Alveolar macrophage accumulation rates, for 28 nm and 250 nm PSL, are mediated by separate mechanisms.

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Abstract. When macrophages accumulate 28 nm and 250 nm diameter polystyrene latex (PSL) beads, the accumulation rates should reflect differences in molecular and cellular function. We used a confocal microscope to measure the accumulation rates of nanoparticles by F344-rat-alveolar macrophages (~25,000 cells adhered to a 0.7 cm² surface). Over the cells were layered 0.1 ml of media, and 0.1 ml of media-with-beads. Fresh cells were introduced for each exposure scenario. The maximum possible individual macrophage exposures were as follows: 8x10⁶, 8x10⁵, and 8x10⁴ 28 nm beads per macrophage; and 8x10⁴ and 1.12x10⁴ 250 nm beads per macrophage. Accumulation rates were estimated over 23 minutes. The increase in bead accumulation-rate matched changes in bead-availability: 7x increase for 250 nm beads; 100x increase for 28 nm beads; and 700x increase for all bead availabilities. The maximum sustained 28 nm bead accumulation rate was > 30,000 /min (for 5 min). Increases in bead accumulation could be explained by two mechanisms: bead-diffusion; and, for the macrophage, macropinocytosis. Also for the highest concentrations of 28 nm beads, we saw a colligative threshold -- possibly due to beads masking the cell surface or obstructing cellular mechanisms.

1. Introduction:
Human exposures to nanoparticles occur through injection, ingestion, skin contact, or inhalation: The initial interaction is between nanoparticles and cells. In particular, during inhalation, an airway-system cell may come into contact with nanoparticles of different composition. Each nanoparticle may impact different mechanistic pathways; each may have a different toxicity. Yet these differences may be overshadowed by the cell's mechanistic and toxic responses to the colligative properties of nanoparticles.

Colligative properties are properties that depend on number density. For example, inside a sealed container, the pressure on the walls is due to the number of gas molecules (actually the rate that the gas molecules bounce off of the container walls). Likewise, although the term "pressure" may not be adequate, for nanoparticles, the number of nanoparticles may impact cells in three ways: the number nanoparticles inside a cell may exert a pressure on (or impede) molecular machinery; the total cross-sectional area of nanoparticles per cellular surface area may impede basic functions of the cell surface; and the total volume of nanoparticles per cell volume may impede basic cell mobility and response. In each case, the number of nanoparticles, a colligative property, impacts efforts to compare and rank the cellular-toxicity of different types of nanoparticles.

The inverse should also be true: When colligative effects are kept negligible, comparisons can be made of the mechanism of action and toxicity of different nanoparticles.
We use a laser scanning confocal microscope to measure the rates, that 26 nm, 28 nm, and 250 nm diameter polystyrene latex (PSL) beads are accumulated by macrophages. In our previous work (Moss, 2008) we showed that the confocal laser scanning microscope could be used to measure, in alveolar macrophages, the accumulation-rate of 26 nm polystyrene latex beads.

In this current work, our objectives are threefold: first, to duplicate our previous work (this time with 28 nm and 250 nm diameter beads); second, to use the rate that nano-beads are accumulated by macrophages as an early indicator of the colligative effects of nanoparticles; and third, to associate the accumulation rate with changes in the molecular processes involved in endocytotic and cellular function. The nano-bead accumulation rates are expected to be impacted by three separate mechanisms: nanoparticle diffusion, macropinocytotic vesicle transport, and cell surface modification.

What we found was that, for the F344 rat alveolar macrophage exposed to PSL nano-beads, a 700x increase in bead availability was matched by the same increase in bead accumulation-rate. The maximum sustained 28 nm bead accumulation rate was > 30,000 /min (for 5 min). The 700x increase in bead accumulation could be explained by two mechanisms: bead-diffusion; and, for the macrophage, macropinocytosis.

2. Materials and Methods
Our approach was to measure the rate that freshly harvested (not activated; not synchronized) rat alveolar macrophages accumulate fluorescent Polystyrene Latex (PSL) beads (28 nm and 250 nm in diameter). Bead concentrations were selected to avoid, within the macrophage, possible colligative effects due to number of beads accumulated per cell, total bead cross sectional surface per cell surface, and total bead volume per cell volume.

The three concentrations of 28 nm PSL beads [High (H), Medium (M); and Low (L)] increased by factors of 10. The two concentrations of 250 nm PSL beads [Low (L); and Very low (V)] increased by a factor of 7. For both sizes of beads, the Low concentration was the same. The concentration range between the V and H concentrations was a factor of 700x. Bead-accumulation by the macrophage was measured for up to 30 minutes or until the fluorescence signal saturated or exceeded the maximum fluorescence of the calibration curves. From the slope of the accumulation curve, we calculated the accumulation rate.

2.1. Materials:

2.1.1. PSL beads: The nano-particles used in this study were 28 nm and 250 nm diameter, fluorescent Polystyrene Latex (PSL) beads (Duke Scientific Corp., Palo Alto, CA). In the fluid covering the macrophages, concentrations of the 28 nm beads were $10^{12}$, $10^{11}$, $10^{10}$ beads/ml -- corresponding to the high (H), medium (M), and low (L) concentrations. For the 250 nm beads, concentrations were $10^{10}$ and $1.4 \times 10^9$ beads/ml -- corresponding to the low (L) and very low (V) concentrations (For only the L concentration of 250 nm beads, non-fluorescent beads of the same size were used to dilute the fluorescence; for this dilution we used a ratio of 1 fluorescent bead to 9 non-fluorescent beads.).

2.1.2. Maximum PSL beads available per macrophage: The concentration of PSL beads (beads/ml) does not provide sufficient information about individual macrophage exposure. Such exposures are better expressed as the maximum number of PSL beads available per macrophage. This alternative expression is calculated from the number of cells (~25,000), the volume of liquid layered over the cells (0.2 ml), and the concentration (beads/ml). From this perspective the maximum possible individual macrophage exposures were H=8x10^6, M=8x10^5, L=8x10^4, and V=1.12x10^4 PSL beads/macrophage.

This estimation is still not ideal. The maximum number of beads available per cell does not indicate the rate at which beads come into contact with the cell membrane. In this study we do not know the rate of contact (the tap-tap rate in contacts/second). However, if we assume that, for every
change in bead concentration there is a proportional change in the tap-tap rate, then we can test whether the magnitude of this change is duplicated in the magnitude of the change in the rate that macrophages accumulate beads.

2.1.3 PSL bead exposure limits: The four exposure levels (H, M, L, and V) were selected to minimize the possibility of PSL beads covering the surface of the macrophage: That is, for the maximum number of PSL beads available per cell, we minimized the possibility of the total cross-sectional area of nanoparticles being greater than the surface area of the cell (For a 12 micron diameter "idealized-spherical" macrophage, and for 28 nm diameter beads, the number is approximately $10^6$ beads per macrophage; for 250 nm diameter beads, the number is $\sim 10^4$ beads per macrophage.). The four exposure levels were also selected to assure that the total volume of nanoparticles per macrophage would be less than 6% of the volume of the macrophage: For 28 nm beads this number is $\sim 5 \times 10^6$ beads per macrophage; for 250 nm beads the number is $\sim 7 \times 10^3$ beads per macrophage (Moss, 2008 [1]).

2.2. Methods:

2.2.1. Animals. This study was conducted under federal guidelines for the care and use of laboratory animals (National Research Council, 1996) and was approved by the Hamner Institutes' Institutional Animal Care and Use Committee. For this study, eight to ten week old F344 female rats were purchased from Charles River Laboratories (Raleigh, NC); the rats were housed in standard cage racks in an AAALAC-accredited facility. Animals were given NIH-07 cereal-based diet (Zeigler Brothers, Gardner, PA) and water ad libitum. Room temperature was maintained at 18 to 26°C; relative humidity was maintained at 30 to 70%.

2.2.2. Collection of Macrophages from BAL. Bronchioalveolar lavage (BAL) fluid was collected from approximately 12-week-old F-344 female rats (weight ~230 g). Rat lungs were lavaged with ice cold phosphate-buffered saline (PBS; Invitrogen, Grand Island, NY). Total cells from the BAL were resuspended in medium (Dulbecco’s modified Eagles medium, DMEM-F12K without phenol red; Invitrogen) with 1% fetal bovine serum (FBS; Invitrogen) and 10mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanosulphonic acid; Sigma, St. Louis, MO). Lavage fluid was counted (97-99% macrophages; Bermudez et al. 2003 ) and then diluted to concentrations of $6.6 \times 10^5$ macrophages/ml.

2.2.3. Imaging of particles in cells. Uptake of 28 nm green fluorescent polystyrene latex (PSL) beads was measured using a confocal laser scanning microscope (LSM 510 META confocal microscope; Zeiss, Thornwood, NY). Visualization of particle uptake by macrophages was accomplished by adjusting the focal plane to step, 800 nm thick optical-slice by optical-slice, through the entire volume of a macrophage (Figure 1).
2.2.4. Calculation of beads accumulated per macrophage: From an optical slice captured by the confocal microscope at least six (6) macrophages were selected. Selection was based on whether the macrophage was fully contained within the Z-stack of optical slices. Around each macrophage, a box was drawn in order to define a region of interest. This region of interest was extended upward through each optical slice of the Z-stack. From these images we extracted four types of relative fluorescence (RF): the RF of the medium before bead addition; the RF of the medium after bead addition; the RF of the medium and macrophage before bead addition; and the RF of the medium and macrophage after bead addition. These values allowed an estimation to be made of the number of beads accumulated within a macrophage. By repeating this process for the selected macrophages, we calculated at each time point the average number of beads per macrophage.

2.2.5. Calculation of bead accumulation rates. From the time-series of the average number of beads per macrophage, we calculated, at each time point, the slope of the curve (beads accumulated per minute per macrophage).

3. Results
By using the confocal laser scanning microscope, we measured the rate that freshly harvested (not activated; not synchronized) rat alveolar macrophages accumulated fluorescent Polystyrene Latex (PSL) beads (28 nm and 250 nm in diameter). Bead exposures were selected to avoid possible colligative effects due to number of beads per cell, total bead cross sectional surface per cell surface,
and total bead volume per cell volume. Even with these restrictions, the concentration of beads -- and thus the availability of beads to each macrophage -- varied by a factor of 700x. Our results were as follows:

3.1. Calculation of beads per macrophage:
We found that, in spite of the macrophages not being activated or synchronized, at each PSL bead exposure level and at each time-point, the average number of beads accumulated was reproducible. By 13 minutes, the macrophages, exposed to the high (H) exposure level of 28 nm diameter PSL beads, had accumulated an average of 380,000 beads; while, during the same time period, the macrophages exposed to the very low (V) exposure level of the larger, 250 nm diameter beads, had accumulated only 99 beads (Figures 2 and 3).

**Figure 2** Alveolar macrophage accumulation of 28 nm and 250 nm diameter PSL beads. Exposure levels, as maximum number of beads available per macrophage, were H(8x10^6), M(8x10^5), L(8x10^4), and V(1.12x10^4). Shown are means plus standard deviations for n equal to or greater than 6.

**Figure 3** Expansion of Figure 2 -- Alveolar macrophage accumulations of 28 nm and 250 nm diameter PSL beads. Exposure levels, as maximum number of beads available per macrophage, were L(8x10^4) and V(1.12x10^4). Shown are means plus standard deviations for n equal to or greater than 6.

3.2. Calculation of bead accumulation rates.
In every case, during the first five minutes, when the exposures of PSL beads changed by a factor of 7 or 10, the average bead-accumulation rate by the macrophages changed by the same factor (Figure 4). However during this same time period, for the low (L) exposures of 28 nm or 250 nm beads, the average rate that macrophages accumulated the 250 nm beads was six (6) times slower than the accumulation rate for the 28 nm beads (73 vs. 438 beads per minute per macrophage). After five minutes, for the high (H) exposures of 28 nm beads, the average bead-accumulation rate began to drop from a sustained rate of 31,000 beads per minute per macrophage, reaching approximately half this value by the eleventh minute. Moreover, for the low (L) exposures of 250 nm beads, after five minutes, the bead accumulation rate began to increase from 73 beads per minute per macrophage, doubling the rate by the thirteenth minute.
4. Discussion:

In these experiments we showed that, for 28 nm and 250 nm diameter Polystyrene Latex (PSL) beads, as the maximum possible number of beads available per macrophage increased, the parallel -- and of equal magnitude -- change in the rate that macrophages accumulated beads could be explained by the difference in the rate at which beads were presented to the surface of the cells. For the 28 nm diameter beads, as the bead exposure changed by a factor of 10, the rate that macrophages accumulated the beads also increased by a factor of 10. Likewise, for the 250 nm diameter beads, as the exposure changed by a factor of 7, the accumulation rate also increased by a factor of 7. In these experiments, the macrophages had sufficient capacity to handle an overall increase of 700x in the rate that beads were accumulated.

The low (L) exposures (a possible maximum of $8 \times 10^4$ beads per macrophage) provide data that confirms predictions by Teeguarden [2]. The 28 nm diameter PSL beads were predicted to move by diffusion at a rate 9x faster than that of the 250 nm diameter PSL beads. However, the 250 nm beads move by sedimentation at approximately the same rate as they move by diffusion. The net effect is a prediction that, compared to the 250 nm beads, the rate that the 28 nm diameter beads move towards a macrophage would be 4x to 5x faster: In our measurements (Figure 4), when the starting concentration of beads was the same, the difference in the rate that macrophages accumulated the two sized beads was between 2x and 6x. For the low (L) exposures of 28 nm and 250 nm beads, the 2-to-6x difference in the rate that macrophages accumulated the beads can be explained by the respective differences in the rate of bead- diffusion and sedimentation.

We saw a 700x increase in the rate that macrophages could accumulate PSL beads. This capacity could be explained by a specific nano-particle transport mechanism -- macropinocytosis (Radcliffe [3]). A macropinocytotic vesicle capable of carrying one 250 nm diameter PSL bead would also be capable of carrying approximately 700 of the 28 nm diameter PSL beads. The macropinocytotic vesicle number and transport rate would not have to increase in order to produce the observed bead accumulation rates -- including the highest rates (30,000 to 40,000 beads/minute/macrophage) seen for the 28 nm diameter beads. The capacity was sufficient to handle the increase in the rate that the beads interacted with the macrophage surface membrane. However, after 5 minutes at an accumulation rate...
of 30,000 to 40,000 beads per minute per macrophage, the rate at which the macrophage accumulated the 28 nm beads began to drop -- as predicted due to colligative effects of the total cross sectional area of the beads approaching the area of the surface membrane of the macrophage. If, for the 28 nm PSL beads, the macrophage was able to sustain the observed accumulation rate of 30,000 beads per minute, then, in 165 minutes the beads would occupy 6% of the macrophage volume: Moreover, at this bead-accumulation rate, in 32 minutes there would be enough beads to cover the surface membrane of an idealized spherical macrophage.

There was one unexpected result for the low exposures of 250 nm diameter PSL beads (a possible maximum of $8 \times 10^4$ beads per macrophage). From minute five to minute thirteen, the average rate that the macrophages accumulated beads doubled. Either the capacity doubled -- more macropinocytotic vesicles; or there was a doubling of the rate that the 250 nm diameter PSL interacted with the macrophage surface membrane; or additional endocytotic processes were initiated. However, as discussed above, we expected the 28 nm beads to approach the macrophage surface membrane at a rate that was 4-to-5x faster than that for the 250 nm beads. For the 250 nm beads, the doubling of the accumulation rate most likely represented a statistical variation in diffusion and sedimentation.

Because the freshly collected rat-alveolar macrophages were not synchronized and not specifically activated, our observations primarily provide an indication of the interaction between nanoparticles and macrophages -- a worst case demonstration that reflects the status of macrophages in the body. If the macrophages were activated and synchronized, the standard deviations of the bead accumulations (Figures 2 and 3) would be significantly smaller. The measurement of bead accumulation rates provides a means for future investigation of the initiation, limits, and obstruction of endocytotic processes; it also provides a means for developing and evaluating high throughput, in vitro tests for ranking the toxicity of nanomaterials.

5. Summation.

In this work we measured, in non synchronized, non activated alveolar macrophages, the average rates that macrophages accumulate 28 nm and 250 nm PSL beads. We draw three conclusions:

First, The volume of macropinocytotic vesicles capable of transporting 250 nm beads is sufficient to explain the broad capacity of the rate that the macrophage accumulated 28 nm diameter beads. As the exposure (the maximum possible number of beads available per macrophage) increased, the rate of accumulation also increased: We saw a 7x increase for 250 nm beads; a 100x increase for 28 nm beads; and a 700x increase for all bead availabilities.

Second, a colligative threshold -- possibly due to beads masking the cell surface or obstructing cellular mechanisms -- could explain the observed decrease in the maximum rate that macrophages accumulated 28 nm diameter beads. The maximum sustained 28 nm bead accumulation rate was > 30,000 /min/macrophage (for 5 min). However, after 5 minutes, this accumulation rate began to decrease, as expected for accumulations of greater than 150,000 28 nm diameter beads per macrophage.

Third, the dose-rate (or rate of accessibility) of the beads at the macrophage surface dominated the observed rate of accumulation. For equal exposures of 28 nm and 250 nm PSL beads (a possible maximum of $8 \times 10^4$ beads per macrophage), during the first 5 minutes, the difference in the macrophage rate of accumulation of 250 nm and 28 nm diameter beads (a factor of 2x to 6x) is of the same order of magnitude as the combined difference in bead-diffusion and -sedimentation (a factor of 4x to 5x).

Acknowledgments:

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