



NCL Method ITA-4

Analysis of Nanoparticle Interaction with Plasma Proteins by 2D PAGE

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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Please cite this protocol as:

Neun BW, McLeland CB, Potter TM, Specht S, Guszcynski T, Dobrovolskaia MA, NCL Method ITA-4: Analysis of Nanoparticle Interaction with Plasma Proteins by 2D PAGE.
<https://ncl.cancer.gov/resources/assay-cascade-protocols> DOI: 10.17917/GS4R-4H02

1. Introduction

Proteins bind the surfaces of nanoparticles, and biological materials in general, immediately upon introduction of the materials into a physiological environment. The further biological response of the body is influenced by the nanoparticle–protein complex. The nanoparticle's composition and surface chemistry dictate the extent and specificity of protein binding. In general, charged particles bind more proteins than their neutral counterparts. Protein binding is one of the key elements that effect particle uptake by the phagocytic cells of the immune system, and therefore, the biodistribution of the nanoparticles throughout the body (Figure 1) [1-4]. Protein binding may or may not affect the activity of the protein in the “corona” surrounding nanoparticle surface [5]. As such using protein binding in lieu of specialized immunotoxicity assays is not recommended.

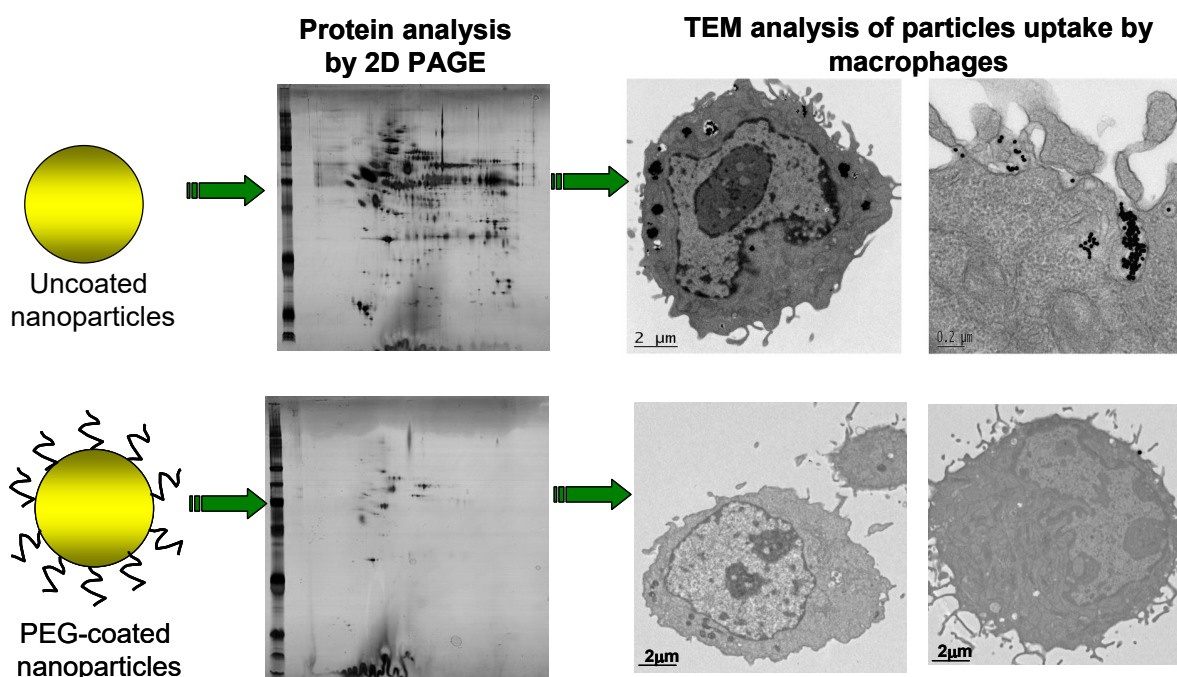


Figure 1. Depiction of how particle characteristics affect protein binding.

Binding of plasma protein influences particle stability, biodistribution, and clearance from the blood stream. Particle surface charge is important for protein binding and uptake by mononuclear phagocytic system (MPS).

2. Principles and Limitations

2.1 Principles

This document describes a protocol for analysis of nanoparticle interaction with plasma proteins by two-dimensional gel electrophoresis (2D PAGE) [6-7].

Nanoparticles are incubated with pooled human plasma derived from healthy donors to allow for protein interaction and binding. Following a separation procedure, bound proteins are eluted from the nanoparticle surface and analyzed by 2D PAGE. Identity of individual proteins separated by this procedure will be evaluated by mass spectrometry. This assay requires at least 1 mg of nanoparticles in 1 mL of vehicle or storage buffer.

2.2 Limitations

This method is limited to nanomaterials with physicochemical properties which allow separation of the particles from plasma by centrifugation (e.g. metal oxides, metal colloids, core-shell materials). This method is not applicable to liposomes, dendrimers and other “soft” nanomaterials. Methods for separation of nanoparticle-bound proteins from the bulk plasma other than centrifugation should be considered to overcome this limitation.

Another limitation is related to comparing images of 2D PAGE to the plasma protein map. The plasma proteome contains over 3000 proteins. The composition of the plasma proteome is affected by physiological conditions and the genetic background of individual donor. Even when pooled plasma is used, some discrepancies may be noted in the position of well-known proteins such as immunoglobulins or albumin on the 2D gel between the plasma protein map and the control plasma sample used in this experiment. Altogether it often complicates the analysis. The alternative to overcome this limitation is to perform mass-spectrometry analysis (see reference 5 for details) after step 5.9 of this protocol.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only.

Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents

- 3.1.1 Normal human plasma, anti-coagulated with sodium-citrate, pooled from at least three donors
- 3.1.2 Nanoparticles
- 3.1.3 PBS, Ca/Mg-free (GE Life Sciences, SH30256.01)
- 3.1.4 Sterile water
- 3.1.5 Destreak Rehydration Solution (GE Healthcare, 17-6003-19)
- 3.1.6 IPG Buffer pH 3-10 (GE Healthcare, 17-6000-87)
- 3.1.7 Dithiothreitol (DTT) (Sigma, D9163-5G)
- 3.1.8 Immobiline Drystrip (IPG) pH 3-10, 11 cm strip (GE Healthcare, 18-1016-61)
- 3.1.9 Immobiline Drystrip cover fluid (GE Healthcare, 17-1335-01)
- 3.1.10 ExcelGel XL SDS 12 – 14 (GE Healthcare, 17-1236-01)
- 3.1.11 ExcelGel SDS Buffer Strips (GE Healthcare, 17-1342-01)
- 3.1.12 SeeBlue Plus2 marker (Invitrogen, LC5925)
- 3.1.13 Tris base (Sigma, T-6791)
- 3.1.14 Urea (GE Healthcare, 17-1319-01)
- 3.1.15 Glycerol (Sigma, G-7893)
- 3.1.16 SDS (Sigma, L-5750)
- 3.1.17 Bromophenol Blue (GE Healthcare, 17-1329-01)
- 3.1.18 Silver Staining Kit (GE Healthcare, 17-1150-01)

3.2 Materials

- 3.2.1 Pipettes covering the range from 0.05 to 10 mL
- 3.2.2 Low retention pipette tips
- 3.2.3 Low retention microcentrifuge tubes
- 3.2.4 Polypropylene tubes 15 mL
- 3.2.5 Immobiline Drystrip Kit (Amersham Biosciences)

- 3.2.6 Immobililine Drystrip Reswelling Tray (Amersham Biosciences)
- 3.3 Equipment
 - 3.3.1 Incubator set at 37°C
 - 3.3.2 Tube rotator
 - 3.3.3 Microcentrifuge
 - 3.3.4 2D Multiphor II System (Amersham Biosciences)
 - 3.3.5 Multitemp III Thermostatic Circulator (Amersham Biosciences)
 - 3.3.6 EPS 3501 XL Power Supply (Amersham Biosciences)

4. Reagent Preparation

- 4.1 2-D Equilibration Buffer

200 mL, 50 mM Tris-Cl pH 8.8, 6 M Urea, 30% glycerol, 2% SDS, bromophenol blue.
- 4.2 2-D Rehydration Buffer

To 3 mL DeStreak Rehydration Solution, add 15 µL IPG Buffer and 8.4 mg dithiothreitol.

5. Preparation of Study Samples

- 5.1 Pipette 1.5 mL of pooled plasma (70-100 mg of total protein) into a microcentrifuge tube. Spin 30 min at 22,000xg, 4°C. Collect supernatant and transfer it into a fresh tube. **Note:** *Two 1.5 mL aliquots will be required for 2 samples. If more than two samples are analyzed, increase the number of aliquots accordingly.*
- 5.2 In a microcentrifuge tube, combine 1 mL of nanoparticles (1 mg/mL) and 1 mL of plasma prepared in step 5.1. This is a “+NP” study sample. Always prepare 1 control tube where nanoparticles are substituted with PBS or other buffer used in nanoparticle formulation. This is a “-NP” control.

Note: *If concentration of 1 mg/mL is not acceptable for a given nanomaterial due, for example, to particle aggregation, then several samples may be prepared and pooled at the end of procedure. For example, three samples with a concentration of 0.333 mg/mL each can be prepared for colloidal gold nanoparticles; at the end*

of purification (step 5.10 outlined below) pellets from three tubes are pooled together and eluted with the same volume of rehydration buffer.

- 5.3 Vortex to mix and incubate 30-35 min at 37°C on a tube rotator.
- 5.4 Centrifuge at 18,000xg, 4°C, for 30 min. Carefully remove and discard supernatant.
- 5.5 Add 1.5 mL of PBS to “+NP” and “-NP” tubes, vortex, and repeat centrifugation as described in step 5.4.
- 5.6 Repeat steps 5.4 and 5.5 three more times.
- 5.7 Add 1.5 mL of PBS diluted with water (1:10) to “+NP” and “-NP” tubes, vortex, and repeat centrifugation as described in step 5.4.
- 5.8 Repeat step 5.7 one additional time.
- 5.9 Centrifuge at 18,000xg, 4°C, for 5 min. Carefully remove and discard traces of supernatant.

***Note:** At this step, the sample can proceed directly to the analysis by mass-spectrometry. The analytical procedure for the mass spectrometry has been described elsewhere [5]. Continue to the next step when analysis by 2D PAGE is desired.*

- 5.10 Add 200 µL of rehydration buffer, vortex and incubate at room temperature for 15-20 min, vortexing occasionally.
- 5.11 Centrifuge at 18,000xg, 4°C, for 20 min. Carefully transfer supernatant to a fresh tube. At this point, one may either proceed to the next step or freeze the sample and store it at -80°C.

***Note:** Steps 5.4 – 5.11 were validated using colloidal gold nanoparticles with sizes of 50, 30 and 10 nm. Other particles may require either different centrifuge speed (e.g. 18,000xg works for 50 and 30 nm Au nanoparticles, while 25,000xg is required for 10 nm Au nanoparticles) or a different procedure (e.g., gel-filtration or magnetic separation). If a different procedure is used, validation experiments should be conducted prior to analysis to ensure optimal assay performance (i.e. minimal loss of nanoparticles during separation, no unspecific protein contamination in minus particle control, etc).*

6. Sample Rehydration

- 6.1 Apply a total of 200 μ L of sample in rehydration solution (sample from step 5.11) into reswelling tray.
- 6.2 Peel cover off IPG strip. Place sticky side down, being careful to remove all bubbles.
- 6.3 Cover with Immobiline Drystrip cover fluid. Place lid on tray and allow to set 10-24 hours to completely reswell.

7. First Dimension

- 7.1 Remove the rehydrated IPG strip from the Immobiline Reswelling Tray. (See reference 6 for more information of 2D gel electrophoresis.)
- 7.2 Position the IPG strip in the Immobiline Drystrip aligner.
- 7.3 Position and attach the electrodes.
- 7.4 Set gradient program as follows:

Phase	Voltage	kVh
1	300	0.001
2	3500	2.9
3	3500	17

- 7.5 Freeze IPG strip overnight in tube at -20°C.

8. Second Dimension

- 8.1 Equilibrate strip in 10 mL of Equilibration Solution with 100 mg DTT for 15 minutes.
- 8.2 Set Multitemp III Thermostatic Circulator to 15°C.
- 8.3 Cover multiphor cooling plate with 2.5 to 3.0 mL of Immobiline Drystrip cover fluid.
- 8.4 Place ExcelGel XL SDS 12 – 14 onto cooling plate.
- 8.5 Position Anode and Cathode ExcelGel SDS Buffer Strips.

- 8.6 Place IPG strip with gel side down on the ExcelGel XL SDS 12 – 14.
- 8.7 Apply 5 μ L of SeeBlue Plus2 marker next to the IPG strip.
- 8.8 Align electrodes on the top of the anode and cathode buffer strips.
- 8.9 Apply current with the following step program:

Step	Voltage (V)	Current (mA)	Power (W)	Duration (hr:min)
1	200	20	20	0:40
2	800	40	40	2:40

9. Silver Staining

Perform silver staining of the gels according to the protocol supplied by manufacturer's instructions [8].

10. Data Analysis

Gel images are analyzed using appropriate 2D analysis software such as for example GellabII (<http://www.lecb.ncifcrf.gov/gellab/>), ImageMaster (Amersham Biosciences) or PDQuest (BioRad Laboratories). Spot identity is then confirmed by mass spectrometry.

11. References

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12. Abbreviations

DTT	dithiothreitol
kVh	kilovolt hours
mA	milliamp
NP	nanoparticle
PAGE	poly acrylamide gel electrophoresis
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate