Visualization of gold and platinum nanoparticles interacting with *Salmonella* Enteritidis and *Listeria monocytogenes*

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Purpose: Rapid development of nanotechnology has recently brought significant attention to the extraordinary biological features of nanomaterials. The objective of the present investigation was to evaluate morphological characteristics of the assemblies of gold and platinum nanoparticles (nano-Au and nano-Pt respectively), with *Salmonella* Enteritidis (Gram-negative) and *Listeria monocytogenes* (Gram-positive), to reveal possibilities of constructing bacteria-nanoparticle vehicles.

Methods: Hydrocolloids of nano-Au or nano-Pt were added to two bacteria suspensions in the following order: nano-Au + *Salmonella* Enteritidis; nano-Au + *Listeria monocytogenes*; nano-Pt + *Salmonella* Enteritidis; nano-Pt + *Listeria monocytogenes*. Samples were inspected by transmission electron microscope.

Results: Visualization of morphological interaction between nano-Au and *Salmonella* Enteritidis and *Listeria monocytogenes*, showed that nano-Au were aggregated within flagella or biofilm network and did not penetrate the bacterial cell. The analysis of morphological effects of interaction of nano-Pt with bacteria revealed that nano-Pt entered cells of *Listeria monocytogenes* and were removed from the cells. In the case of *Salmonella* Enteritidis, nano-Pt were seen inside bacteria cells, probably bound to DNA and partly left bacterial cells. After washing and centrifugation, some of the nano-Pt-DNA complexes were observed within *Salmonella* Enteritidis.

Conclusion: The results indicate that the bacteria could be used as a vehicle to deliver nano-Pt to specific points in the body.

Keywords: morphology, nanoparticles, gold, platinum, bacteria

Introduction

There is an urgent need for new, effective drug delivery systems. In the last few decades, nanotechnology has been increasingly employed in searching for methods to transport drugs to certain organs within the human body. Transporting systems able to transmit cell-insoluble drugs into cells; non-toxic for the surroundings; and with time-prolonged releasing of active substances, have remarkable potential for improving cancer therapy efficacy.¹

Several bacteria isolates may be applied for possible new drug delivery mechanisms. Their unique features including: potential to move; intracellular survival; and ability to settle in precisely programmed points of the body, allow the use of bacteria as specific medicine carriers.² However, the following has still to be elucidated: how can bacteria be enforced to ingest medicines; how can uncontrolled proliferation and distribution of bacteria be avoided within a host organism; and can nanoparticles be used as drugs, or links between drugs and bacteria?

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Nanoparticles derived from noble metals are recognized as being non-genotoxic for the organism.³ Moreover, gold and silver nanoparticles did not reveal proinflammatory activity⁴ and even antiangiogenic properties of Au nanoparticles (nano-Au) were observed.⁵ Nano-Au have been identified as promising candidates for many biological and biomedical applications.⁶ They have been widely used as an inert agent for cell imaging, targeted drug delivery, cancer diagnostics, and various therapeutic applications, due to their small sizes, convenience in bioconjugation, and strong absorbing and scattering properties.^{7–10} They entrap small drugs, or biopharmaceutical agents such as therapeutic proteins and DNA, and can be designed to release these agents at the target site.

Platinum nanoparticles (nano-Pt) are of great interest due to their exceptional catalytic activity, which is affected by the ligands at their external surface. Nano-Pt may display multiple functional groups at the surface, which can be hydrophilic, lipophilic, and chemically reactive.¹¹ Some of the Pt compounds are used as very effective anticancer agents. This property is associated with the inhibition of DNA replication and mitosis by the addition of nano-Pt to DNA strand.¹² On the other side, research of Hikosaka et al¹³ has revealed that nano-Pt have a nicotinamide adenine dinucleotide (NADH): ubiquinone oxidoreductase-like activity. This finding suggests that nano-Pt can be used as a remedy for oxidative stress diseases with suppressed mitochondrial complex I.¹³ Affinity of nano-Pt to DNA strand can also support their activity as carriers locating medicine within bacteria.

Salmonella Enteritidis is a motile, gram-negative (G-), rod-shaped bacterium belonging to the Enterobacteriaceae family. It is adapted to a wide range of hosts. Through flagella, this bacterium can spread from the intestine to the blood and lymph. It proliferates within eukaryotic cells. Recent work has shown that *Salmonella* Enteritidis reside within macrophages.¹⁴

Listeria monocytogenes is a gram-positive (G+), food-borne pathogenic bacterium, which possesses the ability to be internalized by macrophages and non-phagocytic cells, including epithelial cells, endothelial cells, and hepatocytes. It enters the cell using advanced mechanisms, proliferates intracellularly and spreads without contact with the extracellular medium, using elongated protrusions.¹⁵

Methods to deliver functional nano-molecules to specific targets within the body still remain to be investigated. In addition, the impact of noble metals' nanoparticles on bacteria's cells is still unclear. Considering physical and chemical properties of gold (Au) and platinum (Pt) nanoparticles and biological properties of *Salmonella* Enteritidis and *Listeria monocytogenes*,

it is important to evaluate results of their self-organization. Consequently, the objective of the present investigation was to evaluate morphological characteristics of the assemblies of nano-Au and nano-Pt with G+ and G- bacteria, to reveal possibilities of constructing bacteria-nanoparticle vehicles.

Methods

Nanoparticles

Hydrocolloids of nano-Au and nano-Pt were obtained from Nano-Tech Polska (Warsaw, Poland). They were produced by a patented electric non-explosive method (Polish patent 380649) from high purity metals (99.9999%) and high purity demineralized water. Both hydrocolloids had a concentration of 50 ppm. Shape and size of nanoparticles were inspected using a JEM-1220 transmission electron microscope (TEM) at 80 KeV (JEOL, Tokyo, Japan), with a Morada 11 megapixel camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany) (Figure 1). Diameter of particles ranged from 2–29 nm for Au and from 2–19 nm for Pt. Samples of Au and Pt for TEM were prepared by placing droplets of hydrocolloids on to formvar-coated copper grids (Agar Scientific Ltd., Stansted, UK). Immediately after drying of the droplets in dry air, grids were inserted into the TEM.

Bacteria

Bacteria strains (*Salmonella* enterica subspecies enterica serovar Enteritidis ATCC® 13076™ and *Listeria monocytogenes* ATCC 19111) were obtained from LGC Standards (Lomianki, Poland). *Salmonella* Enteritidis were grown on nutritive 2.8% agar (Bio-Rad, Hercules, CA, USA). *Listeria monocytogenes* were grown on brain heart infusion agar (Bio-Rad, Hercules, CA, USA) for 24 hours at 37°C. Bacteria were gently washed out from the agar using sterile distilled water. To remove liquid remains of the medium, bacteria were centrifuged at 4000 rpm for 5 min on an Eppendorf MiniSpin® centrifuge (Eppendorf, Hamburg, Germany) and the sediment (pellet) was re-suspended in Milli-Q water (Millipore, Billerica, MA, USA). To calculate the exact number of bacteria in the suspension, the optical density at

Figure 1 TEM image of nanoparticles manufactured by electric non-explosive method: a) gold b) platinum.

600 nm (OD_{600}) was estimated using a spectrophotometer Helios Epsilon unicam (USA) and serial dilutions of the suspension were prepared. One milliliter of each dilution was transferred to petri dishes with agar. After 24 hours incubation at 37°C, the number of colonies was estimated. Based on the results of measurements carried out in triplicate, the density of bacteria suspension $OD_{600} = 0.130$ corresponded to 2.2×10^8 cfu.mL⁻¹; and $OD_{600} = 0.133$ corresponded to 4×10^8 cfu.mL⁻¹, for *Salmonella* Enteritidis and *Listeria monocytogenes* respectively.

Preparation of samples and microscopy

Experiment 1

Hydrocolloids of nano-Au or nano-Pt (200 µL) were added to two bacteria suspensions (200 µL) in the following order: nano-Au + *Salmonella* Enteritidis; nano-Au + *Listeria monocytogenes*; nano-Pt + *Salmonella* Enteritidis; nano-Pt + *Listeria monocytogenes*. Control samples of bacteria were treated with Milli-Q water. The samples were gently mixed for 15 min and then droplets of the samples were put on formvar-coated 300 mesh copper grids (Agar Scientific Ltd., Stansted, UK). The samples were dried at room temperature, under sterile conditions, and inspected using a JEM-2000EX TEM (JEOL, Tokyo, Japan).

Experiment 2

Hydrocolloids of nano-Pt (200 µL) were added to *Salmonella* Enteritidis suspension (200 µL). To remove unattached nanoparticles, the samples were gently mixed for 15 min and then centrifuged at 4000 rpm for 5 min, washed with ultrapure Milli-Q water, centrifuged at 4000 rpm for 5 min, and re-suspended in Milli-Q water. Samples for microscopic evaluation were prepared using the methods described above. Control samples of bacteria not treated with nanoparticles were prepared using the same methods.

Experiment 3

In the third experiment, we wanted to confirm that the substance realized from *Salmonella* Enteritidis, after nano-Pt treatment, was DNA. Furthermore, the viability of bacteria after incubation with nano-Pt was determined. Bacteria suspended in phosphate buffering solution (PBS) were divided into the control sample, to which 0.5 mL of Milli-Q water was added, and four samples, to which 0.5 mL of colloidal nano-Pt was added. Samples were incubated for 45, 60, and 90 min and then centrifuged at 5000 rpm for 5 min. The supernatant was discarded and the remaining pellet containing bacteria was suspended in 0.5 mL solution with the

fluorescent marker DAPI (4',6-diamidino-2-phenylindol) and PBS in the proportion 1 µL DAPI to 1 mL PBS. Bacteria with the fluorescent dye were incubated for 15 min. After this time, to remove overdose of the dye, samples were centrifuged (5000 rpm for 5 min) and the obtained pellet was suspended in 200 µL PBS. The control was a suspension of bacteria in 200 µL of PBS, subjected to all stages of the experiment, except the incubation with nano-Pt. The resulting suspension was thoroughly stirred and one drop was placed on a microscope slide and immediately covered with a coverslip. The resulting preparations were observed at 300x magnification with an Olympus FV1000 confocal microscope, with FV10-ASW software version 1.4 (Olympus Corporation, Tokyo, Japan). The preparations were observed at the excitation wave length of 405 nm and in the Nomarski contrast. The viability of *Salmonella* Enteritidis was also measured. Samples of *Salmonella* Enteritidis, prepared as in experiment 1, were incubated with hydrocolloids of nano-Pt (1:1) and with Milli-Q water as the control, for 45, 60, and 90 min. Samples of *Salmonella* Enteritidis were then spread and grown on nutritive 2.8% agar (Bio-Rad, Hercules, CA, USA), for 24 hours, at 37°C and bacterial colonies were counted.

Results

Experiment 1

Examination of the control bacteria indicated some changes caused by the used methods (centrifugation, Milli-Q water, drying), but did not show any toxicological or destructive alteration of bacteria. Morphology of interaction between nano-Au and *Salmonella* Enteritidis is presented in Figure 2. Almost all of nano-Au were aggregated and removed from bacterial cells. The aggregates were trapped within flagella network and biofilm. Moreover, some bacteria cells were

Figure 2 TEM image of *Salmonella* Enteritidis and gold (Au) nanoparticles: a) control; b, c, and d) *Salmonella* Enteritidis with Au nanoparticles. Arrows point to nano-Au.

destroyed and their cell walls were disrupted. The cells were malformed, had shrunk, and a loss of cytoplasm was observed. Interestingly, nano-Au was trapped within the flagella-biofilm network and kept at some distance from bacteria.

The results of the morphological interaction of gold nano-particles with *Listeria monocytogenes* also showed that nano-Au were aggregated and non-specifically placed on the cell wall (Figure 3). The cell wall was disintegrated and the cytoplasmic membrane was disrupted. Thereafter, nano-Au were entirely surrounded by biofilm and removed from bacteria.

The visualization of the interaction between nano-Pt and *Salmonella* Enteritidis is shown in Figure 4. The picture shows that nano-Pt disintegrated bacteria cell walls and cytoplasmic membranes; moreover, they entered bacteria and were seen as intensively black spots within the cells. Nano-Pt were located specifically, probably attached to some intracellular compounds (DNA), but under microscope electron beam, these black spots evaporated becoming white (Figure 4c).

The image of interaction between nano-Pt and *Listeria monocytogenes*, shown in Figure 5, indicated that nano-Pt are destructive to these bacteria. The influence of nano-Pt on *Listeria monocytogenes* clearly pointed out that nano-Pt were seen within bacteria, moreover, they were located close to DNA and probably bound to it. Interestingly, these new molecules (nano-Pt with DNA) were removed or escaped from the bacteria cells. The cell wall and cytoplasmic membrane were disintegrated and cytoplasm was lost. As in the case of *Salmonella* Enteritidis, nano-Pt evaporated under the electron beam.

Experiment 2

This experiment was performed to elucidate to what extent nanoparticles, identified from morphological pictures in experiment one, as attached or being inside bacteria, were

Figure 4 TEM image of *Salmonella* Enteritidis and platinum (Pt) nanoparticles: **a**) control; **b**, **c**, and **d**) the same cell of *Salmonella* Enteritidis with Pt nanoparticles. Arrows point to nano-Pt (**b**); and the spots observed with increasing magnification (**b**, **c**, and **d**). Black spots are seen evaporating under the microscope electron beam and are finally seen as white spots.

truly attached or were stably anchored bacteria cells. To remove unattached particles, mixtures with bacteria were centrifuged and washed. For further examination, *Salmonella* Enteritidis was chosen as a better candidate for bacteria-nanoparticle molecules construction. It was revealed that platinum complexes seen as intensively black spots were still within the bacteria (Figure 6).

Experiment 3

This experiment was performed to confirm that nano-Pt is actually removing DNA from *Salmonella* Enteritidis, and whether this process is time-dependent. In this experiment, the average number of bacteria observed by the confocal microscope in the Nomarski contrast and the average number of bacteria labelled with DAPI (DNA-binding fluorochrome) were determined. The average number of bacteria cells (Table 1) seen as a cell wall image observed with the Nomar-

Figure 3 TEM image of *Listeria monocytogenes* and gold (Au) nanoparticles: **a**) control; **b**, **c**, and **d**) *Listeria monocytogenes* with Au nanoparticles. Arrows point to nano-Au (**b** and **d**); and to agglomerate of nano-Au (**c**).

Figure 5 TEM image of *Listeria monocytogenes* and platinum (Pt) nanoparticles: **a**) control; **b**, **c**, and **d**) *Listeria monocytogenes* with nano-Pt. Arrows point to nano-Pt (**b** and **c**); and to black spot partially evaporated under microscope electron beam (**d**).

Figure 6 TEM image of *Salmonella* Enteritidis and platinum (Pt) nanoparticles, after washing and centrifugation to remove nanoparticles unattached to bacteria: a) control; b, c, and d) *Salmonella* Enteritidis with nano-Pt. Arrows point to nano-Pt complexes.

ski contrast (Figure 7), after incubation for 45, 60, and 90 min with nano-Pt, did not change. However, the number of cells containing DNA (seen as a blue) was reduced after 60 and 90 min. Viability of *Salmonella* Enteritidis was also decreased and after 60 and 90 min of incubation, was about one quarter of that measured in the control condition (Table 1).

Discussion

The purpose of the present study was to identify possibilities of using bacteria as a vehicle for transporting nanoparticles and/or nanoparticle-drug molecules. We focused on possibilities of placing nanoparticles of Au and Pt within bacteria, using a simple mechanism of self-organization, but without destroying cell walls of bacteria. The biological action of nanoparticles on mammalian and bacterial cells is highly dependent on their functional groups present on the surface of nanoparticles, which may determine their toxicity.^{16,17} To avoid influence of the additional chemical groups which surround nanoparticles and to determine chemical properties of nanoparticles, we used nano-Au and nano-Pt produced in high purity water by electric methods (the same for Au and

Pt). Consequently, only hydrogen ion (H^+) or hydroxide ion (OH^-) groups could be present on the nanoparticle surface.

The structure of the cell wall of G^+ *Listeria monocytogenes* and G^- *Salmonella* Enteritidis is quite different. *Listeria monocytogenes* has a thick (20–80 nm), continuous cell wall composed of highly cross-linked layers of peptidoglycan, covalently bound to teichoic acids, while the *Salmonella* Enteritidis cell wall is thin (5–10 nm) but more complex. Peptidoglycan is surrounded by an outer membrane containing lipopolysaccharide and also by some non-specific porins, which can potentially transport smaller nanoparticles (around 1 nm). Interestingly, in spite of significant differences in cell walls structure, the self-organization between nanoparticles and bacteria was affected by the type of bacteria to a lesser degree than by the type of nanoparticles.

In our studies, nano-Au formed aggregates trapped within flagella network, when examined with *Salmonella* Enteritidis (Figure 2). The pictures might indicate that this process is initiated by fimbria network creation, which probably promotes the development of biofilm.¹⁸ Formations of biofilm by *Salmonella* Enteritidis increase colonization, but also protect bacteria cells,¹⁹ which in this case could be a system of protection against nanoparticles. The formation of nano-Au aggregates kept the nanoparticles away from the bacteria cells, which might be more safe for the bacteria but unsuitable for constructing a bacteria-nanoparticle drug delivery system. *Listeria monocytogenes* also produced biofilm, but mainly showed abilities to form colonies, making it impossible to observe single cells as it was in the case of *Salmonella* Enteritidis (Figure 3). Probably, building a multi-cells structure was, for *Listeria monocytogenes*, a form of protection against nano-Au, but make it difficult to use *Listeria monocytogenes* as a drug delivery vehicle.

Nanoparticles of Au were mainly placed outside bacteria (Figure 2 and 3), however, some aggregates, containing small

Table 1 Average number and viability of *Salmonella* Enteritidis incubated with nano-Pt for 45, 60, and 90 min. DNA visualization was performed by labelling bacteria with DAPI (DNA-binding fluorochrome)

	Incubation with nano-Pt (min)				ANOVA	
	Control	45	60	90	SEM	P-value
Number of bacteria	56.3	47.0	46.7	59.4	6.34	NS
Number of bacteria stained with DAPI	35.3 ^a	35.0 ^a	14.7 ^b	22.3 ^b	4.57	0.000
Percentage of bacteria stained with DAPI	62.6 ^a	74.5 ^a	31.9 ^b	37.5 ^b	0.04	0.000
Viability, CFU/mL	28.5×10^5	31.2×10^5	10.3×10^5	7.1×10^5		

Notes: Values in each row with different superscript letters (a or b) differ significantly ($P < 0.05$).

Abbreviations: NS, not significant ($P > 0.05$); SEM, standard error of the mean.

Figure 7 Confocal microscope image of *Salmonella* Enteritidis: 1) control; 2, 3, and 4) after incubation with platinum nanoparticles for 45, 60, and 90 min respectively. a) Nomarski contrast; b) stained with DAPI; and c) merged pictures.

(about 5–10 nm) particles of nano-Au, were non-specifically placed on the surface of both bacteria. Bacterial cell walls and cytoplasm membrane in both bacteria were disintegrated, but nano-Au were not seen inside the cells. Pan et al²⁰ observed that particles of 1–2 nm were highly toxic and that nanoparticles larger than 15 nm were not toxic. Consequently, the possibility that the smallest (about 1 nm) nanoparticles could enter the cells could be considered. However, in the present experiment, it was not possible to observe such small nanoparticles.

In the present experiment, the visualization of the association between nano-Pt and bacteria indicated significant effects of nano-Pt on both *Salmonella* Enteritidis and *Listeria monocytogenes*. Cell wall and cytoplasmic membrane of both bacteria were disintegrated, however, *Listeria monocytogenes*, like in the case of nano-Au, created colonies, probably as a defence against nano-Pt. Interestingly, *Salmonella* Enteritidis did not demonstrate the mechanism of fimbria-biofilm production, which was seen as a remedy against nano-Au. Moreover, we noticed that the cell wall and cytoplasmic membrane of both microorganisms were permeable for nano-Pt, which could be seen as intensively black spots, being black even when microscopic pictures were lightened and not existing in the control bacteria.

Moreover, the present pictures (Figure 4, 5) showed that nano-Pt was bound to specific intracellular compounds; probably DNA. Our results are in agreement with previous studies,²¹ revealing that platinum demonstrates effective affinity to DNA strand. These properties may be utilized for using nano-Pt conjugates to inhibit DNA replication, by binding to its strand and therefore acting as potential anticancer agents. The antioxidant activity of platinum complexes, which can penetrate the nucleus membrane and enter the nucleus, has recently gained attention due to its potential for the treatment of a variety of cancers.²² For example, cisplatin and carboplatin are used as chemotherapeutic agents for ovarian and testicular cancer.²³ It is well known that in cancer therapy, tissue-unspecific drug application is a key problem. The possibility to locate nano-Pt inside bacteria ghost, being a locomotive force, could provide target-specific drug delivery. Previously, bacterial cells as ghosts were used as a very promising drug delivery system in a study with *Escherichia coli*.²

In the case of *Listeria monocytogenes*, we observed that nano-Pt-DNA molecules (as intensively black spots) were seen inside the cells, but the majority was placed outside the cells. It is therefore likely that nano-Pt, after binding with DNA, was removed from *Listeria monocytogenes* (Figure 5). In the case of *Salmonella* Enteritidis, the association of nano-Pt and DNA was also visualized as dark spots (Figure 4), but seen rather within bacteria cells. Interestingly, these spots were disappearing under the microscope electron beam, especially when magnification was increasing. In *Salmonella* Enteritidis, in contrast to *Listeria monocytogenes*, the spots were mostly inside the bacterial cells and only a few were removed from bacteria cells.

Considering the results from the first experiment, only *Salmonella* Enteritidis and nano-Pt were chosen in the second experiment, because only nano-Pt was undoubtedly seen within *Salmonella* Enteritidis, and did not flow out from the cells. In this experiment, *Salmonella* Enteritidis were mixed with nano-Pt and then centrifuged and washed to remove nanoparticles being unbound or unattached to bacteria's structures. As we supposed, dark spots placed within bacteria could be observed (Figure 6). However, only a part of bacterial cells still had the spots while the other bacterial bodies were empty.

In experiment 3, we confirmed our hypothesis that after treatment with nano-Pt, DNA is removed from cells of *Salmonella* Enteritidis. However, after 60 min of incubation, only about half of the cells lost their DNA, moreover, this process did not increase in the following 30 min. Abilities of nano-Pt entering *Salmonella* cells and binding with DNA seem to be very promising for a drug delivery system,

even when only part of bacterial cells contain nano-Pt-DNA molecules. A fundamental problem seems to be determination of optimal time and other conditions of incubation allowing location of nano-Pt within bacteria and binding with DNA. We have also examined viability of *Salmonella* Enteritidis after nano-Pt treatment, showing that after 90 min of incubation, about 25% of bacteria was still able to replicate. It can be suspected that if about half of bacteria after incubation for 60–90 min with nano-Pt still contained DNA, and about a quarter of bacteria was still alive, the other quarter of bacteria contains nano-Pt-DNA molecule, but it is not able to replicate.

Conclusion

Visualization of morphological interaction between nano-Au and *Salmonella* Enteritidis and *Listeria monocytogenes*, showed that nano-Au were aggregated within flagella or biofilm network, but were not specifically attached. In addition, nano-Au visible under the microscope did not penetrate inside the bacterial cell. The analysis of morphological effects of interaction between nano-Pt and bacteria revealed that nano-Pt entered cells of *Listeria monocytogenes* but were removed from the cells. In the case of *Salmonella* Enteritidis, nano-Pt were seen inside bacteria cells, indicating that the bacteria cells could be used as a vehicle to deliver nano-Pt, and active substances attached to Pt, to specific points in the body.

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Disclosure

The authors declare that they have no competing interests in this work.

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