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Towards resolution of antibacterial mechanisms in metal and metal oxide nanomaterials: a meta-analysis of the influence of study design on mechanistic conclusions

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Environmental Significance Statement

The antibacterial activity of metal and metal oxide nanomaterials is of great environmental relevance; while these materials have been touted as promising candidates to alleviate the mounting crisis of antibiotic resistance, concerns have also been raised that accidental release may have a detrimental impact on environmental systems. Designing nanomaterials to maximize desired functionality while minimizing undesired environmental consequences requires thorough understanding of their mechanisms of antibacterial activity. Despite widespread pursuit of mechanistic understanding through research efforts spanning more than a decade, there remains a significant amount of controversy and purported uncertainty in the published literature. This review elucidates the underlying sources contributing to these disagreements including experimental conditions to assess the mechanistic basis for metal and metal oxide engineered nanomaterial antibacterial activity .

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Abstract

While the antibacterial potency of metal and metal oxide engineered nanomaterials (MMO ENMs) has been well-established in the literature, the underlying mechanisms of antibacterial activity are regarded by many as uncertain, despite a considerable volume of publications on this subject. In order to illuminate sources of perceived uncertainty and disagreement in the mechanistic nanotoxicology literature, 318 articles pertaining to the mechanism of antimicrobial activity of Ag, Cu, CuO, TiO_2 and ZnO ENMs were analyzed. The 318 studies all aimed to assess one or more of eight mechanistic questions, and both positive (i.e. affirmative) and negative conclusions were reported for each mechanistic question for each of the five core compositions. Differences in study design, including the exposure conditions and experimental methods used, were found to statistically significantly correlate with differences in reported mechanistic conclusions. Further analysis of studies which investigated two or more mechanisms revealed how assumptions about which mechanisms predominate for a given core composition may influence study design and, in turn, conclusions. Finally, 181 distinct experimental methods were identified, many of which are relatively untested and have not been evaluated in the published literature, while many frequently-used methods were found to have limitations that may obscure interpretation and mechanistic insight.

1. Introduction

Over the past two decades, the antibacterial properties of metal and metal oxide engineered nanomaterials (MMO ENMs) have garnered significant interest [1-3]. While these materials may be promising alternatives to conventional antibiotics in some applications, with the potential to alleviate the antibiotic resistance crisis [4-7], there remain concerns of possible unintended consequences upon environmental release, including their potential adverse impacts on essential processes mediated by bacteria, such as nitrogen cycling [8, 9] and wastewater treatment [10, 11]. Many researchers, therefore, aim to design and select MMO ENMs with desirable functionality and minimal environmental hazard, a goal which requires an understanding of not only the magnitude of ENM antibacterial activity but also the underlying mechanisms [12-14]. However, the significant complexities inherent to the study of MMO ENM antibacterial mechanisms may inhibit mechanism-informed design. Specifically, the selection and interpretation of experimental methods presents many opportunities for ambiguous, confounding, and even contradictory results in identifying likely mechanisms, even for a given core composition.

The possible antibacterial mechanisms in various MMO ENM core chemistries were explored in a 2016 book chapter [15]. Briefly, existing mechanistic literature encompasses investigations into four main areas: 1. the physicochemical processes determining ENM exposure and bioavailability, 2. the role of intact ENMs *versus* dissolved ions and/or reactive oxygen species (ROS), 3. the transport mechanisms and outcomes (i.e. where particles localize and/or accumulate) of ENMs and dissolved species within the cell, and 4. the physical and chemical effects on cellular components including membranes, DNA, enzymes, and others. Given the vast range of points of inquiry along a mechanistic pathway from initiation to outcome, the very framing and articulation of the research question(s) can, increasingly, be a source of complexity within the mechanistic literature.

As mechanistic knowledge has accumulated across different core chemistries, a divergence in research aims has emerged. Researchers interested in mechanism as it pertains to nano-design for specific applications often ask whether or not a given process, transport outcome, or cell effect contributes significantly to antibacterial activity for a given core composition [16-32]. In contrast, biologists, nanotoxicologists, and ecosystem scientists often ask to what extent and in what conditions a given mechanistic pathway contributes to a toxicity outcome [33-38], inquiries with nuances that are not as readily captured within such a binary framework. These different framings of mechanistic questions may create apparent uncertainty in what is “known” and “unknown” about antibacterial mechanisms for a given core chemistry; while some studies report that long-established mechanistic questions have yet to be resolved in even well-studied core chemistries [39-44] and others report seemingly definitive but contradictory conclusions [45-51], new questions continue to be raised about the conditions under which various mechanistic pathways may predominate [52, 53].

A 2014 study examined over 600 published articles to understand the disparities between ENM concentrations used in laboratory-scale ecotoxicity assessments and modeled or measured

concentrations of ENMs in the environment [53]. While a main finding was the dearth of ecotoxicity studies conducted at environmentally-relevant (e.g., sufficiently low dose) ENM concentrations, the review also identified numerous experimental conditions that could potentially impact the conclusions drawn from experimental work. The impact of study conditions on reported conclusions was investigated in more detail in a 2016 follow-up report, which determined that exposure conditions such as aqueous chemistry, temperature, pH, and exposure duration play a large role in elucidating the mechanistic basis of antibacterial activity [52], in addition to the known effects of ENM size, shape, and surface coating [17, 54-59]. These variables, which are often underreported in mechanistic studies, introduce additional complexity to the interpretation of mechanistic conclusions reported in the literature, as well as the design of new mechanistic studies. In response, efforts by communities of experts have aimed to harmonize and standardize both exposure conditions [60] and ENM characterization [61] in ecotoxicological studies of ENMs.

In addition to diverging research aims and dissimilar exposure conditions between studies, a third source of complexity is the variety and lack of standardization of experimental methods used to evaluate antibacterial mechanisms. While some methods are specifically tailored to provide mechanistic insight (e.g. those targeting the gene transcription level [52]), others are adaptations of existing cellular or acellular methods which are not necessarily representative of real exposure conditions (e.g., using a growth inhibition or CFU count assay to compare ENMs to scaled ion controls [62], or assessing ion release from ENMs in a cell-free environment [63-65]).

Just as mechanistically-relevant research questions may differ by disciplinary orientation, preferred experimental methods for mechanistic investigation may also be related to familiarity and acceptance within ones' research community. The limitations of different methods [62, 66-71], as well as the nuances of what information they can and cannot provide, may not be obvious to those who are expert in nano-related topics but may not have sufficient depth, through training or collaboration, in microbiology. Proposed frameworks for producing high-quality studies on the magnitude of antibacterial activity for ENMs [72-77] may prove to be invaluable guides for refining and standardizing mechanism-targeted experimental methods, but further work remains to adapt these frameworks for the complexity and variety of mechanistic investigations. Similarly, recent studies and expert reviews have provided new insights into accurate interpretation and artifact avoidance in ENM toxicity studies across a range of organisms [69-71], which presents an opportunity to connect what is known about the limitations of experimental methods with mechanistic conclusions reported in the literature thus far.

The factors discussed above introduce complexity into mechanistic research at three stages: articulating areas of inquiry, obtaining relevant empirical results, and interpreting results to yield conclusions. Accordingly, even when two studies ask the same question about a given core chemistry, seemingly contradictory conclusions may be reported for different reasons. In some cases, differences in study conditions or ENM properties between two studies (which may or may not have been reported) cause a different mechanism to predominate; the two sets of

conclusions taken together thus represent valuable information for the community's understanding of that core chemistry. In other cases, rather than a "true" difference in mechanism between the two studies, the difference arises because the method(s) used could not elucidate a conclusive answer for the research question being investigated, contributing to a perception of disagreement or ambiguity in the literature and serving to confound mechanistic resolution. Understanding the prevalence and influence of these two sources of disagreement would enhance both the interpretation of existing studies and the design of new ones. Such understanding requires, however, a relatively comprehensive list of the many mechanism-targeted experimental methods currently in use, as well as an analysis of how method choice affects reported mechanistic conclusions, as compared to the known effects of ENM properties and exposure conditions.

As such, this study applies statistical analyses to assess the potential influence of study design, including method choice, on reported mechanistic conclusions. To this end, a dataset of studies which reported conclusions on eight straightforward, frequently studied mechanistic questions (described in Table 1) is systematically retrieved. Five of the most heavily studied core compositions (Ag, Cu, CuO, TiO_2, and ZnO) are selected to produce a robust dataset. The first phase of the analysis quantifies the frequency with which different exposure conditions were reported by studies in the dataset, as well as the impact of exposure conditions on reported mechanistic conclusions. Studies that reported conclusions to multiple mechanistic questions are also examined in order to capture the effect that presumed relationships between mechanistic investigations may have on the reported conclusions. Subsequently, the effects of experimental method choice on reported conclusions are quantified, and these findings are presented alongside a review of the advantages and limitations of methods currently documented in the literature. Finally, experimental methods are evaluated based on their tendency to produce consistent or inconsistent mechanistic conclusions for a given core composition, demonstrating how methods may differ in terms of their sensitivity, appropriateness, or both. Through these analyses, this study will facilitate method selection and interpretation of results, while also supporting the refinement and standardization of experimental methods aimed at elucidating antibacterial mechanisms for ENMs.

Table 1. Eight binary mechanistic questions relating to multiple topics of interest in MMO ENM antibacterial activity [15] were selected for analysis. While the goal of quantitative analysis necessitated representing conclusions as binary variables and therefore formulating mechanistic questions in a "yes-or-no" format, many studies incorporate nuances not captured in this binary framework; common examples are noted below, along with points of clarification on question definitions.

Name	Question Reviewed	Notes
Ion	"Are dissolved metal ions, released from particles into the exposure medium, necessary for antibacterial activity?"	• A related question not reviewed here is whether ions exert toxicity directly or indirectly through another antibacterial pathway (e.g., ROS production) [9, 78-81] • Proposed mechanisms predicated on the local release of ions from an ENM in close proximity with the cell would instead constitute a positive conclusion to the "Contact" question described below; this is because the release of ions into the exposure medium alone may not be sufficient to cause antibacterial activity in these cases (i.e., a "nano-specific" effect can be said to exist).
Contact	"Is close association between the ENM and cell necessary for antibacterial activity?"	• Example mechanisms include mechanical damage to the cell envelope [82, 83], internalization of particles [84-86], local ion release [87, 88], and ROS production *via* direct electron transfer [89]
Internalization	"Does internalization of intact ENMs across an intact cell membrane contribute significantly to antibacterial activity?"	• A related question not reviewed here is whether and under what conditions nano-sized particles can cross bacterial membranes without membrane permeabilization as a prerequisite [90, 91]. • When intracellular objects are observed, it may be difficult to discern whether these truly represent intact ENMs internalized across the membrane, as some studies have reported the internalization of ions into the cytoplasm followed by oxidation to metal oxide [19, 92, 93] or reduction to metal [47, 94, 95], giving the appearance of intracellular particles.
ROS	"Does the production or accumulation of ROS, intracellularly or extracellularly, contribute significantly to antibacterial activity?"	• ROS may be generated directly from ENM surfaces, potentially through a light-mediated mechanism, and/or may accumulate naturally in cells as a result of other stresses exerted by ENMs [96]. A related question, not reviewed here, is whether ROS generated endogenously is sufficient to cause cell death [96]. • If ROS accumulation is driven by endogenous production, ROS levels may continue to rise even after the stressor is removed, leading to delayed cell death [97] that may not be captured depending on the timeframe of the study. • This question overlaps with the "Ion" and "Contact" questions as some proposed mechanisms involve the generation of ROS by dissolved ions [9, 78-81] or through direct electron transfer between ENMs and cells [89]

Photoactivity	"Is the presence of light, of any wavelength, necessary for antibacterial activity?"	• While many studies treat "light-mediated mechanisms" as interchangeable with "photoactivated production of ROS," ion-driven mechanisms involving photo-dissolution processes have also been proposed [98-100]. • Since photoactivated ROS production can occur at both visible and UV wavelengths, light-mediated mechanisms may be overlooked when ambient lighting is not considered [101].
Membrane	"Does permeabilization of the cell membrane contribute significantly to antibacterial activity?"	• Although membrane permeability has long been used as an indicator for cell death [102], membrane permeability may occur without antibacterial activity, and vice versa [103, 104]. • Related questions not reviewed here include whether membrane permeability contributes to cell death *per se* [105] or simply increases cell vulnerability to other injuries (e.g. internalization of toxic components and/or ROS accumulation) [91, 106], as well as the different responses of the outer membrane (OM) and inner membrane (IM) in Gram negative bacteria [107, 108]. • This question overlaps with the "Contact" and "ROS" questions when the proposed pathway involves mechanical abrasion [82, 83] or lipid peroxidation [20, 47], respectively.
DNA	"Does damage to bacterial DNA contribute significantly to antibacterial activity?"	• Related questions not reviewed here include whether DNA damage occurs via a primary or secondary pathway (i.e., from interaction of ENMs themselves with DNA or from ion- or ROS-mediated damage) [109] as well as which types of DNA damage contribute most significantly to antibacterial activity [96]. • This question overlaps with the "Protein" question, since DNA repair enzyme abundance and activity were found to be key determinants of antibacterial activity for some antibiotics [110, 111], and ENMs may bind and deactivate cellular proteins, including enzymes [112, 113], as discussed below.
Protein	"Does the binding or inactivation of intra- or extracellular proteins by ENMs or their dissolved ions contribute significantly to antibacterial activity?"	• Many bacterial proteins have been shown to bind specifically to ENMs of one or more compositions [113, 114], and the dissolved ions of both silver and copper have been shown to target thiol groups present in amino acids [88, 115, 116]. • Enzymes are of particular interest due to their importance in bacterial metabolic processes [117] and may be inactivated by ENMs in several ways including direct blockage of the active site [113] or alteration of enzyme structure [114]. Additionally, the relationship between ENM concentration and enzyme activity may not be straightforward [114]. • Protein damage is often investigated alongside holistic metabolic effects using techniques such as transcriptome and proteome analysis [118], incorporating a variety of questions not captured within this binary framework.

2. Methods

2.1 Data Retrieval

A literature search was conducted in the Web of Science database using search terms listed in Table S1, and an initial screening of titles and abstracts was performed in order to identify experimental (rather than review) articles that pertained to antibacterial activity of the core compositions of interest (i.e., Ag, ZnO, TiO_2,Cu, CuO). Articles were separated by core composition because this study aims to investigate reported conclusions within ENM "classes" as they are typically defined in the literature, and different core compositions are typically treated as different "classes" of ENMs for the purposes of mechanistic investigation. Studies on nanocomposites (i.e., materials in which the "nano" component of the system is deliberately engineered to be of heterogeneous core composition) could not be accommodated within this framework and were excluded. However, ENMs composed of a metal or metal oxide core shell with a capping agent or MMO ENMs embedded on or within a non-nanostructured surface or matrix were not excluded; this is because studies comparing capped and uncapped or colloidal and immobilized particles often do not treat these as separate classes of ENMs in discussions of antibacterial mechanism. For the same reason, metal ENMs which are known to form an outer layer of oxide were included.

Since this analysis involves tabulating reported mechanistic conclusions and attributing differences in conclusions to ENM properties, exposure conditions, and experimental method choice, articles in the dataset must also meet three additional criteria: 1) one or more mechanistic conclusion(s) must be reported, 2) mechanistic conclusion(s) must be supported by at least one experiment, and 3) ENM properties must be documented. Therefore, only articles which specifically articulated a conclusion about the mechanism of antibacterial activity (as defined by the respective authors), supported this conclusion with at least one experimental method, and documented some type of ENM "characterization" (as defined by the respective authors) were included. No restrictions were imposed on definitions of "mechanism" and "characterization" because, in addition to affecting the ease of comparison across studies, it is possible that assumptions about which mechanistic pathways and ENM properties are relevant may influence or prematurely restrict the conclusions reached. Therefore, it was important to capture how discrepancies in the way these terms are defined by different authors may contribute to confusion or disagreement. As summarized in Table 2, the initial search returned more than 11,000 articles, but applying the screens described above yielded a final dataset of 318 articles.

Table 2. Number of articles included after each stage of the screening process (note: the sum of the final column is greater because studies which examined multiple core compositions are "double-counted" in multiple composition groups).

Core Composition	Total returned in search	Passed initial screening	Reached mechanism conclusion	Performed mechanism-targeted experiment	Included ENM characterization
Cu/CuO	1623	96	72	66	60
TiO_2	2434	81	61	54	44
Ag	4780	359	205	178	160
ZnO	2211	191	133	119	99
Total	*11048*	*727*	*471*	*417*	*363*

2.2 Correlating Study Design Variables with Mechanistic Conclusions

The data collected from each article were represented as binary "conclusions" and "study design variables" (Table 3). For each mechanism question (Table 1) investigated in each study, a "positive" conclusion was recorded if the authors of the study reported an affirmative answer to the question and a "negative" conclusion was recorded if the authors of the study reported a negative answer. In order to accurately represent authors' interpretations of their results, no re-interpretation of experimental findings was performed. For example, if reported data appeared to support enhanced antibacterial activity in the presence of light, but the authors of the study did not explicitly discuss the mechanistic implications of this finding, no conclusion was recorded for "photoactivity."

The study design variables analyzed here are listed in full in Table S2. Examples of study design variables include "ENM had at least one dimension smaller than 10nm as measured by TEM" (part of the "ENMs… Properties… Size" set of variables), "Used a Gram-negative bacterium" (part of the "Bacterium… Gram type" set), and "Performed mechanism-targeted experiments under ambient (visible-spectrum) light" (part of the "Methodology… Exposure Conditions… Presence (and wavelength) of light" set). Within the "Mechanism-targeted experimental methods" category of study design variables, the 181 distinct methods identified (referred to as "Techniques" hereafter) were further sorted into 30 "Approaches" and seven "Groups." This categorization aims to lend coherence to the long list of Techniques by highlighting key similarities and differences. Different Techniques within one Approach utilize different instruments or reagents but purport to measure similar endpoints; different Approaches within the same Group aim to measure different endpoints, but researchers apply similar rationales to interpret results and draw mechanistic conclusions. Aggregation of individual Techniques into Approaches and Groups also provided larger sample sizes for comparing methods *via* statistical tests, which was desirable because the majority of Techniques were used in a small number of (i.e. fewer than five) studies.

For statistical analyses, a binomial distribution of conclusions was constructed for each of the eight mechanism questions (Table 1) within each core composition. The influence of each study design variable on reported conclusions was then assessed as follows. First, the distribution of conclusions reported in studies where a given study design variable was true was compared to the distribution of conclusions reported in studies where a given study design variable was false. If the difference between these distributions was found by Fisher's exact test to have a less than five percent probability of occurring by coincidence (i.e. $p < 0.05$), the study design variable was determined to affect conclusions to a statistically significant degree. Since core composition is expected to influence mechanism, this analysis was performed within core compositions only. To complement statistical analyses, a review of the available literature pertaining to the advantages and limitations of the reviewed Techniques, Approaches, and Groups of methods was conducted.

Further analysis was directed at studies which investigated multiple mechanisms. In order to reveal correlations between reported conclusions for different mechanism questions as they exist in the literature, the "null hypothesis" adopted for this analysis was that the eight

mechanism questions are independent of one another. When this "null hypothesis" is shown to be untrue, it may be because a relationship between mechanisms actually exists (e.g., an ENM internalization-driven mechanism is necessarily also a contact-mediated mechanism) or because a relationship is only assumed to exist (e.g. ion- and contact-mediated mechanisms are sometimes believed to be mutually exclusive). Metrics used include 1) the percent of studies which investigated two mechanisms simultaneously (of studies which investigated one or the other), and 2) the percent of studies which reported positive conclusions for two mechanisms (of studies which reported positive conclusions for one or the other).

Table 3. Data collected from each article in the dataset. Each entry in the green zone corresponds to a category of binary "study design variables" (Table S2 lists the specific variables within each category). The blue zone lists the eight included mechanistic questions (Table 1), the reported answers to which were coded as binary "conclusions" that could be "positive" or "negative."

ENMs		**Bacterium**	**Methodology**			**Conclusions**
Core Compositions	*Properties*			*Exposure Conditions*	*Mechanism-targeted methods*	Ions
Ag Cu CuO TiO2 ZnO	Size Surface coating and charge Mode of delivery (colloidal or immobilized)	Gram type	ENM characterization (16 methods) Non-mechanistic antibacterial activity assessment (8 methods)	Presence (and wavelength) of light during exposure ENM aggregation in exposure media	181 "Techniques" ⇩ 30 "Approaches" ⇩ 7 "Groups"	Contact Internalization ROS Photoactivity Membrane DNA Protein

2.3 Assessing the Consistency of Mechanism-Targeted Experimental Methods

Subsequent analyses aimed to quantify the tendency of experimental methods to yield similar or different conclusions for a given mechanism question, within a given core composition. Such differences are a potentially important consideration when evaluating experimental methods, since they may stem from different degrees of sensitivity and/or ease of interpretation between methods. A new metric, termed the "Consistency Score," was defined as:

$$\left|0.5 - \frac{Number\ of\ positive\ conclusions}{Total\ number\ of\ conclusions}\right| \times 200$$

Since the absolute value of the average of a binomial distribution captures the "spread" of the distribution (similar to a standard deviation), the Consistency Score provides a metric for the degree of consensus that exists within a set of studies without regard for whether that consensus favors positive or negative conclusions. This allows the consistency of conclusions to be quantified even across sets of studies which are expected to differ in terms of the "true" conclusion. The highest possible Consistency Score is 100, which corresponds to unanimous agreement within a set of studies, while a score of zero corresponds to equal numbers of positive and negative conclusions within a set of studies.

For each of the eight mechanism questions, Consistency Scores were calculated for subsets of studies which used each Group, Approach, and Technique to evaluate the relevant question. This was first done within each core composition. Weighted averages were then calculated across all core compositions to yield an overall Consistency Score for each Group, Approach, and Technique as applied to each mechanism. As described above, the weighted average could be used because the Consistency Score depends only on the degree of spread within the distribution of conclusions, not on whether the consensus favors positive or negative conclusions, rendering the differences in conclusions that would be expected to arise between different core compositions irrelevant. A bootstrapping technique, in which the original dataset was randomly sampled with replacement to generate 200 "re-samples" of the same size as the original dataset and a Consistency Score was calculated for each of these re-samples, was used to obtain distributions of Consistency Scores. The mean and standard error of the resultant distributions were then used to obtain 95% confidence intervals for the overall Consistency Score of each Group, Approach, and Technique.

3. Results and Discussion

3.1 Summary of Reported Mechanistic Conclusions and Relationship with Study Design

Figure 1 summarizes the mechanistic conclusions reported by studies in the dataset. Within the body of literature analyzed, all eight mechanistic questions have yielded both positive and negative conclusions within all five core compositions. The number of studies for each core composition is unequally distributed, with nAg the subject of significantly more studies than other ENM core compositions. The prevalence of nAg studies is particularly clear for less-studied questions including internalization, DNA damage, and protein damage. The proportion of studies investigating each mechanistic question is roughly equal across core compositions, with the exception of photoactivity, where nTiO2 and nZnO account for the majority of studies.

A majority of studies in the dataset investigated questions of ion-, contact-, ROS-, and membrane permeabilization-mediated toxicity, with each of these questions being the subject of more than 150 studies across the five core compositions. Questions of particle internalization, photoactivity, DNA damage, and protein damage have received less attention, with a total of between 30 and 60 studies each. This discrepancy could have several explanations. One reason may be that some questions are investigated less frequently because they are perceived to be "settled" already, that is, they are widely believed to either contribute or not contribute to the

antibacterial activity of a given core composition. Alternatively, the complexity and expense of the experiments required to test for some mechanistic questions may cause these questions to be investigated less frequently.

The ratio of positive to negative conclusions for most mechanism questions across all core composition groups suggests considerable influence from ENM properties, exposure conditions, and/or experimental methods. Exceptions include membrane permeabilization and protein damage, for which there is near-unanimous agreement in conclusions within the more commonly studied core compositions, Ag and ZnO. For other questions, all cases in which conclusions appear to agree unanimously draw on fewer than 10 studies, indicating that the question may be settled or may not yet be sufficiently well-studied. Subsequent analysis aims to explore the causes of disagreement as they relate to study design variables outlined in Table 3.

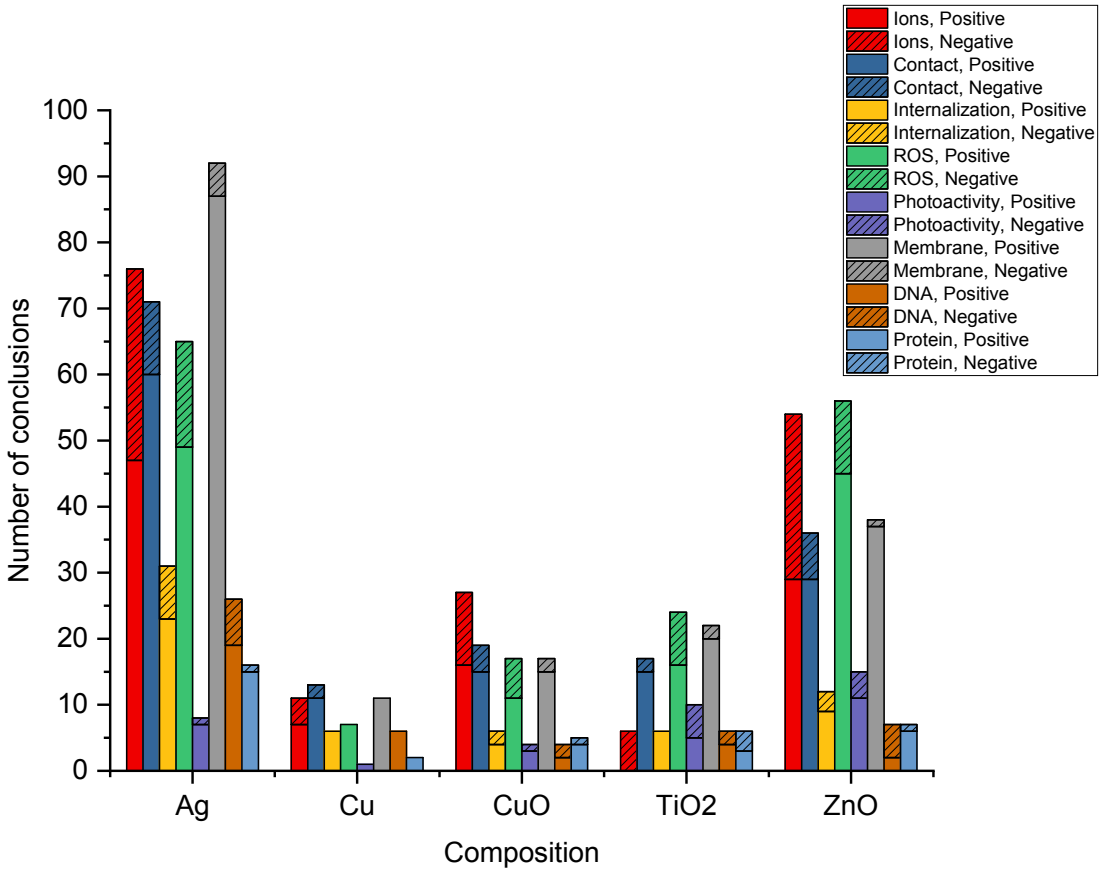


Figure 1. Number of positive and negative conclusions for each mechanistic question, by core composition. In some cases, a single study reported multiple conclusions across several core compositions and/or mechanism questions.

Figure 2 summarizes three important indicators of the relationship between study design and reported conclusions. First, the percent of conclusions for each question which were supported by experiment is given in the leftmost column, since studies may have performed an

experiment for one question but based conclusions for other questions on assumptions and/or previous studies, which may have utilized dissimilar exposure conditions. Second, the percentage of studies in the dataset which reported any information for each category of study design variable is given, because not all studies specified all ENM properties or exposure conditions. Finally, for each of the eight mechanism questions, categories of study design variables containing at least one variable that generated statistically significant differences in conclusions (as defined in section 2.2, "Correlating Study Design Variables with Mechanistic Conclusions") are highlighted.

Despite the criterion that included studies must include at least one experiment that targeted antibacterial mechanism, the number of studies that reported a conclusion for a given mechanism was generally found to be higher (usually by 10-20%, but more in some cases) than the number of studies which performed an experiment to test for that mechanism. This was found to result from the aforementioned practice of investigating some questions experimentally while drawing conclusions about others via "process of elimination" or a review of previous literature. These common practices may compound disagreement and perceived ambiguity in the literature by, for the former, prematurely assuming the mutual exclusivity of mechanisms and, for the latter, combining conclusions that were reached under different conditions. Conclusions reached through these means are therefore excluded from Figure 1 and subsequent analyses.

Additionally, reporting of potentially relevant ENM properties and exposure conditions was found to be incomplete. Since comprehensive reporting of exposure conditions [52] and characterization of ENMs [73] have both been identified as important to the interpretation and comparison of results, this gap in reporting is a likely contributor to real or perceived mechanistic uncertainty. While the vast majority of studies specified ENM core composition and size as measured by TEM or equivalent method, as well as bacterium used, fewer studies characterized the aggregation state of ENMs in exposure media and the surface charge or presence of any capping agents. Furthermore, only a small minority of studies which did not specifically investigate light-mediated mechanisms indicated the lighting conditions used in toxicity or mechanistic experiments, suggesting that the importance of light in some mechanistic pathways may be underexplored.

Contrary to expectation, few statistically significant differences in conclusions were identified between core compositions, which may indicate that the five core compositions reviewed overlap more in antibacterial mechanism than their separate treatment in the literature may suggest. In contrast, other study design variables were found to yield many statistically significant differences in conclusions. Differences in conclusions which emerge based on ENM size, capping agent, bacterium, antibacterial activity assessment methods, and lighting conditions suggest that different mechanisms may predominate in different conditions (e.g., a separate mechanism actually exists for Gram positive versus Gram negative bacteria, or for small versus large ENMs, or for antibacterial activity in UV light versus in darkness). In contrast, differences in conclusions which emerge based on characterization methods suggest that researcher's subjective interpretations of results may influence conclusions (e.g., information gleaned from

characterization may influence how the results of mechanism-targeted methods are interpreted by researchers to formulate conclusions).

Taken together, these results confirm that some perceived ambiguity surrounding mechanism is likely attributable to differences in study design that are not reported or not considered when researchers design experiments and interpret their results to yield conclusions. However, it is difficult to differentiate between cases of different conclusions representing real differences in mechanism (due to dissimilar ENM properties, model organisms, or exposure conditions) and cases of different conclusions resulting from choice of experimental methods and/or subsequent interpretation of results. Simple measures to promote clarity include more complete reporting of ENM properties and exposure conditions as previously suggested [52, 73], as well as separating mechanistic conclusions reached through experiment from mechanistic conclusions inferred from previous literature.

		ENM Properties				*Study Methodology*					
Percent of conclusions supported by experiment	**Mechanism Question**	**Composition**	**Size (dry)**	**Size (hydrodynamic)**	**Capping agent, surface charge**	**Bacterium**	**Characterization methods**	**Antibacterial activity assessment methods**	**Lighting conditions**	**Mechanism-targeted methods**	**Co-investigation of multiple questions**
89%	**Ion**	100%	92%	72%	49%	90%	100%	100%	20%	100%	N/A
81%	**Contact**	100%	92%	64%	50%	89%	100%	100%	21%	100%	N/A
69%	**Internalization**	100%	93%	67%	38%	93%	100%	100%	18%	100%	N/A
84%	**ROS**	100%	89%	60%	53%	95%	100%	100%	31%	100%	N/A
87%	**Photoactivity**	100%	89%	57%	46%	94%	100%	100%	97%	100%	N/A
90%	**Membrane**	100%	93%	57%	57%	95%	100%	100%	17%	100%	N/A
86%	**DNA**	100%	94%	47%	53%	98%	100%	100%	2%	100%	N/A
42%	**Protein**	100%	91%	58%	39%	100%	100%	100%	18%	100%	N/A

Figure 2. The leftmost column indicates the percent of reported conclusions for each mechanism question which were found to be supported by at least one experiment. The remaining columns show statistically significant differences in conclusions for each question based on variables related to ENM properties and study methodology, overlaid with the percent of studies that reported any information about each variable. Highlighted cells indicate that dividing the dataset based on the variable on the horizontal axis created a difference in conclusions about the mechanism question on the vertical axis that was large enough to be considered statistically significant ($p < 0.05$ according to Fisher's exact test). With the exception of core composition, all variables were examined within core composition groups; a highlighted cell therefore indicates that a statistically significant difference was identified within one or more compositions. No statistically significant differences in conclusions emerged for immobilized *versus* colloidal

delivery methods, so this variable category is not displayed. In Figure S1 and S2, the analysis is disaggregated by core composition and whether the variable in question skewed conclusions towards positive or negative is indicated.

Among studies in the dataset, a tendency to frame possible mechanisms as mutually exclusive was noted. One common example is the perceived "ion" versus "particle" dichotomy also noted in previous reviews [62]. In order to better understand whether and how this may affect conclusions, studies which investigated more than one mechanistic question within a given core composition were examined (Figure 3). This investigation also sought to identify areas in which the relationships between mechanisms may warrant further exploration, a development which would complement moving away from "binary" definitions of questions to introduce greater nuance into mechanistic discussions.

The patterns that emerge highlight existing preconceptions of the relationships between mechanisms which may, in turn, shape study design. For example, ion- and particle-driven mechanisms are frequently investigated together, and photoactivity is always investigated alongside ROS, which aligns with the general consensus that these two pairs of mechanisms are related. On the other hand, mechanisms related to specific cellular targets (i.e., membrane damage, DNA damage, and protein damage) are usually not investigated alongside ion- or particle-driven antibacterial activity. These relationships could be explored more in future work in order to relate the "origins" of ENM antibacterial activity to specific cellular effects. Additionally, future membrane studies can focus on protein damage in order to obtain more detailed information about the nature and origins of membrane permeabilization, as well as the relationship between membrane effects and metabolic effects. Further, the link between ions and photoactivity remains underexplored, and may yield insights about proposed "photo-dissolution" processes in some core compositions [98-100]. Finally, while studies on internalization often feature investigations of particle-driven antibacterial activity, other mechanisms can be explored more extensively in combination with internalization. For example, studies on DNA and protein damage can provide insights to the intracellular targets of ENMs following internalization.

Interestingly, it appears that studies which reached a positive conclusion for one mechanism were, to varying degrees, more likely to draw negative conclusions for any other mechanism. This is especially true for ion- and particle-mediated antibacterial activity. While one possible explanation is that this trend is reflective of reality (i.e. that one mechanism actually does dominate at a given time), a more plausible explanation is that the trend is not reflective of reality (i.e. that belief in a single, dominant mechanism continues to guide many investigations). In this case, reaching a positive conclusion about one mechanism would introduce bias that would lead to other mechanisms being prematurely ruled out. While it was not possible to test these hypotheses with greater statistical rigor using this dataset, the questions raised merit further investigation, which would be supported by more studies examining multiple mechanistic questions.

a.

	Ion	Contact	Internalization	ROS	Photoactivity	Membrane	DNA	Protein
Ion		72%	53%	50%	58%	42%	32%	45%
Contact	57%		75%	40%	44%	47%	36%	33%
Internalization	20%	31%		17%	6%	24%	26%	21%
ROS	52%	51%	48%		100%	53%	62%	61%
Photoactivity	10%	11%	3%	16%		6%	0%	6%
Membrane	42%	58%	77%	56%	33%		77%	61%
DNA	10%	13%	22%	18%	0%	21%		18%
Protein	8%	9%	12%	12%	6%	11%	13%	

b.

	Ion	Contact	Internalization	ROS	Photoactivity	Membrane	DNA	Protein
Ion		-59%	-32%	-46%	-35%	-48%	-29%	-39%
Contact	-38%		-12%	-17%	0%	-10%	-18%	-21%
Internalization	-28%	-19%		-19%	-20%	-22%	-19%	-16%
ROS	-32%	-29%	-19%		-17%	-22%	-15%	-21%
Photoactivity	-8%	-37%	-33%	-17%		-20%	0%	-50%
Membrane	-13%	-3%	-8%	-4%	0%		0%	-9%
DNA	-29%	-33%	-19%	-19%	0%	-29%		-15%
Protein	-4%	-6%	-5%	-6%	0%	-7%	-6%	

Figure 3. Frequency with which different mechanistic questions are co-investigated and the extent to which this affects the mechanistic conclusions. (a) Of the studies that investigated the question on the horizontal axis, what percent investigated the question on the vertical axis? Red indicates less overlap and green indicates more overlap. (b) Within studies that investigated both the question on the vertical axis and the question on the horizontal axis, what was the percentage point difference between studies that drew positive conclusions for both the question on the horizontal axis and the question on the vertical axis, versus studies that drew a positive conclusion for the question on the horizontal axis but a negative conclusion for the question on the vertical axis? Darker color indicates greater difference.

3.2 Summary and Assessment of Mechanism-Targeted Methods

The remainder of the analysis aims to further illuminate the role that method choice may play in sowing perceived uncertainty. 181 distinct experimental "Techniques" of mechanistic investigation, further categorized into 30 "Approaches" and seven "Groups," are summarized in Table 4. The "Uses" column lists the full set of mechanistic questions to which each Technique was applied in studies in the dataset, which demonstrates how a single method is often applied to multiple questions. While some methods aim to provide binary information about the presence or absence of a given mechanism, others are tailored towards more detailed insight. For example, the propidium iodide (PI) stain (Group 5, Approach 18, Technique 97) is commonly used to establish loss of membrane integrity, but Fourier-transform infrared spectroscopy (FTIR) of the cell mass (Group 3, Approach 11, Technique 53) and scanning electron microscopy (SEM)

(Group 4, Approach 13, Technique 64) can be used to understand chemical changes in the cell membrane and the extent and character of membrane permeabilization, respectively. All methods are expected to have advantages and limitations, although these may not be documented in the literature; those which were found to be documented in the literature are summarized in Table S5.

Statistically significant differences in conclusions were identified between studies which used certain Groups, Approaches, and Techniques as compared to studies which did not; these Groups, Approaches, and Techniques are highlighted in Table 4. These differences were sometimes, but not always, identified in methods for which advantages or limitations were reported in the literature (Table S5). Some methods which produced differences in conclusions were the same methods for which multiple limitations were reported (e.g., Group 1 "elimination" methods and Group 4 "ENM localization or visualization" methods) while others were methods for which multiple advantages were reported (e.g. some Group 2 "genomic" and Group 3 "chemical analysis" methods).

In some cases, relatively untested methods for which no advantages or limitations are yet reported yielded a difference in conclusions. Examples include the membrane barrier and filtration methods for ion isolation (Group 1, Approach 2, Techniques 6 and 7). A possible explanation may be "bio-dissolution" processes (**Error! Reference source not found.**) in which cells mediate the release of ions from ENMs, which would not be captured in these techniques [63-65]. Additionally, some methods were reported to have strong advantages but did not yield any statistically significant difference in conclusions from other methods; an example of this phenomenon is electron paramagnetic resonance (EPR) spectroscopy, Group 5, Approach 20, Technique 119, as compared to other Group 5 "cell effects" methods. It is possible that this is due to small sample size, since statistically significant differences tended to emerge only for the most commonly used methods. While it is not possible to identify with certainty the source of differing conclusions between methods, these instances confirm that the experimental methods used may influence the conclusions reached, and that further exploration by microbiologists of the advantages and limitations of commonly used methods would be beneficial.

Figure 4 demonstrates that a small set of methods, many of which are facile and yield binary yes/no answers about the presence or absence of mechanisms, dominate mechanistic investigations. Several of the most-used assays, including comparison to soluble salts (Group 1, Approach 5, Technique 30), correlation with ion release (Group 7, Approach 27, Techniques 145-150), electron microscopy (Group 4, Approaches 13 and 14, Techniques 64 and 67), and DCFH-DA (Group 5, approach 18, Technique 93), have significant, documented limitations (Table S5) that may impede resolving the intended mechanistic question(s). Additionally, nearly 150 of 181 Techniques are used in fewer than 10 studies; statistical analysis of these methods is difficult given the small sample size, and no attention has been devoted to their advantages and limitations in the literature. Taken together, these observations suggest a need to critically evaluate the wide breadth of mechanism-targeted methods currently in use and promote a smaller set of methods that are known to be reliable and tailored to provide the insight needed drive

forward understanding. This shift may entail moving away from long-standing and commonly used assays in favor of those which may yield more information or be subject to fewer confounding variables.

Table 4. Summary of Techniques used to study mechanistic questions, categorized into 30 "Approaches" and seven "Groups." The "Endpoint" and "Type of Assay" columns give basic information about the Technique, while the "Uses" column lists all the mechanistic questions to which the Technique was applied within the dataset. A highlighted Group, Approach, or Technique indicates that the distribution of conclusions produced by that method differed from those produced by other methods to a statistically significant degree ($p < 0.05$ according to Fisher's exact test). Methods that were used to target multiple mechanistic questions were compared to other methods within each question group separately (e.g. a method which was used to investigate both ions and ROS was compared first to all other methods used to study ions, and then to all other methods used to study ROS). Comparisons were performed within the five core compositions only. Highlighted methods did not necessarily produce statistically significant differences in conclusions across all uses or across all core compositions. Only Techniques used in three or more studies are displayed here, with others listed in Table S3. A list of the abbreviations used here can be found in Table S4.

Group	Approach	Technique	Endpoint	Type of Assay	Uses	References
1 "Elimination" methods: Modifications of antibacterial activity or other assays designed to isolate the contribution from a single mechanism (i.e., by blocking the action of that mechanism or alternative mechanisms)	1 Isolate dissolved ion effect	2 Insoluble salt-forming agent addition, including orthophosphate, sulfide, sodium thiosulfate, sodium chloride	difference in cell death relative to control	(dependent on antibacterial activity assay)	ion, contact	[119-122]
		3 Chelator addition, including NAC, EDTA, bathocuproine, neocuproine			ion, contact	[45, 78, 119, 123-129]
	2 Isolate particle effect	5 Compare colloidal to immobilized particles			ion, contact, internalization	[122, 130, 131]
		6 Compare to ENM-free filtrate			ion, contact	[18, 20, 51, 54, 120, 132-138]
		7 Membrane barrier			ion, contact	[87, 139-141]
		8 Induce aggregation, including extracellular polymeric substance (EPS) addition			ion, contact	[49, 51, 141-143]
		9 Compare to inert ENMs of same size and morphology			contact	[81, 88, 144-146]
		10 Compare antibacterial activity in aerobic and anaerobic environment			ion, contact, ROS	[28, 119, 147]
	3 Isolate ROS effect	11 Cysteine (e.g. NAC) addition			ROS	[20, 28, 81, 135, 148-151]
		12 Ascorbic acid addition			ROS	[149, 152, 153]
		16 GSH addition			ROS	[121, 139, 152]
		17 SOD addition			ROS	[126, 135, 151, 154]
		18 CAT addition			ROS	[126, 151, 155, 156]
	4 Isolate light exposure effect	27 NP pre-irradiation			photoactivity	[9, 99, 157-159]
		28 Compare antibacterial activity in light vs dark conditions			photoactivity	[85, 129, 154, 160-183]
	6 Compare to positive control	30 Soluble salt (e.g. AgNO3, CuSO4, ZnCl2)			ion, contact	[1, 2, 23, 25, 45, 50, 55, 56, 64, 78, 81, 83, 87, 88, 99, 112, 123, 125, 129, 132, 139-142, 145,

							158, 163, 165, 174, 180, 184-219]
		32	H2O2			ROS	[20, 129, 141, 216]
		33	Detergent or bacteriolytic agent			membrane	[17, 191, 220, 221]
2 "Genomic" methods: Using information about bacterial genomes to infer the mechanism of antibacterial activity based on cellular responses to ENMs	7 Cellular methods: bioreporters and knockout strains	34	Recombinant bioluminescent reporter strain	various (incl. intracellular ROS species, bioavailable metal, DNA damage, membrane damage)	chemiluminometric	ion, ROS, membrane, DNA, protein	[1, 55, 56, 64, 78, 126, 138, 148, 175, 184, 215, 222-225]
		35	Single-gene deletion ("knockout") strain	difference in antibacterial activity	(dependent on antibacterial activity assay)	ion, ROS, DNA	[55, 56, 64, 225, 226]
	8 High-throughput methods: transcriptome and proteome analysis	36	Transcriptome analysis	quantity of RNA produced relative to control (indicator for gene up- and down-regulation)	various, usually microarray or high-throughput sequencing	ion, contact, ROS, membrane, DNA, protein	[8, 20, 88, 137, 148, 175, 178, 192, 209, 214, 226-238]
		37	Proteome analysis	quantity of protein produced relative to control (indicator for gene up- and down-regulation)	various, usually mass spectrometry	ion, contact, ROS, membrane, DNA, protein	[127, 152, 174, 191, 194, 195, 219, 232, 234, 235, 239-242]
	9 Enzyme activity	39	Ammonium molybdate assay	CAT activity	colorimetric	ROS, protein	[45, 127, 135, 152, 221, 243]
		43	DTNB assay	GR activity		ROS, protein	[129, 135, 244]
		44	NADH assay (with INT or resorufin)	Dehydrogenase, including LDH, activity	colorimetric or fluorometric	protein, ROS	[32, 127, 198, 208, 217, 239, 243-246]
3 "Chemical analysis" methods: Using information about the chemical and electronic state of metals or biomolecules following ENM exposure to bacteria in order to pinpoint the origin of antibacterial activity and relevant chemical processes	10 Chemical analysis of metals	50	XAS analysis of metal in cell mass or supernatant	Local geometric and electronic structure of metal atoms	XAS	ion, contact, internalization, ROS	[19, 92, 93, 247, 248]
	11 Chemical analysis of cellular components	53	FTIR of cellular fraction or extracellular polymeric substances (EPS)	Chemical changes in cellular components	FTIR	contact, membrane, protein, DNA	[26, 85, 93, 155, 174, 185, 206, 249-256]
		54	Raman spectroscopy of cellular fraction		Raman spectroscopy	protein, DNA	[85, 136, 257]
		56	TBA/MDA assay	Degree of lipid peroxidation	colorimetric or fluorometric	ROS, membrane	[20, 47, 121, 135, 151, 161, 162, 169, 184, 202, 243, 254, 258-267]
4 "ENM localization or visualization" methods: Probing the localization of ENMs and/or ions and cell morphology changes after cell exposure to ENMs	12 Metal partitioning study	61	Metal concentration in cellular fraction, including sucrose gradient centrifugation assay	Quantity of metal associated with cells	ICP-MS	ion, contact, internalization	[11, 24, 50, 112, 219, 238, 247, 268, 269]
	13 High-resolution imaging of cell surfaces	64	SEM	Qualitative attributes of cell/particle interaction	Image of cell exterior	contact, membrane	[26, 27, 32, 84, 121, 129, 136, 141, 143, 150, 151, 155, 166, 173, 198, 204, 238, 262, 264, 265, 270-296]
		65	SEM with elemental mapping (EDX or synchrotron XFM)		Image of cell exterior with elemental mapping	contact, membrane	[16, 217, 218, 253, 297-300]
		66	AFM		Image of cell surface	contact, membrane	[27, 55, 56, 125, 131, 224, 252, 255, 301-303]
	14 High-resolution imaging of cell	67	TEM		Image of cell interior	membrane, contact,	[17, 32, 47, 55, 56, 82, 86, 92, 128, 129, 141,

	interiors					internalization, DNA	152, 165, 167, 171, 185, 204, 207, 210, 229, 237-239, 249, 254, 265, 288, 304-318]
		68	TEM with elemental mapping (EDX or synchrotron XFM)		Image of cell interior with elemental mapping	contact, internalization, membrane	[29, 31, 50, 54, 126, 184, 193, 195, 237, 250, 253, 260, 297-299, 319-321]
	15 Light microscopy and light scattering techniques	69	CLSM	Qualitative attributes of cell/ENM interaction (for fluorescently labeled or intrinsically fluorescent ENMs)	fluorescence microscopy	contact, internalization, membrane, DNA	[183, 309, 322-325]
		72	Dark-field microscopy (may be equipped with HSI)	Relative strength of interactions between ENMs and cell surfaces	light microscopy	contact, membrane	[50, 192, 269, 326]
		74	ENM tracking with intrinsic fluorescence or fluorescent label (e.g. rhodamine B)	Localization of NPs within cells	fluorescence microscopy	contact, internalization	[196, 242, 281]
		75	Light scattering method for particle internalization	Ratio of forward- to side-scattered light (varies with cell granularity)	light microscopy	internalization	[86, 129, 276, 327]
5 "Cell effects" methods: Assays which gauge the degree of disruption caused to various cellular systems, thereby elucidating the specific effects that ENMs have on the cell	16 Membrane permeability via release of cellular components into extracellular space	76	ONPG hydrolysis assay	GAL leakage	colorimetric	membrane	[153, 242, 251, 265, 271, 283, 328]
		77	K+ and/or Mg2+ leakage	K+ and/or Mg+ in supernatant	AAS/AES or selective electrode	membrane	[129, 139, 197, 213, 232, 249, 265, 295, 316]
		80	Nucleic acid leakage	Nucleic acids in supernatant	UV-Vis	membrane	[16, 112, 124, 151, 153, 221, 242, 251, 270, 280, 287, 311, 323, 329-331]
		81	Sodium pyruvate assay	LDH in supernatant		membrane	[11, 23, 49, 195, 253, 256, 260, 324, 332, 333]
		82	Lowry method	Protein in supernatant	colorimetric	membrane	[153, 245, 276, 325, 328]
		83	Bradford method			membrane	[32, 155, 208, 221, 243, 246, 251, 290, 294, 295, 300, 312, 330, 332, 334-336]
		84	Miller method	Reducing sugar in supernatant		membrane	[208, 217, 243, 245, 246, 312, 330, 334-336]
		85	DNA release, including diphenylamine and PicoGreen assays	DNA in supernatant	fluorometric	membrane	[112, 124, 143, 293, 326]
	17 Genomic DNA extraction and analysis	89	DNA ladder assay	Degree of gDNA fragmentation	gel electrophoresis	DNA, protein	[47, 92, 153, 198, 258-261, 291, 295, 333, 337, 338]
	18 Intracellular probes for stress markers	93	DCFH-DA (including variants such as CM-H2DCFDA, ab113851-DCFDA, H2DCFDA, DCF-DA)	Intracellular ROS	fluorometric	ROS	[8, 9, 16-18, 20, 21, 23, 24, 28, 50, 84, 86, 121, 135, 143, 150-152, 166, 167, 170-172, 178-180, 184, 188, 211, 219, 236, 238, 243, 245, 253-256, 258, 260-264, 267-272, 276, 281, 282, 287, 288, 292, 310, 320, 324, 325, 327, 333, 339-343]

		94	NBT assay	Usually superoxide anion concentration; inhibition of stain is also used as a measure for SOD activity	colorimetric	ROS	[16, 99, 112, 127, 135, 152, 154, 253, 256, 259, 324, 330, 340, 344]
		95	Rhodamine dyes, including DHR6G and dihydrorhodamine 123	Intracellular ROS	fluorometric	ROS	[26, 262, 345, 346]
		97	Propidium iodide (PI) stain	Membrane permeability (enters cells with compromised membranes)		membrane	[16, 18, 19, 23, 24, 50, 84-86, 92, 93, 124, 128, 129, 132, 143, 151, 171, 180, 199, 204, 205, 217, 226, 238, 250, 253, 255, 264, 272, 274, 280, 288, 292, 331, 337, 341, 342, 347, 348]
		99	DiBAC4 stain	Membrane potential		membrane	[205, 217, 236]
		100	diSC3(5) assay for membrane potential			membrane	[47, 191, 276, 284, 326]
		101	1-NPN	Outer membrane permeability		membrane	[124, 197, 242, 264, 270, 287]
		102	DPH membrane fluidity assay	Membrane fluidity		membrane	[17, 276, 295, 341]
		104	TUNEL assay	Degree of apoptosis		DNA	[236, 276, 292]
		107	Annexin V			protein	[152, 236, 349]
		109	RedoxSensor assay	Reductase activity		ROS	[19, 92, 93]
	19 Other assays targeting general cell respiration or disturbance to cellular processes	110	DTNB assay for cellular GSH	Quantity of disulfide-containing molecules	colorimetric	ROS, protein	[129, 243, 255, 260, 262]
		115	Luciferin/trichloroacetic (TCA) assay for ATP content	Cell respiration	chemiluminometric	protein	[47, 51, 82, 151, 184, 191, 217, 243, 295]
		118	Intracellular K+ or Ca2+	Cell respiration (as a reflection of membrane damage and metabolic disruption)	flame AES	protein	[47, 191, 236]
		119	EPS quantification	EPS production (as a measure of cell viability or metabolic activity)	colorimetric	protein	[11, 16, 93]
		121	Phag-GFP expression assay	Degree of Phag-GFP expression relative to control	chemiluminometric	protein	[58, 92, 93]
	20 Other ROS quantification methods	124	EPR/ESR	ROS quantity (species depends on spin trap used)	ESR	ROS	[30, 154, 170, 174, 181, 184, 192, 235, 238, 254, 280, 292, 343, 350]
	21 Reverse mutation assay	125	Ames test	Number of mutations caused by material	number of colonies	DNA	[270, 287, 351, 352]
6 "Acellular" methods: Acellular techniques which examine interactions between ENMs and biomolecules	23 Acellular ENM-biomolecule interaction study	132	in vitro enzyme activity assay	Inhibition of enzyme activity in vitro	various, usually colorimetric	protein	[23, 47, 49, 195]
	24 Acellular DNA damage assays	136	in vitro DNA fragmentation assay	Degree of plasmid fragmentation	gel electrophoresis	DNA	[24, 259, 334]

7	"Correlation" methods: Establishing correlations between magnitude of antibacterial activity and other measured variables to identify what attributes of ENMs are relevant to antibacterial activity	27 Correlate antibacterial activity with ion release	145	ICP-MS, -OES, -AES	Concentration of ions in exposure media	ICP-MS, -OES, -AES	ion	[45, 49, 54, 56, 123, 125, 130, 132, 145, 161, 162, 185, 247, 259] [2, 8, 17, 24-26, 29, 30, 50, 55, 64, 88, 127-129, 147, 150, 151, 164, 165, 167, 174, 175, 180, 182, 191, 192, 194, 199, 201-203, 205, 207, 211-213, 216, 218, 219, 254-256, 285, 286, 292, 293, 298, 320, 327, 329, 340, 346, 347, 350, 353-364]
			146	AAS	Concentration of ions in exposure media	AAS	ion	[11, 19, 55, 56, 87, 99, 126, 138, 140, 143, 154, 155, 204, 206, 215, 223, 226, 274, 275, 280, 365, 366]
			150	silver/sulfide electrode	Concentration of ions in exposure media	selective electrode	ion	[58, 87, 140, 151, 188, 345]
		28 Correlate antibacterial activity with in vitro redox assay	152	GSH oxidation (DTNB)	ROS quantity	colorimetric	ROS, protein	[54, 161, 162, 256, 274, 282, 364]
			153	XTT	Superoxide radical quantity	colorimetric	ROS	[84, 95, 163-165, 182, 270, 274, 287, 364]
			155	Methyl orange	ROS quantity	colorimetric	photoactivity, ROS	[254, 357, 358]
			156	Methylene blue	ROS quantity	colorimetric	photoactivity, ROS	[157, 171, 172, 233, 329]
			157	3'-(p-aminophenyl) fluorescein (APF)	Hydroxyl radical quantity	fluorometric		[20, 188, 233]
			166	Terepthalic acid (or Phth)	Hydroxyl radical quantity	fluorometric	ROS	[154, 294, 344, 363]
			167	Luminol	Superoxide radical quantity	chemiluminometric	ROS	[184, 363]
			169	p-chlorobenzoic acid (pCBA)			ROS	[163-165, 182, 362]
			170	FFA			ROS	[163-165, 182]
		29 Correlate antibacterial activity with ENM property	176	PL spectroscopy for defect sites/oxygen vacancies	difference in antibacterial activity	(dependent on antibacterial activity assay)	ROS	[233, 344, 361, 367, 368]
			177	Morphology			ion, contact	[337, 356, 364]
			178	Zeta potential			contact, membrane	[2, 125, 149, 168, 223, 224, 253, 255, 256, 268, 320, 358]
			179	Surface coating			ion, contact	[46, 144, 189, 252, 369]

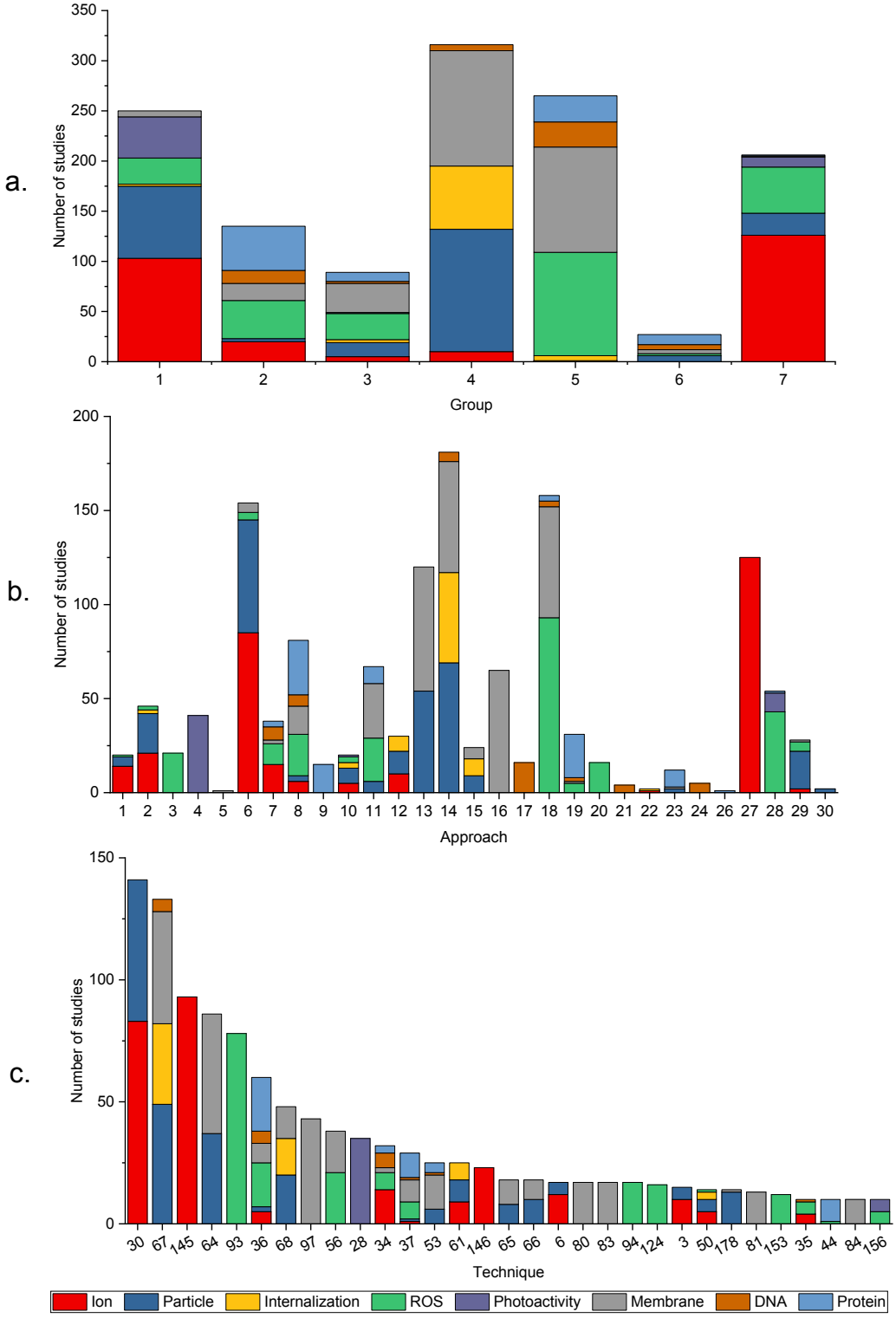


Figure 4. Number of times each (a) Group, (b) Approach, and (c) Technique was used, and the mechanistic question to which each was applied, as indicated by color. Note different y-axis scales for part (a), (b), and (c). For 3c (Technique), only Techniques which were used in 10 or more studies are shown.

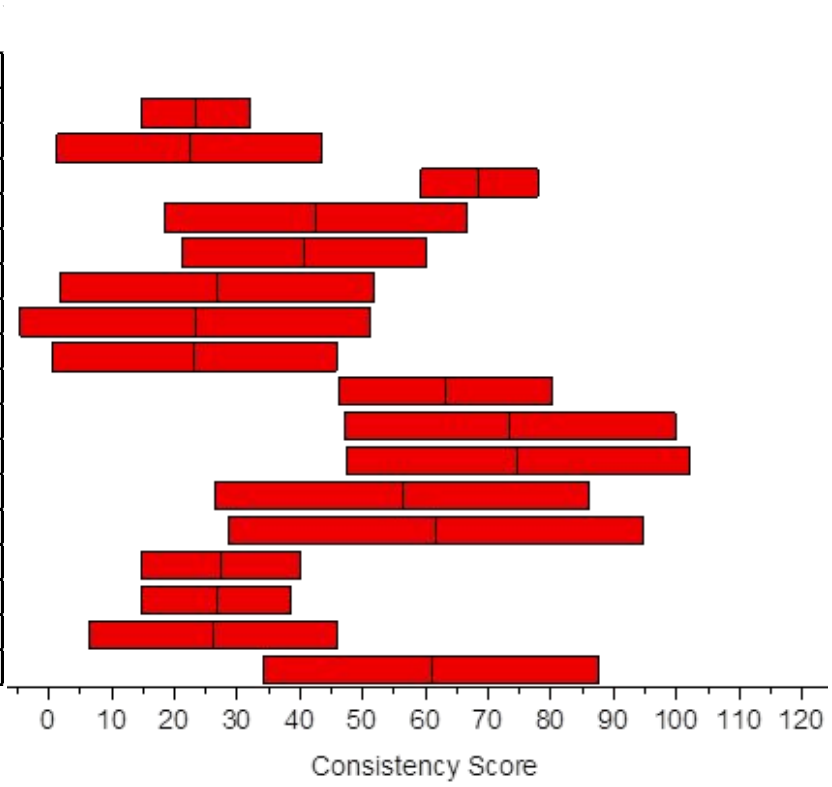
Figure 5 compares the Consistency Score (weighted average across all core compositions, with 95% bootstrap confidence intervals) of method Groups, Approaches, and Techniques used in 10 or more studies. A high Consistency Score for a method indicates that studies which used that method tended to draw the same conclusion about a given mechanism within a given core composition, regardless of whether that conclusion was positive or negative. A high Consistency Score does not, however, indicate that the method tends to produce the "correct" conclusion.

As previously noted, there are several possible explanations for the observed differences in Consistency Score between methods. The first explanation is that disagreement between studies is reflective of real differences in mechanism that may arise from, for example, differing ENM properties or experimental conditions; this would imply that methods with a low Consistency Score are relatively sensitive (i.e. precise and accurate). Another explanation is that disagreement between studies is not reflective of real differences in mechanism and arises instead from differing interpretations of a method's output; this would imply that methods with a lower Consistency Score are relatively difficult to interpret. Finally, a third explanation is that some methods are uninformative (i.e. precise but not necessarily accurate), consistently yielding the same, seemingly unambiguous result even when it does not reflect the "true" antibacterial mechanism for the core composition and exposure conditions of interest.

Identifying the underlying reasons for varying Consistency Scores will require further investigation. Possible avenues might include examining the statistically significant differences (as indicated by non-overlapping confidence intervals) between methods applied to the ions (Figure 5a) and ROS (Figure 5d) questions, as well as why the same method may have different Consistency Scores when applied to different questions, as in the case of Group 4 methods for particle (Figure 5b) versus internalization (Figure 5c) questions (although these differences are not statistically significant). Additionally, since some methods applied to the membrane permeabilization question (Figure 5f) had the highest possible Consistency Score of 100, corresponding to unanimous agreement among the studies reviewed within every core composition, it may be beneficial to examine more closely those methods for which disagreement was present. If this disagreement is found to be attributable to real mechanistic differences, in line with the first explanation described above, this may reveal facets of the membrane permeabilization question which remain to be resolved despite relative consensus about its general role in antibacterial activity (as discussed in section 3.1, "Summary of Reported Mechanistic Conclusions and Relationship with Study Design").

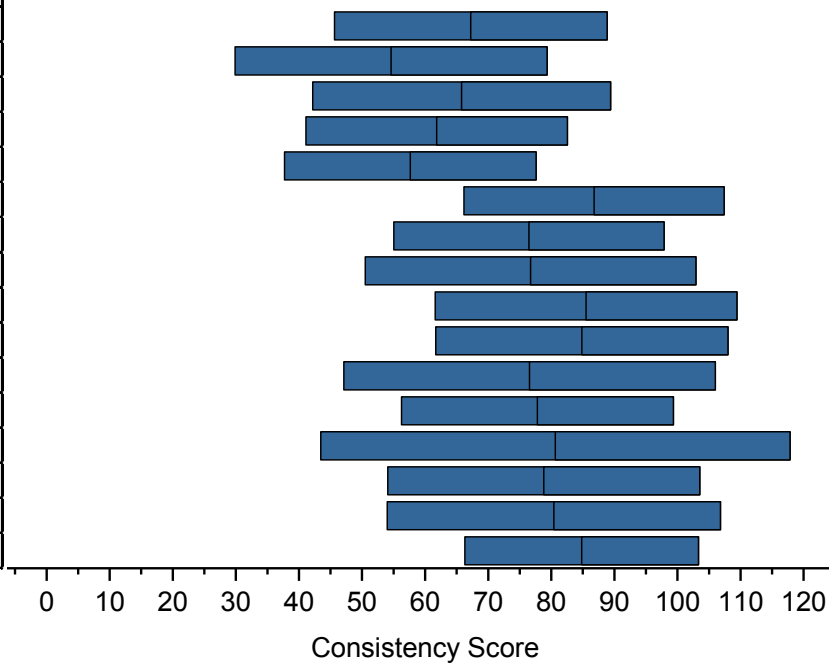
a. Ion

Group	Approach	Technique
		All studies
1		All Group 1
	1	All Approach 1
		Technique 3
	2	All Approach 2
		Technique 6
	6	All Approach 6
		Technique 30
2		All Group 2
	7	All Approach 7
		Technique 34
4		All Group 4
	12	All Approach 12
7		All Group 7
	27	All Approach 27
		Technique 145
		Technique 146



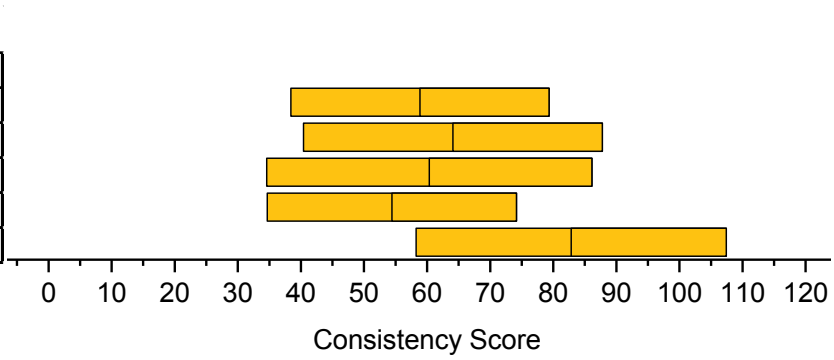
b. Contact

Group	Approach	Technique
		All studies
1		All Group 1
	2	All Approach 2
	6	All Approach 6
		Technique 30
3		All Group 3
4		All Group 4
	12	All Approach 12
	13	All Approach 13
		Technique 64
	14	All Approach 14
		Technique 67
		Technique 68
7		All Group 7
	29	All Approach 29
		Technique 178



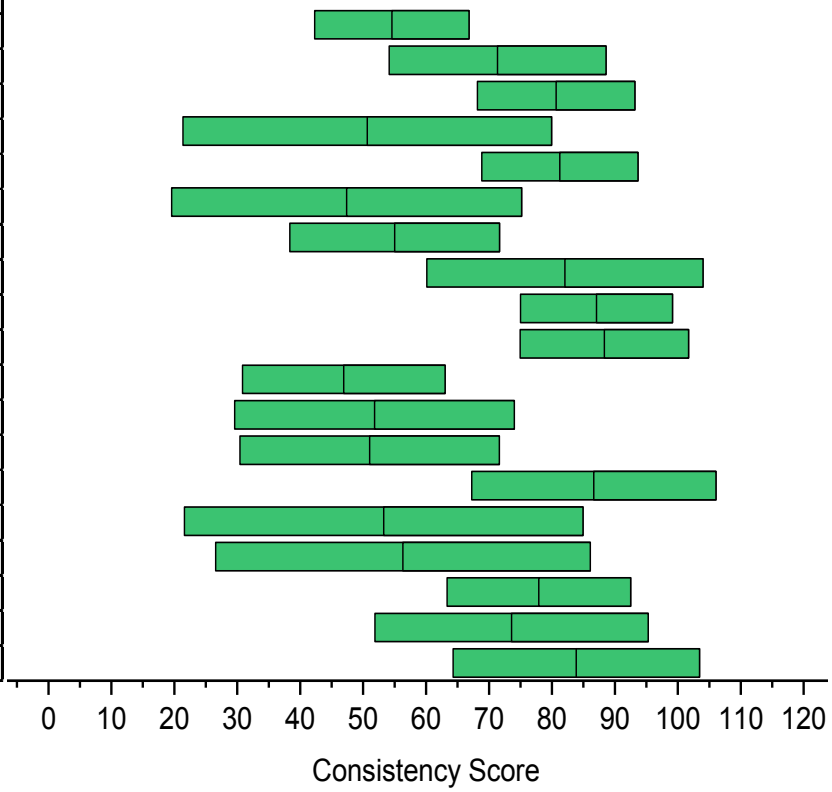
c. Internalization

Group	Approach	Technique
		All studies
4		All Group 4
	14	All Approach 14
		Technique 67
		Technique 68



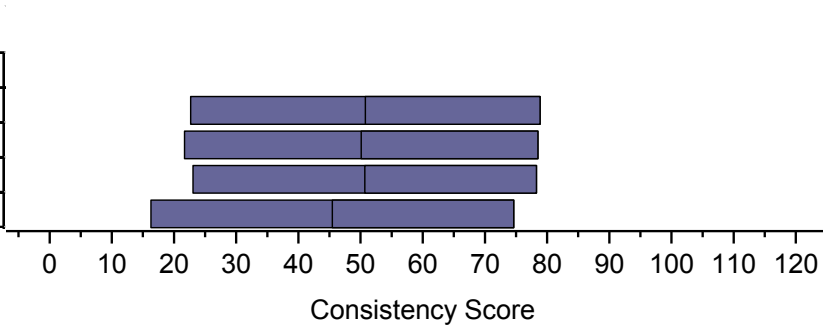
d. ROS

Group	Approach	Technique
		All studies
1		All Group 1
	3	All Approach 3
2		All Group 2
	7	All Approach 7
	8	All Approach 8
		Technique 36
3		All Group 3
	11	All Approach 11
		Technique 56
5		All Group 5
	18	All Approach 18
		Technique 93
		Technique 94
	20	All Approach 20
		Technique 124
7		All Group 7
	28	All Approach 28
		Technique 153



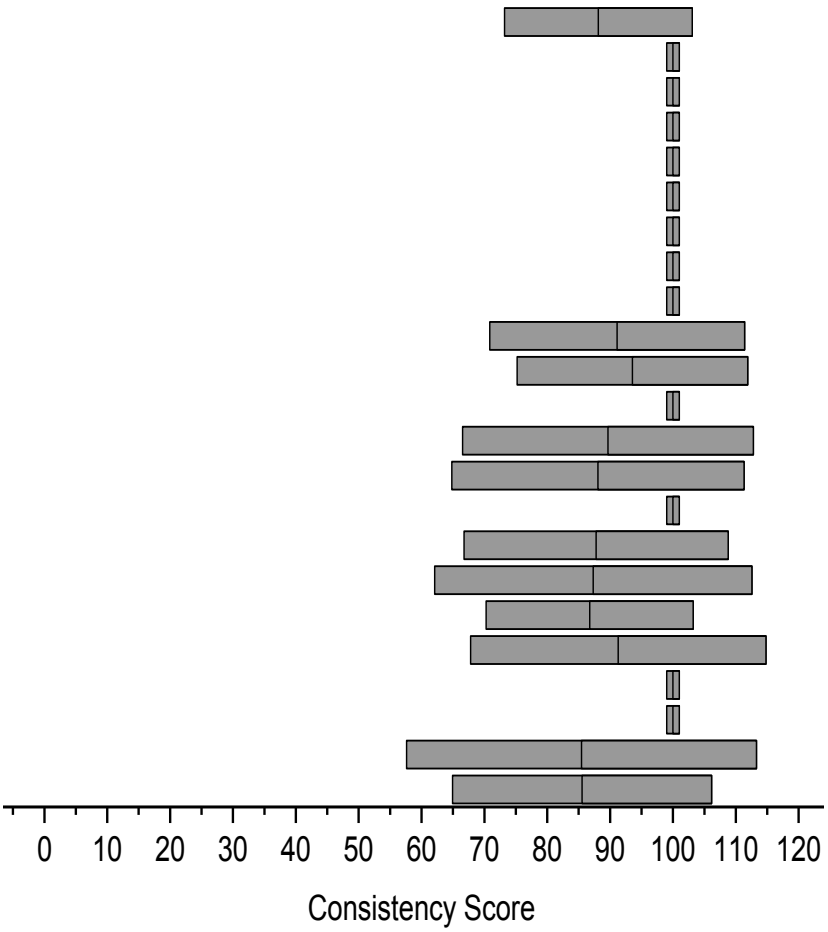
e. Photoactivity

Group	Approach	Technique
		All studies
1		All Group 1
	4	All Approach 4
		Technique 28



f. Membrane

Group	Approach	Technique
		All studies
2		All Group 2
	8	All Approach 8
		Technique 36
		Technique 37
3		All Group 3
	11	All Approach 11
		Technique 53
		Technique 56
4		All Group 4
	13	All Approach 13
		Technique 64
	14	All Approach 14
		Technique 67
		Technique 68
5		All Group 5
	16	All Approach 16
		Technique 80
		Technique 81
		Technique 83
		Technique 84
	18	All Approach 18
		Technique 97



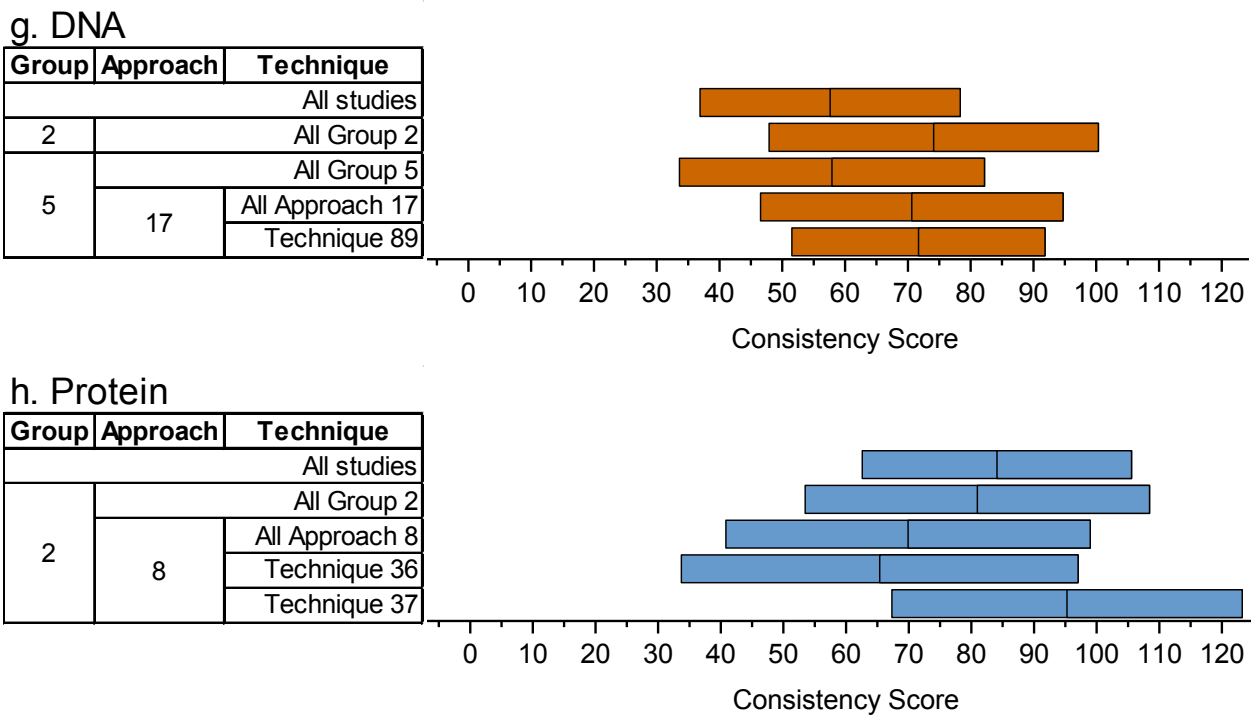


Figure 5. Change in the weighted average Consistency Score (a measure of the level of consensus among studies) across core compositions, for each mechanistic question (a-h) tested using different mechanism-targeted methods at the Group, Approach, and Technique level. Only Groups, Approaches, and Techniques which were used in 10 or more studies (across all core compositions) are shown. The left and right edges of each box represent 95% confidence intervals around the mean, indicated by the line in the center.

4. Implications

The purpose of this study was two-fold: to synthesize the mechanism(s) of antibacterial activity that have been previously reported for five core compositions, and to investigate the underlying reasons that ENM antibacterial mechanisms continue to appear ambiguous. All of the mechanisms considered here were reported to contribute, to some extent and in some conditions, to the antibacterial activity of all of the core compositions reviewed. Contrary to expectation, differences in conclusions between core compositions were found to be minimal, suggesting that considering these core compositions together may yield useful insights that may also be transferable to other MMO ENMs. Taken together, these findings indicate a need to move beyond models which assume that mechanisms are mutually exclusive and re-focus on the conditions under which each mechanism might predominate, as these have so far eluded succinct articulation.

While the contributions of ions, ENM contact, ROS, and membrane permeabilization have been the subject of hundreds of studies, questions of internalization, photo-activation, DNA damage, and protein damage remain relatively under-studied, which may stem partly from assumptions about which mechanisms apply to which core compositions. Additionally, even seemingly well-studied questions (e.g. membrane permeabilization) are often framed in binary

terms (i.e., yes or no), to the detriment of nuanced understanding. Rather than continuing to conduct new research according to the status quo, a more fundamental shift in the framing and methodology of studies may be necessary to advance understanding of ENM antibacterial mechanisms and inform advanced ENM design.

With regard to the impact of the selected study design parameters (which included ENM properties, experimental conditions, choice of experimental methods, and investigation of multiple mechanisms), almost all of the parameters investigated were shown to influence conclusions to a statistically significant degree. It is not, however, possible to discern the origin of these differences based on this analysis alone. A closer investigation of experimental method choice reveals that there is much variety in methods used to study mechanism, some of which have documented advantages and limitations, but the majority of which have yet to be evaluated. Additionally, many methods are applied to the study of multiple questions, for which they may not be equally suited. Both the conclusions reached and the level of disagreement between studies were found to vary based on the methods chosen, occasionally to a statistically significant degree, although the reasons for this cannot yet be explained.

Ultimately, this analysis highlights the need for the experimental methods used to support mechanistic investigation to match the growing level of nuance in mechanistic discussions, as binary questions of "does X mechanism contribute or not to Y core composition's antibacterial activity?" are being gradually replaced with lines of inquiry that recognize the importance of ENM properties, study conditions, and possible interactions between multiple mechanisms of antibacterial activity. It is only with this level of knowledge that the full functional potential of ENMs can be realized while minimizing unintended, adverse impacts.

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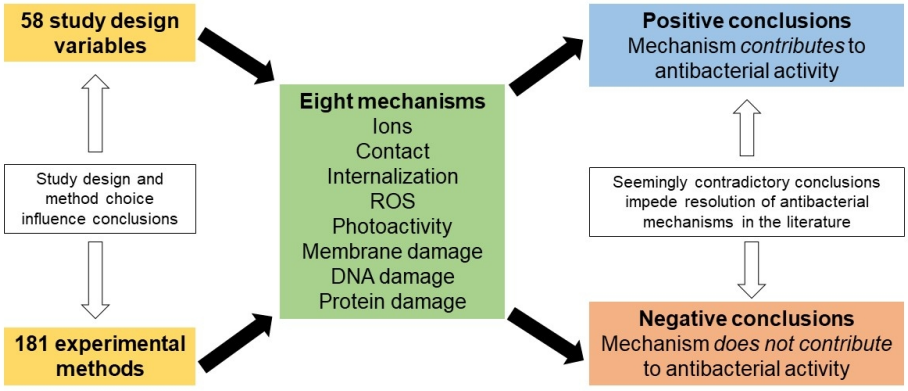

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