin (HSA), vitronectin, etc.^[5] The layers of bound or adsorbed proteins around NOAAs are called protein corona^[8]. Physicochemical characteristics of the nanomaterials (i.e. size, surface area, hydrophobicity, charge density, surface chemistry, morphology) could affect the interaction with surrounding biological compounds. The possible interaction between nanomaterials and these compounds depends on protein association and dissociation kinetics. Nanomaterial–ligand complexes have a lifespan ranging from microseconds to days^{[3][4][5][6][7][8][9]}. Nanomaterial–protein interaction could lead to reversible or irreversible conformational changes on their secondary structures. Slight changes in the secondary structure of proteins following the interaction with nanomaterials are potentially reversible, whereas substantial changes could be irreversible. These changes can be monitored by recording UV-CD spectra^[9]. UV-CD spectroscopy has its origin in the photophysical process by raising an electron from ground state to an electronically excited state. UV-CD spectroscopy is extensively used in the characterization of secondary structure, folding and binding properties of proteins^{[4][5][6]}. The technique uses polarized light and measures the difference in absorbance between the left- and right-handed circularly polarized light result in a UV-CD signal. Absorptions below 240 nm

are due to peptide bonds and absorptions in the range of 260 nm to 320 nm are due to aromatic amino acid side chains. α -Helix, β -sheet, and β -turns are the most common secondary structural elements (see [Annex A](#) and [Figure C.1](#)). It should be noted that aromatic amino acid side chains can also contribute to the CD spectrum below 240 nm. It should also be noted that disulfide bonds can contribute to the CD spectrum in both wavelength regions. Protein tertiary structure characterization is beyond the scope of this document.

The structural alteration of critical human proteins following interaction with NPs has been reported using UV-CD (see [Table B.1](#)). For instance, the irreversible structural changes caused by interaction of human transferrin with superparamagnetic iron oxide NP (SPION), and fibrinogen with Au-NPs, led to the loss of their primary biological function^[10]. The role of physical force on NP-cell interactions investigated by studying interactions between Ag-NPs and HSA using UV-Vis, transmission electron microscopy (TEM) and UV-CD measurement methods. It has been revealed that Ag-NPs-HSA binding is mediated by hydrogen bonding, electrostatic and hydrophobic interactions that causes α -helices decrease, and β -sheets increase, thereby changing protein biological function^[11]. The interaction and stability of HSA-AgNP has been studied by SRCD spectroscopy and results showed reduction of α -helix content of protein structure^{[12][13]}. PAA-GNP binding produced misfolding of Mac-1 protein, which promotes interaction with the integrin receptor. Activation of this receptor increase the NF- κ B signalling pathway, resulting in the release of inflammatory cytokines^[14].

6 Sample preparation

6.1 General

For recording spectra, an UV-CD instrument is needed with a data-acquiring range from 175 nm to 700 nm with a temperature control unit. A quartz glass cell (either rectangular or cylindrical) with path lengths ranging from 0,5 mm to 10,0 mm is required. For recording UV-CD spectra, all material, solvents and buffers should have low absorption in UV range. They should be as transparent as possible. Working with optically active buffers creates additional challenges and is not recommended (see [Tables C.1](#), [C.2](#) and [C.3](#)). For handling the proteins, special functionalized glassware with low-binding affinity to protein should be used. All solutions shall be prepared with deionized water.

6.2 Desired properties of the UV-CD quartz cell

The UV-CD spectra should be recorded in highly transparent quartz cells. The cells shall have no optical activity and desired path lengths ranging from 0,5 mm to 10,0 mm (rectangular or cylindrical). The cells shall be thoroughly cleaned between the individual measurements (see [Annex C](#)).

6.3 Preparation of protein solution

Use a low-surface protein affinity test tube to weigh the protein and add a buffer solution to make a stock solution with a suitable concentration. The required concentration can be determined using molar extinction coefficients by the spectrophotometric method^[11]. The buffer shall be chosen according to the type of protein and the type of NP used in the study. Prepare a stock solution of protein in the concentration range of 1,0 mg/ml to 5,0 mg/ml. The stock solution can then be diluted for the UV-CD measurements. The UV-CD spectra of proteins are recorded in 0,5 mm to 10,0 mm cells, a concentration of 0,005 mg/ml to 5,0 mg/ml depending on the path length and the type of buffer. An acceptable UV-CD spectrum should be obtained with desired protein contents between 0,005 mg/ml to 0,300 mg/ml depending on the cell path length. For typical UV-CD measurements:

- in a 0,5 mm cell: 0,2 mg/ml to 1,0 mg/ml protein and required volume 0,025 ml to 0,050 ml;
- in a 1,0 mm cell: 0,05 mg/ml to 0,2 mg/ml protein and required volume 0,3 ml to 0,4 ml;
- in a 2,0 mm cell: 0,1 mg/ml to 0,3 mg/ml protein and required volume 0,9 ml to 1,0 ml;
- in a 10,0 mm cell: 0,005 mg/ml to 0,01 mg/ml protein and required volume 3,0 ml to 4,0 ml.

The protein should produce a sufficient UV and UV-CD signal. The desired UV level for protein solutions at the wavelength and path-length of interest should range from 0,5 AU to 1,0 AU. The optimum absorbance level is 0,89 AU (see Figure C.2).

6.4 Instrumental setting condition

The equipment needs to be purged with nitrogen gas about 30 min to 60 min before starting the machine (manufacturer's suggested time). A water circulation bath is required for controlling the temperature of analyses using a water-jacketed cell holder/software-controllable Peltier. The bath should be set at the desired temperature, which shall be constant throughout the measurement. The lamp should be turned on before experiments and allowed to stabilize the output (30 min to 40 min).

6.5 Recording UV-CD spectra procedure

6.5.1 General

Before starting UV-CD measurements, set the temperature to the desired value (25 °C)^{[3][4]}. To obtain proper signal-to-noise ratio to adequate spectral resolution, set the bandwidth between 1,0 nm and 1,5 nm. The wavelength range adjustment depends on the sample and cell used:

- from 190 nm to 260 nm for 0,2 mg/ml to 0,8 mg/ml protein samples in a 0,1 mm cell;
- from 190 nm to 260 nm for 0,1 mg/ml to 0,2 mg/ml protein samples in 1,0 mm and 2,0 mm cells;
- from 190 nm to 260 nm for 0,01 mg/ml to 0,02 mg/ml protein samples in a 10,0 mm cell.

Data collection interval of 1,0 nm sets should be used for samples with low ellipticity and with a signal to noise ratio and 0,10 nm to 0,25 nm. The recommended interval for measurement of UV-CD spectrum ranges from 190 nm to 260 nm. Data should be collected at one nm per s.

6.5.2 Buffer

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Record the spectrum of the buffer to make sure it does not have any ellipticity. Make sure that measuring parameters such as slit width, scanning step, integration time and scanning speed/integration time are the same as those that will be used for measuring the samples. The presence of any buffer-related UV-CD effect by overlapping on the protein UV-CD could lead to a misleading result. The increases absorbance of the buffer in comparison with deionized water will decrease the signal-to-noise ratio. The spectrum of the buffer and deionized water should overlay each other, within the experimental error, but the spectrum of the buffer usually has a lower signal-to-noise ratio than the spectrum of deionized water at low wavelengths^[4].

- The recommended buffer for dissolving protein is sodium or potassium phosphate with an optimal concentration of 10 mM, which is used as blank sample.
- Avoid using buffers with interfering with UV-CD spectrum such as citrates, MOPS (3-(N-morpholino) propanesulfonic acid), imidazole and dithiothreitol (DTT). The list of buffers and UV cut off are presented in [Table C.3](#).
- Record a CD spectrum of the buffer alone before starting with a sample. The obtained spectrum of blank should be relatively flat to ensure the buffer absorbance is not a concern.

6.5.3 Protein sample

After cleaning the cell, it is filled with protein solution and UV-CD spectra are recorded. Repeat the measurement five to six times. Overlay each spectrum and average the data sets. Smooth the spectra of the sample and blank^[4]. A number of approaches are available for spectral smoothing. Typically, the Savitsky-Golay smoothing algorithm with polynomial order of 3 and smoothing window of 20 pts is

used. Subtract the smoothed baseline from the smoothed spectrum of the sample^[4] to ^[15]. The ellipticity for most proteins should be close to zero between 250 nm and 260 nm.

NOTE The data are inspected to avoid the introduction of distortion in the pre- and post-smoothing process.

6.5.4 Stability of NP suspension in the protein solution

The stability of NPs in the buffer used for dissolving protein needs to be tested to avoid any agglomeration of NPs suspension. Ideally the agglomeration state should be the same in sample and control. However, it is noted that many studies have shown that proteins enhance particle agglomeration and thus this might not always be practical. Preliminary studies addressing NP agglomeration should be undertaken prior to UV-CD analysis as part of experimental design, as these can inform decisions on the choice of buffers and particle concentrations. The measurement can be achieved by DLS techniques (see Reference ^[15] and ISO 22412^[16]).

6.6 Preparation of protein-NPs conjugated suspension

Prepare the protein-NPs conjugated suspension as follows:

- a) Use glassware with low affinity to proteins.
- b) Pipette the pre-calculated amount of protein stock solution into each vial at the same concentration.
- c) Fill the glassware with sufficient amount of water to have a constant protein concentration.
- d) Gently shake the vials and incubate them for about 5 min at room temperature (25 °C).
- e) Add the fixed volume of nanomaterials suspension (10 µg/ml to 100 µg/ml) to the protein solution of constant concentration, followed by gentle mixing (the correct ratio of protein and NPs can be found in [Annex E](#)).
- f) Incubate the prepared samples for 4 h at room temperature (25 °C).
- g) Transfer the solution to the UV-CD cells.
- h) Record spectra using a UV-CD cell over a range of 190 nm to 260 nm at room temperature (25 °C). Collect data at 1,0 nm with a bandwidth of 1 nm, at 50 nm/min and averaging over five to six scans. The final spectra should be baseline-corrected and data presented as mean residue ellipticity (MRE), which is explained in [6.8](#).

6.7 UV-CD spectra measurement

Measure the UV-CD spectra as follows:

- a) Record a UV-CD spectrum of the desired buffer (see [Tables C.1](#) and [C.2](#)).
- b) It is possible that reagents remain in the NP dispersion. Record UV-CD spectra of the corresponding solution.
- c) Test the UV absorbance of the used NPs in the range of 190 nm to 260 nm. If the absorbance of the NP in this region is greater than 1,0 AU, the particle is not suitable for UV-CD experiment (see [Figures C.1](#) and [C.2](#)).
- d) Carry out UV-CD measurements with the incubated protein–NP dispersion for at least five to six replicates to test the reproducibility. If the reproducibility is not acceptable, it can point to an insufficient equilibration time or a destabilized suspension.
- e) Subtract the background/blank spectrum from sample data.

The CD spectra of NOAA in the range of 190 nm to 250 nm shall be recorded. The achiral NOAA shows no CD effect in range of interest (see [C.3](#)). The NOAA which show a strong CD effect will not be suitable

for this type of investigation. All procedures for the recording of CD experiment shall be identical for the NOAA, blank and protein sample.

6.8 Calculation of molar ellipticity

Quantitative analysis of the α -helix content in the protein can be calculated by converting the UV-CD signal (see Figure C.2 and Table D.1) to the MRE using Formula (1):

$$[\theta] = \frac{\theta}{10Cnl} \tag{1}$$

where

- $[\theta]$ is the MRE in 222 nm;
- θ is the observed ellipticity in mdeg;
- C is the molar concentration of the protein;
- n is the number of amino acid residues;
- l is the path length.

The percentage of helicity is calculated using Formula (2):

$$H = \frac{([\theta] - 3000)}{(-36000 - 3000)} \tag{2}$$

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where

- H is the α -Helix (%); [ISO/PRF TS 23459](https://standards.iteh.ai/catalog/standards/sist/591f96e4-05e0-42f1-8178-330df9c28e1d/iso-prf-ts-23459)
- $[\theta]$ is the observed MRE at 222 nm;
- 3 000 is the MRE $[\theta]$ of the random coil and β -form conformation cross at 222 nm;
- 36 000 is the MRE $[\theta]$ value of pure α -helix at 222 nm.

6.9 Data analysis

The estimation of protein secondary structure is carried out by quantitative analysis of UV-CD spectra. Many validated reference spectra are publicly available from the Protein Circular Dichroism Data Bank (PCDDDB)[17]. The deconvolution of UV-CD spectra can be carried out via different methods (see Table E.1). There are a number of platforms which can be used for the estimation proteins structural contents, such as CDPro[18], ValiDichro[19], PDB2CD, K2D3, DichroCalc, DICHROWEB, CCA+[20] and Beta Structure Selection (BeStSel)[21][22]. There are also other simplified procedures for the determination of the structural content.[4][23][24][25]. By applying such methods, the secondary structure of proteins can be estimated and the changes made to the protein's three dimensional structure as a result of interactions with NPs can be measured. The changes above 5 % in the secondary structure of the proteins are significant and ≥ 90 % on secondary structure of proteins will be denatured[57].

The measured UV-CD spectra can be transferred to the web server as a text file (see Annex G) or it can be copied into the window in two data columns: data in units of either delta epsilon, MRE or mdeg. The wavelength interval for input data at intervals of 1,0 nm is recommended. The output of structural content estimation is obtained as a graphical presentation superposition of experimental and estimated spectra, the listing of the secondary structure composition, total content and the spectral fitting with root mean square deviation (RMSD) and normalized RMSD (NRMSD) data. It can be saved either in the graphical form or in text format for further data processing or figure preparation. The users can adjust