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Comparative study between chemostat and batch reactors to quantify membrane permeability changes on bacteria exposed to silver nanoparticles

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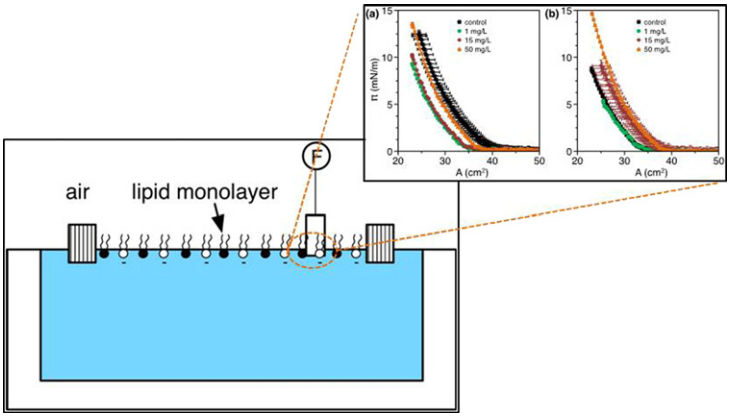
HIGHLIGHTS

- *E. coli* was exposed to AgNPs (D = 44.8 nm) under continuous and batch conditions.
- Lipid monolayer was extracted from *E. coli* membrane to assess chronic AgNPs exposure.
- Bacteria synthesized lipids to stabilize membranes after rupture due to NP exposure.
- In batch systems AgNPs inhibited growth by 20% at 1 mg/L and 0% at 15 mg/L in terms of OD670 reduction.
- In chemostat systems AgNPs from 1 mg/L to 50 mg/L inhibited the growth from 0% to 16%

GRAPHICAL ABSTRACT



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ABSTRACT

Continuous and batch reactors were used to assess the effect of the exposure of casein-coated silver nanoparticles (AgNPs) on *Escherichia coli* (*E. coli*). Additionally, *E. coli* membrane extracts, membrane permeability and Langmuir film balance assays were used to determine integrity and changes in lipid composition in response to AgNPs exposure.

Results showed that batch conditions were not appropriate for the tests due to the production of exopolymeric substances (EPS) during the growth phase. After 5 h of contact between AgNPs and the used growth media containing EPS, the nanoparticles increased in size from 86 nm to 282 nm reducing the stability and thus limiting cell-nanoparticle interactions. AgNPs reduced *E. coli* growth by 20% at 1 mg/L, in terms of Optical Density 670 (OD670), while no effect was detected at 15 mg/L. At 50 mg/L of AgNPs was not possible to perform the test due to aggregation and sedimentation of the nanoparticles. Membrane extract assays showed that at 1 mg/L AgNPs had a greater change in area ($-4.4 cm^2$) on bacteria compared to 15 mg/L ($-4.0 cm^2$). This area increment suggested that membrane disruption caused by AgNPs had a *stabilizing/rigidifying effect* where the cells responded by shifting their lipid composition to more unsaturated lipids to counteract membrane rigidification. In chemostats, the constant inflow of fresh media and aeration resulted in less AgNPs aggregation, thus increased the AgNPs-bacteria interactions, in comparison to batch conditions. AgNPs at 1 mg/L, 15 mg/L, and 50 mg/L

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inhibited the growth (OD670 reduction) by 0%, 11% and 16.3%, respectively. Membrane extracts exposed to 1 mg/L, 15 mg/L, and 50 mg/L of AgNPs required greater changes in area by −0.5 cm^2, 2.7 cm^2 and 3.6 cm^2, respectively, indicating that the bacterial membranes were disrupted and bacteria responded by synthesizing lipids that stabilize or strengthen membranes.
This study showed that the chemostat is more appropriate for the testing of nanotoxicological effects when testing bacteria at growing conditions.

1. Introduction

Silver nanoparticles (AgNPs) are one of the most commonly used nanomaterials in consumer products due to their antimicrobial properties (Kasaraneni et al., 2014; Schifman et al., 2015; Zhang and Oyanedel-Craver, 2012, 2013). While the antimicrobial properties of AgNPs are beneficial for several medical applications (Prabhu and Poulose, 2012; Li et al., 2008), the accidental release of them can negatively affect bacterial populations responsible to important biogeochemical cycles (Marambio-Jones and Hoek, 2010; Panyala et al., 2008). Several studies have demonstrated the antimicrobial properties of AgNPs and the critical role of membrane integrity; however, changes in the composition and properties of bacterial membranes due to AgNPs exposure, is not yet completely understood (Guzmán et al., 2012).

Cell membrane acts as a permeability barrier to the cytoplasm and is able to regulate the transport of macro- and micro- nutrients from the media to the cytoplasm as well as the osmotic pressure through the plasma membrane. The osmotic pressure influences the integrity and hydration of cells and their intracellular compartments. Inflowing of water and swelling is governed by a decrease in external osmotic pressure (Wood, 2015), whereas an increase in osmotic pressure results in outflowing of water and dehydration. However, water fluxes coming simultaneously from opposite directions can disturb several cellular properties, including cell volume, turgor pressure, strain and cytoplasmatic membrane tension. Attenuation of water fluxes, by the accumulation or release of solutes, is one mechanism by which cells will respond to changes in external osmotic pressure (Wood, 2015).

AgNPs can damage bacterial membrane *via* three mechanisms. First, the electrostatic interaction between cell membranes and nanoparticles can interrupt transmembrane electron transfer, and produce break formation (pit formation). Through this mechanism, AgNPs can also penetrate into the cell membrane producing an increase in the permeability, resulting in an uncontrolled plasma-membrane transport and even leading to cell death (Prabhu and Poulose, 2012). Secondly, AgNPs can release silver ions (Ag^+) through cooperative oxidation with both protons and dissolved O_2 (Liu and Hurt, 2010). Ag^+ can be transported in the bacterial membrane by the potential disruption of nanoparticles to the cell wall and membrane. Bacterial membrane permeability can be affected by the Ag^+ mechanism described above and cause the release of lipopolysaccharides (LPS) and membrane proteins (Losasso et al., 2014). Moreover, Ag^+ can cause the release of phosphate, mannitol, succinate, proline and glutamine from the cytoplasm and disrupt the respiration cycle by inhibiting the uptake of phosphorous, thus impairing the formation of energy-regulating compounds such as nicotinamide adenine dinucleotide (NADH) or damaging molecules, such as DNA (Rai et al., 2012).

Finally, the interaction between Ag^+ and thiol groups in proteins, in addition to inactivating the respiratory enzymes, can lead to the production of undesirable compounds, such as reactive oxygen species (ROS) (Li et al., 2008). Intracellular oxidative stress can then occur as a result of high amounts of ROS, which can cause changes in the permeability of the cell membrane, protein structure, mitochondrial activity, and DNA replication (Manke et al., 2013; Eckhardt et al., 2013; Prabhu and Poulose, 2012).

Bioreactors are used to grow bacteria in continuous or batch mode. Previous studies have provided insight into the potential use of continuous reactors (chemostats) to assess stress conditions on bacteria. The effects of pH, osmotic stress, antibiotic resistance, and temperatures on bacteria have been studied extensively using chemostats (King et al., 2006; Leenheer and Cogan, 2008). In chemostats, bacteria response in terms of cell growth and adaptation can be studied under single and multiple conditions, such as competition with nutrient recycling and antibiotic treatment (Lin et al., 2012; Ziv et al., 2013; Miller et al., 2013; Gresham and Hong, 2015). Comparatively, batch reactors have been used broadly to quantify the antimicrobial properties of nanoparticles in terms of their impacts on metabolic functions and cell structure such as, viability, membrane permeation, growth and respiration (Anaya et al., 2015; Mirzajani et al., 2011; Roe et al., 2008; Choi et al., 2008; Zhang and Oyanedel-Craver, 2012, 2013).

The objective of this work is to compare batch and chemostat systems to assess the toxicity of casein-coated AgNPs on *Escherichia coli* (*E. coli*) based on membrane permeability, which is an indicator of membrane integrity and cells ability to adapt its membrane lipid composition. The effects of AgNPs on *E. coli* have been widely studied, and thus the results produced in this research can be compared to those obtained previously. Spherical casein-coated AgNPs have been characterized and used in our research group and others (Zhang et al., 2012; Kvitek et al., 2009). To our knowledge, cell membrane changes due to nanoparticle exposure have not been studied and compared as a function of bacterial growth conditions, batch or continuous growth. The rate of substrate utilization and product formation are dependent on the growth conditions and can influence the nature and magnitude of the effect of AgNPs on the cell membrane, and thus, bacterial metabolism.

To gain additional mechanistic insight into AgNPs-membrane interactions, we have coupled bioreactor experiments with Langmuir film balance analysis of lipid monolayers formed using *E. coli* membrane extracts. Film balance studies have used to examine nanoparticle interactions with synthetic lipid monolayers, but they have not been used to examine lipid monolayers from membrane extracts to assess AgNPs exposure (Guzmán et al., 2013; Peetla and Labhasetwar, 2008; Torrano et al., 2013). Monolayer film balance analysis yields surface pressure-area isotherms that can be used to assess the biophysical properties of lipids as well as lipid composition (Kurniawan et al., 2013; Bothun et al., 2016; Venkataramanan et al., 2014). The influence of the bacteria growth conditions coupled with membrane permeability assays and membrane extract analysis provides new methodologies and testing conditions that may be used to more accurately examine the response of microorganisms to nanoparticle exposure.

2. Materials and methods

2.1. Materials

A non-pathogenic strain of *E. coli* K-12 (ATCC 23716) was selected for this study. *E. coli* is a gram-negative bacterium that has been extensively used in nanotoxicological studies (Venieri et al., 2014; Choi et al., 2008; Pratap Reddy et al., 2007). Reagents used to prepare the growth media for the bacteria — sodium chloride (NaCl), yeast extract, and tryptone; and phosphate buffer solution (PBS) — monobasic potassium phosphate, dibasic potassium phosphate and ethylenediaminetetraacetic acid (EDTA) — were purchased from Sigma Aldrich. The chemical oxygen demand (COD) was measured using the TNT 822 kit from Hach Company. Dichloromethane, methanol, and chloroform used for