Research Article

Microcalorimetric Method to Assess Phagocytosis: Macrophage-Nanoparticle Interactions

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Abstract. This study evaluated the use of isothermal microcalorimetry (ITMC) to detect macrophage–nanoparticle interactions. Four different nanoparticle (NP) formulations were prepared: uncoated poly (isobutyl cyanoacrylate) (PIBCA), polysorbate-80-coated PIBCA, gelatin, and mannoseylated gelatin NPs. Changes in NP formulations were aimed to either enhance or decrease macrophage–NP interactions via phagocytosis. Alveolar macrophages were cultured on glass slabs and inserted in the ITMC instrument. Thermal activities of the macrophages alone and after titration of 100 μ L of NP suspensions were compared. The relative interactive coefficients of macrophage–NP interactions were calculated using the heat exchange observed after NP titration. Control experiments were performed using cytochalasin B (Cyto B), a known phagocytosis inhibitor. The results of NP titration showed that the total thermal activity produced by macrophages changed according to the NP formulation. Mannoseylated gelatin NPs were associated with the highest heat exchange, 75.4 ± 7.5 J, and thus the highest relative interactive coefficient, $9,269 \pm 630$ M⁻¹. Polysorbate-80-coated NPs were associated with the lowest heat exchange, 15.2 ± 3.4 J, and the lowest interactive coefficient, 890 ± 120 M⁻¹. Cyto B inhibited macrophage response to NPs, indicating a connection between the thermal activity recorded and NP phagocytosis. These results are in agreement with flow cytometry results. ITMC is a valuable tool to monitor the biological responses to nano-sized dosage forms such as NPs. Since the thermal activity of macrophage–NP interactions differed according to the type of NPs used, ITMC may provide a method to better understand phagocytosis and further the development of colloidal dosage forms.

KEY WORDS: flow cytometry; isothermal microcalorimetry; macrophages; nanoparticles; phagocytosis.

INTRODUCTION

Since the emergence of colloidal delivery systems, phagocytosis mediated by macrophages has been extensively studied (1–4). This phenomenon is an innate immune defense mechanism that has a substantial impact on the success or failure of any treatment that involves colloidal delivery systems, including nanoparticles (NPs) (5–7). Macrophages are considered targets and sites of action in specific diseases,

such as AIDS and Leishmania infections and TB, in which phagocytotic cells are hosts for the virulent agent (8–10). In other diseases such as cancer, macrophages are normally considered obstacles to be avoided in order to enable NPs to have longer circulation times, thus increasing their chance of reaching cancerous cells (11,12). Therefore, different strategies were developed to manipulate the structure of NPs and, consequently, to control the cellular response of macrophages to enhance or inhibit phagocytosis (13,14). Coating the surface of NPs with a hydrophilic surfactant such as polysorbate-80 was a strategy extensively studied to decrease phagocytosis to obtain long-circulating NPs (15–18). On the other hand, phagocytosis can be enhanced by targeting special receptors on the surface of macrophages, especially the mannose receptor. This was achieved by tagging NP surfaces with mannose moieties via surface association and/or chemical linkage (19–21). Monitoring changes in phagocytosis is essential to assess the macrophage cellular response to NPs of different structures.

Fluorescence microscopy and flow cytometry are normally used to assess phagocytosis. Both technologies require NPs to possess fluorescence properties in order to be detected intracellularly after being phagocytosed by macrophages as they estimate the percentage of cell populations that express the fluorescence properties of the probe (22–24). Therefore,

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NPs or any other colloidal delivery system must be tagged with fluorescent probes before detection by fluorescence microscopy or flow cytometry (25,26). Moreover, these techniques can assess phagocytosis that has been accomplished, but cannot monitor phagocytosis in real time.

Isothermal microcalorimetry (ITMC) is a universal analytical technique that measures heat exchange to monitor physical, chemical, and biological reactions (27). Samples tested by ITMC are surrounded by an isothermal heat sink that compensates for any thermal activity going on in the sample to maintain an isothermal environment (28). During a reaction, heat exchange between the sample and the heat sink is monitored and recorded continuously in real time (29). Results are expressed in thermal activity profiles where power (usually in microwatts) is plotted against time.

Most biological functions are associated with changes in cellular metabolism and, consequently, heat exchange with the surrounding environment (30). Similar to other biological function, metabolism is the only source of energy required to perform phagocytosis (31). Therefore, ITMC has two advantages over other techniques that monitor NP phagocytosis: (1) there is no need to tag NPs with a fluorescent probe for intracellular detection, and (2) ITMC provides a continuous thermal record of the phagocytosis process (32). This study assessed the feasibility of using ITMC to monitor phagocytosis and investigated macrophage responses to different NP formulations and compared the results with flow cytometry data.

METHODS

Materials

Cytochalasin B (Cyto B), dextran 70,000, fluorescein isothiocyanate (FITC)-dextran, rat-tail collagen, gelatin type B, and mannose were purchased from Sigma (Ontario, Canada). DME medium, trypsin-EDTA solution 1%, and all other cell culture supplements were supplied by Invitrogen (Ontario, Canada). Isobutyl cyanoacrylate monomer (Lot. 02GD9236) was a gift from Loctite Ltd. (Dublin, Ireland). Murine alveolar macrophages (MH-S) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA).

A thermal activity monitor (TAM III) from TA instruments (Newcastle, USA) was used to monitor changes in the thermal activity of macrophages throughout the study. A titration cell equipped with a 3 mL stainless steel ampoule, a Hamilton syringe pump, and special cell culture glass slabs, 1.5×3.5 cm, were purchased from TA instruments. Characterization of NPs (size, polydispersity index, and zeta potential) was performed using a Zetasizer HSA 3000 (Malvern, Worcestershire, UK). FACSCalibur (BD biosciences, USA) was used to perform flow cytometry analysis.

Experimental

Cell Culture

MH-S cells, a continuous cell line of murine alveolar macrophages, were cultivated in 25 mL ventilated flasks (Corning, USA) using DME medium supplemented with

100 mM sodium pyruvate solution, 100 mM non-essential amino acid solution, 1 mM HEPES buffer, 17.8 mM sodium bicarbonate, 100 IU/mL penicillin, 10 µg/mL streptomycin, and 10% (v/v) heat inactivated fetal bovine serum. Macrophages were maintained at 37°C in a 5% CO₂ humidified incubator. In order to prepare the cell culture glass slabs to receive macrophages, the surface of each slab was covered with 250 µL of collagen dissolved in isopropyl alcohol. The glass slabs were left under the laminar flow hood until the alcohol totally evaporated. After the cell culture slabs were prepared, passage #12 of macrophages, cultivated previously in flasks, were trypsinized, counted, and seeded on the surface of the glass slabs; each glass slab received approximately 250,000 cells to achieve a density of about 50,000 cell/cm². The glass slabs, with macrophages seeded on the surfaces, were placed in Petri dishes filled with 15 mL of DME medium and maintained in a cell culture incubator for 24 h.

Preparation and Characterization of NPs

Poly(isobutyl cyanoacrylate) (PIBCA) NPs were prepared using an emulsion polymerization method. Briefly, 100 mg dextran was dissolved in 10 mL of 0.01 N hydrochloric acid. One hundred microliters of isobutyl cyanoacrylate monomer was added dropwise to the dextran solution with continuous stirring at 500 rpm. After 4 h of stirring, the formed NP dispersion was filtered using 0.8 µm nucleopore® membrane filter (Whatman, Ontario, Canada) under vacuum (33). Polysorbate-80-coated PIBCA NPs were prepared by adding 0.1 mg of polysorbate-80 to previously prepared uncoated PIBCA NPs under continuous stirring for 4 h (34).

Gelatin NPs were prepared using a two-step desolvation method reported previously (35). Briefly, 2.5 g of gelatin was dissolved in distilled water under constant stirring (500 rpm) and heating (40°C). The high molecular weight fraction of gelatin was precipitated in the first desolvation step using acetone. The supernatant was discarded and the precipitated gelatin was dissolved again using distilled water. The pH of the high molecular weight gelatin solution was adjusted to 2.5 using 0.1 M hydrochloric acid and acetone was added dropwise until NPs formed. One hundred microliters of an 8% aqueous solution of glutaraldehyde was added as a cross-linker to stabilize the *in situ* formed NPs. Acetone remaining in the gelatin NP dispersion was removed by evaporation under vacuum followed by dialysis for 48 h. Mannosylated gelatin NPs were synthesized using the gelatin NP dispersion prepared previously. The synthesis process includes the ring opening of mannose followed by Schiff's base formation (13). Briefly, a calculated amount of D-mannose was dissolved in 0.1 M acetate buffer (pH 4.0) and added to a gelatin NP dispersion to form a 1:1 w/w ratio. The mixture was shaken continuously at room temperature for 48 h to insure reaction completion. Excess unreacted mannose was removed by dialysis against double distilled water using dialysis tubing (12–14 kDa molecular weight cut-off) for 48 h. The synthesis of mannosylated NPs was confirmed with IR spectroscopy (Nicolet Magna 550 IR spectrometer). After freeze-drying, a small amount of mannosylated gelatin NPs powder was ground with potassium bromide crystals using a mortar and pestle to form a fine, homogeneous powder. A small portion

of the mixture was mechanical pressed to form a translucent, thin film. The film was held using two discs of potassium bromide and inserted in the IR spectrometer.

The concentration of NPs dispersions was determined using a gravimetric method. A 1 ml volume of NP dispersion was placed in a porcelain pan and the sample was heated until totally dry. The weight of NPs was calculated by subtracting the weight of the pan from the total weight of pan plus dried NPs. Appropriate dilutions were made to maintain the four different types of NP dispersions at a concentration of 0.1 mg/mL. The size, polydispersity index, and zeta potential of all types of NPs were assessed using a Zetasizer HSA 3000 ((Malvern, Worcestershire, UK).

Four different NP formulations were labeled with FITC by adding FITC-dextran during the first step of preparation procedure. The amount of FITC-dextran was calculated to be 1% of the dry weight of the final NPs. Excess free FITC-dextran was removed by dialysis against double distilled water using dialysis tubing (12–14 KDa molecular weight cut-off) for 48 h. The loading efficiency of FITC-dextran was calculated according to the fluorescence difference between the supernatant separated after NP precipitation and the unwashed NP dispersions at 488 nm excitation wavelength and 530 nm emission wavelength.

ITMC and Thermal Activity

A TAM III was calibrated using a stainless steel ampoule filled with 3 mL of DME medium and the baseline was adjusted accordingly. A cell culture glass slab with macrophages seeded on the surface was transferred to the stainless steel ampoule of the titration cell. The ampoule was filled with 3 mL of DME medium and sealed to the shaft of the titration cell, then inserted gradually in the TAM III channel according to the manufacture's recommendations. The average ($n=3$) thermal activity recorded during this experiment was considered to be the basic thermal activity of the macrophages. To assess the thermal activity associated with phagocytosis, 100 μ L of different NP dispersions of equal concentrations was titrated at 50 μ L/min to the ampoule in order to be in direct contact with the macrophages seeded on the surface of the glass slab. This was performed with a Hamilton syringe filled with 250 μ L of NP dispersion and connected to the stainless steel ampoule of the titration cell. The experiment was repeated for the four different types of NP-PIBCA (coated and uncoated) and gelatin (mannosylated and non-mannosylated). Each experiment was done in triplicate. The average ($n=3$) heat exchange associated with each NP formulation was used to calculate the relative interactive coefficient as described later. Control experiments were performed by (1) titrating the same amount of NPs to a stainless steel ampoule containing a glass slab with no macrophages seeded on the surface and (2) titrating 100 μ L of phosphate buffered saline (PBS) into the stainless steel ampoule containing macrophages seeded on a glass slab. The control experiments measured the heat flow associated with the titration process itself, effects of the titration on the thermal activity of macrophages, and side reactions unrelated to macrophage–NP interactions, such as dissolution effect. The thermal activity of control experiments was excluded from the total thermal activity before calculating the relative interactive

coefficient. Four positive control experiments were performed using the following mixtures: (1) uncoated PIBCA NPs mixed with 1% w/w of polysorbate-80 for 1 min and used directly, (2) 1% w/w polysorbate-80 solution in distilled water, (3) a 1:1 w/w mixture of mannose and gelatin NPs not chemically bonded, and (4) 0.1 mg/mL mannose solution.

Experiments that included different NP formulations were repeated under the same conditions after adding 2.5×10^{-6} M Cyto B to the medium in the stainless steel ampoule. This amount of Cyto B was shown previously to inhibit phagocytosis by 50% (36).

The total heat exchange for each experiment was determined by calculating the area under the curve of the thermal activity profiles using the TMA III assistant® program. The relative interactive coefficient of each NP formulation toward macrophages was calculated using the same program after subtracting the basal thermal activity of non-treated macrophages and the thermal activity of other control experiments. In the present study macrophage–nanoparticle interactions were described by the following equation:

Where M stands for macrophages and L for NPs.

The interaction between one macrophage with one or more NPs can be considered to proceed in an overall or stepwise reaction path. An overall reaction path is given by:

$$M + 2L \leftrightarrow ML_2 \quad \beta_2, \Delta H_2 \quad \text{where} \quad \beta_1 = \frac{[ML_1]}{[M][L]} \quad \beta_2 = \frac{[ML_2]}{[M][L]^2} \quad \beta_n = \frac{[ML_n]}{[M][L]^n}$$

Where β_n refers to the overall relative interactive coefficient and ΔH_n is the reaction enthalpy for the overall reaction.

A stepwise reaction path is given by:

$$ML_1 + L \leftrightarrow ML_2 \quad K_2, \Delta H_{2s} \quad \text{where} \quad K_1 = \frac{[ML_1]}{[M][L]} \quad K_2 = \frac{[ML_2]}{[ML_1][L]} \quad K_n = \frac{[ML_n]}{[ML_{n-1}][L]}$$

Where K_n is the stepwise relative interactive coefficient and ΔH_{ns} is the reaction enthalpy for the stepwise reaction.

The relation between the stepwise and the overall relative interactive coefficient can be found by comparing K_n and β_n :

$$\beta_1 = K_1 \quad \beta_2 = K_1 K_2 \quad \beta_n = K_1 K_2 K_{n-1} K_n$$

The relation between the reaction enthalpies for stepwise and overall reactions is described by:

$$\Delta H_{1s} = \Delta H_1 \quad \Delta H_{2s} = \Delta H_2 - \Delta H_1 \quad \Delta H_{ns} = \Delta H_n - \Delta H_{n-1}$$

NP concentration and initial cell count were input in the TMA III assistant® program to calculate the relative interactive coefficient.

Flow Cytometry Analysis

The cellular uptake of different NP formulations by macrophages was assessed using flow cytometry. Appropriate dilutions of FITC-labeled NPs were made to obtain the four different types of labeled NP dispersions at a concentration of 0.1 mg/ml using cell culture media. Five milliliter of each diluted FITC-labeled NP dispersion were added MH-S alveolar macrophages cultivated in 75 ml vented flasks. The treatment was discarded after 1 h and macrophages were washed three times with PBS and trypsinized using 1% trypsin-EDTA to obtain a single-cell dispersion. The cells were centrifuged, the supernatant was discarded, and the cells were resuspended in 2 mM EDTA-PBS solution. The final cell suspensions were counted using trypan blue and hemocytometer and the counts were adjusted to 1×10^6 cell/ml. Cell suspensions were filtered with BD Falcon, 5 mL polystyrene round-bottom tube with cell-strainer cap (BD biosciences, USA), and injected into the FACSCalibur. Flow cytometric analyses were performed using a FACSCalibur flow cytometer equipped with a 15 mW argon ion laser emitting at 488 nm for excitation. FITC was detected using FL1 detector, 530 ± 15 nm band-pass filter. Cells were gated according the forward scattering and side scattering to exclude dead cells and cell debris from the analysis. Auto-fluorescence of untreated macrophages on flow cytometric analysis was used as negative control. All parameters were collected as logarithmic signals. The intensity cut-off of fluorescence was set so that 99% of the untreated macrophages auto-fluorescence was considered negative. The percentage of the macrophages that show positive fluorescence properties was expressed by counting the macrophages that exceed the cut-off value. Data analysis was carried out with CellQuest software obtained from BD Biosciences. Generally, a total of 20,000 cells were analyzed for each one of the four NP dispersions.

Statistical Analysis

Data were expressed as mean $\pm$ standard deviation. A one-way ANOVA was used to test for differences among heat exchange and relative interactive coefficient values for different NP formulations. NP characteristics, particle size, and zeta potential were compared using the Student's *t* test. A result was considered statistically significant at $P < 0.05$.

RESULTS

NP Characteristics

Table I shows the physical characteristics of different NP formulations. Loading efficiency of FITC-dextran was about 90% in all NP formulations with no significant difference between them. Please refer to the Electronic Supplementary Material (ESM 1) for the IR spectrum of mannosylated gelatin NPs.

Thermal Activity of Macrophages

The thermal activity profiles of macrophages alone and after titration with 100 μ L of uncoated PIBCA NPs and polysorbate-80-coated NPs are shown in Fig. 1. The thermal activity of macrophages alone (Fig. 1(a)) consists of two main phases, ascending and descending. The ascending phase extends for the first 37 h and comprises two subphases, the first subphase is a linear increase in heat production for the first 10 h followed by an exponential incline in the thermal exchange for the rest of the ascending phase to reach a maximum of 221 μ W after 37 h. The descending phase shows a sharp linear decline for the first 5 h followed by a slower exponential decline that extends to near stagnation. The total heat exchanged during the 100 h was 9.8 ± 2.3 J.

The thermal activity of macrophages recorded after 100 μ L of uncoated PIBCA NPs were titrated is shown in Fig. 1(b). Although two main ascending and descending phases are also present, the linear increase of the exothermic phase has a higher slope, which reflects a faster increase in heat production. The exponential subphase of the heat exchange has a slower start, resulting in a shift of the whole heat flow profile to the right, with a maximum heat flow of 386 μ W after 60 h. The total heat exchanged during 100 h was 37.6 ± 5.6 J, which is significantly higher than what was observed with macrophages alone, 9.8 ± 2.3 J ($P < 0.05$). The relative interactive coefficient of uncoated PIBCA NPs toward macrophages was calculated to be $4,356 \pm 350$ M⁻¹.

The results of using polysorbate-80-coated PIBCA NPs instead of uncoated NPs are shown in Fig. 1(c). The thermal activity of polysorbate-80-coated PIBCA NPs falls between values of the thermal activity of macrophages alone and after titrating uncoated NPs. The total heat exchanged during 100 h after titrating coated NPs (15.2 ± 3.4 J) was significantly less than what was observed using uncoated NPs (37.6 ± 5.6 J) and higher than the thermal activity of macrophages alone (9.8 ± 2.3 J). Coating the surface of PIBCA NPs with polysorbate-80 resulted in a significantly lower relative interactive coefficient (890 ± 120 M⁻¹) compared with the relative interactive coefficient of uncoated NPs ($P < 0.05$). Titrating the same concentration of uncoated NPs after mixing them with 1% w/w of polysorbate-80 for only 1 min resulted in a thermal activity profile very similar to what was observed with uncoated NPs. Similarly, titration of 100 μ L of 1% w/w polysorbate-80 solution in PBS resulted in a thermal activity profile very similar to that observed with macrophages alone.

Adding Cyto B to the cell culture medium produced macrophage thermal activity profiles as shown in Fig. 2. The thermal activity profile of macrophages alone (Fig. 2(a)) was

Table I. Characteristics of Poly(Isobutyl Cyanoacrate) and Gelatine Nanoparticles

NPs' formulation	Particle size (nm)±SD	Polydispersity±SD	Zeta potential (mV)
Uncoated PIBC NPs	137±19	0.082±0.009	-25.6±0.32
Polysorbate-80-coated NPs	140±25	0.073±0.011	-24.2±0.41
Gelatin NPs	225±23	0.098±0.013	12.2±0.25
Mannosylated gelatin NPs	305±35	0.125±0.015	7.3±0.12

Values are mean±SD ($n=3$)

significantly different from the thermal activity of macrophages in the presence of Cyto B. The thermal activity profile of macrophages plus Cyto B was shorter in time (60 h) and lower in terms of heat exchange (3.9 ± 1.3 J) than when the experiment was performed with macrophages alone (9.8 ± 2.3 J). Moreover, the presence of Cyto B in the medium inhibited cellular responses of macrophages toward coated or uncoated NPs (Fig. 3(b, c)). The total heat exchange after titrating NPs in the presence of Cyto B was not significantly different from the heat exchange of titrating macrophages alone.

Figure 3 shows the thermal activity profile of macrophages alone after titrating 100 μ L of gelatin NPs and mannosylated gelatin NPs. Similar to what was seen with uncoated PIBCA NPs, non-mannosylated gelatin NPs (Fig. 4(b)) induced a significant increase in the total thermal activity when compared with macrophages alone, 45.5 ± 8.2 J and 9.8 ± 2.3 J, respectively. The relative interactive coefficient of non-mannosylated gelatin NPs was significantly higher than the relative interactive coefficient of uncoated PIBCA NPs, $5,120\pm410$ M⁻¹ and $4,356\pm350$ M⁻¹, respectively (Table II). This can be attributed to the difference in NP characteristics (size and zeta potential) and to their polymer nature. Mannosylated gelatin NPs changed the thermal activity profile and the total heat exchange dramatically (Fig. 3(c)). The thermal activity of macrophages increased from 0 to almost 100 μ W in less than 1 h. The thermal activity associated with mannosylated gelatin NP titration, 75.4 ± 7.5 J was not predicted by experiments performed previously. The high amount of heat produced resulted in a significantly higher relative interactive coefficient, $9,269\pm630$ M⁻¹, for mannosylated NPs.

Fig. 1. The thermal activity profiles of A macrophages alone, B macrophages after titration of 100 μ L of uncoated poly(isobutyl cyanoacrylate) nanoparticles, and C macrophages after titration of 100 μ L of polysorbate-80-coated poly(isobutyl cyanoacrylate) nanoparticles

Figure 4 shows the heat production associated with the titration of 100 μ L of a 1:1 w/w mixture of not chemically bonded mannose and gelatin NPs (A) and 100 μ L of 0.1 mg/mL mannose solution (B). The thermal activity recorded with not chemically bonded mixture of mannose and gelatin NPs (Fig. 4(a)) was similar to what was observed with mannosylated NPs, which are chemically bonded (Fig. 3(c)). However, using mannose solution resulted in a different thermal activity profile (Fig. 4(b)). The same early, sharp increase in heat production was noticed (Fig. 4(b)), but the rest of the thermal profile was very similar to what was observed with macrophages alone.

Adding Cyto B to the cell culture medium resulted in phagocytosis inhibition of mannosylated gelatin NPs. However, it did not affect the sharp increase in the heat production associated with ligand–receptor interaction of mannose and mannose receptor as shown in Fig. 4(c).

Flow Cytometry Analysis

Representative dot plots and histograms of four different FITC-labeled NP formulations along with the negative control are shown in Figs. 5 and 6, respectively. More than 99% of the untreated macrophages are located mainly in the lower left (LL) quadrant and showed a mean fluorescence intensity (MFI) of 3.19 on an arbitrary channel as shown in Figs. 5a and 6a, respectively. After treating macrophages with polysorbate-80-coated PIBCA only 1% of these macrophages were detected in the lower right (LR) quadrant Fig. 5b with

Fig. 2. The thermal activity profiles of A macrophages alone, B macrophages after titration of 100 μ L of non-coated poly(isobutyl cyanoacrylate) nanoparticles, and C macrophages after titration of 100 μ L of polysorbate-80-coated poly(isobutyl cyanoacrylate) nanoparticles after the cytochalasin B was added at the same concentration ($2-5\times10^{-6}$ M) to the medium used in all experiment

Fig. 3. The thermal activity profiles of *A* macrophages alone, *B* macrophages after titration of 100 μ l of gelatin nanoparticles, and *C* macrophages after titration of 100 μ l of mannoseylated gelatin nanoparticles

low MFI value of 5.07. 14.12% of macrophages were shifted to the LR quadrant when they were exposed to the uncoated PIBCA NPs, the MFI was 16.8 as shown by Figs. 5c and 6c.

Higher percentage (54.96%) of macrophages were detected in the LR when gelatin NPs were used as treatment with MFI value of 18.39 as shown in Figs. 5d and 6d, respectively. Mannoseylated gelatin NPs were associated with highest uptake as shown by Figs. 5e and 6e. 58.69% of macrophages were shifted to the LR quadrant and the MFI was 46.08 on an arbitrary channel.

DISCUSSION

NP characteristics found in this study were similar to those reported previously (13,33–35). The positive zeta potential of gelatin NPs can be explained by using a pH lower than the isoelectrical point of gelatin during NP preparation. The positively charged primary amine groups

Fig. 4. The thermal activity profiles of *A* macrophages after titration 100 μ l of 1:1 mixture of gelatin nanoparticles and mannose, *B* macrophages after titrating 100 μ l of mannoseylated solution of 0.1 mg/ml, and *C* macrophages after titration of 100 μ l of mannoseylated gelatin nanoparticles with cytochalasin B added to the cell culture medium

($-\text{NH}_3^+$) result in a positive zeta potential. Mannoseylated gelatin NPs were bigger than non-mannoseylated NPs and had less positive charge on the surface (Table I). This can be attributed to the formation of a Schiff's base ($-\text{C}=\text{N}^-$) between the primary amine groups on the surface of NPs and the aldehyde groups of the ring-opened mannose moieties (13). An IR spectrum of mannoseylated gelatin NPs confirms the success of the synthesis. The stretches between 1,465–1,500 and 1,505–1,550 cm^{-1} correspond to Schiff's bases ($-\text{C}=\text{N}^-$) and secondary amine groups, respectively (13). Please refer to the Electronic Supplementary Material (ESM 1) for the IR spectrum of mannoseylated gelatin NPs.

The thermal activity profile of macrophages alone, Fig. 1 (a), is different from what was reported previously (30,32). The thermal activity of living cells is comprised of an ascending phase followed by a descending phase. Previous reports describe both phases to be linear with no exponential subphase. The observations in this study can be explained by a difference in experimental settings. In the previous reports, experiments were performed on macrophages suspended in cell culture media with no surface to adhere to; this is not optimal. In the present study, macrophages were seeded on special cell culture slabs that provided optimum conditions for the expression of biological functions. The ascending phase of the thermal activity profile represents a period in which the macrophages are metabolically active, with oxygen and nutrients still abundant in the cell culture medium. The exponential increase in heat exchange can be attributed to growth and multiplication of macrophages. Later, due to the increasing number of macrophages, oxygen and other essential nutrients will be depleted and cellular death will follow. This is represented by a decrease in thermal activity during the descending phase.

The aim of this study was not to compare different types of NPs prepared from different polymers, but to investigate the impact that pharmaceutical manipulation of NPs, such as surface decorating or coating, might have on biological interactions such as phagocytosis monitored by ITMC.

Prephagocytosis, phagocytosis, and postphagocytosis processes may explain the initial fast increase in heat production after the titration of uncoated PIBCA NPs (Fig. 1(b)). The observation that the heat exchange was slower in the beginning of the exponential subphase of the ascending phase might be explained by the transient conversion in the metabolic energy source required to finish the phagocytosis process. The source may have changed from oxidative phosphorylation to glycolysis, as it is known that glycolysis is associated with less heat production than oxidative phosphorylation (37). Heat production associated with uncoated PIBCA NPs was significant compared with heat production of macrophages alone, 37.6 ± 5.6 J and 9.8 ± 2.3 J, respectively. Since macrophage count, NP concentration, and volume titrated were constant, the relative interactive coefficient of different NP formulations was used to evaluate the phagocytosis process and compare different NP formulations.

Titration with polysorbate-80-coated PIBCA NPs resulted in a significant decrease in the total heat production as shown in Fig. 1(c). The polysorbate-80 coating material decreased the macrophage cellular response to PIBCA NPs. Polysorbate alone or in a physical mixture with NPs had no effect on the thermal activity profiles. This suggests that the inhibition of NP

Table II. Values of Total Heat Exchange and the Relative Interactive Coefficients of Different NP Formulations

NPs' formulation	Total heat exchange (J)	Relative interactive coefficient (1/M)
Macrophages alone	9.8±2.3	NA
uncoated PIBCA NPs	37.6±5.6	4,356±350
Polysorbate-80-coated NPs	15.2±3.4	890±120
Gelatin NPs	45.5±8.2	5,120±410
Mannosylated gelatine NPs	75.4±7.5	9,269±630

Values are mean±SD ($n=3$)

phagocytosis by polysorbate-80 is limited to the formation of a surfactant layer on the NP surface.

Cyto B is a mycotoxin with high cell permeability properties. It inhibits the formation of contractile actin microfilaments which results in inhibition of cellular movement and phagocytosis functionality (38). Cyto B has been used as a phagocytosis inhibitor to study this cellular function (36,39). Cyto B was included in this study design to confirm that the increase in heat exchange observed after NP titration is a direct result of the phagocytosis process. If the increase in heat exchange observed after NP titration is inhibited by Cyto B, this would imply that phagocytosis is responsible for the heat exchange. As shown in Fig. 2(a), in the presence of Cyto B the macrophage thermal activity profile was significantly lower and there was a significant decrease in the total heat exchange in the medium compared with experiments performed without Cyto B, 3.9 ± 1.3 J and 9.8 ± 2.3 J, respectively. In addition to its effect on phagocytosis, Cyto B could affect other aspects of cellular function such as

inhibition of glucose transportation and induction of nuclear extrusion (39), and this would impact the thermal activity profile. However, Cyto B totally inhibited an increase in heat production after NP titration, as shown in Fig. 3(b, c). On the other hand, Cyto B had no effect on the ligand–receptor reaction of mannose and mannose receptor as shown in Fig. 4 (c). Therefore, it could be concluded that the observed increase in heat exchange induced by NP titration (Figs. 1(b) and 4(b, c)) was directly connected to the phagocytosis process.

Targeting mannose receptors on the macrophage cellular surface using mannose-decorated NPs is an established strategy to enhance phagocytosis (19). Therefore, mannosylated NPs are expected to be associated with enhanced phagocytosis and, consequently, with higher heat production as detected by ITMC. The initial sharp increase in heat production associated with mannosylated gelatin NPs (Fig. 3 (c)) can be explained by ligand–receptor interaction between mannose moieties and mannose receptors on the surface of

Fig. 5. Flow cytometric dot plots of (a) untreated macrophages, (b) macrophages treated with polysorbate-80-coated PIBCA nanoparticles, (c) macrophages treated with uncoated PIBCA nanoparticles, (d) macrophages treated with gelatin nanoparticles and (e) macrophages treated with mannosylated gelatin nanoparticles

Fig. 6. Flow cytometric analysis of the macrophage uptake of nanoparticles: (a) represent the macrophages auto-fluorescence MFI=3.19, the horizontal line was set to calculate the percentage of positive cells, (b) polysorbate-80 coated PIBCA nanoparticles MFI=5.07, (c) uncoated PIBCA nanoparticles MFI=16.81, (d) gelatin nanoparticles MFI=18.39 and (e) mannosylated gelatin nanoparticles MFI=46.08

macrophages. The similarity between the heat activity profiles of mannosylated gelatin NPs and the non-chemically bonded mixture of mannose and gelatin NPs (Figs. 4(c) and 5a) suggests that the presence of mannose in solution is enough to provoke and enhance the phagocytotic functionality of macrophages. This observation is in contrast to what was noticed with polysorbate-80-coated PIBCA NPs. The fact that mannose solution induced an initial sharp increase in heat production implies that the similar sharp increase observed with mannosylated gelatin NPs is a result of interaction between mannose moieties and mannose receptors. Moreover, the initial sharp increase in the heat production observed in the presence of Cyto B after titrating 100 μ L (Fig. 4(c)) implies that Cyto B affects phagocytosis process, yet has not effect on ligand–receptor interaction.

Flow cytometry analysis was used to validate the data obtain from ITMC. Macrophages were exposed to different NP formulations and the results were expressed in relative to the untreated macrophages. The locations of the macrophages on the dot plot along with the value of MFI were used to assess the uptake of different NP formulations. The percentage of macrophages that relocated from LL to LR quadrant represents the portion of macrophages that has been taken up NPs, however, the MFI value represents the extent of uptake.

Mannosylated gelatin NPs were associated with the highest uptake. 58.69% of the macrophages were detected in LR quadrant Fig. 6e. These portion of macrophages showed a high MFI values, 46.08. Polysorbate-80-coated PIBCA showed the lowest uptake. Only 1% of macrophages

expressed an increase in the fluorescence properties with low MFI value of 5.07. Macrophages treated with uncoated PIBCA and gelatin NPs showed similar MFI values of 16.8 and 18.39, respectively. However, gelatin NPs were associated with higher percentage of macrophages in the LR quadrant and, consequently, a higher uptake.

The results of the flow cytometry highly confirm the data collected via ITMC. The higher uptake of mannosylated gelatin NPs detected by flow cytometry was associated with the highest heat exchange and relative interactive coefficient as discussed earlier. Moreover, titrating polysorbate-coated NPs into the ITMC was associate with insignificant increase in the heat exchange detected via ITMC as shown in Fig. 1(c). This fact was supported by flow cytometry as these NPs showed almost no cellular uptake. Flow cytometry showed that gelatin NPs had a higher uptake compared with uncoated PIBCA NPs and a lower uptake in comparison with mannosylated gelatin NPs. These results are in agreement with what was observed using ITMC. ITMC showed that heat exchange and relative interactive coefficient of gelatin NPs falls in between compared with uncoated PIBCA and mannosylated gelatin NPs as shown in Table II.

CONCLUSIONS

Understanding the cellular responses of macrophages to different NP formulations is essential in the design of colloidal drug delivery systems. This study concludes that ITMC is able to differentiate between the macrophage cellular responses to different NP formulations in terms of

heat exchange in real-time. The results showed that thermal activity was dependent on the type of NP present. The flow cytometry confirmed cellular nanoparticle uptake.

ITMC, as a biological monitor, proved to be a valuable tool to understand macrophage–NP interactions.

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REFERENCES

- Oh Y-K, Swanson JA. Different fates of phagocytosed particles after delivery into macrophage lysosomes. *J Cell Biol.* 1996;132(4):585–93.
- Schafer V, von Briesen H, Rubsamen-Waigmann H, Steffan AM, Royer C, Kreuter J. Phagocytosis and degradation of human serum albumin microspheres and nanoparticles in human macrophages. *J Microencapsul.* 1994;11(3):261–9.
- Roser M, Fischer D, Kissel T. Surface-modified biodegradable albumin nano- and microspheres. II: effect of surface charges on *in vitro* phagocytosis and biodistribution in rats. *Eur J Pharm Biopharm.* 1998;46(3):255–63.
- Al-Hallak KM, Azarmi S, Anwar-Mohamed A, Roa WH, Lobenberg R. Secondary cytotoxicity mediated by alveolar macrophages: a contribution to the total efficacy of nanoparticles in lung cancer therapy? *Eur J Pharm Biopharm.* 2010;76(1):112–9.
- Soma CE, Dubernet C, Barratt G, Benita S, Couvreur P. Investigation of the role of macrophages on the cytotoxicity of doxorubicin and doxorubicin-loaded nanoparticles on M5076 cells *in vitro*. *J Control Release.* 2000;68(2):283–9.
- Chiannilkulchai N, Ammoury N, Caillou B, Devissaguet JP, Couvreur P. Hepatic tissue distribution of doxorubicin-loaded nanoparticles after i.v. administration in reticulosarcoma M 5076 metastasis-bearing mice. *Cancer Chemother Pharmacol.* 1990;26(2):122–6.
- Azarmi S, Roa WH, Lobenberg R. Targeted delivery of nanoparticles for the treatment of lung diseases. *Adv Drug Deliv Rev.* 2008;60(8):863–75.
- Lobenberg R, Kreuter J. Macrophage targeting of azidothymidine: a promising strategy for AIDS therapy. *AIDS Res.* 1996;12(18):1709–15.
- Bender A, Schfer V, Steffan AM, Royer C, Kreuter J, Rubsamen-Waigmann H, *et al.* Inhibition of HIV *in vitro* by antiviral drug-targeting using nanoparticles. *Res Virol.* 1994;145(3–4):215–20.
- Gaspar R, Preat V, Opperdoes FR, Roland M. Macrophage activation by polymeric nanoparticles of polyalkylcyanoacrylates: activity against intracellular *Leishmania donovani* associated with hydrogen peroxide production. *Pharm Res.* 1992;9(6):782–7.
- Bazile D, Prud'homme C, Bassoullet MT, Marlard M, Spelnhauer G, Veillard M. Stealth Me.PEG-PLA nanoparticles avoid uptake by the mononuclear phagocytes system. *J Pharm Sci.* 1995;84(4):493–8.
- Moghimi SM, Szebeni J. Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. *Prog Lipid Res.* 2003;42(6):463–78.
- Jain SK, Gupta Y, Jain A, Saxena AR, Khare P, Jain A. Mannosylated gelatin nanoparticles bearing an anti-HIV drug didanosine for site-specific delivery. *Nanomedicine.* 2008;4(1):41–8.
- Peracchia MT, Fattal E, Desmaele D, Besnard M, Noel JP, Gomis JM, *et al.* Stealth PEGylated polycyanoacrylate nanoparticles for intravenous administration and splenic targeting. *J Control Release.* 1999;60(1):121–8.
- Gulyaev AE, Gelperina SE, Skidan IN, Antropov AS, Kivman GY, Kreuter J. Significant transport of doxorubicin into the brain with polysorbate 80-coated nanoparticles. *Pharm Res.* 1999;16(10):1564–9.
- Moghimi SM, Hunter AC, Murray JC. Long-circulating and target-specific nanoparticles: theory to practice. *Pharmacol Rev.* 2001;53(2):283–318.
- Alyautdin RN, Petrov VE, Langer K, Berthold A, Kharkevich DA, Kreuter J. Delivery of loperamide across the blood-brain barrier with polysorbate 80-coated polybutylcyanoacrylate nanoparticles. *Pharm Res.* 1997;14(3):325–8.
- Al-Hallak MH, Azarmi S, Sun C, Lai P, Prenner EJ, Roa WH, *et al.* Pulmonary toxicity of polysorbate-80-coated inhalable nanoparticles; *in vitro* and *in vivo* evaluation. *AAPS J.* 2010;12(3):294–9.
- Irache JM, Salman HH, Gamazo C, Espuelas S. Mannose-targeted systems for the delivery of therapeutics. *Expert Opin Drug Deliv.* 2008;5(6):703–24.
- Cui Z, Hsu CH, Mumper RJ. Physical characterization and macrophage cell uptake of mannan-coated nanoparticles. *Drug Dev Ind Pharm.* 2003;29(6):689–700.
- Dutta T, Agashe HB, Garg M, Balakrishnan P, Kabra M, Jain NK. Poly (propyleneimine) dendrimer based nanocontainers for targeting of efavirenz to human monocytes/macrophages *in vitro*. *J Drug Target.* 2007;15(1):89–98.
- Rolland A, Merdrignac G, Gouranton J, Bourel D, Le Verge R, Genetet B. Flow cytometric quantitative evaluation of phagocytosis by human mononuclear and polymorphonuclear cells using fluorescent nanoparticles. *J Immunol Methods.* 1987;96(2):185–93.
- Panyam J, Sahoo SK, Prabha S, Bargar T, Labhasetwar V. Fluorescence and electron microscopy probes for cellular and tissue uptake of poly(D, L-lactide-co-glycolide) nanoparticles. *Int J Pharm.* 2003;262(1–2):1–11.
- Cunningham RE. Overview of flow cytometry and fluorescent probes for cytometry. *Methods Mol Biol.* 1994;34:219–24.
- Gasco MR, Bocca C, Caputo O, Cavalli R, Gabriel L, Miglietta A. Fluorescent non-stealth and stealth solid lipid nanoparticles as markers of phagocytosis by macrophages. In: *Proceedings of the Controlled Release Society.* 1997; p. 26.
- Chiellini F, Dinucci D, Bartoli C, Piras AM, Chiellini E. Intracellular fate investigation of bio-eliminable polymeric nanoparticles by confocal laser scanning microscopy. *J Bioact Compat Polym.* 2007;22(6):667–85.
- Al-Hallak MHDK, Xu Z, Ghaffari F, Löbenberg R. The effect of compression forces on the stability of dibasic calcium phosphate dihydrate tablets in the presence of glutamic acid hydrochloride monitored by isothermal calorimetry. *Thermochim Acta.* 2007;467(1–2):86–90.
- Velazquez-Campoy A, Ohtaka H, Nezami A, Muzammil S, Freire E. Isothermal titration calorimetry. *Curr Protoc Cell Biol.* 2004; Chapter 17, Unit 17: 8.
- Wasdo I. Isothermal microcalorimetry in applied biology. *Thermochim Acta.* 2002;394:305–11.
- Backman P. Effects of experimental factors on the metabolic rate of T-lymphoma cells as measured by microcalorimetry. *Thermochim Acta.* 1990;172:123–30.
- Sbarra AJ, Karnovsky ML. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. *J Biol Chem.* 1959;234(6):1355–62.
- Gebreselassie D, Kasravi B, Bäckman P, Jeppson B. A microcalorimetric method to study the activation of murine peritoneal macrophages. *Thermochim Acta.* 1996;275(1):109–15.
- Azarmi S, Tao X, Chen H, Wang Z, Finlay WH, Lobenberg R, *et al.* Formulation and cytotoxicity of doxorubicin nanoparticles carried by dry powder aerosol particles. *Int J Pharm.* 2006;319(1–2):155–61.
- Alyautdin R, Gothier D, Petrov V, Kharkevich D, Kreuter J. Analgesic activity of the hexapeptide dalargin adsorbed on the surface of polysorbate 80-coated poly(butyl cyanoacrylate) nanoparticles. *Pharm Res.* 1999;16(10):1564–9.

- crylate) nanoparticles. Eur J Pharm Biopharm. 1995;41(1):44–8.
35. Azarmi S, Huang Y, Chen H, McQuarrie S, Abrams D, Roa W, *et al.* Optimization of a two-step desolvation method for preparing gelatin nanoparticles and cell uptake studies in 143B osteosarcoma cancer cells. J Pharm Pharm Sci. 2006;9(1):124–32.
36. Axline SG, Reaven EP. Inhibition of phagocytosis and plasma membrane mobility of the cultivated macrophage by cytochalasin B. Role of subplasmalemmal microfilaments. J Cell Biol. 1974;62(3):647–59.
37. Cifarelli A, Pepe G, Paradisi F, Piccolo D. The influence of some metabolic inhibitors on phagocytic activity of mouse macrophages *in vitro*. Res Exp Med. 1979;174(2):197–204.
38. Zigmond SH, Hirsch JG. Effects of cytochalasin B on polymorphonuclear leucocyte locomotion, phagocytosis and glycolysis. Exp Cell Res. 1972;73(2):383–93.
39. Malawista SE, Gee JB, Bensch KG. Cytochalasin B reversibly inhibits phagocytosis: functional, metabolic, and ultrastructural effects in human blood leukocytes and rabbit alveolar macrophages. Yale J Biol Med. 1971;44(3):286–300.