NCL Method ITA-9

Phagocytosis Assay

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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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1. Introduction

This document describes a protocol for evaluation of nanoparticle internalization by phagocytic cells, where the foreign substances are isolated via a cell membrane enclosure and degraded by the phagolysosome. Phagocytosis is a receptor mediated endocytosis specific to phagocytic cells, e.g. cells of the mononuclear phagocytic system (MPS). Phagocytosis is an active process and requires actin polymerization. There are four main receptors which mediate phagocytic uptake. Phagocytosis via three of these receptors (complement receptor (CR), FcγR receptor, and mannose receptor (MR)) is accompanied by inflammatory reactions (e.g., cytokine secretion). Phagocytosis via the fourth receptor (scavenger receptor (SR)) is not accompanied by inflammatory responses [1-5].

2. Principles and Limitations

This assay utilizes a luminescent-based approach in which nanoparticles are incubated with HL-60 promyelocytic cells, and the phagocytic activity is visualized with luminol. Luminol is a dye that is not luminescent unless exposed to the low pH of the phagolysosome.

This protocol may not be applicable for certain types of nanomaterials. For example, nanoparticles with fluorescent capabilities such as quantum dots should be studied using confocal microscopy or flow cytometry. Modification(s) of the current procedure and/or changes in the detection dye may be required for particles that interfere with luminol-dependent chemiluminescence.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

3.1 Reagents

1. HL-60 promyelocytic cells (ATCC, CCL-240)
2. Human AB serum or plasma pooled from at least three donors.
3. Phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)
4. Zymosan (Sigma, Z4250)
5. Fetal bovine serum (FBS) (GE Life Sciences, Hyclone , SH30070.03)

6. RPMI-1640 (Invitrogen, 11875-119)
7. Pen/Strep solution (Invitrogen, 15140-148)
8. Trypan Blue solution (Invitrogen, 15250-061)
9. Luminol (Sigma-Aldrich, 123072)

3.2 Materials

1. Pipettes, 0.05 to 10 mL
2. Flat bottom 96-well white luminescence plates
3. Polypropylene tubes, 50 and 15 mL

3.3 Equipment

1. Centrifuge, 400x*g* and 2000x*g*
2. Refrigerator, 2-8ºC
3. Freezer, -20ºC
4. Cell culture incubator, 5% CO_2 and 95% humidity
5. Biohazard safety cabinet approved for level II handling of biological material
6. Inverted microscope
7. Vortex
8. Hemocytometer
9. Plate reader capable of working in luminescence mode

   Note: The plates used for this assay have a solid white bottom; therefore the plate should be read from the top. Depending on the type of the plate reader one may need to use a plate adaptor to provide optimal conditions for the top read.
10. Warm gel-pack

    Note: This material is optional and may be omitted. It is used to keep the plate warm for optimal phagocytosis. If it takes longer than 2 min to transfer the plate to the plate reader after addition of all reagents, the phagocytosis process will start before one can start analyzing the plate on the plate reader.

4. Preparation of Reagents and Controls

4.1 Heat-Inactivated Fetal Bovine Serum

Thaw a bottle of FBS at room temperature, or overnight at 2-8ºC and allow to equilibrate to room temperature. Incubate 30 minutes at 56ºC in a water bath mixing

every five minutes. Single use aliquots may be stored at 2-8ºC for up to one month or at a nominal temperature of -20ºC indefinitely.

4.2 Complete RPMI-1640 Medium

The complete RPMI medium should contain the following reagents: 20% FBS (heat inactivated); 4 mM L-glutamine; 100 U/mL penicillin; and 100 µg/mL streptomycin sulfate. Store at 2-8ºC, protected from light for no longer than one month. Before use, warm in a 37ºC water bath.

4.3 Zymosan A Stock

Prepare Zymosan A stock at a final concentration of 2 mg/mL in PBS. Use fresh.

4.4 Positive Control (Read Section 5 to determine which approach to use.)

4.4.1 Approach A

Combine Zymosan A stock and human AB serum or plasma. Use 1 mL of serum/plasma per each 0.5 mL of Zymosan A stock. Incubate Zymosan A with serum/plasma for 30 minutes at 37°C. Wash Zymosan A particles two times with PBS (use 1 mL of PBS per each 0.5 mL of original Zymosan stock and a centrifuge setting of 2000x*g* for 2 min) and resuspend in PBS to a final concentration of 2 mg/mL.

4.4.2 Approach B

Reconstitute Zymosan A in PBS with 20% human AB serum/plasma to a final concentration of 2 mg/mL.

4.5 Negative Control

Use PBS as the negative control. Process this control the same way as test the samples.

4.6 Luminol Stock (10 mM in DMSO)

Dissolve luminol in DMSO to a final concentration of 10 mM, e.g., dissolve 17.7 mg of luminal in 10 mL of DMSO. Prepare single use aliquots and store at -20°C. Protect from light.

4.7 Luminol Working Solution (250 µM in PBS)

On the day of the experiment, thaw an aliquot of luminol stock solution and dilute with PBS to a final concentration of 250 µM, e.g., add 250 µL of 10 mM stock into 9.750 mL of PBS. Protect from light. Discard unused portion.

4.8 Vehicle Control

Vehicle control is the buffer or media used to formulate test nanomaterials. Common excipients used in nanoformulations are trehalose, sucrose, and albumin. However, other reagents and materials are also used alone or in combination. Vehicle control should match formulation buffer of the test nanomaterial by both composition and concentration. This control can be skipped if nanoparticles are stored in PBS.

5. Preparation of Study Samples

This assay requires 2 mL of nanoparticle solution dissolved/resuspended in PBS, at a concentration 3X the highest final test concentration. Test concentrations are based on the calculated plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol this concentration is called “theoretical plasma concentration”. Considerations for estimating theoretical plasma concentration have been reviewed elsewhere [6] and are summarized in Box 1 below.

This assay evaluates four concentrations: 10X (5X if 10X cannot be achieved, or 100X or 30X when feasible) of the theoretical plasma concentration, the theoretical plasma concentration, and two serial 1:5 dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, 1 mg/mL is used as the highest concentration. Alternatively, the highest reasonably achieved concentration can be used if 1 mg/mL is unattainable.

For example, if the theoretical plasma concentration to be tested is 0.2 mg/mL, a stock of 6 mg/mL is prepared. This sample is then diluted 10 fold (0.6 mg/mL), followed by two 1:5 serial dilutions (0.12 and 0.024 mg/mL). When 0.1 mL of each of these sample dilutions are combined in a culture well plate and mixed with 0.1 mL of luminol and 0.1 mL of cell suspension, the final nanoparticle concentrations tested in this assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL.

Box 1. Example Calculation to Determine Nanoparticle Theoretical Plasma Concentration

In this example, we are assuming a known efficacious mouse dose of 123 mg/kg. Therefore, the scaled equivalent human dose would be:

$$\text{human dose} = \frac{\text{mouse dose}}{12.3} = \frac{123 \text{ mg/kg}}{12.3} = 10 \text{ mg/kg}$$ (see reference [5])

The blood volume of a human is approximately 8% of the body weight. Therefore, an average human of 70 kg body weight has approximately 5.6 L of blood. Assuming all the nanoparticle injected goes into the systemic circulation, this provides a rough approximation of the potential maximum nanoparticle concentration in a human. The theoretical plasma concentration, i.e. in vitro test concentration, is calculated by:

$$\text{theoretical plasma concentration} = \frac{\text{human dose}}{\text{human blood volume}} = \frac{70 \text{ kg x } 10 \text{ mg/kg}}{5.6 \text{ L}} = 0.125 \text{ mg/mL}$$

Human AB serum or plasma used for opsonisation of the positive control is also used to opsonize the nanoparticles. Opsonization of nanoparticles should be done following the same procedure used for the opsonization of the positive control. Nanoparticles:Serum/Plasma volume ratio and incubation conditions are the same as described for the positive control in section 4.4. If centrifugation to separate nanoparticles from bulk plasma is possible, Approach A (described in section 4.4.1) is used to prepare both the positive control and nanoparticles. However, when centrifugation is not applicable to the nanoparticles, both particles and positive control should be prepared according to Approach B (described in section 4.4.2).

Note: If approach B is used, nanoparticle opsonization can be performed either using the stock nanoparticle or each tested dilution. When nanoparticle stock is used for opsonization, further dilutions of this stock should be performed in the medium containing serum or plasma.

6. Cell Preparation

HL-60 is a non-adherent promyelocytic cell line derived by S.J. Collins, et al. from a patient with acute promyelocytic leukemia [5]. Cultures can be maintained by the addition of fresh medium or replacement of medium. Alternatively, cultures can be established by centrifugation with subsequent resuspension at $1x10^5$ viable cells/mL. **Do not allow cell concentration to exceed $1x10^6$ cells/mL.** Maintain cell density between $1x10^5$ and $1x10^6$ viable cells/mL. On the day of the experiment, count cells using trypan blue. If the cell viability is ≥90% proceed to the next step.

7. Experimental Procedure

1. Turn on plate reader, allowing it to warm up to 37°C. Place an empty white 96-well test plate inside the plate reader chamber, allowing it to warm to 37°C as well. Set up the instrumental parameters.
2. Adjust cell concentration to $1x10^7$ cells/mL by spinning cell suspension down and reconstituting in complete medium (refer to section 4.2 for details). Keep at room temperature.
3. Add 100 μL of controls and test-nanoparticles in PBS to appropriate wells of the pre-warmed test plate. Prepare three duplicate wells for each sample and two duplicate wells for positive and negative control.

   Note: Always leave duplicate wells for each of the following controls: 1) luminol only control (no cells); 2) nanoparticles only (no cells); and 3) nanoparticles plus luminol (no cells). See Appendix for an example plate map.
4. Add 100 μL working luminol solution in PBS to each sample-containing well. Do not forget to prepare two "luminol only" control wells.

   Note: Keeping the plate warm during sample aliquoting (e.g., using a plate warmer or warm gel pack) may be helpful in achieving optimal assay performance and reproducibility.
5. Plate 100 μL of cell suspension per well on the 96 well white plate.
6. Start kinetic reading on a luminescence plate reader immediately.

   Note: If using a plate reader with both top and bottom read capabilities, do not forget the plate adaptor before proceeding with the plate analysis on the plate reader.

8. Calculations

8.1 Using Excel or other relevant software, compare area under the curve (AUC) for all samples. An increase in the AUC at least 2-fold above the negative control (baseline) is considered a positive response. Use relevant statistical analysis to compare AUC values for test samples with that of the baseline.

8.2 A percent coefficient of variation is used to control precision and calculated for each control or test sample according to the following formula:

$$\%CV = (SD/Mean) \times 100\%$$

9. Acceptance Criteria

1 The %CV for each control and test sample should less than 30%.

2 Samples demonstrating higher variability should be re-analyzed.

10. References

1. Antonini JM., van Dyke K., ye Z., DeMatteo M., Reasor MJ. Introduction of luminol-dependent chemiluminescence as a method to study silica inflammation in the tissue and phagocytic cells of rat lung. *Environ. Health Perspect*. 1994;102(suppl10):37-42.
2. Gref R., Luck M., Quellec P., et al. Stealth corona-core nanoparticles surface modified by PEG: influences of corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein absorption. *Colloids and surfaces B: Biointerfaces*, 2000;18:301-313.
3. Leroux JC., Gravel P., Balant L., et al. Internalization of poly(D,L-lactic acid) nanoparticles by isolated human leukocytes and analysis of plasma proteins absorbed onto particles. *J.Biomed.Materials Res*. 1994;28:471-481.
4. Mold C., Gresham HD., DuClos TW. Serum Amyloid P component and C-reactive protein mediate phagocytosis through murine FcγRs. *J.Immunol*. 2001;166:1200-1205.
5. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. *Proc Natl Acad Sci U S A*. 1978;75(5):2458-62.
6. Dobrovolskaia MA, McNeil SE. Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. *J Control Release*. 2013;172(2):456-66.

11. Abbreviations

AUC	area under the curve
CV	coefficient of variation
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
PBS	phosphate buffered saline
SD	standard deviation
VC	vehicle control

12. Appendix

Example Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	PC	NC	**TS1 (0.008 mg/mL)**	**TS1 (0.008 mg/mL)**	**TS1 (0.008 mg/mL)**	**TS1 (0.04 mg/mL)**	**TS1 (0.04 mg/mL)**	**TS1 (0.04 mg/mL)**	**TS1 (0.02 mg/mL)**	**TS1 (0.02 mg/mL)**	**TS1 (0.02 mg/mL)**
B	Blank	PC	NC	**TS1 (0.008 mg/mL)**	**TS1 (0.008 mg/mL)**	**TS1 (0.008 mg/mL)**	**TS1 (0.04 mg/mL)**	**TS1 (0.04 mg/mL)**	**TS1 (0.04 mg/mL)**	**TS1 (0.02 mg/mL)**	**TS1 (0.02 mg/mL)**	**TS1 (0.02 mg/mL)**
C	**TS1 (2 mg/mL)**	**TS1 (2 mg/mL)**	**TS1 (2 mg/mL)**	Blank	PC	NC	VC	VC				
D	**TS1 (2 mg/mL)**	**TS1 (2 mg/mL)**	**TS1 (2 mg/mL)**	Blank	PC	NC	VC	VC				
E												
F												
G	Luminol	TS (0.008 mg/mL) + Luminol	TS (0.04 mg/mL) + Luminol	TS (0.2 mg/mL) + Luminol	TS (2.0 mg/mL) + Luminol	TS (0.008 mg/mL) NO LUMINOL	TS (0.04 mg/mL) NO LUMINOL	TS (0.2 mg/mL) NO LUMINOL	TS (2.0 mg/mL) NO LUMINOL			
H	Luminol	TS (0.008 mg/mL) + Luminol	TS (0.04 mg/mL) + Luminol	TS (0.2 mg/mL) + Luminol	TS (2.0 mg/mL) + Luminol	TS (0.008 mg/mL) NO LUMINOL	TS (0.04 mg/mL) NO LUMINOL	TS (0.2 mg/mL) NO LUMINOL	TS (2.0 mg/mL) NO LUMINOL			

PC: Positive Control; NC: Negative Control; TS: Test Sample; VC:vehicle control

No cells