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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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Foreword

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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].

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*

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-kB 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 experiment. 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 of 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

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 260 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 at 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 in cm.

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

$$H=\frac{([\theta]-3\,000)}{(-36\,000-3\,000)} \tag{2}$$

where

H is the α-helix (%);

$[\theta]$ 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 (PCDDB)[17]. The deconvolution of UV-CD spectra can be carried out via different methods (see Table F.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

the wavelength range and use a scaling factor to recalculate the results. The form of the output data can be modified for the convenience of the user.

7 Test report

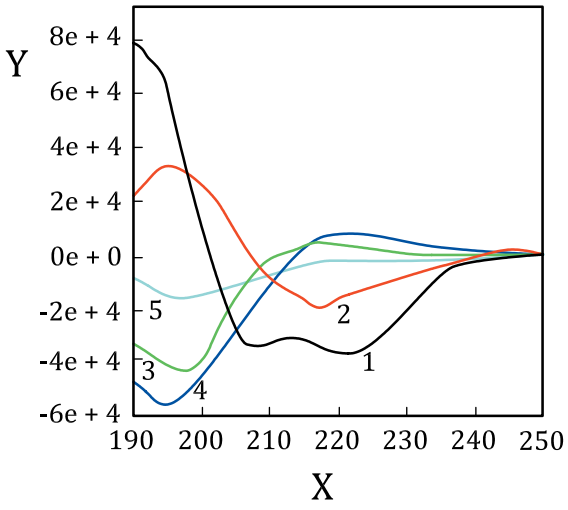
The test report shall include the information given in Table 1.

Table 1 — Test report

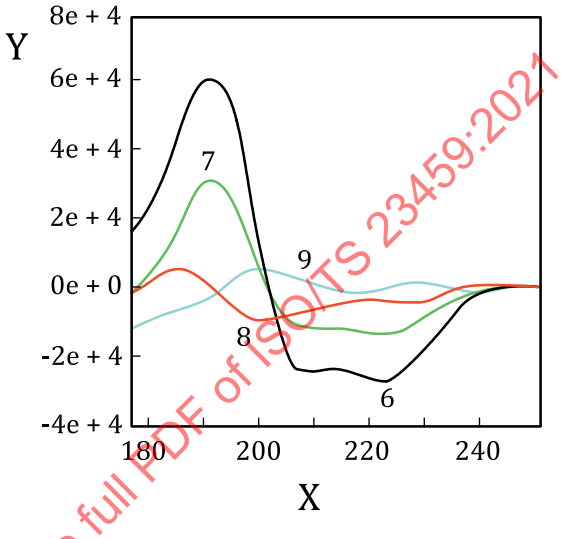
General			
Product name:		Product application:	
Batch no.: Lot no.:		Manufacturing method:	
Lab. name:		Lab. address:	
NOAA characteristics			
Size		Chemical composition	
Shape		Surface chemistry	
NOAA concentration (M)[a]			
Protein characteristics			
Protein concentration (mg/ml)		pH	
Estimated secondary structure content			
Structural content	The protein before interaction with NOAA (%)	The protein after interaction with NOAA (%)	
Helix			
Antiparallel			
Parallel			
Others			
RMSD			
NRMSD			
Type of database used for protein structure content analysis:			
[a] The concentration is expressed in µg/ml.			

Annex A
(informative)

Typical UV-CD spectra of proteins



a) The various secondary structure of proteins in far-UV



b) Representative proteins with varying conformations

Key

X	wavelength (nm)	6	myoglobin
Y	$[\theta]$ (deg cm^2dmol^{-1})	7	LDH
1	α-helix	8	chymotrypsin
2	antiparallel β	9	Bence Jones
3	extended		
4	collagen (triple helix)		
5	collagen (denatured)		

Figure A.1 — UV-CD spectra[4]

Annex B
(informative)

Literature survey on structural changes of NOAA and proteins

Table B.1 — Proteins conformational changes by NOAAs

Type of NP	Type of protein	Shape	Size	Surface properties	NP concentration	Protein concentration	pH	Conclusion	Ref.
AgNP	Lysozyme	Spherical	4 nm 20 nm 100 nm	Negative charge	1:1 000 (1:250 for the 100 nm) $2,3 \times 10^{18}$ 4 nm $5,9 \times 101^{6}$ 20 nm $6,4 \times 10^{14}$ 100 nm	50 µg/ml	9,2 6,9 5,0	Irreversible, for all particle size, the adsorption of lysozyme decreases as the pH decrease. The most protein is unfolding for 100 nm.	[26]
AgNP	Cytochrome C	—	4 nm 15 nm 35 nm	Negative charge		20,0 µg/ml 200 µg/ml	7,0	Reversible, little loss of second structure on larger AgNPs.	[27]
Au NP	Cytochrome	Solid surface	2 nm to 4 nm 16 nm	—	0 nm to 18 nm length	3,5 µM	7,2	Hydrophobic interactions cause the adsorption of Cyt c on 2 nm to 4 nm. The lowest ratio of cyt c to 2 nm to 4 nm is 20 cyt c per gold NP and electrostatic interactions for cyt c on 16-nm the ratio is 200 cyt c per Au NP.	[28]
SiO_2	Ribonuclease A	Spherical	4 nm 15 nm	Negative charge		10 mg/ml	7,4	Reversible unfolding, the stability of the enzyme is decreased further on larger NPs with smaller surface curvature	[29]
AgNP	(HCAI) Human carbonic anhydrase I	Solid surface	6 nm 9 nm 15 nm	Negative charge	$1,12 \times 10^{17}$ 15 nm $5,09 \times 10^{17}$ 9 nm $7,30 \times 10^{17}$ 6 nm (particles/ml) HCAI: AgNP [1:0,25, 1:0,5, 1:0,75, 1:1] (for all size of particles)	50 µM	8,4	Ultracentrifugation and gel permeation chromatography is used for desorption, Particles with 15 nm diameter cause a ~6-fold higher change in secondary structure than 6 nm particles.	[30]
PAA-AuNP	Fibrinogen	Spherical	5 nm 10 nm 20 nm	Negative charge	10 µg/ml 20 µg/ml 30 µg/ml 40 µg/ml	10 µg/ml	7,4	Negatively charged NPs can unfold fibrinogen and the addition of PAA-GNP resulted in a loss of protein secondary structure.	[31]

Table B.1 *(continued)*

Type of NP	Type of protein	Shape	Size	Surface properties	NP concentration	Protein concentration	pH	Conclusion	Ref.
AgNP	Jack Bean Urease (JBU)	Spherical	10 nm	Positive charge	1 000 µM JBU: AgNP [1:1, 1:2, 1:3, 1:4, 1:5 (v/v)]	40,0 µM	8	Irreversible change, Formation of protein-nano bio conjugate takes place at the higher concentration. The protein corona formed with a higher concentration of AgNP exhibits a total inhibition of urease enzymatic action.	[32]
CuNPs	BSA (bovine serum albumin)	Spherical	7,5 nm	Cubic structure	$1{,}58 \times 10^{-4}$µM $3{,}53 \times 10^{-4}$ µM $7{,}34 \times 10^{-4}$ µM $11{,}66 \times 10^{-4}$ µM	5,0 µM		The α-helicity of the BSA decreases due to its interaction with Cu NPs.	[33]
AgNPs	Fungal protein (Aspergillus foetidus)	—	5 nm	—	1: 0 µM 2: 12,5 µM 3: 25 µM (protein:AGNP)	1 mg/ml	7,4	It can be concluded that binding of silver NP to fungal extracellular protein can cause irreversible change in protein.	[34]
Ag-PVT NPs	Human serum albumin (HSA)	—	—	—	$2{,}4 \times 10^{-5}$ mol/dm^3 4×10^{-5} mol/dm^3 8×10^{-5} mol/dm^3 16×10^{-5} mol/dm^3 24×10^{-5} mol/dm^3 32×10^{-5} mol/dm^3 40×10^{-5} mol/dm^3 60×10^{-5} mol/dm^3 80×10^{-5} mol/dm^3	1,5 × µM	7,4	Ag-PVT NPs slightly change the 285-secondary structure of protein.	[35]
ZnO NPs	α-lactalbumin	Spherical	4 nm to 7 nm	Positive charge	1:1 to 1:10 (protein: NPs)	10,0 µM	7,4	The binding between ZnO and protein can be Electrostatic, hydrophobic, and mixed and hydrophobic interaction is deleterious to protein structure while electrostatic interaction stabilizes the protein	[36]

Table B.1 *(continued)*

Type of NP	Type of protein	Shape	Size	Surface properties	NP concentration	Protein concentration	pH	Conclusion	Ref.
C_{60} NPs	Human serum albumin (HAS)	Spherical	50 nm to 110 nm	—	3,38 µM 5,63 µM 11,8 µM	7,3 µM	7,4	The percentage of α-helicity of HSA increase and the protein becomes more compact upon association with nC_{60}.	[37]
CdS-QD NPs	Lyz	Spherical	2 nm to 3 nm	MPA- capped, GSH- capped, Lcys- capped negative charge	0,16 µM 0,40 µM 8,0 µM $1,2 \times 10^{-6}$ 1,6 µM 24.0 µM	0.11 µM	7,4	The sudden decline in the α-helix fraction of LZY was observed when the concentration of the three CdS QDs reached $2,4 \times 10^{-5}$ mol/l and LZY began to denaturize.	[38]
CdS-QD NPs	BSA	Spherical	2 nm to 3 nm	MPA- capped, GSH- capped, Lcys- capped negative charge	0,16 µM 0,4 µM 0,8 µM 1,2 µM 1,6 µM 24,0 µM	3,0 µM	7,4	The concentration of MPA-CdS QDs increased to $2,4 \times 10^{-5}$ mol/l α -helix fraction of BSA did not revert to the value in the native molecule of MPA-CdS QDs. While the α-helix content of BSA declined with the concentration of GSH-CdS QDs increased.	[38]
HO-SWCNT NPs	Haemoglobin Myoglobin	Solid cylindrical	OD 1 nm to 2 nm Length 5 µm to 30 µm	Electron (hydrogen) donor	0,5 to 10,0 mg/l	5,0 µM	7,4	HO-SWCNTs induced the denaturation and unfolding of Hb and Mb.	[39]
SWCNT NPs	Tau protein	Tubular shapes	OD 1 nm to 2 nm	Negative charge less hydrophobic	10 µg/ml 50 µg/ml 100 µg/m	200 µg/ml	7,8	The binding of tau and SWCNT induces the protein folding and more compact structure.	[40]
MWCNT NPs	Tau protein	Tubular shapes	OD 10 nm to 20 nm	Negative charge more hydrophobic	10 µg/ml 50 µg/ml 100 µg/ml	200 µg/ml	7,8	The binding of MWCNT has not altered the secondary structure of tau protein.	[40]

Table B.1 *(continued)*

Type of NP	Type of protein	Shape	Size	Surface properties	NP concentration	Protein concentration	pH	Conclusion	Ref.
SWCNT NPs	Lysozyme	Tubular shapes	—	Hydrophobic	30 µg/ml	$6{,}9 \times 10^{-6}$ M	—	The confirmation of LYS bound to nanotubes is closer to native than to denatured form since no shifts in minimum wavelength were observed.	[41]
GQDs NPs	HSA	Spherical	1,2 nm	High surface area	8,55 µM 17,1; µM 34,2 µM	2,0 µM	7,4	The α-helices content decreased, but other secondary structure contents increased with the increment of the concentration of GQDs and biological activity of HAS reduce upon the interaction with GQDs	[42]
Ag NPs	BHb	—	5 nm to 10 nm	—	18,7 µM 37,4 µM 74,8 µM	0,25 µg/ml	7,4	Binding of silver NP leads to alpha-beta transition and causes to a partial unfolding of BHb.	[43]
Morin-Au NPs	BSA	Spherical	30 nm to 40 nm	Reduced	1,5 µM	5,0 µM	7,4	The structure of BSA is predominantly helical, and the secondary structure of the BSA changed dramatically in the presence of M–Au NPs.	[44]
CdS NPs	HSA	Platelet-like	5 nm to 10 nm	High surface area	0 µM to 4 µM	4,0 µM	7,4	The structure of serum albumins is predominantly α-helical even after the binding of CdS NPs but α -helicity of serum albumins due to the binding with CdS NPs	[45]
ZnO NPs	ToxR	Spherical	2,5 nm	Positive charge	1:1 (protein/NPs)	10,0 µM	8	Binding to ZnO NPs can result in major structural changes of the ToxRp, and a significant percentage of secondary structure was lost	[46]
ZnO NPs	Lysozyme	Spherical	4 nm to 7 nm	Positive charge	—	10,0 µM	7,4	ZnO NPs bind to the largest cleft on the protein surface and can retain the secondary structures to a greater degree and exhibit enzymatic activity even under denaturing conditions.	[47]
ZnO NPs	BSA	Spherical	5 nm to 11 nm	Hexagonal wurtzite structure	0,09 µM 0,12 µM	—	—	The basic structure of the protein is kept intact after binding with ZnO NPs but there is a decrease in α-helical contents.	[48]

Table B.1 *(continued)*

Type of NP	Type of protein	Shape	Size	Surface properties	NP concentration	Protein concentration	pH	Conclusion	Ref.
CdTe QD NPs	α -Chymotrypsin	Spherical	3 nm to 4 nm	—	0,6 µM	1,6 µM	7,02 9,05	Hydrogen bonds are involved when pH is 9·05 while the hydrophobic and electrostatic interactions are involved when pH is 7·20. CdTe can change the conformation of α-Chy but it could maintain its high activity under different pH values for 24 h in the presence of CdTe.	[49]
CdSe/ZnS QD	HSA	Quasi-spherical	4,43 nm	Negative charge	2,5 µM 5,0 µM 0,1 µM	0,1 µM	7,4	HSA underwent conformational changes at both secondary and tertiary structure levels and changes were more significant at a higher concentration of QDs. The biological activity of HSA was weakened in the presence of QDs.	[50]
Glutathione-CdTe-QDs	HSA	—	5 nm	Negative charge	0,5 µM 1,0 µM 2,0 µM	0,1 µM	7,4	QDs can induce the conformational changes of HSA, which may then change the the biological activity of HSA.	[51]
CdTe-QDs	HSA	—	2 nm 3,1 nm 3,7 nm 4,8 nm	MPA capped GSH capped CA capped NAC capped	1,0 µM (20:1) (10:1) (5:1) (HAS: QDs)	0,1 µM	7,4	The interaction between HSA and negatively charged QDs has nothing to do with the surface modification. QDs can influence the α-helical content in protein and the conformational changes of HAS can change the biological activity of HSA.	[52]

Annex C
(informative)

Description of buffers that can be used for protein solubility

C.1 Buffer

Recommended buffer properties are shown in Table C.1. Buffers not recommended are listed in Table C.2.

Table C.1 — Recommended buffer properties[4]

Buffer[a]	Approximate lower wavelength limit (nm)
10 mM potassium phosphate, 100 mM potassium fluoride	185
10 mM potassium phosphate, 100 mM $(NH_4)_2SO_4$	185
10 mM potassium phosphate, 50 mM Na_2SO_4	185
10 mM potassium phosphate, 100 mM KCl	195
20 mM sodium phosphate, 100 mM NaCl	195
Dulbecco's phosphate buffered saline (PBS): 9,33 mM potassium phosphate, 136 mM, NaCl, 2,7 mM, KCl, 0,6 mM, $MgCl_2$, 0,9 mM $CaCl_2$.	200
2 mM hepes, 50 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol	200
50 mM tris, 150 mM NaCl, 1 mM dithiothreitol, 0,1 mM EDTA	201
[a] DMSO and formamides have high absorbance and cannot be used for CD measurements.	

Table C.2 — Not recommended buffers

Buffer[a]	Approximate lower wavelength limit (nm)
hexafluoro-2-propanol (HFIP)	200
trifluoroethanol (TFE)	200
[a] DMSO and formamides have high absorbance and cannot be used for CD measurements.	

C.2 Cleaning agent

The following steps are recommended when using a cell cleaning agent.

a) The stock solution of the cell cleaning agent should be mixed with de-ionized water as described by the supplier.

b) Sink the cell in the cleaning solution and heat the solution between 50 °C and 60 °C for about 20 min. Then wash the cell with de-ionized water several times.

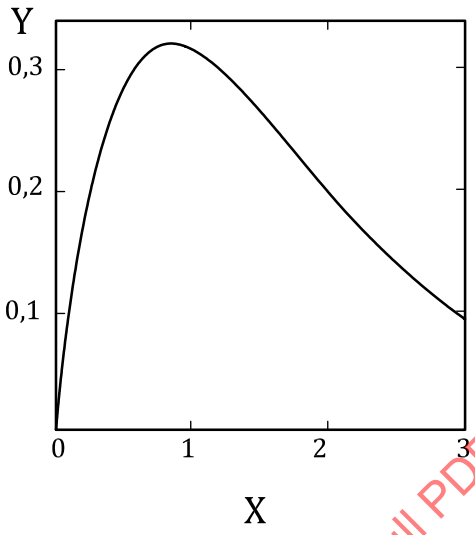
c) For removing NPs from the cell, aqua regia can be used, which is produced by mixing hydrochloric acid and nitric acid according to the following reaction:

$$HNO_{3(aq)} + 3HCl_{(aq)} \rightarrow NOCl_{(aq)} + 2Cl^{nasc}\,(g) + 2H_2O$$

d) Mixing the two acids produces aqua regia, which can be identified by a golden yellow colour. Aqua regia is suitable for most metallic NPs. However, for some other NPs, depending on the NP, more suitable solvents are advisable.

e) Boil the cell in a small amount of aqua regia for about 5 min.

f) Wash the cell with deionized water four times. To make sure that all of aqua regia is washed away, check the pH of the rinsing water and keep washing until a neutral pH is reached.

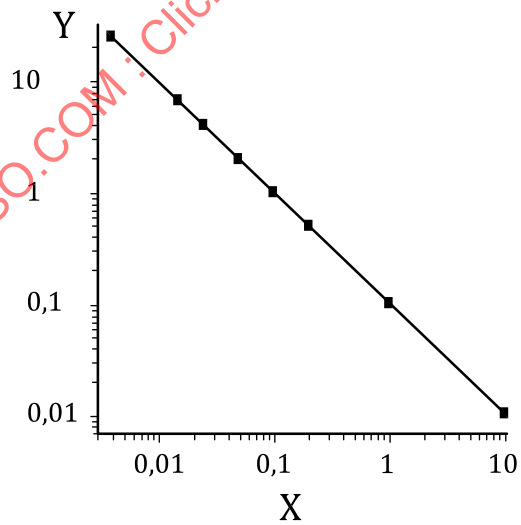


Key

X absorbance unit

Y S/N ($-2.303 I_0 \Delta\varepsilon/\varepsilon$)

Figure C.1 — Signal-to-noise ratio in a CD measurement as function of the absorbance unit[5]



Key

X path length (mm)

Y [protein] (mg/ml)

NOTE The plot shows that the protein concentration required to produce an absorbance of 0,5.

Figure C.2 — Optimal protein concentration as a function of the path length of the cell[5]

C.3 Absorbance of NP in CD experiments

Most NOAAs with a size of > 10 nm are achiral and will not exhibit quantifiable CD signals[53]. When NMs are conjugated with chiral molecules or biomolecules, they will be active in CD technique[54]. NMs with a size smaller than 10 nm have the potential to absorb CD signals by surface plasmonic resonances of the metallic structure in the visible region. In addition, NMs can produce clusters (0,5 nm to 2,0 nm) that will convert to the chiral objects and be able to absorb CD signals[55].

C.4 Absorbance of buffers in far-UV region

See Table C.3.

Table C.3 — Absorbance of various salt and buffer substances in the far-UV region[56]

Compound	pH	No absorbance above	Absorbance of a 10 Mm solution in a 1,0 mm cuvette at:			
			210 nm	200 nm	190 nm	180 nm
$NaClO_4$		170	0,00	0,00	0,00	0,00
NaF, KF		170	0,00	0,00	0,00	0,00
Boric acid		180	0,00	0,00	0,00	0,00
NaCl		205 nm	0,00	0,02	> 0,50	> 0,50
Na_2HPO_4		210 nm	0,00	0,05	0,30	> 0,50
Na_2HPO_4		210 nm	0,00	0,05	0,30	> 0,50
Na acetate		220 nm	0,03	0,17	> 0,50	> 0,50
Glycine		220 nm	0,03	0,10	> 0,50	> 0,50
Diethylamine		240 nm	0,40	> 0,50	> 0,50	> 0,50
NaOH	12,0	230 nm	> 0,50	> 2,00	> 2,00	> 2,00
Boric acid, NaOH	9,1	200 nm	0,00	0,00	0,09	0,30
Tricine	8,5	230 nm	0,22	0,44	> 0,50	> 0,50
TRIS	8,0	220 nm	0,02	0,13	0,24	> 0,50
HEPES	7,5	230 nm	0,37	0,50	> 0,50	> 0,50
PIPES	7,0	230 nm	0,20	0,49	0,29	> 0,5
MOPS	7,0	230 nm	0,10	0,34	0,28	> 0,50
MES	6,0	230 nm	0,07	0,29	0,29	> 0,50
Cacodylic acid	7,0	210 nm	0,01	0,20	0,22	

C.5 Control for sample preparation and recording good quality spectra

When using CD:

a) Buffers/samples in cuvettes should be inspected by eye before and after the CD measurement:

   1) Are any air-bubbles present (samples can out-gas during the measurement)?

   2) Is the sample stable in solution?

   3) Is the sample scattering an excessive amount of light?

b) The buffer shall be checked for the presence of a CD signal. If present, this can be subtracted if care is taken to ensure that the buffer and sample are matched.

c) Consider whether the cuvette is placed in the instrument in a reproducible way. If it is not, then the buffer and sample spectra can appear to be vertically offset from each other. The region between 250 nm and 260 nm can be expected to be close to zero mdeg.

d) Consider whether the sample is photolyzing in the measuring beam. Record repeat scans and check them carefully for reproducibility. A decreasing signal intensity can be due to sample photolysis.

e) When using an instrument which requires a scan speed, it is possible to accidently over-smooth or distort the data. The instrument's user manual will explain how to select an appropriate scan speed.

Annex D
(informative)

Unit conversions in CD measurements

Table D.1 — Units conversion in CD measurements

Original unit	Absorbance[a]	Milliabsorbance[b]	Molar extinction[c]	Degrees[d]	Millidegrees[e]	Molar ellipticity[f]
(A)	A	A × 1 000	A × M/ (C × L)	A × 32,98	A × 32 980	A × M × 3298/ (L × C)
(mA)	mA/1 000	mA	A × M/ (C × L × 1 000)	mA × 0,03298	mA × 32,98	mA × M × 3,298/ (L × C)
(ε)	ε × C × L/M	ε × C × L × 1 000/M	ε	ε × C × L × 32,98/M	ε × C × L × 32 980/M	ε × 3298
(°)	°/32,98	°/0,03298	m° × M/ (C × L × 32,98)	°	° × 1 000	° × M × 100 /(L × C)
(m°)	m°/32 980	m°/32,98	m° × M/ (C × L × 32 980)	m°/1 000	m°	m° × M/ (10 × L × C)
[Θ]	[Θ] × C × L/ (3 298 × M)	[Θ] × C × L/ (3,298 × M)	[Θ]/3 298	[Θ] × C × L/ (100 × M)	[Θ] × C × L × 10/M	[Θ]

[a] Units are absorbance (Abs).

[b] Units are milliabsorbance (mAbs).

[c] Units are A × L/mol × cm.

[d] Units are degrees (°).

[e] Units are millidegrees (m°).

[f] Units are deg × cm^2/dmol.

Annex E
(informative)

Calculating the concentration range of the sample

The appropriated concentration of the NPs relative to the protein shall be calculated by the number of proteins needed to form a monolayer on the surface NP. Select NP concentration assuring that the total NP surface of in the mixture will be theoretically sufficient to accommodate all proteins present. The stability of the NP suspension in the buffer is tested by DLS[16].

The surface site concentration can be determined by simply multiplying this value by the number of NPs in 1 L and dividing by the Avogadro constant (N_A = 6,022 × 10 mol^{-1}), as shown by Formula (E.1):

$$C_{\text{surface site concentration}}\left[\frac{\text{mol}}{\text{L}}\right] = \frac{C_{\text{surface sites}}\left[\text{L}^{-1}\right]}{N_A\left[\text{mol}^{-1}\right]} \quad \text{(E.1)}$$

Annex F
(informative)

Methods for estimation of secondary structures of protein

Table F.1 — Reliability of different methods for estimation of secondary structure by the CD spectra[21]

Method	Failures	Helix	Corr	Antiparallel		Parallel		β-sheet		Turn+Others	
		RMSD		RMSD	Corr	RMSD	Corr	RMSD	Corr	RMSD	Corr
BeStSel	—	0,038	0,99	0,050	0,98	0,032	0,97	0,039	0,99	0,033	0,95
VARSLC	5	0,089	0,97	0,155	0,62	0,860	−0,08	0,133	0,73	0,130	0,74
LINCOMB	—	0,119	0,91	0,214	0,45	0,198	0,59	0,230	0,51	0,232	0,59
CDNN	—	0,083	0,97	0,122	0,83	0,076	0,91	0,102	0,89	0,115	0,81
SELCON	—	0,147	0,86					0,122	0,82	0,077	0,73
CONTIN	2	0,095	0,95					0,068	0,96	0,074	0,73
CDSSTR	—	0,201	0,76					0,139	0,75	0,099	0,71
K2D	—	0,198	0,84					0,152	0,79	0,153	0,55
K2D2	—	0,222	0,70					0,162	0,71	0,088	0,68
K2D3	—	0,136	0,87					0,184	0,64	0,143	0,65
CAPITO	—	0,260	0,57					0,161	0,85	0,147	0,70