Chapter 34

Single Cell Gel Electrophoresis Assay (Comet Assay) for Evaluating Nanoparticles-Induced DNA Damage in Cells

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Abstract

Comet assay provides a rapid and reliable way to screen genotoxic effects of a wide variety of nanoparticles. Single cell gel electrophoresis assay (SCGE) or comet assay gained in popularity as a standard technique and is widely used for testing novel particles or chemicals for genotoxicity, monitoring environmental contamination with genotoxins, human biomonitoring and molecular epidemiology, and fundamental research in DNA damage and repair. Here, we describe the method of alkaline comet assay to detect nanoparticle-induced DNA damage.

Key words: Nanoparticles, DNA damage, Genotoxicity, Reactive oxygen species, Comet assay

1. Introduction

Nanotechnology is the manipulation of matter on a near-atomic scale to produce nanoparticles with unique properties, allowing new commercial applications. Since nanoparticles exhibit unique physicochemical properties, evaluation of the genotoxic effects of nanoparticles is essential. Genotoxic activities may be produced by direct interaction of particles with the genetic material or by secondary damage from particle-induced reactive oxygen species (ROS). Both pathways may relate to surface properties, the presence of transition metals, intracellular iron mobilization, or lipid peroxidation processes. Other aspects relevant to primary genotoxicity are particle size, shape, particle uptake, and the presence of mutagens carried with the particles (1). Secondary genotoxicity is characterized by excessive and persistent formation of ROS by inflammatory cells. In vitro genotoxicity tests, such as the comet assay, are sensitive tools for the detection of the mutagenic potential of a test material (2). Nanomaterials that stimulate generation of ROS are suggested to test its genotoxicity.

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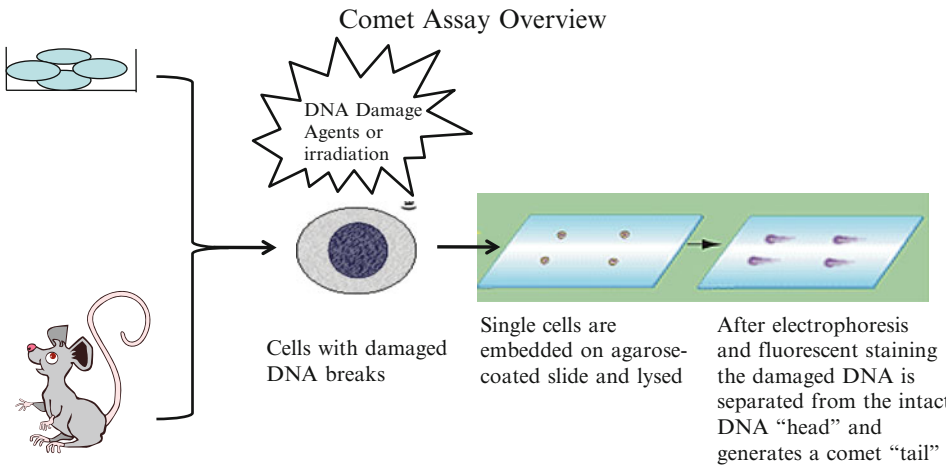

Fig. 1. An overview of single cell gel electrophoresis assay (comet assay).

Single cell gel electrophoresis assay (SCGE) or comet assay was first described by Singh et al. in 1988 (3). The principle of the comet assay is that the damaged DNA fragments will migrate out of the cell when an electric current is applied, whereas the undamaged DNA will remain in the cell nucleus. The results resemble a comet with a head (intact DNA) and a tail (damaged DNA). The size and shape of the tail and the distribution of DNA within the comet correlate with the extent of DNA damage (4–6). Treated cells are embedded in an agarose gel on a microscope slide. The cells are lysed to remove cellular protein. Cells are then treated with alkali to denature the DNA. The damaged DNA is allowed to migrate away from the nucleus by undergoing electrophoresis. The samples are stained with a DNA-specific fluorescent dye. The gel is then analyzed for the amount of fluorescence in the head and tail and the tail length (6–11) as shown in (Fig. 1).

The image analysis measures the overall intensity of the fluorescence for the whole nucleoid and the fluorescence of the migrated DNA and compares the two signals. The stronger the signal from the migrated DNA, the more damage there is present (12). The brighter and longer the tail, the higher the level of damage (Fig. 2).

Comet assay is an extremely sensitive DNA damage assay. This sensitivity needs to be handled carefully as it is also vulnerable to physical changes, which can affect the reproducibility of the results. Essentially, anything that can cause DNA damage or denaturation except the factor(s) being researched is to be avoided (4). Due to its simple and inexpensive setup, it can be used in conditions where more complex assays are not available.

Comet assay kits, reagents, control cells, and electrophoresis systems are available from Trevigen, Inc. Trevigen has developed a complete comet assay system which includes comet assay kits,

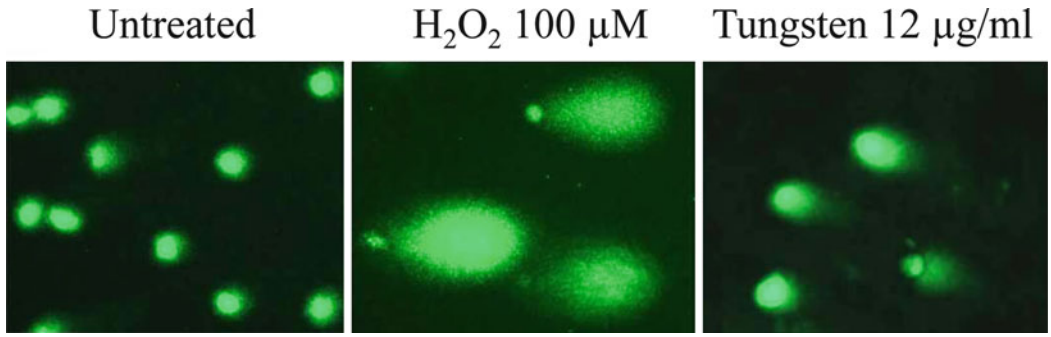

Fig. 2. Photo micrographs showing the DNA damage of JB6 cells. *Left*: untreated cells *center*: damage by H_2O_2 (positive control); *Right*: damage by nanoparticles of tungsten carbide-cobalt (WC-Co).

comet slides, comet assay control cells, and a specialized electrophoresis unit. These kits and reagents were successfully used in our lab for screening the genotoxicity of occupational related dusts, chemicals, and nanoparticles.

2. Materials

1. Comet assay kit (Trevigen) containing Lysis Solution (2×500 mL, store at room temperature), comet LM agarose (LMA) (15 mL, store at room temperature), Trevigen Comet Slide™ (25 slides, store at room temperature), 200 mM EDTA (pH 10) (12.5 mL, store at room temperature), and SYBR® Green I nucleic acid gel stain (store at −20 °C).
2. DMSO.
3. Phosphate Buffered Saline (PBS 1×, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, adjust to a final pH of 7.4), calcium and magnesium free.
4. 500 mM EDTA solution, pH 8.
5. Alkaline DNA denaturing solution (200 mM NaOH, 1 mM EDTA, pH >13). To prepare 50 mL of alkaline unwinding solution, combine: 0.4 g of NaOH pellets, 100 µL of 500 mM EDTA, 49.75 mL distilled H_2O. Stir until fully dissolved. The solution will warm during preparation. Allow to cool to room temperature before use (see Note 1).
6. Alkaline electrophoresis solution (NaOH 200 mM, EDTA 1 mM, pH >13). To prepare 1.5 L, dissolve 12 g of NaOH pellets in distilled H_2O, add 3 mL of 500 mM EDTA (pH = 8), and make up to 1.5 L with H_2O (see Note 1).
7. TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH = 7.5.
8. SYBER Green 1 staining solution. Add 1 µL of SYBER Green I to 10 mL of TE Buffer, pH = 7.5

9. Suitable cell line. Here, JB6 cell line was used in this Lab.
10. Eagle's minimal essential medium (EMEM).
11. 200 mM L-glutamine.
12. Penicillin–Streptomycin (10,000 penicillin/mL, 10 mg streptomycin/mL).
13. Heat-inactivated fetal bovine serum (FBS).
14. Complete EMEM medium for JB6 cells: EMEM medium containing FBS (5 %), L-glutamine (2 mM), and penicillin–streptomycin (1 %).
15. Trypsin–EDTA: 0.5 % Trypsin, 5.3 mM. Dilute to 0.01 % in 1× PBS for JB6 cells.
16. Tungsten carbide-cobalt nanoparticles (Inframat Advanced Materials): prepare 10 mg/mL stock solution in sterile PBS.
17. Boiling water bath (or a large beaker) and 37 °C water bath.
18. Horizontal DNA electrophoresis apparatus.
19. Epifluorescence microscope equipped with fluorescein filter or light transmission microscope when using a silver staining kit (Olympus AX-70).
20. 15 mL sterile conical centrifuge tubes.
21. 1 L graduated cylinder.
22. 4 °C refrigerator/cold room.
23. Hemacytometer.
24. Heating block.
25. Sonicator with microprobe set at output voltage of 150 W and constant duty cycle (Branson Sonifier 450).

3. Methods

3.1. Preparation of Nanoparticles (See Note 2)

1. Add 10 mg of nanoparticles to 1 mL sterile PBS in 15 mL sterile conical centrifuge tube to prepare a stock solution.
2. Keeping sample on ice, sonicate for 30 s, then rest 15 s. Repeat cycle to sonicate for a total of 3 min.
3. Make serial dilutions of the nanoparticles with complete EMEM medium to cover a range of concentration (typically 0–100 μg/cm^2) for cell treatment (see Notes 3–5).

3.2. Cell Treatment and Preparation

1. Seed JB6 cells at 5×10^4 cells/well in 24-well (growth area, 1.9 cm^2/well) tissue culture plate in complete medium (EMEM medium with 5 % FBS, 2 mM L-glutamine, 1 % penicillin–streptomycin).

2. Incubate the cells overnight in a 37 °C, 5 % CO_2, 80 % humidity incubator.
3. Prepare nanoparticles and make a series dilutions of nanoparticles in complete EMEM medium (see Subheading 3.1).
4. Remove cells from incubator.
5. Remove media from the cells by aspiration.
6. Replace medium with desired concentration of nanoparticle suspended in complete EMEM medium, return the plate to incubator, and incubate cells for 0.5–24 h (see Note 6).
7. After incubation, remove the medium and wash 1× with PBS, remove PBS.
8. Add 0.5 mL 0.01 % Trypsin–EDTA and incubate at 37 °C for 5–10 min to detach the cells. Pipette up and down several times to separate cells, and then add 0.5 mL complete EMEM medium containing FBS (5 %), L-glutamine (2 mM), penicillin–streptomycin (1 %).
9. Transfer cells to 1.5 mL Eppendorf microcentrifuge tube, obtain a cell count by using hemacytometer, and then pellet cells by centrifuging at 1,000 rpm for 3 min.
10. Remove supernatant. Wash once in ice cold 1× PBS (Ca^{++} and Mg^{++} free). Resuspend cells at 1×10^5 cells/mL in ice cold 1× PBS (see Note 7).

3.3. Alkaline Comet Assay

All steps are performed at room temperature unless otherwise specified. Work under dimmed or yellow light to prevent damage from UV.

1. Prepare lysis solution without DMSO and chill on ice for at least 20 min before use (see Note 8).
2. Melt LMA agarose in a beaker of boiling water for 5 min with cap loosened, then place bottle in a 37 °C water bath for at least 20 min to cool (see Note 9).
3. Add 500 µL melted agarose to 1.5 mL microcentrifuge tube and keep at 37 °C.
4. Add 50 µL cell (~500 cells) suspensions to the Eppendorf microcentrifuge tube containing melted agarose and keep at 37 °C. Mix well with a pipette (see Note 10).
5. Immediately pipette 50 µL cell suspensions with agarose on Comet Slide that is kept at 37 °C by placing on top of heating block or incubator. Cover entire sample area of slide by spreading with pipette tip (see Note 11).
6. Place slide flat in box to keep in the dark at 4 °C (e.g., place in refrigerator or on ice) for 30 min. A 0.5 mm clear ring appears at edge of Comet Slide area.

7. Immerse slide in prechilled lysis solution and leave at 4 °C in the dark for 30–60 min.
8. Drain excess buffer from slides and immerse in freshly prepared alkaline DNA denaturing solution (see Note 12).
9. Leave Comet Slide in alkaline DNA denaturing solution for 40 min at room temperature, in the dark.
10. For the comet assay ES tank, add 950 mL prechilled alkaline DNA denaturing solution, place slides in electrophoresis slide tray, and cover with slide tray overlay. Set power supply to 21 V and apply voltage for 30 min.
11. Gently drain excess electrophoresis solution, rinse by dipping several times in distilled H_2O.
12. Then place in 70 % ethanol for 5 min.
13. Air dry samples at room temperature in the dark for 10–15 min. Drying brings all the cells in a single plane to facilitate observation. Samples may be stored at room temperature with desiccant prior to scoring at this stage.
14. Place 100 μL of diluted SYBR® Green I onto each circle of dried agarose and place in refrigerator for 5 min. Gently tap slide to remove excess SYBR solution. Allow slides to dry completely at room temperature in the dark.
15. View slide under fluorescence microscope equipped with Fluorescein filters in the dark (see Note 13 and 14).

4. Notes

1. Use of freshly made solution is recommended. Prechill the alkaline electrophoresis solution at 4 °C. The alkaline DNA denaturing solution should be kept at room temperature. Wear gloves when preparing and handling the alkaline solution.
2. Most commercially available nanoparticles, such as titanium dioxide (TiO_2) nanoparticle, single-walled carbon nanotubes (SWCNT), and tungsten carbide-cobalt nanoparticles (WC-Co), are water-insoluble. For in vitro testing, nanoparticles should be suspended in culture medium. Since nanoparticles tend to form coarse agglomerates in physiological media, nanoparticles have to be sonicated to improve dispersion.
3. We recommend using 0–100 μg/cm² in adhesion cell system. To do so, the volume of particle-containing medium added to each well should be adjusted according to the growth area of each well. For example, a final nanoparticle concentration of 5 μg/cm² may be achieved by adding 1 mL of medium with the nanoparticle concentration of 10 μg/mL or 2 mL of

medium containing 5 μg/mL nanoparticles. These values should also be adjusted for different plates, i.e., for a 24-well plate, the growth area of each well is 2 cm^2 if purchased from Falcon Inc., but it is 1.9 cm^2 if obtained from Costar Inc.

4. The nanoparticle doses chosen for cellular or animal testing systems need to be relevant to anticipated workplace exposures. We used 0–100 μg/cm^2 for JB6 or lung fibroblast V79 cells.
5. Solutions of nanoparticles need to be freshly prepared on the day of treatment.
6. For best results, start with the range of doses (0–100 μg/cm^2) and incubation times (0.5–24 h). A sample of untreated cells should always be processed as a negative control for assay.
   To generate positive samples for comet tails, we used H_2O_2 (100 μm) to treat JB6 cells for 20 min.
7. The media in the cell culture needs to be washed away since it can reduce the adhesion of the agarose on the Comet Slide. Cell samples should be prepared immediately before starting the assay and should be handled under dimmed or yellow light to prevent DNA damage from ultraviolet light. All buffers should be chilled to 4 °C or on ice to inhibit endogenous damage occurring during sample preparation and to inhibit repair in the unfixed cells. PBS should be calcium and magnesium free to inhibit endonuclease activities. The appropriate controls should also be included (see below).
8. The addition of DMSO is optional and is required only for samples containing heme, such as blood cells or tissue samples. The buffer formulation is proprietary.
9. The temperature of the agarose is critical or the cells may undergo heat shock. Heat blocks are not recommended for regulating the temperature of the agarose. However, once the agarose and cells have been spread on the slides, it works very well to maintain 37 °C.
10. For best results, use 500–1,000 cells per Comet Slide sample area. Using 50 μL of a cell suspension at 1×10^5 cells/mL combined with 500 μL of LM agarose will provide the correct agarose concentration and cell density for optimal results when spreading 50 μL per well. Control cells and treated cells should be handled in an identical manner. All steps are performed at room temperature unless otherwise specified.
11. When working with many samples, it may be convenient to place aliquots of the molten agarose into prewarmed microcentrifuge tubes and place the tubes at 37 °C. Add cells to one tube, mix by gently pipetting once or twice, then transfer 50 μL aliquots onto each sample area as required. Then proceed with the next sample of cells. If sample is not spreading evenly on the slide, warm the slide at 37 °C before application.

12. Wear gloves when working with alkaline solution.
13. Qualitative analysis: The comet tail can be scored according to DNA content (intensity). The control (untreated cells) should be used to determine the characteristics of data for a healthy cell. Scoring can then be made according to nominal, medium, or high intensity tail DNA content. At least 50 cells should be scored per sample.
14. Quantitative analysis: There are several image analysis systems that are available for quantitation of comet assay data from Trevigen Inc. The more sophisticated systems include the microscope, camera, and computer analysis package. These systems can be set up to measure the length of DNA migration, image length, nuclear size and calculate DNA damage parameters. At least 50 randomly selected cells should be analyzed per sample.

Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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