BRIEF REPORT

Quantitative cytotoxicity analysis of antibacterial Janus nanoparticles in immune and cancer cells

[version 1; peer review: 1 approved with reservations, 1 not approved]

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Abstract

Background

Nanoparticles (NPs) hold promise as alternatives to antibiotics in the fight against multi-drug-resistant bacteria. However, concerns about their cytotoxicity, particularly their effects on mammalian cells, must be thoroughly addressed to ensure therapeutic safety. Amphiphilic Janus NPs, which have segregated hydrophobic and polycationic ligands on two hemispheres, have previously been shown to exhibit potent antibacterial activity.

Methods

In this study, we evaluated the cytotoxicity of amphiphilic Janus NPs in immune and cancer cell lines. Cytotoxicity assays were performed to assess the effects of Janus NPs on cell viability and membrane integrity, with a particular focus on how internalization of the nanoparticles influenced cellular responses.

Results

The results revealed that both immune and cancer cells exhibited negligible cytotoxic effects when exposed to Janus NPs. However, phagocytic immune cells demonstrated greater susceptibility to membrane damage and viability loss, suggesting that internalization plays a significant role in nanoparticle-induced cytotoxicity.

Conclusions

Amphiphilic Janus NPs show great potential as highly effective

Open Peer Review

Approval Status ? ✗

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version 1 08 Nov 2024	? view	✗ view

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2. **Wing-Hin Lee**, Universiti Kuala Lumpur, Kuala Lumpur, Malaysia

Any reports and responses or comments on the article can be found at the end of the article.

antibacterial agents with minimal cytotoxicity. While immune cells may be more vulnerable to nanoparticle-induced damage due to their internalization capacity, these findings support the further investigation of Janus NPs for clinical applications.

Keywords
Janus nanoparticles, cell cytotoxicity, anti-bacterial nanomaterials, cell internalization, cell viability

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Author roles: Johnson B: Data Curation, Formal Analysis, Writing – Original Draft Preparation; **Peck M**: Data Curation, Formal Analysis, Writing – Original Draft Preparation; **Richman H**: Data Curation, Formal Analysis, Writing – Original Draft Preparation; **Bhattacharyya S**: Data Curation, Formal Analysis, Writing – Original Draft Preparation; **Yu Y**: Conceptualization, Project Administration, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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Introduction

The rise of multi-drug resistant bacteria poses a significant threat to human health. In 2019 alone, antimicrobial resistance (AMR) caused an estimated 1.27 million deaths globally.[1] The AMR crisis is expected to worsen with the slowdown in new antibiotic development. To address the urgent need for effective antibacterial agents, nanoparticles (NPs) have emerged as a promising alternative to traditional antibiotics.[2–4] Owing to their unique physicochemical properties, antibacterial NPs are known to interact with bacterial cells through a combination of multiple mechanisms, including physical disruption of cell membranes and oxidative stress induction, which collectively minimize the likelihood of bacterial resistance development.[5–8]

However, alongside their potential benefits, the cytotoxicity of antibacterial NPs, particularly their impact on mammalian cells, must be thoroughly evaluated, to ensure their therapeutic viability and safety. Previous research has shown that nanoparticle cytotoxicity can vary widely depending on their physiochemical properties, such as size, shape, surface charge, and composition.[9,10] For instance, NPs with certain surface chemistries, including positive charges and hydrophobicity, may induce oxidative stress or inflammation in mammalian cells, potentially leading to adverse effects.[11–14]

Our previous work demonstrated that amphiphilic Janus NPs, designed with hydrophobic and polycationic ligands on separate hemispheres, are highly effective against both gram-positive and gram-negative bacteria.[15] We showed that these Janus NPs effectively inhibit bacterial growth by physically disrupting the bacterial outer membranes and inducing oxidative stress in bacterial cells. Building upon these findings, the current study extends our investigation to assess the cytotoxicity of these NPs in mammalian cell cultures, focusing on immune cells and cancer cells. Using quantitative cytotoxicity assays with single-cell precision, we establish the relationship between nanoparticle concentration and cytotoxicity *in vitro*.

Methods

Reagents and cells

RAW264.7 macrophage cells and HeLa cells were purchased from ATCC. Gibco advanced Dulbecco's Modified Eagle Medium (DMEM) 12491015, Gibco FluoroBrite™ Dulbecco's Modified Eagle Medium (FluoroBrite DMEM) A1896701, Gibco L-Glutamine 200 mM (100x) 25030081, propidium iodide (PI) P21493 and Hoechst ™ H21486 fluorescent dyes were purchased from ThermoFisher™ (Waltham, MA, USA). 12-well glass-bottomed cell culture plates (P12G-1.5-11-F) were purchased from Mattek (Ashland, MA, USA). 100x Penicillin-streptomycin (10,000 mg/mL) MT30001CI and fetal bovine serum (MT35010CV) were purchased from Corning (Corning, NY, USA). Amine-functionalized silica nanoparticles (100 nm) SISN100-25M were procured from Nanocomposix (San Diego, CA, USA). Gold (99.99% purity) EVMAU40QXQ and chromium (99.99% purity) EVMCR35 were purchased from Kurt J. Lesker, Co. (Jefferson Hills, PA, USA). Octadecanethiol (L04123-22), colistin sulfate, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water (18 MΩ·cm) was used for all experiments.

Janus nanoparticle (NP) fabrication

Amphiphilic cationic/hydrophobic Janus NPs (+/pho JPs) were fabricated as described below. Briefly, a sub-monolayer of 100 nm cationic silica NPs was coated on piranha-etched microscope slides. Chromium (5 nm thickness) and gold (25 nm thickness) were coated onto one hemisphere of the particles using an Edwards thermal evaporation system. The metal-coated NPs were immediately functionalized with 2 mM 1-octadecanethiol in ethanol to render the gold side hydrophobic. The resulted NPs were sonicated off the slides within 24 h before use. Aggregates from metal bridging were removed by two steps of differential centrifugation (100 rcf four times, 500 rcf four times).

For colistin conjugation, Janus NPs were washed 3 times with 10 mM HEPES buffer (pH 7.40) and resuspended with 10 % glutaraldehyde in 10 mM HEPES buffer for 90 min at room temperature under gentle rotation. The particles were then washed three times with 10 mM HEPES buffer to remove excess glutaraldehyde. Particles were then resuspended and incubated in 1 mg/mL colistin in 10 mM HEPES buffer for 60 min at room temperature under gentle rotation, followed by washing three times in 10 mM HEPES and then three times in 70% ethanol to remove excess colistin. Functionalized Janus NPs were then stored in 70% ethanol at 4°C until use, when ethanol was replaced with cell imaging buffer.

Hydrodynamic size and zeta potential of colistin conjugated amphiphilic Janus NPs (col/pho JPs) were characterized using Dynamic Light Scattering (DLS) and Malvern Zetasizer). Gold coating on the NPs were assessed using scanning electron microscopy. For more detail on the fabrication and properties of col/pho Janus NPs, see methods from Wiemann et al.[15]

Dual-staining cytotoxicity assay

Mammalian cells (RAW264.7 macrophage and HeLa) were seeded onto glass-bottomed 120-well cell culture plates at a concentration of 0.125 million cells/mL (RAW264.7 cells) or 0.0156 million cells/mL (HeLa cells) and grown for 24h at 37 °C, 5% CO_2. Both mammalian cells lines were grown in 400 μL of DMEMα containing advanced DMEM with penicillin-streptomycin (1x), L-glutamine (2 mM), and fetal bovine serum (10% v/v). Following 24h incubation, cell media was replaced with serum free media (advanced DMEM with no fetal bovine serum) containing various concentrations of Janus NPs. Janus NPs were stored in 70% ethanol and washed multiple times with DMEMβ containing advanced DMEM with penicillin-streptomycin (1x) and L-glutamine (2 mM) before addition to cell samples. Janus NPs were incubated with cells for 24h before the dual-staining cytotoxicity assay.

Following exposure to Janus NPs, cells were washed once with DMEM. Then a total volume of 400 μL staining mixture containing propidium iodide (2.5 μg/mL) and Hoechst (25 μg/mL) was added to the cell media in each well. Approximately 10 min after addition of the staining mixture, fluorescence and differential interference contrast (DIC) images were acquired using a Nikon Ti-E inverted microscope equipped with a 40x/0.95 NA Nikon air objective. Hoechst was imaged at excitation/emission 350/450 nm and propidium iodide was imaged at excitation/emission 535/615 nm. Cell samples were incubated at 37 °C, 5% CO_2 throughout imaging process using a UNO-T-H-CO2 stage top incubator.

Results and Discussion

The amphiphilic Janus NPs (100 nm in diameter) have distinct surface coatings on two hemispheres (Figure 1A), same as we reported previously, and were fabricated using our established protocol.[15,16] Briefly, we first coated one hemisphere of aminated silica NPs with thin chromium (5 nm thickness) and gold layers (25 nm thickness) via directional vapor deposition, and then conjugated octadecanethiol on the gold cap to render it hydrophobic. The other hemisphere of the aminated silica NPs was conjugated with colistin using glutaraldehyde crosslinking chemistry. Colistin, typically reserved as a last-resort antibiotic, functions here as a polycationic ligand that enhances the strong electrostatic interaction of Janus NPs with bacterial membranes, as we reported previously.[15]

Based on our previous findings demonstrating the effective disruption of model lipid membranes and bacterial outer membranes by Janus NPs,[15,17–20] we proceeded to investigate their impact on plasma membrane integrity and subsequent cell viability. This investigation utilized a dual staining protocol involving propidium iodide and Hoechst, a widely accepted method for assessing cell cytotoxicity.[21,22] The assay was conducted in HeLa cells and RAW264.7 macrophage cells, two commonly used cell lines to represent cancer and immune cells, respectively. In this protocol, Hoechst dye, which penetrates intact membranes, stains the DNA of all cells, whether alive or dead (Figure 1B). Conversely, propidium iodide, a membrane-impermeable dye that binds to nucleic acids, selectively stains non-viable cells with compromised membranes. This approach allows us to not only identify cells with nanoparticle-induced plasma membrane permeabilization, but also quantify cytotoxic effects by comparing the number of propidium iodide-stained non-viable cells to the total Hoechst-stained cell population.

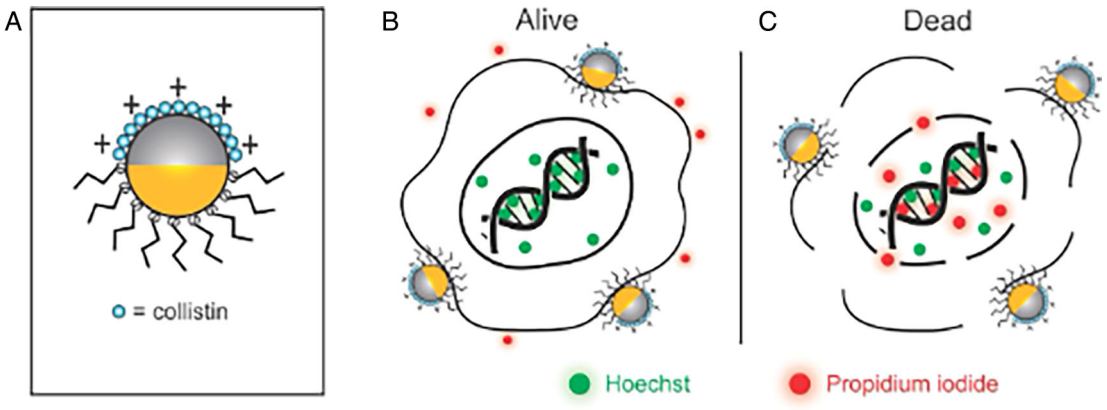

Figure 1. Schematic illustration of Janus nanoparticles (NPs) and the cell viability assay. (A) Amphiphilic Janus NPs exhibit distinct surface makeups on two hemispheres. One side is conjugated with hydrophobic octadecane thiol ligands on a gold cap, while the other side is conjugated with the polycationic ligand colistin. (B) In the cytotoxicity assay, nuclei of all cells are stained with membrane permeable Hoechst dye (green). (C) Nuclei of non-viable cells with damaged plasma membrane are stained with the membrane impermeable dye propidium Iodide (red).

Initially, HeLa cells were incubated for 24h with Janus NPs at concentrations ranging up to 64 pM. The 0-64 pM concentration range was selected because it covers and exceeds the range of half maximal effective concentrations (EC50) of these NPs against four different bacterial strains (10-47 pM).[15] We previously demonstrated that 64 pM Janus NPs induce substantial membrane damage and production of reactive oxygen species in almost all *E. coli* cells.[15]

Using both differential interference contrast (DIC) and widefield fluorescence microscopy, we observed that the morphology of HeLa cells remained unchanged in terms of both shape and size following exposure to Janus NPs (Figure 2A, C). There was a slight increase in the number of cells stained with propidium iodide, as nanoparticle concentration increased, indicating minor damage to the cell plasma membrane (Figure 2B). To quantify the impact on cell viability, we calculated the percent viability at each particle concentration using the equation: $\text{Percent Viability} = \frac{\textit{Number of Viable Cells}}{\textit{Number of Total Cells}} \times 100\%$. As shown in Figure 2D, even with 64 pM NPs, approximately 94.8% of cells remained viable. This reduction in cell viability is much less than the cytotoxic threshold defined by EN ISO 10993-5 as a reduction in cell viability by more than 30% compared to an untreated control.[23] Therefore, Janus NPs exhibited minimal effect on overall viability of HeLa cells.

After assessing the impact of JNP treatment on HeLa cells, we investigated the cytotoxicity of the Janus NPs to professional phagocytes, a type of immune cells specialized in internalizing and digesting foreign particles including

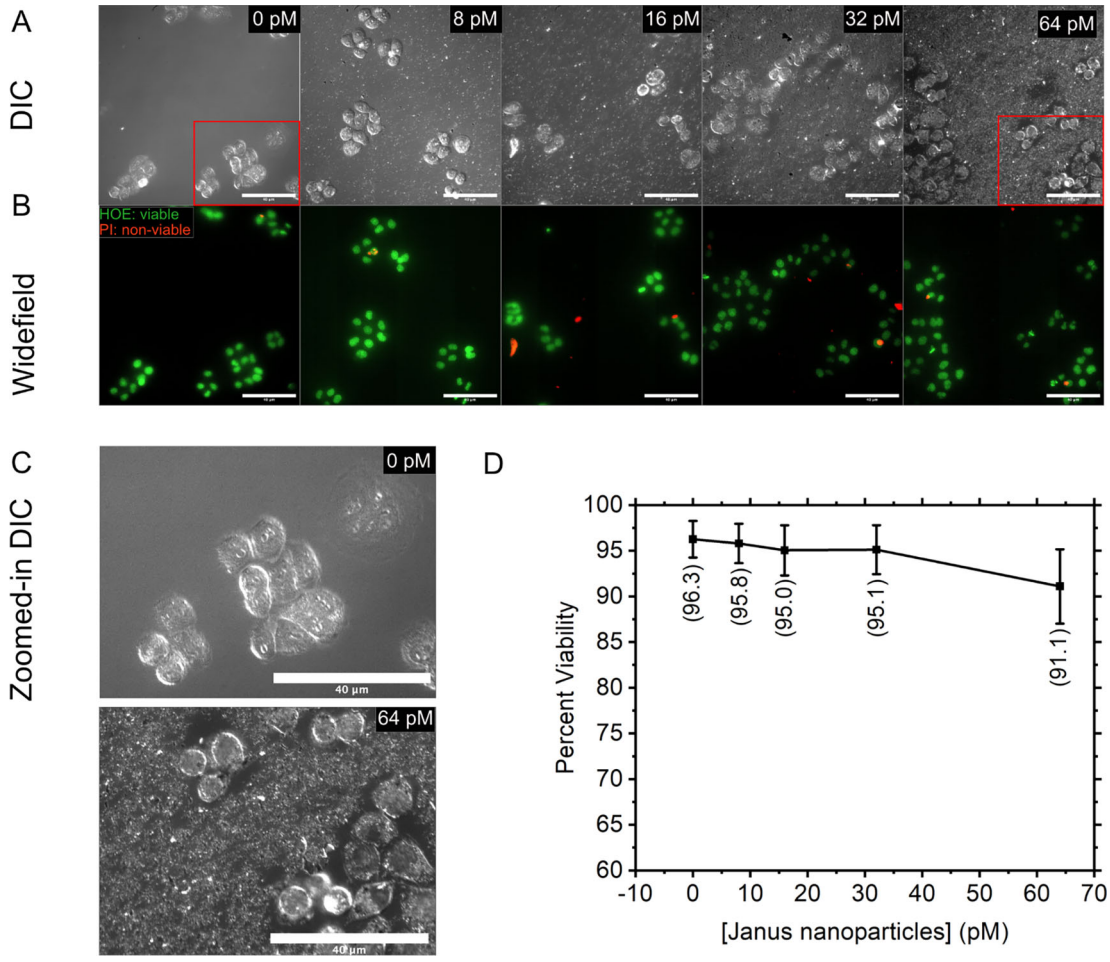

Figure 2. Viability assay results of HeLa cells after interaction with varying concentrations of Janus NPs. Differential interference contrast (DIC) (A) and widefield epi-fluorescence (B) images showing cells stained with Hoechst (green) and propidium iodide (red) after 24h incubation with Janus NPs at varying concentrations. (C) Zoomed-in DIC images of areas outlined in red rectangles in (A). Quantification of viability of HeLa cells as a function of Janus nanoparticle concentration (D). Each data point represents the average of two independent samples and each error bar represents the standard error between samples. Scale bars: 40 µm.

pathogens and synthetic NPs.[24–28] To this end, we evaluated the cytotoxicity of the NPs against RAW264.7 cells using the same dual staining protocol.

Using both DIC and widefield fluorescence imaging, we observed that some NPs were internalized by the macrophage cells, resulting in regions immediately adjacent to cells showing almost no NPs compared to background levels (Figure 3A, C). This phenomenon is more pronounced at higher nanoparticle concentrations. Meanwhile, the cell morphology changed slightly upon nanoparticle exposure. Post-interaction with NPs, cells exhibited increased heterogeneity in shape, characterized by enhanced membrane protrusions and asymmetry (Figure 3C).

Consistent with these observations, there was a slight increase in the number of cells stained with propidium iodide at higher nanoparticle concentrations (Figure 3B), indicating concentration-dependent damage to the cell plasma membrane and subsequent cell death. As shown in Figure 3D, Janus NPs up to 16 pM had negligible effects on macrophage cell viability. However, cell viability decreased by 22.9% and 26.6% after exposure to 32 pM and 64 pM NPs, respectively. It is important to note that, even though higher concentrations of Janus NPs reduced cell viability, it did not fall below the threshold of a 30% reduction, which is considered cytotoxic as defined by the EN ISO 10993-5 standard.[23]

By comparing the results from HeLa cells and RAW264.7 macrophage cells, it is evident that the cytotoxic effect of the Janus NPs is more pronounced in phagocytic immune cells, which inherently internalize a larger number of NPs. This is

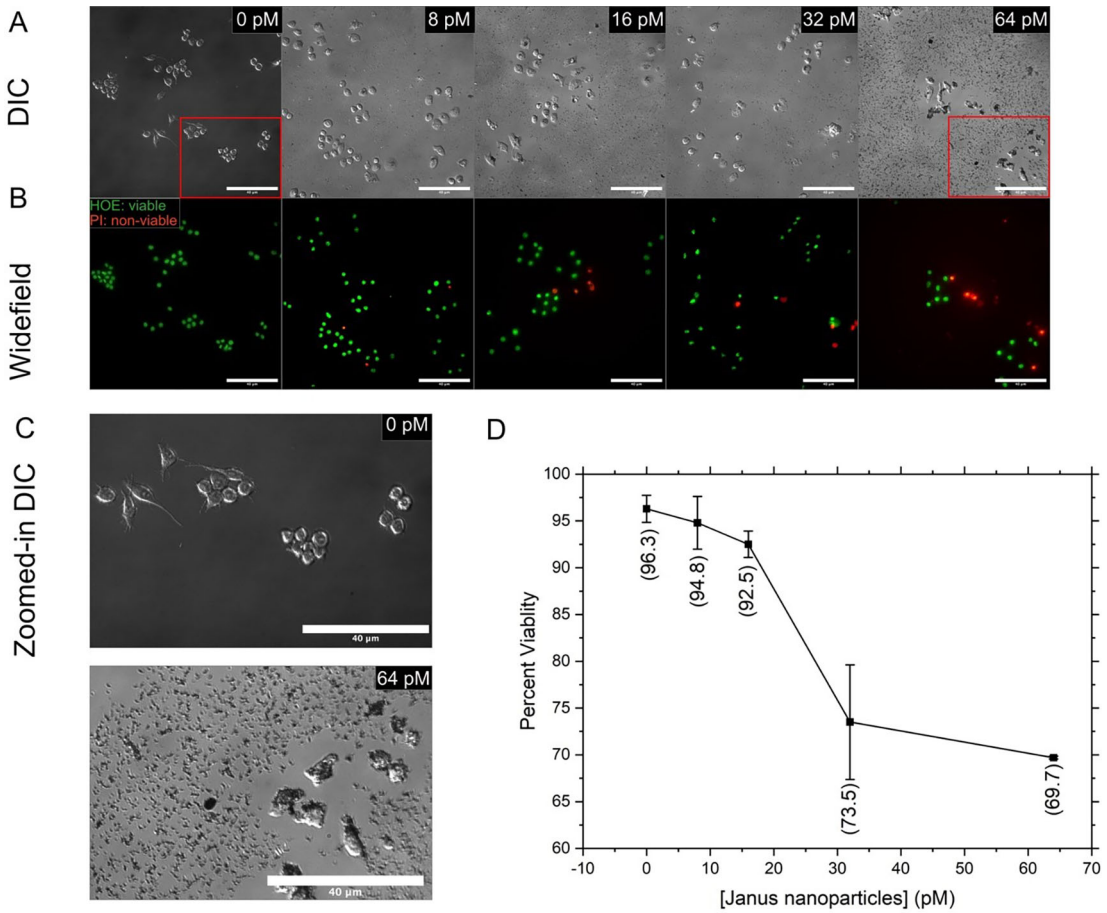

Figure 3. Viability assay results of RAW264.7 macrophage cells after interaction with varying concentrations of Janus NPs. Differential interference contrast (DIC) images (A) and Widefield epi-fluorescence images (B) showing cells stained with Hoechst (green) and propidium iodide (red) after 12h interaction with Janus NPs of varying concentrations. (C) Zoomed-in DIC images of areas outlined in red rectangles in (A). Quantification of viability of RAW264.7 macrophage cells as a function of Janus nanoparticle concentration (D). Each data point represents the average from three independent samples and each error bar represents the standard error between samples. Scale bars: 40 µm.

supported by viability quantification and our observation of changes in macrophage cell morphologies. This finding aligns with previous reports that internalization is a contributing factor in the cytotoxicity of NPs.[29–32]

Importantly, neither cell type shows a significant cytotoxic effect from the Janus NPs, based on the established threshold of a 30% reduction in cell viability as indicative of toxicity. The nanoparticle concentrations we tested ranged from 0 to 64 pM, exceeding the EC50 values of the NPs against various bacteria (e.g., an EC50 of ~10 pM for E. coli).[15] These results validate the potential of Janus NPs as antibacterial agents. This research opens doors to further studies, including more comprehensive assessments of both *in vitro* and *in vivo* cytotoxicity of the NPs using additional markers, such as the generation of reactive oxygen species and the inflammatory response of immune cells.

Author contribution statement

B.J., M.P., H.R., and S.B. carried out the experiments. All authors wrote the manuscript. Y.Y. conceived the project idea and supervised the project.

Ethics & consent

Ethical approval and consent were not required.

Data availability statement

All study data are included in the manuscript and/or supporting information. Raw data used to generate main figures and source code for simulation have been deposited at https://doi.org/10.6084/m9.figshare.27092086.v3.

FigShare: Janus-particle Cytotoxicity Raw Data, https://doi.org/10.6084/m9.figshare.27092086.v3.[33]

This project contains the following underlying data:

- Raw Data. (All Raw264.7 and HeLa cell microscopy images used for figures and analyzed for graphs)

Acknowledgements

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Version 1

Reviewer Report 22 February 2025

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✗ **Wing-Hin Lee**
Universiti Kuala Lumpur, Kuala Lumpur, Federal Territory of Kuala Lumpur, Malaysia

This manuscript is not suitable for indexing. This is due to multiple reasons:
1) No bacteria experiment works are included in this manuscript.
2) This manuscript is unclear as to why HeLa and Raw cells were used.
3) The amount of drug loaded was not reported.
4) In general, the main objective of this manuscript was not clear.

Is the work clearly and accurately presented and does it cite the current literature?
No

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
No

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Drug delivery

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 13 February 2025

https://doi.org/10.5256/f1000research.171921.r362489

?

Atanu Singha Roy
Department of Chemical and Biological Sciences, National Institute of Technology Meghalaya, Shillong, India

The authors have fabricated the nanoparticles. In this regard, no data/plots are provided at least as the supplementary information. JNPs exhibits interesting features and I want to get the following details related to the characterization of the JNPs:

1. PXRD data before and after fabrication of all the NPs systems.
2. FT-IR related to surface fabrication.
3. XPS to get the details after fabrication related to existence of various metals in JNPs.
4. TEM/FESEM images of the NPs (if possible with EDX).

These results/plots etc as applicable must be supplied in the supplementary information.

Also the results from DLS and Zeta potential are not mentioned. It is also needed to be added in the SI. Is there any changes of zeta potential after interaction with the cell lines? Is it determined? Any related discussion is possible?

Is the work clearly and accurately presented and does it cite the current literature?
No

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biophysical Chemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.