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# Interaction between silver nanoparticles of 20 nm (AgNP<sub>20</sub>) and human neutrophils: induction of apoptosis and inhibition of *de novo* protein synthesis by AgNP<sub>20</sub> aggregates

Michelle Poirier, Jean-Christophe Simard, Francis Antoine and Denis Girard

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#### ABSTRACT

Cytotoxic and proinflammatory properties of silver nanoparticles (AgNPs) have been reported in few studies but the direct interaction between AgNPs and neutrophils, which play a key role in inflammation, has never been documented. Here, we examined the role of AgNPs with a starting size of 20 nm (AgNP<sub>20</sub>) in human neutrophils. Using dynamic light scattering for the characterization of NPs suspended under identical conditions to those used for *in vitro* experiments, we found that, at 10 µg ml<sup>-1</sup>, 92% of AgNP<sub>20</sub> possess a diameter of 17.1 nm but, at 100 µg ml<sup>-1</sup>, a tri-modal size distribution with large aggregates was observed (> 500 nm). Neutrophil cell size increased when treated with AgNP<sub>20</sub> and transmission electronic microscopy experiments revealed that AgNP<sub>20</sub> can rapidly interact with the cell membrane, penetrate neutrophils, localize in vacuole-like structures, and be randomly distributed in the cytosol after 24 h. Treatment with 100 µg ml<sup>-1</sup> AgNP<sub>20</sub> for 24 h (but not 10 µg ml<sup>-1</sup>) increased the neutrophil apoptotic rate and inhibited *de novo* protein synthesis. We conclude that AgNP<sub>20</sub> induced apoptosis and can act as potent inhibitors of *de novo* protein synthesis at 100, but not 10 µg ml<sup>-1</sup> in human neutrophils. Copyright © 2013 John Wiley & Sons, Ltd.

#### Introduction

Research in the field of nanotechnology has increased tremendously in the last few years due to the expectation that nanomaterials continue to be incorporated into practically all types of products; more than 1000 nanoproducts already exist on the market, many of which contain silver nanoparticles (AgNPs, or nanosilver) (Arora *et al.*, 2012). Owing to their well-reputed antimicrobial properties, AgNPs are presently among the leading nanotechnology products (Pratsinis *et al.*, 2013; Sotiriou and Pratsinis, 2010). For example, many of these products are used in the textile industries, including clothes made of AgNP-containing fabric (Kulthong *et al.*, 2010). In addition, AgNPs are found in personal hygiene products, including toothpastes (Nowack *et al.*, 2011). Thus, certain AgNP-containing products may be in close

contact with the human body and may potentially reach the bloodstream, via inhalation or absorption through the skin or gastrointestinal tract (Chen and Schluesener, <u>2008</u>; Korani *et al.*, <u>2011</u>; Quadros and Marr, <u>2011</u>).

Although several studies have reported that NPs can induce inflammation *in vivo*, as evidenced by an increased number of polymorphonuclear neutrophil cells (PMNs) in lungs/brochoalveolar lavages (Noel *et al.*, 2012; Rossi *et al.*, 2010; Roursgaard *et al.*, 2010; Srinivas *et al.*, 2011), the direct interaction between NPs and PMNs, key players in inflammation, is poorly documented. We have recently shown that titanium dioxide (TiO<sub>2</sub>) NPs activate human PMNs *in vitro*, and can delay apoptosis and increase the production of the proinflammatory cytokine interleukin-8 (Goncalves *et al.*, 2010). Thus, the possibility that other NPs could alter PMN biology need to be determined, particularly with regard to the modulation of apoptosis, given that resolution of inflammation occurs largely by elimination of apoptotic PMNs by professional phagocytes, including macrophages (Fox *et al.*, 2010).

Studies investigating the interaction of AgNPs with human PMNs have not been documented. However, interactions between AgNPs and macrophages have been reported (Haase *et al.*, 2011; Park *et al.*, 2011; Pratsinis *et al.*, 2013; Shavandi *et al.*, 2011; Wasowicz *et al.*,2011). Physiological changes induced by different types and sizes of NPs have been investigated, and different responses according to both characteristics have been observed. In fact, AgNPs and gold NPs (AuNPs) were shown to be taken up by cells and located in vesicles in the cytosol; however, AuNPs adopt a curious circular pattern in comparison with the diffuse localization of AgNPs. Moreover, there is variation in the expression of proinflammatory cytokines observed with different diameters of the same NPs, as well as between AuNPs and AgNPs (Yen *et al.*, 2009). In addition, it was observed that AgNPs caused mitochondrial damage, resulting in induction of apoptosis and death of macrophages (Singh and Ramarao, 2012).

Although it is known and highly predictable that a given NP can exert different cytotoxic effects and/or alter cell physiology according to its diameter (Bartneck *et al.*, 2010), the aim of the present study was to determine whether or not NPs of a given diameter, namely AgNP with a starting diameter of 20 nm (AgNP<sub>20</sub>), could alter the biology of human PMNs when tested at different concentrations. Therefore, we characterized AgNP<sub>20</sub> at different concentrations using dynamic light scattering (DLS), under conditions identical to those used in *in vitro*experiments (culture medium, human serum, etc.). We demonstrated that AgNP<sub>20</sub> increased the cell size of freshly isolated human PMNs, rapidly interacted with the cell membrane and penetrated inside cells, induced cell apoptosis and inhibited *de novo* protein synthesis only when present as large aggregates (> 500 nm, in this study).

#### Materials and Methods

#### Chemicals

The cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF) was purchased from PeproTech Inc. (Rocky Hill, NJ, USA). The plant lectin *Viscum album* agglutinin-I (VAA-I), lipopolysaccharides from *Escherichia coli*, trypan blue and dextran were from Sigma Aldrich Ltd. (St. Louis, MO, USA). AgNP<sub>20</sub> (Pelco® citrate Biopure<sup>™</sup> silver) were purchased from Ted Pella (Redding, CA, USA). Ficoll-Hypaque was purchased from GE Healthcare (Uppsala, Sweden) and RPMI-1640, HEPES, penicillin and streptomycin were from Life Technologies, Grand Island, NY, USA). The lactase dehydrogenase (LDH)– Cytotoxicity Assay Kit II was purchased from Abcam® (Toronto, ON, Canada).

#### Size Distribution and Zeta Potential Measurements

The size distribution and surface charge (zeta potential) of  $AgNP_{20}$  were determined by DLS using a Malvern Zetasizer Nano-ZS (model ZEN3600; Malvern Instruments Inc., Westborough, MA, USA). Measurements were performed at 10 and 100 µg ml<sup>-1</sup> AgNPs in RPMI-1640 with HEPES and penicillin/streptomycin + 10% heat-inactivated human serum.

#### Neutrophil Isolation

Neutrophils were isolated from venous blood of healthy volunteers by dextran sedimentation followed by centrifugation over Ficoll-Hypaque as previously described (Binet *et al.*, 2008; Pelletier *et al.*, 2004). Blood donations were obtained from informed and consenting individuals according to institutionally approved procedures. Cell viability was monitored by trypan blue exclusion and was always > 98%. Purity was confirmed by cytology from cytocentrifuged preparations colored by the Hema 3 stain set (Fisher Scientific company L.L.C., Kalamazoo, MI).

#### **Cell Viability and Cell Shape Changes**

Freshly isolated human PMNs ( $10 \times 10^6$  cells ml<sup>-1</sup> in RPMI-1640 HEPES-P/S, supplemented with 10% heatinactivated autologous serum) were treated for 0–24 h with or without increasing concentrations of AgNP<sub>20</sub> ranging from 0 to 100 µg ml<sup>-1</sup>, as reported by others (Arora *et al.*, 2009; Rosas-Hernandez *et al.*, 2009). Cell viability was monitored by trypan blue exclusion and by LDH assays. The morphological cell shape changes were observed under light microscopy (× 400), and photomicrographs were taken using a Nikon Eclipse TS100 camera (Melville, NY).

#### Evaluation of Cell Size and Inner Complexity by Flow Cytometry

Freshly isolated human PMNs (10 × 10<sup>6</sup> cells ml<sup>-1</sup> in RPMI-1640 HEPES-P/S) were treated with or without 10 or 100 µg ml<sup>-1</sup> AgNP<sub>20</sub> for 30 min and data for cell size (forward scattered light height (H)) and inner complexity (side scattered light height (H)) were acquired by flow cytometry using a FACScan (BD Biosciences, San Jose, CA).

#### Confocal Microscopy

PMNs (10 × 10<sup>6</sup> cells ml<sup>-1</sup>) were resuspended in RPMI 1640 HEPES Pen/Strep without phenol red containing 10% serum albumin. Cells were then incubated with the indicated agonist in poly-D-coated glass bottom

Microwell dishes (Mat Tek Corporation, Ashland, MA, USA). After 24 h, NucBlue® Live ReadyProbes™ Reagent (Life Technologies) was added to micro-well dishes for microscopy. Images were captured with a Zeiss LSM 780 confocal microscope. Cell sizes were measured using Zen 2011 Blue Edition (Carl Zeiss, Oberkochen, Germany). One hundred randomly selected cells were measured using three different fields for each condition. Results were confirmed with at least four blood donors.

#### Transmission Electron Microscopy

Freshly isolated human PMNs ( $10 \times 10^6 \text{ ml}^{-1}$ ) were incubated with  $100 \mu \text{g ml}^{-1} \text{ AgNP}_{20}$  for 0–24 h, fixed with glutaraldehyde (2.5%) and examined using a Hitachi H-7100 (Dallas,TX) transmission electron microscope (TEM) at different periods of time.

#### Assessment of Neutrophil Apoptosis

Apoptosis was evaluated by cytology, as previously published (Girard *et al.*, <u>1997</u>; Goncalves *et al.*, <u>2010</u>). PMNs ( $10 \times 10^6$  cells ml<sup>-1</sup>) were incubated at 37 °C in 5% CO<sub>2</sub> in 96-well plates for 24 h in the presence of buffer (control), anti-apoptotic cytokine GM-CSF ( $65 \text{ ng ml}^{-1}$ ), pro-apoptotic plant lectin VAA-I (1 µg ml<sup>-1</sup>) or the indicated concentrations of AgNP<sub>20</sub>. Cells were cytocentrifuged on microscope slides and colored with the Hema 3 staining kit. Apoptosis evaluation was based on the nucleus morphology. The results were expressed as the percentage of PMNs in apoptosis. In some experiments, Annexin-V binding was used to confirm the ability of AgNP<sub>20</sub> to induce neutrophil apoptosis. After 24 h of incubation, the cells were suspended at concentrations of  $10 \times 10^6$  cells ml<sup>-1</sup>, washed and then resuspended at  $1 \times 10^6$  cells ml<sup>-1</sup> and incubated with 2 µl of fluorescein isothiocyanate-annexin-V for 15 min at room temperature (light protected) before fluorescence-activated cell sorting analysis.

#### Metabolic Labeling and *de novo* Protein Synthesis Assay

Cells ( $10 \times 10^6$  cells ml<sup>-1</sup> in RPMI-1640 medium supplemented with 10% autologous serum) were metabolically labeled with 4.625 MBq of the Redivue Pro-Mix L-[<sup>35</sup>S] *in vitro* cell labeling mix (GE Healthcare, Baie d'Urfé, QC, Canada) in the presence or absence of 10 or 100 µg ml<sup>-1</sup> AgNP<sub>20</sub>, 5 µM arsenic trioxide (ATO) or 10 µg ml<sup>-1</sup> cycloheximide (CHX) for 24 h as previously described (Binet et al., <u>2006</u>).

#### **Statistical Analysis**

The data are reported as means  $\pm$  SEM and were analyzed by one-way ANOVA, and differences between tested groups and control were assessed using the Dunnett's multiple comparison test or analyzed by Student's *t*-test (for Fig. <u>3</u>B only), with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was established at *P* < 0.05, and each experiment was performed and validated at least three times.

#### Results

## Nanoparticle Characterization and Lack of Necrotic Activity in Human Polymorphonuclear Neutrophil Cells

The results of AgNP<sub>20</sub> characterization are summarized in Table <u>1</u>. According to the manufacturer's data sheet, the starting diameter of the AgNPs was 20 nm. We confirmed this using DLS, where a bimodal size distribution was observed (17.1 nm, 92%; 2.2 nm, 8%) for a concentration of 10  $\mu$ g ml<sup>-1</sup>, and by TEM, where each NP was close to 20 nm (Fig. <u>1</u>A). However, at a concentration of 100  $\mu$ g ml<sup>-1</sup>, a tri-modal size distribution was observed, as was the presence of large aggregates (505 nm, 59.5%; 1135 nm, 37.3%; and 1576 nm, 3.2%). We next monitored cell viability by trypan blue exclusion assay by testing different concentrations of 0, 5, 10, 20, 50 or 100  $\mu$ g ml<sup>-1</sup> AgNP<sub>20</sub> for up to 24 h of incubation. For all conditions tested, cell necrosis never exceeded 2% (data not shown). We next determined cell necrosis by evaluating LDH release. Previous experiments revealed that AgNP<sub>20</sub> did not interfere with the assay (data not shown). As illustrated in Fig. <u>1</u>(B), after 24 h, 10 or 100  $\mu$ g ml<sup>-1</sup> AgNP<sub>20</sub> did not significantly increase LDH release from neutrophils vs. untreated cells, despite the fact that the positive technical control of the assay led to a marked release of LDH, as expected.

Characterization		Starting material	$AgNP_{20} (10 \ \mu g \ ml^{-1})^{b}$	$AgNP_{20} (100 \ \mu g \ ml^{-1})$
	1. TEM, transmissi	on electronic microscopy.		
2.	а			
Data are representative of three different lectures.				
3.	b			
NPs were suspended in RPMI-1640 + 10% human serum as detailed in Materials and methods.				
4.	С			
Number in parentheses are the proportion of NPs with the indicated sized measured by DLS.				
5.	d			
Results are means $\pm$ SD ( $n = 3$ ).				
				505 (59.5%)
Size (nm	h)	19.9 (TEM) as reported by the manufacturer	17.1 (92%)° 2.2 (8%)	1135 (37.3%)
				1576 (3.2%)

Zeta potential (mV)d

N/A

 $-9.4\pm1.4$ 

 $-9.7\pm0.4$ 

#### Table 1. Characterization of the silver nanoparticle used in this study<sup>a</sup>



**Figure 1.** Transmission electronic microscopy image of the primary solution stock of AgNP<sub>20</sub> and evaluation of cell necrosis in activated human polymorphonuclear neutrophil cells. (A) Sample of the solution stock from the manufacturer was used for characterization by transmission electronic microscopy to confirm that the primary diameter of the NP was close to 20 nm. This was also confirmed by dynamic light scattering (Table <u>1</u>). (B) Freshly isolated human polymorphonuclear neutrophil cells were incubated for 24 h in the presence of buffer (controls), 65 ng ml<sup>-1</sup> GM colony-stimulating factor, 10 and 100 µg ml<sup>-1</sup> AgNP<sub>20</sub> and cell necrosis was evaluated by measuring the level of LDH released in the supernatants. Results are means ± SEM (n = 3). Both low and high controls were used according to the manufacturer's recommendations. AgNP<sub>20</sub>, silver nanoparticles of 20 nm; GM, granulocyte–macrophage; LDH, lactic dehydrogenase.

#### Silver Nanoparticles of 20 nm Increased the Neutrophil Cell Size

Next, as a marker of PMN activation, cell shape changes were monitored by cytology after AgNP<sub>20</sub> treatment, as done routinely in our laboratory (Girard et al., 1996; Goncalves et al., 2010). As illustrated in Fig. 2, after 24 h, AgNP<sub>20</sub> increased the cell size of PMNs in vitro(Fig. 2B, black arrows) when compared to control cells (Fig. 2A). However, the cell shape remained relatively round or spherical in all experimental conditions, in contrast to classical neutrophil activators such as cytokines, including GM-CSF or interleukin-15 (Girard et al., 1996), where the round shape was replaced by an irregular shape. Of note, cells that appeared less well-conserved in controls or in response to AgNP<sub>20</sub> probably corresponded to apoptotic PMNs known to spontaneously undergo apoptosis in ~ 30-50% of cells (spontaneous apoptosis) after 24 h of incubation. Results were confirmed by flow cytometry, where AgNP<sub>20</sub> increased not only the cell size (forward scattered light H), but also the inner complexity (side scattered light H) (Fig. 2C). Next, confocal microscopy, using Hoescht (NucBlue® Live ReadyProbes™ Reagent, Eugene, OR), was performed to confirm the effect of AgNP<sub>20</sub> on PMN cell size. As illustrated in Fig. 3, after 24 h without treatment (spontaneous apoptosis), two populations of cells (about 50% each) brightly or diffusely blue stained, corresponding to normal and apoptotic cells, respectively, were observed. In Fig. 3, c and f are shown at higher magnification (c' and f') to better illustrate that untreated PMNs display approximately the same cell size (panel c') whether they are brightly or diffusely stained with the blue dye. In contrast, only one population of PMNs (100% of cells) were observed after AqNP<sub>20</sub> treatment; these cells exhibited a larger surface area, confirming the previous data (Fig. 2), and are weakly stained with the blue dye that is diffuse throughout the cells (f'). During these experiments, cell size (surface area) was measured and the data were plotted as a bar graph (Fig. <u>3</u>B). AgNP<sub>20</sub> increased the surface area by a factor of ~ 2.3, as the value increased from  $68.8 \pm 1.4 \mu m^2$  (mean  $\pm$  SEM, n = 4 donors) to  $160.8 \pm 5.4 \mu m^2$ .



**Figure 2.** AgNP<sub>20</sub> increase the cell size and inner complexity of human polymorphonuclear neutrophils. Cells were treated with buffer (Ctrl), or the indicated concentrations of AgNP<sub>20</sub> and cell morphology (A,B) was observed for up to 24 h by optical microscopy. Cell size and inner complexity were determined by flow cytometry (C) as detailed in the experimental section. Note that the vast majority of cells treated with AgNP<sub>20</sub> (B, black arrows) possess a larger size than control cells (A). For simplicity, only the results obtained with 100 µg ml<sup>-1</sup>, after 24 h, are illustrated. Results are representative of five different experiments. (C) Results are means  $\pm$  SEM (*n*=3). \**P*<0.05 vs Ctrl. AgNP<sub>20</sub>, silver nanoparticles of 20 nm; Ctrl, controls; FSC-H, forward scattered light; SSC-H, side scattered light.



**Figure 3.** Cell size measurements of AgNP<sub>20</sub>-activated human polymorphonuclear neutrophils by confocal microscopy. Polymorphonuclear neutrophils were treated with buffer (Ctrl) or 100  $\mu$ g ml<sup>-1</sup> AgNP<sub>20</sub>, stained with Hoescht and observed by confocal microscopy (A). Cell size was measured as detailed in the experimental section (B). Representative images of immunofluorescence staining of the DNA/nucleus (A, panels a,d) and corresponding DIC images (A, panels b,e) as well as the merge images (A, panels c,f). The panels c and f were enlarged (c' and f') to show that the dye is diffusely distributed throughout the cytosol in response to AgNP<sub>20</sub> (f'), but not for control cells (c'), and to show the increased cell size, easily observable. (B) Results are means ± SEM (*n*=4). \*, *P* < 0.05 vs Ctrl. AgNP<sub>20</sub>, silver nanoparticles of 20 nm; Ctrl, controls; DIC, differential interference contrast.

## Silver Nanoparticles of 20 nm are Ingested by Human Polymorphonuclear Neutrophil Cells

The above results indicated that  $AgNP_{20}$  increased PMN cell size, suggesting that they can penetrate the cells. We next performed TEM experiments and, as indicated in Fig. <u>4</u>,  $AgNP_{20}$  rapidly interacted with the plasma membrane.  $AgNP_{20}$  penetrated inside neutrophils into vacuoles (even within 1 min in some cases), were localized in vacuole-like structures, and were randomly distributed in the cytosol after 24 h (Fig. <u>5</u>).



**Figure 4.** Silver nanoparticles of 20 nm (AgNP<sub>20</sub>) can rapidly interact with human polymorphonuclear neutrophil cell membrane and are internalized in vacuole-like structures. Freshly isolated human polymorphonuclear neutrophils were incubated with 100 µg ml<sup>-1</sup> AgNP<sub>20</sub> for 1 min and prepared for transmission electron microscopy analysis. AgNP<sub>20</sub> are localized close to the cell membrane (arrows in A) and some are readily observed in vacuole-like structures (square in A, enlarged in A') or ingested by the cells and observed in cell membrane protrusion (square in B, enlarged in B'); note that some AgNP<sub>20</sub> are clearly observed in cell membrane protrusion (arrows in B') and that some other are not in contact with the membrane (circle). n=nucleus.



**Figure 5.** Silver nanoparticles of 20 nm (AgNP<sub>20</sub>) are randomly distributed throughout the cytosol in human polymorphonuclear neutrophils over time. Freshly isolated human polymorphonuclear neutrophils were incubated with 100  $\mu$ g ml<sup>-1</sup> AgNP<sub>20</sub> for 24 h and prepared for transmission electron microscopy analysis. Note the presence of AgNP<sub>20</sub> not only in the vacuole-like structure, but also freely distributed throughout the cytoplasm (arrows).

#### Silver Nanoparticles of 20 nm Induce Neutrophil Apoptosis

Because of the importance of regulating PMN apoptosis in inflammation (Fox *et al.*, 2010; Savill and Haslett, <u>1995</u>), and as apoptotic PMNs excluded trypan blue, we next determined whether AgNP<sub>20</sub> could regulate neutrophil apoptosis. As illustrated in Fig. <u>6</u>(A), the basal apoptotic rate of  $45.8 \pm 2.9\%$  (mean ± SEM, *n* = 4) increased to  $81.8 \pm 4.1\%$  and decreased to  $18.0 \pm 5.28\%$  when cells were incubated with either 100 µg ml<sup>-1</sup> AgNP<sub>20</sub> or the anti-apoptotic cytokine GM-CSF, respectively, as evidenced by the presence of an increased number of cells harboring pycnoctic nuclei (black arrows) versus typical polylobed nuclei (white arrows) observed in viable PMNs. As expected, almost all PMNs (97.5 ± 0.5%) were apoptotic when incubated with VAA-I, a positive proapoptotic control (Lavastre *et al.*, 2002). Of note, concentrations of 5, 10, 20 or 50 µg ml<sup>-1</sup> did not significantly alter the basal apoptotic rate (Fig. <u>6</u>B). The proapoptotic activity of 100 µg ml<sup>-1</sup>AgNP<sub>20</sub> was confirmed by determining the percentage of fluorescein isothiocyanate-annexin-V-positive cells by flow cytometry (Fig. <u>6</u>C).



**Figure 6.** AgNP<sub>20</sub> induce apoptosis in human PMNs. Cells were treated with buffer, 65 ng ml<sup>-1</sup> of the antiapoptotic cytokine GM colony-stimulating factor,  $1 \mu g m l^{-1}$  of the proapoptotic plant lectin VA or the

indicated concentration of AgNP<sub>20</sub> and apoptosis was evaluated by cytology as detailed in the experimental section. (A) The ability of AgNP<sub>20</sub> to alter the apoptotic rate in PMNs is compared to VA. Results are means  $\pm$  SEM (*n*=4). Inset, representative images illustrating normal viable (white arrows) and apoptotic (black arrows) PMNs. (B) Dose–response effect of the proapoptotic activity of AgNP<sub>20</sub>. \**P*<0.05 vs. Ctrl. (C) In two separate experiments, the proapoptotic activity of AgNP<sub>20</sub> was confirmed by flow cytometry by determining the number of annexin-V-positive cells. AgNP<sub>20</sub>, silver nanoparticles of 20 nm; Ctrl, controls; GM, granulocyte–macrophage; PMNs, polymorphonuclear neutrophils; VA, *Viscum album* agglutinin-I.

# Silver Nanoparticles of 20 nm Particles are Potent Inhibitors of *de novo* Protein Synthesis

Knowing that several agents that modulate apoptosis in PMNs also alter *de novo* protein synthesis (Girard *et al.*, <u>1997</u>; Savoie *et al.*, <u>2000</u>; Stringer *et al.*, <u>1996</u>), we investigated here whether or not AgNP<sub>20</sub> could alter *de novo* protein synthesis, as this has never been investigated in response to NPs, to the best of our knowledge. As illustrated in Fig. <u>7</u>, AgNP<sub>20</sub> acted as potent inhibitors of *de novo* protein synthesis when PMNs were treated with 100 µg ml<sup>-1</sup> (lane 5, panel A), similar to CHX (lane 1, panel A), a potent inhibitor of protein synthesis. Although no new polypeptides were synthesized in response to 100 µg ml<sup>-1</sup> AgNP<sub>20</sub> (and CHX), the corresponding Coomassie blue-stained gel (Fig. <u>7</u>B) clearly illustrated equivalent loading of proteins (see lanes 5 and 1, panel B vs. lanes 5 and 1, in panel A), except for a particular protein (?, in the bottom panel). In these experiments, ATO was used as a positive control (lane 3, panels A and B) known to induce *de novo* protein synthesis in human PMNs (Binet *et al.*, <u>2006</u>).



**Figure 7.** AgNP<sub>20</sub> inhibit de novo protein synthesis in human PMNs. PMNs were metabolically labelled with the Redivue Pro-Mix L-[35S] in vitro cell labelling mix and incubated in the presence of cycloheximide (CHX, lane 1); buffer (Ctrl, lanes 2 and 6); 5  $\mu$ M arsenic trioxide (ATO, lane 3); 10 or 100  $\mu$ g/ml AgNP<sub>20</sub> (lane 4 and 5, respectively) for 24 h and cell lysates were prepared for one-dimensional sodium dodecyl sulphate polyacrylamide gel as described in the experimental section. Upper panel is the autoradiogram and the bottom panel is the corresponding Commassie blue stained gel illustrating an equivalent charge of proteins loaded. Note however that the level of expression of one polypeptide is decreased in response to 100  $\mu$ g/ml AgNP<sub>20</sub> (see bottom panel).

#### Discussion

The present study is the first to investigate the direct effect of AgNPs in primary human neutrophils. AgNP<sub>20</sub> was tested at different concentrations and characterized by DLS under conditions identical to the experimental conditions used for investigating PMN cell physiology. This led to the observation that at a concentration of 100 µg ml<sup>-1</sup>, AgNP<sub>20</sub> induced large aggregate formation and altered human PMN cell physiology. However, at a concentration of 10 µg ml<sup>-1</sup> AgNP<sub>20</sub>, devoid of large aggregates, as assessed by DLS, these effects were not observed. Our results that 100 µg ml<sup>-1</sup> AgNP<sub>20</sub> increased the cell size of PMNs concur with other studies that have reported that carbon black nanoparticles increased the cell size of macrophages, which, like neutrophils, are cells of myeloid origin (Reisetter et al., 2011). We confirmed this effect of AgNP<sub>20</sub> on cell size using three distinct procedures: microscopic observation, flow cytometry, and confocal microscopy using Hoescht staining. AgNP20 were rapidly internalized in human PMNs, as evidenced by TEM analysis, in accordance with that observed in cell size. AgNP<sub>20</sub> were localized in vacuolelike (or membrane-bound vesicles), as well as in cytosol. These results are in agreement with those previously reported for carbon black NPs that were internalized by macrophages (Reisetter et al., 2011). In another study conducted with murine macrophages (Yen et al., 2009), AgNPs, as well as AuNPs, were observed inside the cells, as assessed by TEM, in agreement with our present data with human PMNs. Although the authors mentioned that NPs observed inside vesicles tend to make clusters, we did not observe any such a phenomenon in the vesicles. Using 45 and 13 nm AuNPs at concentrations of 20 µg ml-<sup>1</sup> (AuNP<sub>45</sub>) and 142  $\mu$ g ml<sup>-1</sup> (AuNP<sub>13</sub>), Mironava *et al.* (2010) reported that, for both particle sizes, the NPs were sequestered inside large vacuoles and did not penetrate the nucleus or mitochondria in primary human dermal fibroblasts (Mironava et al., 2010). These findings are similar to our results, as we did not observe a distribution of AgNP<sub>20</sub> in the nucleus but, as human PMNs possess very few mitochondria (Geering and Simon, 2011), it remains difficult to analyze possible association/penetration of NPs with/in mitochondria. Hence, we have no evidence that AgNP<sub>20</sub> interacts with mitochondria in human PMNs; this remains to be determined and represents certain challenges. Although research regarding the interactions of AgNPs with cells is limited, AgNPs are known to enter cells of some macrophage cell lines where Ag\* metal can be degraded intracellularly, leading to the release of Ag<sup>+</sup> ions, which then alter mitochondrial activity and cause cell death (Pratsinis et al., 2013; Singh and Ramarao, 2012). Whether or not this occurs in human PMNs is unknown and remains to be determined. Of note, the concentrations of AgNPs used in the present study were in the range used by others in in vitro studies (Arora et al., 2009; Rosas-Hernandez et al., 2009). We are aware that such concentrations might be high, but, as yet no study has clearly indicated what the concentrations of AgNPs are that humans could be exposed daily. Such information is further complicated by the fact that AgNPs are included in several different compounds and at different NP sizes. In a health surveillance case study on two workers who manufacture silver nanomaterials, the blood levels of silver were ~ 0.02–0.03 µg dl<sup>-1</sup> with no significant findings on their health status (Lee et al., 2012). Although these concentrations are low when compared to those used in *in vitro* studies, this indicates that silver could at least reach the blood circulation. Further studies, however, are needed for drawing any conclusion.

To the best of our knowledge, our previous study reporting the anti-apoptotic property of TiO<sub>2</sub> NPs is one of the only ones dealing with NPs and human PMN apoptosis (Goncalves et al., 2010). Here, in contrast to TiO<sub>2</sub>, AgNP<sub>20</sub> induced apoptosis in human PMNs. This indicates that not all NPs act in a similar fashion, and that there is a potential need to identify the pro- or anti-apoptotic activity of NPs. In general, agents known to delay human PMN apoptosis are known to induce de novo protein synthesis, while pro-apoptotic molecules inhibit de novo protein synthesis (Bouchard et al., 2004; Cox and Austin, 1997; Girard et al., 1997; Savoie et al., 2000). However, the anticancer drug ATO induced apoptosis in human PMNs and induced de novo protein synthesis, the only agent thus far shown to act like this in these cells (Binet et al., 2006). This can be explained by the fact that ATO induced the production of heat-shock proteins involved in the unfolded protein responses and endoplasmic reticulum stress-mediated apoptotic pathway of cell apoptosis (Binet et al., 2010). How NPs, acting as pro-apoptotic agents, would alter de novo protein synthesis in human PMNs has never been investigated until now. The fact that AgNP20 aggregates inhibit de novo protein synthesis is important to better understand the mode of action of AgNPs. Interestingly, when used at 10 µg ml<sup>-1</sup>, AgNP<sub>20</sub> did not alter *de novo* protein synthesis, nor were other effects reported in this study observed, suggesting an association with the observed effects and the formation of large aggregates. Although the field of study of interactions between NPs and human neutrophils is in its infancy, the results of the present study indicate that, at 100 µg ml<sup>-1</sup>, AgNP<sub>20</sub> alters human PMN physiology resulting in increased cell size and inner complexity, induction of apoptosis and inhibition of de novo protein synthesis. We are aware that the effects could be somewhat related to the potential release of Ag<sup>+</sup> ions, but the present study was aimed at investigating commercially available AgNPs. All of these effects were not observed at 10 µg ml<sup>-1</sup>. It is tempting to conclude that the only variable explaining this discrepancy is related to the appearance of large aggregates at a concentration of 100 µg ml<sup>-1</sup>. However, this is presently only speculative, as in addition to the different size distribution observed, we have to consider that other criteria could be involved, including the total surface area of the NPs and the probable liberation of Ag+ into the cytosol. This remains to be determined. Nevertheless, our observations suggest that the characterization of NPs should be performed using conditions identical to those used for evaluating a given biological effect. For example, serum is frequently added to the culture medium; given the existence of many proteins in human serum (~ 500 proteins were detected by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry; Adkins et al., 2002), NPs may be rapidly covered/enveloped by proteins. This is even more significant in *in vivo* studies, especially when the biophysical properties of NPs can be modified by salt concentrations, pH, plasma proteins, etc., all of which can interfere with NP interactions with cells (Baumann et al., 2012). There are presently numerous studies in which characterization of NPs using TEM and/or DLS is performed in pure water or other diluents, but the NPs are then used in experimental conditions completely different from those used for characterization. The results of the present study attests to the importance of characterizing NPs under the same conditions (or as closely as possible) as the experimental conditions, particularly when the aim of a study is to compare, for example, the effects of a

given NP at different diameters (starting size), as the biological activity observed may be caused by the presence of aggregates rather than simply the starting NP size

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# **Conflict of Interest**

The Authors did not report any conflict of interest.