Detecting the acellular oxidative reactivity of nanoparticles

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1

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Synthetic coral HDR

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This protocol was designed to detect acellular oxidative reactivity of nanoparticles

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Health and safety precautions

All work should be conducted in a fume hood wearing standard personal protection equipment (lab coat, nitrile gloves, safety glasses).After use NP suspensions are filtered through a 0.2 micron syringe filter using a 10 mL plastic syringe.Dispose of the NP laden syringe filter in the Biohazard box for incineration.The filtered aqueous buffer can be disposed in the sink.
NOTE: 10M sodium hydroxide is highly corrosive.

Materials

- Hydrogen peroxide solution (30 wt% in water, Sigma Aldrich cat. no. 216763)
- 2',7'-Dichlorodihydrofluorescein diacetate (>97%, Sigma Aldrich cat. no. D6883)
  NOTE: Once opened, the solid DCFH-DA must be kept under argon at -20°C.
- Methanol (HPLC grade)
  NOTE: Can substitute with pure Ethanol 190 Proof (Decon Labs cat. no. V1101)
- PBS, 1X Phosphate-Buffered Saline (ThermoFisher cat. no. 10010023)
- Horseradish peroxidase (~150 units/mg, 100mg, MW ~40,000 Da, Sigma Aldrich cat. no. 77332)
- Sodium hydroxide (10M in water, Sigma Aldrich cat. no. 72068)

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DCFH-DA stock solution preparation

2 - Add 24.4 mg DCFH-DA (MW 487.29 g/mol) powder to a 50 mL volumetric flask.
- Fill flask to 50 mL volume line with methanol.
- This makes a 1 mM DCFH-DA stock concentration.
- Wrap flask with aluminum foil.
- This stock solution can be stored in the freezer at -20°C for 4 months.

H2O2 stock solution preparation

3 - Add 114 µL of 30 wt% $H_2O_2$to a 10 mL volumetric flask.
- Fill flask to 10 mL volume line with Milli-Q water.
- This makes a 0.1 M H_2O_2 stock solution.
[This stock solution should be made fresh]

NaOH stock solution preparation

4 - Add 50 µL of 10 M NaOH to a 50 mL volumetric flask.
- Fill flask to 50 mL volume line with Milli-Q water.
- This makes a 10 mM NaOH stock solution.
[This stock solution can be stored at 22°C for 1 month]

H2O2 working solution preparation

5 - Add 20 µL of 0.1 M H_2O_2 stock solution to a 10 mL volumetric flask.
- Fill flask to 10 mL volume line with Milli-Q water.
This makes a 200 µM H_2O_2 working solution

DCFH working solution preparation

6 - Add 4 mL of 10 mM NaOH and 1 mL of 1mM DCFH-DA stock solution to a 20 mL volumetric flask.
- Wrap flask in aluminum foil.
- Let the mixture react at room temperature for 30 min.
- Quench the reaction by diluting the DCFH-DA in NaOH solution with phosphate buffer saline (1X PBS, pH 7.2-7.4) up to the 20 mL mark on the volumetric flask.
This makes a 50 µM DCFH-DA concentration.

DCFH-HRP working solution preparation

7 - Add 1 mg of horseradish peroxidase (HRP) powder (~150 units/mg) to a 50 mL volumetric flask.
- Add 10 mL of freshly prepared 50 µM DCFH solution to the flask.
- Fill the flask to the volume line with 1X PBS.
This makes a 10 µM DCFH with 3 unit/mL HRP working solution.

Analyte preparation (for analytes in suspension)

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8 **- If the concentration is known:** prepare 1 mL of a 100 µg/mL analyte suspension by diluting the analyte stock suspension using 1X PBS as the diluent.
If the concentration is unknown: prepare 1 mL of a 1:10 dilution analyte suspension by adding 100 µL of the analyte stock suspension to 900 µL 1X PBS.

- Mix thoroughly using a vortex mixer or bath sonicator.

Analyte preparation (for powder analytes)

9 - Place a 2.5 mL Eppendorf tube inside the static eliminator built into the ultramicrobalance and run to dissipate any electrostatic charge in the plastic.
- Weigh 0.2 mg of powder analyte directly into the Eppendorf tube.
- Add 0.1 mL of dimethyl sulfoxide (DMSO) to disperse the powder analyte.
- Add 1.90 mL 1X PBS.
- This makes a 100 µg/mL analyte suspension from powder.

Dosing plate preparation

10 Using a round bottom 96-well plate, prepare the dosing plate according to the following layouts.

		H2O	H2O2 Dilutions	Final / Total
Row Number	Chemical Dose	Volume (µL)	Volume (µL)	Volume (µL)
B	0 µM	50	0	50
C	12.5 µM	50	50	50
D	25 µM	50	50	50
E	50 µM	50	50	50
F	100 µM	50	50	50
G	200 µM	0	100	50
		PBS	Analyte Dilutions	Final / Total
Row Number	Analyte Dose	Volume (µL)	Volume (µL)	Volume (µL)
B	0 µg/mL	50	0	50
C	1 µg/mL	90	10	50
D	10 µg/mL	60	40	50
E	25 µg/mL	50	50	50
F	50 µg/mL	50	50	50
G	100 µg/mL	0	100	50

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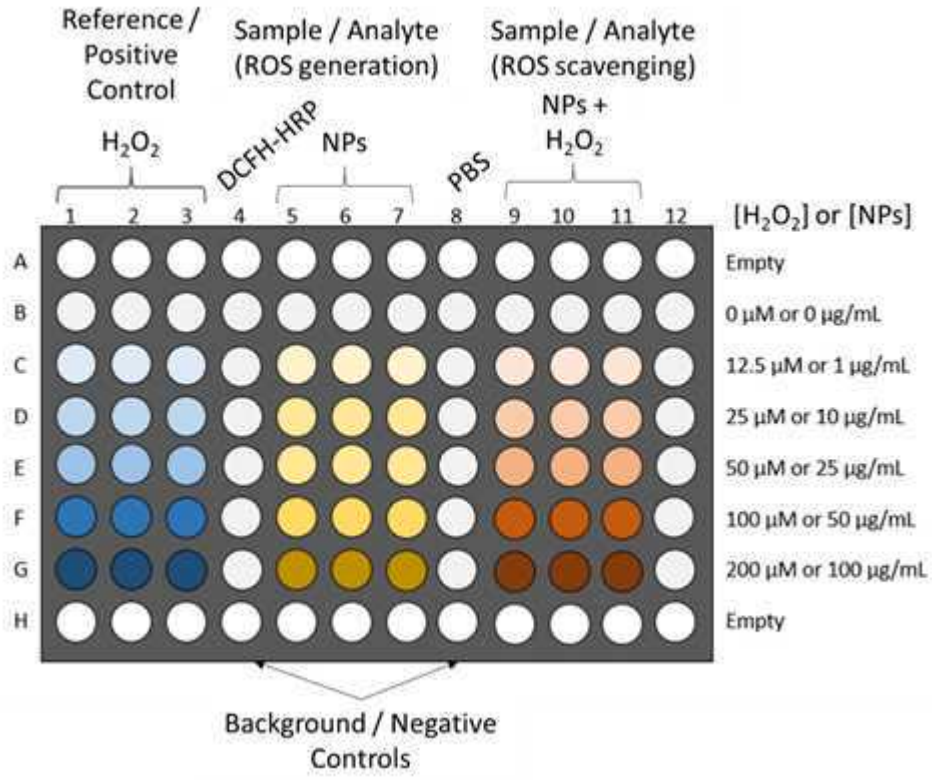

Fluorescence measurement

11 - Using a multipipette, transfer 20 µL from each well in the dosing plate to a black 96-well plate.
Note: Rows A & H and Column 12 will not be filled.
- Using a 8-channel multipipette, add 200 µL of the DCFH – HRP working solution to wells in Columns 1-3, 5-7, 9-11.
- Add 220 µL of the DCFH – HRP working solution to wells in Column 4.
- Add 220 µL of 1X PBS to wells in Column 8.
- Place the 96-well plate into the multiplate reader (Cytation 3) thermostatted at 37°C and after shaking the platefor 5 seconds, wait 2 minutes thenstart fluorescent intensity measurements.
- The fluorescence signal is measured every minute for 60 minutes. The spectroscopic reading occurs at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Note: Data is presented by first background correcting for the DCHF-HRP signal then plotting the signal increase compared to the blank (0 µM or 0 µg/mL) at specific times or through the change in fluorescence response over time at a given concentration.

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