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In-Situ TEM Studies on Nanoparticle Interactions with Bacterial Cells

Abhijit H. Phakatkar[1], Pankaj Ghildiyal[2], Yujie Wang[2], Michael R. Zachariah[2], Tolou Shokuhfar[1*] and Reza Shahbazian-Yassar[3*]

[1.] Department of Biomedical Engineering, University of Illinois at Chicago, Chicago, IL, USA.
[2.] Department of Chemical Engineering and Materials Science, University of California Riverside, CA, USA.
[3.] Department of Mechanical and Industrial Engineering, University of Illinois at Chicago, Chicago, IL, USA.
* Corresponding authors: tolou@uic.edu, rsyassar@uic.edu.

Increasing antimicrobial resistance (AMR) in bacteria is a continuously emerging global healthcare challenge. Recently, at the global healthcare platform it received utmost attention being as a potential 'hidden pandemic' [1]. In 2019, more than 1.2 million people died worldwide due to multi-drug resistant bacterial infections [2]. Metal nanoparticles lie at the core for developing the novel strategies for combatting AMR in bacteria. Metal nanoparticles demonstrate bactericidal efficacy via various pathways such as the generation of reactive oxygen species (ROS), metal cations release, physical damage to bacterial cell membrane, and chemical binding with functional membrane proteins[3]. The understanding of metal nanoparticles endocytosis is very important in the nanomedicine field attributed to their potential as a nano-carriers for the targeted drug delivery purposes [4].

Over the period, transmission electron microscopy (TEM) has played critical role in evaluating nanoparticles internalization mechanisms in bacteria [5]. Conventionally, resin embedded ultramicrotome and cryo-TEM ultrathin cross-sections upon nanoparticles interaction with bacteria are widely studied [6, 7]. Nanoparticles internalization – endocytosis pathways are described based on the observations of these ultrathin cross-sections of bacterial cells, where physical and chemical characteristics of nanoparticles, mainly elemental composition, shape, and size were considered [4, 8]. Although these conventional TEM techniques have provided fundamental insights of possible nanoparticles internalization mechanisms into bacterium, there exists a limitation with the speculations made based on the aliquots from the temporal study. To address this existing knowledge gap in-situ liquid TEM techniques possess huge potential to study real time nanoscale endocytosis events, which can be essential for developing novel drug delivery systems to counter multidrug resistant bacteria.

In the present work, for the very first time, we propose a novel approach based on in-situ graphene liquid cell (GLC) TEM to study metal nanoparticles interaction with bacteria. GLC possess tremendous potential to study biological processes attributing to its advantages with capability of acquiring atomic resolution imaging with higher signal to noise ratio and with graphene conductivity resulting in mitigating charging effects and radiolysis damage.[9] For preparing the sample, the cultured *Escherichia Coli* bacteria (100 CFU/ml concentration) and (Fe/Ni/Cu) ternary metal nanoparticles (10 μg/ml concentration) were mixed in the ultrapure water as a medium for 5 minutes. 0.3 μL of prepared solution was encapsulated between two graphene coated TEM grids to prepare GLC. The GLC was immediately loaded on single tilt holder for further high-resolution TEM study. Aberration corrected ARM200CF 200 kV scanning transmission electron microscope (STEM) in the TEM mode was used for performing in-situ GLC TEM nano-bio interaction study. The results indicate dynamic event of the real time nanoparticle uptake by bacterial cell. **Figure 1** shows the dynamic event of (Fe/Ni/Cu) nanoparticle

internalization into bacterial cell outer membrane. Bacterial outer cell membrane is composed of phospholipids bilayer with hydrophilic and hydrophobic polar components. Additionally, functional membrane proteins are present for regulating the ions transfer into the bacterial cell. **Figure 1a** shows nanoparticle attached with the bacterial outer cell membrane where cytoplasmic region and extracellular regions are highlighted. Additionally, organic corona around a nanoparticle can be observed. **Figure 1b** and **1c** show dynamics of nanoparticle internalization process. The various nanoscale phenomenon during the endocytosis process such as rotation of nanoparticle, simultaneous release of metal ions from a nanoparticle, splitting of inner and outer phospholipids from outer membrane lipid bilayer, subsequent rupture of inner phospholipid layer, membrane pore formation, and finally the reinstatement of outer lipid layer upon internalization of nanoparticle were studied. Further the investigation of organic corona around a nanoparticle was performed using STEM-energy dispersive X-ray spectroscopy (EDS) technique, as represented in **Figure 2**. STEM-EDS micrographs confirm the presence of O, P, N, K, Ca, and Cl elements which are diagnostic ions from the bacterial cell membrane and cytoplasmic components. The localized leakage of cytoplasmic cellular components can be confirmed. The study sheds valuable insights on a nanoparticle internalization mechanism highlighting the importance of organic corona around a nanoparticle for developing novel drug delivery systems to overcome AMR in bacteria [10].

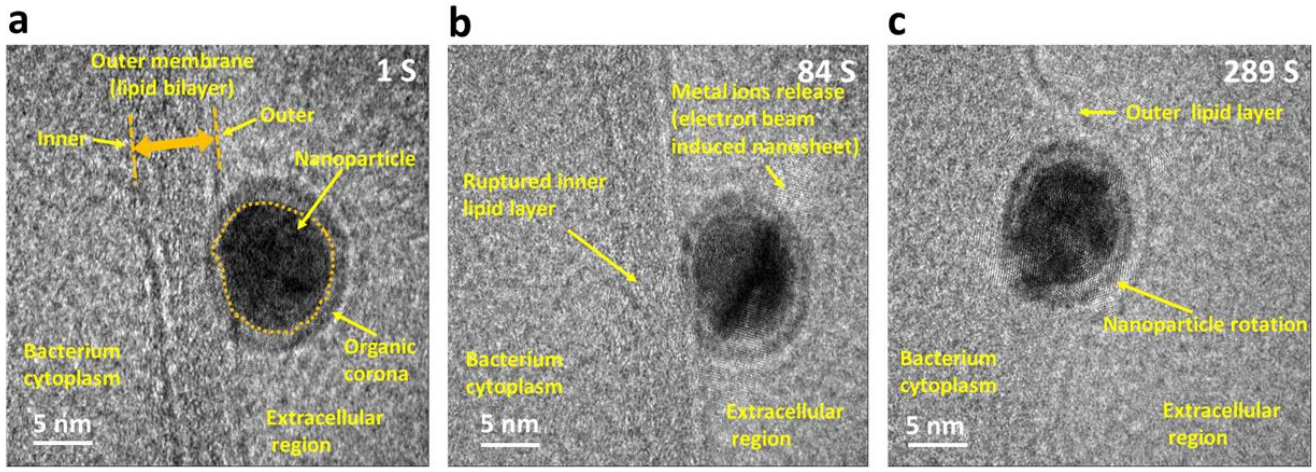

Figure 1. In-situ graphene liquid cell HR-TEM micrographs representing the event dynamics of (Fe/Ni/Cu) ternary nanoparticle internalization pathway by *Escherichia Coli* bacterial cell membrane. (a) HR-TEM micrograph indicating the outer bacterial cell membrane (lipid bilayer) and a nanoparticle with organic corona attached with the bacterial cell membrane. (b) HR-TEM micrograph confirming the ruptured inner lipid layer and split outer lipid layer during the event of nanoparticle internalization. (c) HR-TEM micrograph confirming the complete internalization of nanoparticle by bacterial cell with the surrounding outer lipid layer.

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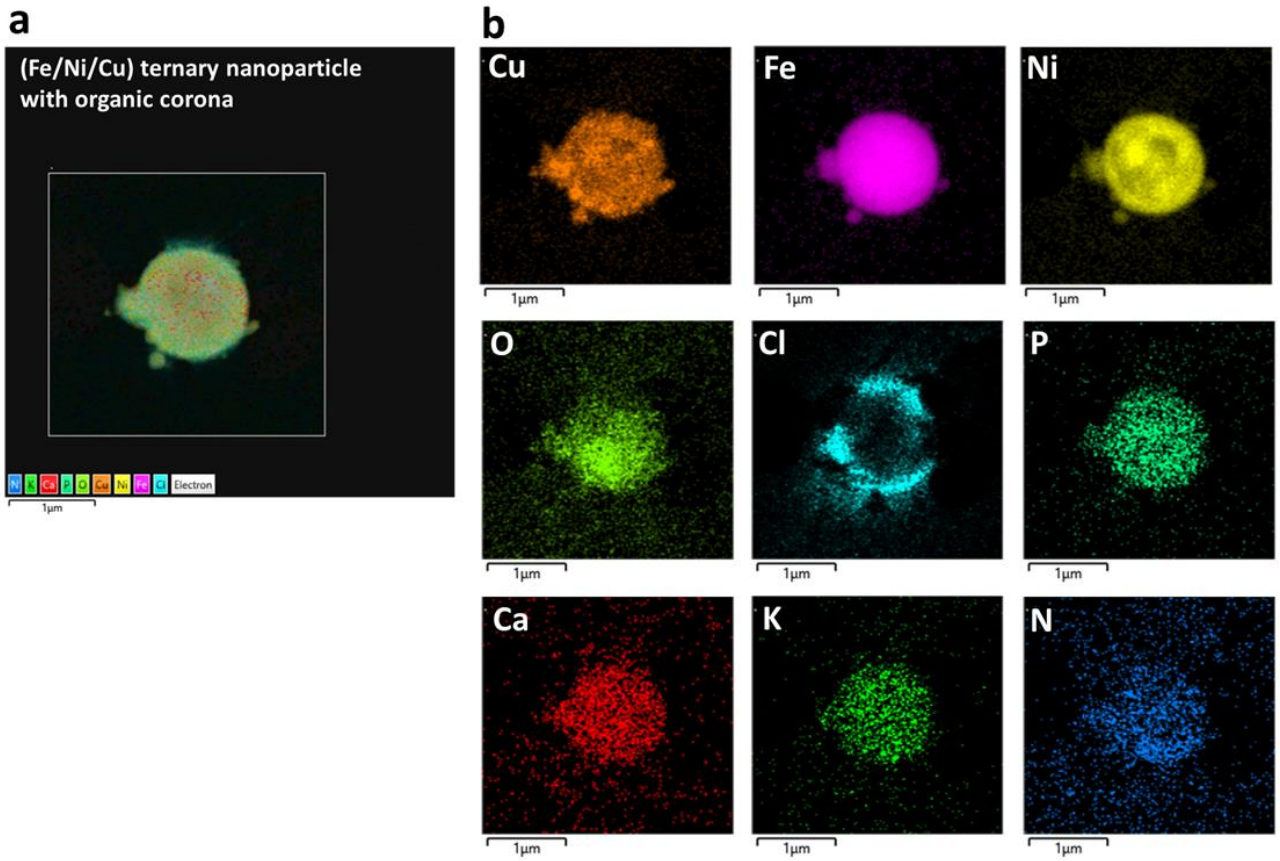

Figure 2. In-situ graphene liquid cell STEM-EDS micrographs evaluating the elemental mapping of the formed organic corona around the nanoparticle upon interacting with *Escherichia Coli* bacterium. (a) Layered elemental mapping of (Fe/Ni/Cu) ternary nanoparticle with organic corona. (b) Respective individual elemental mapping of organic corona around the nanoparticle confirming the bacterium cytoplasmic contents.

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