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A high-throughput method to characterize the gut bacteria growth upon engineered nanomaterial treatment †

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Abstract

Human are increasingly exposed to various types of engineered nanomaterials (ENMs) via dietary ingestion of nano-enabled food products, but these ENMs' impact on the gut bacteria health is still poorly understood. Current efforts in understanding the impact of these ENMs are hampered by their optical interferences in conventional quantification and viability assays, such as optical density and whole cell fluorescence staining assays. Therefore, there is a need to develop a more reliable bacteria quantification method in the presence of ENMs to effectively screen the potential adverse effects arising from the exposure of increasing ENMs on human gut microbiome. In this study, we developed a DNA-based quantification (DBQ) method in a 96-well plate format. Post-spiking method was used to correct the interference from ENMs on the reading. We showed the applicability of this method for several types of ENMs, i.e., cellulose nanofiber (CNF), graphene oxide (GO), silicon dioxide (SiO₂), and chitosan, both in pure bacterial culture and *in vitro* human gut microbiome community. The detection limit for the highest dosing of CNF, GO, SiO₂, and chitosan ENMs was approximately 0.18, 0.19, 0.05, and 0.24 as OD₆₀₀, respectively. The method was also validated by a dose response experiment of *E. coli* with chitosan in the course of 8 hr. We believe that this method has great potential to be used in screening the effect of ENMs on the

†Electronic Supplementary Information (ESI) available:

5. Conflicts of interest

There are no conflicts to declare.

growth of gut bacteria or any other *in vitro* models and normalization for metabolites or proteins analysis.

Graphical Abstract

1. Introduction

The main text of the article should appear here with headings as appropriate.

The last decade has been marked with the tremendous growth of nanotechnology which is poised to hold over \$3 trillion global market share with 6 million jobs being created worldwide by 2020¹. Dramatic nanotechnology growth has also been noted in agriculture and food industry where engineered nanomaterials (ENMs) were added into food and food related products to improve the production efficiency, food characteristics, taste, appearance and safety^{2–6}. For example, TiO₂ (E171) and SiO₂ (E551) have been directly added in various food products as food pigment and anti-caking agent, respectively. In other cases, food packaging and other food contact materials have been formulated with antimicrobial silver ENMs and copper ENMs to minimize food spoilage, which could unwarrantedly get released and migrated to the food⁷. Therefore, human oral exposure to ENMs are unavoidable⁸. Emerging nanotoxicology research shows that the nanoscale size of ENMs provides them unique physicochemical properties compared to bulk materials, which makes them more bioactive⁹. Although there are some recent studies documenting the adverse effects of ingested ENMs on human small intestinal epithelium^{10–14}, much is still unknown with regard to their potential impact on the gut microbiome.

The gut microbiome, is considered to be an essential organ that strongly interacts with its host, including nutrient synthesis and metabolism, epithelial development, and immune response^{15–18}. Several recent studies have revealed that the exposure to environmental chemicals and pharmaceuticals can directly alter the composition and functionality of indigenous gut microbiome^{19–22}. Clinically relevant outcomes such as colitis, obesity, and immunological changes may ensue²³. There are also some additional studies reporting the effect of ENMs on the bacteria. For example, silver ENMs were proved to have antibacterial effect²⁴, while ingestion of cellulose nanofibers (CNF) at physiologically relevant concentration and exposure to ZnO, CeO₂, and TiO₂ at environmentally relevant

concentrations caused non-lethal yet significant changes to gut microbial community's phenotype^{25, 26}.

Among all the biological end points, change in the bacterial growth kinetic is one of the most significant and the most discernible end points in toxicological assessment. In addition, bacteria numbers are very important for the normalization of metabolite or protein levels. Without knowing the bacteria counts or at least the relative change of bacteria number, it is difficult to figure out how exposure stress may alter bacterial function. Optical methods including standard optical density at 600 nm (OD₆₀₀) and fluorescence-based whole cell staining assays like LIVE/DEAD BacLight viability assay using SYTO9 and propidium iodide, are widely used as they can be easily performed on standard plate readers, allowing high-throughput screening of bacterial growth upon exposure to different chemical stressors. However, many ENMs exhibit optical interference with such assays by absorption, scattering, or auto-fluorescence, resulting in unreliable assay result. On the other hand, cultivation-based assays, especially agar plate-based colony counting assay, are unlikely to suffer from optical interference by ENMs; however, they are hard to adapt into a high-throughput format. Efforts such as automated image analysis, have been made to improve the colony counting efficiency. Nevertheless, the experimental procedure for plating remains labour-intensive²⁷. A few recent studies have explored novel methods to quantify the bacterial growth under the nanoparticle treatment. In one study, a series of dilutions were conducted to correct the ENM effect on the OD reading²⁷. However, this method could not meet the requirement of high-throughput screening and the coefficient of variation could reach over 25%, which reduced the reliability of the assay. In another study, flow cytometry was adopted to quantify fluorescence stained bacterial culture²⁸. The method was demonstrated to be more accurate and efficient than colony counting method and optical density method in the quantification of bacterial samples after 1 hr ENM exposure. Nevertheless, inadequate information was given about the linear range for the flow cytometry quantification method. Considering the limitation of the available methods, we believe that there is an inherent need to develop a robust high-throughput screening method to assess the bacterial growth in the presence of ENMs.

In our preliminary study, we have found great interference from graphene oxide (GO) and chitosan with conventional optical density reading method (see results) and the interference effect varied greatly between ENMs. To address this problem, we developed a DNA-based quantification assay and further scaled it up to 96-well plates to increase the overall procedure efficiency, reduce labor intensity, i.e. high-throughput. The method has been demonstrated to be applicable to the kinetic study of both pure bacteria culture as well as gut microbiome culture in the presence of four selected model ENMs, chitosan, CNF, GO and SiO₂. We believed the method can have great potential in the toxicity screening of ENMs in terms of growth kinetics and normalization for metabolites or proteins analysis with high throughput.

2. Method

2.1 Bacterial strains, *in vitro* gut microbiome, and cultivation conditions

The experiment of this study used two types of bacterial cultures: *Escherichia coli* K12-MG1655 (*E. coli*) and *in vitro* human gut microbiome. *E. coli* was cultured in reinforced clostridium medium (RCM) (Oxoid, Thermo Scientific Microbiology, Singapore) at 37 °C in a shaking incubator at 120 rpm. *In vitro* human gut microbiome was inoculated with the fresh feces of a healthy adult. The sampling protocol was in compliance with the bioethical consideration of NTU IRB and approved with NTU IRB reference number of IRB-2017-02-023. The gut microbiome was maintained in a compact chemostat system (Winpact model FS-05) with controlled temperature, dissolved oxygen, flow rate, and pH. The chemostat was operated with a working volume of 2 L and residence time of 2.8 days at 37 °C. Modified Gifu Anaerobic broth (mGAM; HyServe GmbH & Co.KG, Germany) was used as the growth media. The inoculation process was provided in detail in the supplementary information (SI) and the reactor set up was shown in Supplementary Figure S1. The gut reactor was sampled three times a week for routine monitoring of OD₆₀₀, chemical oxygen demand (COD), and short-chain fatty acid (SCFA). The gut microbiome sequence was conducted after one month's operation when the nutrient profiles were stable. The result showed that the microbial composition of the gut reactor was able to reasonably represent that in the fecal inoculum.

2.2 Nanomaterials

To investigate the interference of ENMs in bacterial quantification and to validate our method, different types of ENMs were used in this study, including nature-derived organic ENMs (CNF and chitosan), food-grade inorganic ENMs (SiO₂ known as E551), and 2-dimensional ENMs (GO). The ENMs used in this study were synthesized and characterized extensively. The work is part of the Harvard-NIEHS Reference ENM repository established at part of the National Institute of Environmental Health Sciences (NIEHS) Nanotechnology Health Implication Research (NHIR) Consortium. To verify the sterility, ENMs were tested for endotoxin level using the EndoZyme™ recombinant factor C (rFC) assay (Hyglos, Germany) according to the manufacturer's instruction as described in detail by the authors²⁹. Microbiological sterility was also assessed using WHO protocol in the international pharmacopoeia³⁰ as previously described³¹

Detailed synthesis method and characterization of cellulose nanofibers (CNF) were previously described by the authors²⁹. Briefly, CNF was synthesized by mechanical grinding of dried sheets of softwood bleached kraft fiber (St. Felicien Mill, Canada). As measured by scanning electron microscope (SEM), the length of CNF is 671 ± 561 nm and the diameter is 64 ± 29 nm. The density is 1.312 ± 0.016 g cm⁻³ and the specific surface area (SSA) is 34 m² g⁻¹. The endotoxin level is less than 0.5 EU mg⁻¹ and the microbiological result is zero colony forming unit (CFU) per gram, demonstrating the sterility of the material. Vegetables contain cellulose from 0.3 to 3.6%. Micron-sized cellulase has been designated "Generally Recognized As Safe" (GRAS) and allowed by the United States Department of Agriculture (USDA) to be added in food production at up to 0.25~3.5% (2,500 ~ 35,000 ppm)³². However, no regulations were referred to nanocellulose, i.e., CNF. Whereas referring to

regulations for other nano-enabled food additives, *i.e.*, Title 21 of Code of the Federal Regulations (21 CFR §73.575 and §172.480), the allowable maximum concentrations of the color additive TiO₂ (E171), and the anticaking agent SiO₂ (E551), are 1% and 2% w/w (*i.e.*, 10,000 ppm and 20,000 ppm) of the food, respectively. Therefore, considering the viscosity of the 10 times stock solution, 1,000 ppm of CNF was employed in this study.

Chitosan ENMs were fabricated by electrospray synthesis as previously described³³. Detailed characterization was also reported by the authors¹³. Briefly, the size measured by Brunauer-Emmett-Teller (BET) is 159 ± 2.3 nm and the SSA is 23.66 ± 0.32 m² g⁻¹. The density is 1.587 ± 0.009 g cm⁻³. The endotoxin level is less than 0.5 EU mg⁻¹ and the microbiological result is 0 CFU g⁻¹. Chitosan has shown the potential of reducing fat absorption³ and is marketed for fat reduction in the food industry³⁴. Based on the existing product for human consumption (3,300 ppm as ingested concentration³⁵) and a study for a potential therapeutic application (0.03%, 300 ppm)

³⁶, an upper concentration of 2,000 ppm was selected.

Food-grade SiO₂ (E551) were purchased from Spectrum Chemical MFG. Corp. (New Brunswick, NJ, USA). Detailed physiochemical characterization was reported previously by the authors³⁷. In brief, X-ray Diffraction (XRD) patterns revealed an amorphous morphology of SiO₂ (E551). The mean size measured by the Brunauer-Emmett-Teller (BET) is 12 nm and the SSA is 190.4 m² g⁻¹. The endotoxin level is less than 0.5 EU mg⁻¹ and the microbiological result is 0 CFU g⁻¹. SiO₂ (E551) is primarily used for direct addition to food as anticaking agent, stabilizer, adsorbent, or adjuvant. Referring to Title 21 of Code of the Federal Regulations (21 CFR §172.480), the maximum allowable concentration of the anticaking agent SiO₂ (E551) is 2% w/w (*i.e.*, 20,000 ppm) in food³⁸. Considering the viscosity of the 10 times stock solution, 2,000 ppm was utilized in this study as the highest dosing level.

Endotoxin-free synthesis of graphene oxide (GO) was performed according to an improved Hummer's method previously presented by the authors^{14, 39}. Complete characterization data, including atomic force microscopy (AFM), X-Ray photoelectron spectroscopy (XPS), endotoxin and microbiological test are presented in Supplementary Table S1. Briefly, based on AFM measurements, GO was organized in single layers with a lateral size of 242 ± 118 nm and a thickness of 0.8 ± 0.1 nm. As measured by XPS, the C/O ratios of GO was 1.53. The endotoxin level is less than 0.5 EU mg⁻¹ and the microbiological result is 0 CFU g⁻¹. Increased interests have been shown in exploring GO's potential application in the food, agriculture, biomedical, and water purification industries^{40, 41}. Due to the limited exposure data and available *in vitro* oral toxicology data¹⁴, 250 ppm as a relatively high concentration had been decided as an upper testing limit.

2.3 Development of high-throughput DNA based quantification (DBQ) assay

Step 1: Plate layout and design of controls.—The first section of the developed method comprised of the plate layout design and the preparation of controls as illustrated in Figure 1(a). To achieve high throughput screening, deep 96-well plates (96 × 2 mL square well sample collection plates with pyramid bottom; Waters, 186002482) were used for this

method. The matching mat caps (Waters, 186002484) were applied to protect the samples against spillage and contamination in the subsequent freeze & thaw cycles and sonication step. The pyramid bottom aided the phase separation during centrifugation. For every “ENM + bacteria” combination, three identical wells each containing 225 μL culture and 25 μL ENM stock solution were prepared in the same plate as biological replicates. The total working volume (250 μL per well in this study) may be increased if lower cell density was expected. ENM solutions were sonicated in the ultrasonic bath (Elmasonic E30H; Germany) for 10 min to achieve homogenous distribution before use in the experiment. In addition, triplicates of negative control and blank control were prepared in the same plate by adding water instead of ENM solution and blank medium instead of bacterial culture, respectively (Figure 1(a)). The negative control was prepared for each “ENM + bacteria” combination while blank control was prepared for a whole batch and only when nutrient-rich medium was used for bacterial cultivation. All plates were autoclaved for sterilization prior to experiment.

Step 2: Sample treatment.—The second section to post-treat the samples was shown in Figure 1(b). After ENM treatment and incubation of bacterial culture in the deep 96-well plate for designed exposure/incubation time, the plate was taken out of the incubation chamber. Following that, the respective negative controls was post-spiked with the same amount of ENM solution and mixed by pipetting before the subsequent treatment. The plate was then centrifuged at 4,000 rpm ($\sim 3,220\times g$) to separate the bacteria/ENM mixture from the growth medium. The supernatant was removed by directly flipping over and shaking the plate. After that, the residual medium component in the pellet was washed twice with saline solution (0.85% NaCl). Saline was chosen as washing buffer as it reduced the occurrence of ENM aggregation as compared to the conventional washing buffer, phosphate-buffered saline (PBS) (data not shown).

Step 3: Partial DNA extraction.—Figure 1(c) shown the third part of the method that involved partial DNA extraction. Briefly, 200–250 μL lysis buffer per well (0.1M NaCl, 0.01M EDTA, 0.1M Tris-HCl at pH = 8.0 and 1% SDS) was added to the bacteria/ENM pellets (obtained from Step 2), and mixed by pipetting a few times to ensure complete dispersion of the pellets. The degree of homogeneity of the bacterial pellets in lysis buffer may affect the overall DNA extraction efficiency. Thereafter, the lysed solution was frozen and thawed two cycles by soaking the plate into liquid N₂ and boiling water for at least 1 min each. Equal volume of tris-EDTA (TE) buffer having 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA (pH 8.0) was added to the samples and mixed before 15 min sonication in the ultrasonic bath (Elmasonic E30H; Germany) to homogenize the mixture. The plate was then centrifuged and 150 μL supernatant containing the genomic DNA was transferred to a separate transparent 96-well plate (Greiner, 655180). The added TE buffer was to dilute the SDS concentration, reduce the overall viscosity of the final solution and minimize the bubble formation during sample handling. This would ensure convenient and efficient transfer of the supernatant and minimize the contamination from ENM or bacterial debris in the pellet.

Step 4: PicoGreen assay.—The final step of the developed method is to utilize the PicoGreen assay for the DNA quantification as shown in Figure 1(d). In this step, the supernatant was diluted 25–100 times with TE buffer in another transparent 96-well plate. The dilution number was adjusted based on the bacterial concentration. The diluted samples were then directly transferred to black 96-well plates (Greiner, 655086) at 50 μ L per well and at least two wells per sample were prepared as technical replicates. For each black 96-well plate, a series of λ DNA dilutions at 0–500 ng/ml in TE were prepared to construct the fluorescence Vs. DNA calibration curve for DNA calculation. The same concentration of lysis buffer as in the diluted samples was added in the standard DNA solutions to correct the effect of lysis buffer. After all samples and λ DNA standards were transferred to the black 96-well plate with replicates, PicoGreen working reagent was prepared as 1:200 dilution of PicoGreen (Invitrogen, P11496) in TE buffer and transferred to the samples in black 96-well plate at 50 μ L per well with pipette mixing. After 8–10 min incubation in the dark at room temperature, the samples in black 96-well plate were measured with microplate reader (Tecan, MPro1000) at excitation/emission wavelengths of 485/535 nm. To minimize potential interference from medium components, the measured fluorescence value was corrected against the blank control data, as illustrated in Supplementary Figure S2. Standard DNA for calibration curve construction was prepared containing the same concentration of lysis buffer as in the diluted samples. For better accuracy, trial experiment may be required to maintain the measured DNA concentration in the range of 100–500 ng/mL by adjusting the dilution number as mentioned earlier in this section.

2.4 Method optimization

Testing on pure DNA adsorption by ENM.—ENM solutions were mixed into TE buffer with λ DNA at 0, 100, 200, 300, 400 and 500 ng/mL in the transparent 96-well plate. Final ENM concentrations were maintained at the level of 1,000 ppm, 250 ppm, 2,000 ppm and 2,000 ppm for CNF, GO, SiO₂ (E551) and chitosan, respectively. Three replicate wells of 200 μ L each were prepared for every “ENM + λ DNA concentration” combination. The plate was then centrifuged at 4,000 rpm (~3,220Xg) and 100 μ L supernatant per well was transferred to a black 96-well plate for PicoGreen assay as described above (see Section 2.3, Step 4).

Testing on effect of lysis buffer.—To examine the effect of lysis buffer residual on PicoGreen quantification, λ DNA was diluted to 0–500 ng/mL with 0, 1, 5, and 10% lysis buffer in TE buffer. The DNA solution with lysis buffer was then quantified by PicoGreen assay as described in the previous section. The quantification result with different concentrations of lysis buffer was compared with blank control (0% lysis buffer group).

Testing on biomass change after washing.—Fresh culture obtained from *in vitro* gut microbiome reactor (OD₆₀₀ ~ 1.5) was washed twice with sterile PBS to remove medium components that may affect the quantification process in PicoGreen assay. The pellet was resuspended in PBS to serve as the stock culture (OD₆₀₀ ~ 0.8) and thereafter further diluted with PBS to give final concentrations of 100, 80, 60, 40, and 20% of the stock concentration. The diluted culture suspensions were then aliquoted into the deep 96-well plate at 300 μ L per well. Three replicates were prepared for each concentration. At least 700 μ L at each

concentration remained unchanged to serve as the unwashed group. The plate was then centrifuged, and the supernatant was removed by inverting the plate and shaking. The cell pellets were washed twice with PBS and resuspended in the original volume of PBS. Both the unwashed and washed culture suspension were transferred to a new transparent 96-well plate at 200 μ L per well with triplicates. The plate was then measured in the microplate reader for OD₆₀₀ readings to check the change of biomass before and after washing.

Testing on plate layout effect and DNA extraction process optimization.—To test whether there was any effect from different plate layout design, we compared the readout from replicates at different locations in the plate. Specifically, 200 μ L per well of fresh culture suspension in PBS (prepared as described in the previous section) was aliquoted to Row A and Column 7 of the deep 96-well plate as shown in Supplementary Figure S3. The plate was centrifuged for 5 min and the liquid portion was discarded. Subsequently, 200 μ L lysis buffer per well was added to the pellets in Row A and Column 7 and mixed by pipetting. To optimize the DNA extraction processes, we varied the numbers of freeze-thaw cycles and the time of sonication. The resultant DNA extraction efficiency was then determined accordingly. Briefly, two cycles of freeze-thaw in liquid N₂ and boiling water were applied to the plate. After that, 200 μ L TE buffer was added to each sample well and mixed by pipetting. The plate was then sonicated for 15 min and centrifuged for 5 min, and 40 μ L of supernatant per well was transferred to PCR tubes and stored at 4 °C. The rest of the samples were pipette mixed and processed through the third freeze-thaw cycle. A second 40 μ L supernatant was transferred for storage after centrifugation. After pipette mixing the rest samples in the plate, the samples were subjected to another 15 min sonication and 5 min centrifugation steps, and the final 40 μ L supernatant was collected. The supernatant collected through three rounds were then diluted 100 times with TE buffer and quantified with PicoGreen assay.

2.5 Standard curve construction and limit of detection calculation

Fresh culture obtained from *in vitro* gut microbiome reactor was washed twice with PBS and then diluted to 100, 80, 60, 40, and 20% of the original concentration. The diluted culture suspensions were further mixed with either pre-sonicated ten-times ENM stock or water, which was pre-aliquoted to deep 96-well plate at 25 μ L per well, following the pattern of Supplementary Figure S4a. At least three replicates were tested for each dilution. Then the same procedures as described in finalized DBQ method section from Step 2 (Sample treatment) onwards were applied.

Limit of Detection.—The standard curves were used to estimate the limit of detection of the method using the following equation⁴²:

$$\text{LOD} = 3.3 \frac{\sigma}{m}$$

where LOD is limit of detection, σ is standard error of the regression, and m is slope of linear regression curve.

2.6 Dose response for the growth of *E. coli* with chitosan

The optimized method was validated by examining the growth of *E. coli* under chitosan treatment, which adsorbed the highest amount of λ DNA and produced the most deviated standard curve compared with the non-ENM control in the experiment result. Three chitosan dosage (500, 1000 and 2000 ppm) was adopted in this experiment. Pre-sonicated, ten-times concentrated chitosan stock solution (5, 10 and 20 mg/mL, 25 μ L per well) was added into nine wells in each of three deep 96-well plates. Another nine wells per plate was filled with 25 μ L water and served as negative controls. The positions of the total 18 wells on each plate were identical. Since the growth change at presence of ENMs was more interested in this study, fresh overnight *E. coli* culture was diluted to 5% in its original growth medium (RCM) instead of specially prepared exposure solution and added to the wells containing chitosan ENM/water at 225 μ L per well. Nine more wells were prepared in one of the plates with RCM and chitosan only (no bacteria) at three wells for each chitosan dosage to serve as blank controls. After sample preparation, two of the plates were covered with caps and incubated at 37 °C. The plate with blank controls were taken as 0 hr sampling and processed immediately. Specifically, the controls were post-spiked with the respective ten-times chitosan stock and mixed by pipetting. The plates were then processed following the subsequent steps described in Section 2.3 from Step 2 onwards. At 4 hr and 8 hr, the rest two plates also followed the same treatment procedures.

3. Results and discussion

3.1 Optical interference of four ENMs on the bacteria counting

We first evaluated the interference of selected ENMs on the OD₆₀₀ reading at different concentrations. Concentration dependent interference on the measured OD₆₀₀ was observed across the four tested ENMs (Figure 2). Among them, GO produced the highest interference on the OD₆₀₀ reading, followed by CNF and chitosan. Significant interference on the OD₆₀₀ reading was noted at concentration as low as 2.5, 10, and 20 ppm for GO, CNF, and chitosan, respectively. The measured OD₆₀₀ of GO and chitosan at the highest tested concentrations even reached similar level as the highest culturable bacterial concentration (OD₆₀₀~1.5) as depicted in Figure 2b and 2d. This optical interference could be expected as ENMs have shown nonspecific interactions (i.e., scattering, absorption, reflection) with light. For example, GO has been reported to have ultrabroad absorption spectrum⁴³ and ultrafast response towards light⁴⁴. The least interference was observed for the inorganic ENM SiO₂ (E551). However, the maximum allowable level of SiO₂ (E551) as the food additives (20,000 ppm) indicated perceptible interference at this level. Collectively, these data suggest that it was not possible to obtain reliable and comparable data with traditional optical method to screen the toxicity of different ENMs.

3.2 Method optimization

DNA adsorption affected by ENMs.—Results showed that tested ENMs had different DNA adsorption capacity (Figure 3a). Among the four tested ENMs, CNF and SiO₂ (E551) had minimal DNA adsorption, registering less than 5% reduction in the measured DNA content in the supernatant. In contrast, GO and chitosan ENMs reduced the measured DNA concentration by 66% and 76%, respectively, indicating the ability of these ENMs to adsorb

soluble DNA 45 and consequently reduce the DNA content in the liquid phase after centrifugation. Based on the result in Figure 3a, the fluorescence signals of detected DNA after ENM treatment showed good linearity at different original DNA concentrations. The good linearity reflected that the DNA adsorption to ENMs was highly proportional to the original DNA concentration at give ENM dose. Therefore, the same amount of ENM post-spike into negative controls after incubation/exposure would adsorb the same proportion of DNA as the testing samples, allowing the direct comparison of the control and test samples. In this sense, all the testing samples should have negative controls having ENM dosing of the same type and amount after incubation/exposure.

Effect of lysis buffer.—As mentioned previously, in the developed DBQ assay the DNA was directly quantified in the assay following their extraction without additional DNA purification step. Therefore, the lysis buffer residual remained in the assay medium until the final PicoGreen assay. Based on the result shown in Figure 3b, 1% lysis buffer had negligible effect on the measured DNA concentration. In contrast, increasing lysis buffer concentration to 5% and 10% resulted in the respective 25% and 73% reduction of the measured values, although the regression coefficient still remained at 0.999 for 5% lysis buffer. Therefore, dilution no less than 20 times (i.e., < 5%) before PicoGreen staining and preparing the standard DNA at the same lysis buffer concentration as the diluted samples for fluorescence Vs. DNA calibration were recommended for this study.

Biomass loss after washing.—Loss of biomass was almost inevitable when the washing step was engaged. However, the loss was no more than OD₆₀₀ of 0.11 for all tested culture densities, which would be insignificant if the original biomass was higher (Figure 3c). In addition, the reproducibility of biomass loss between replicates was very good with a relative standard deviation of less than 5% from 40% to 100% of original bacterial concentration. Moreover, ENMs in the culture suspension may improve the strength of the culture pellet after centrifugation, therefore the biomass loss could be further reduced when ENMs were involved. To achieve better quantification accuracy, it was recommended to increase the working volume of bacteria culture when pure bacteria density (without ENM) was less than OD₆₀₀ = 0.2.

Plate layout effect.—While the 96-well plate design greatly improved the efficiency of the quantification process, effect of sample position in the plate was also examined. Since different wells of the 96-well plate would experience centrifugal force of different strength and different temperature gradient during freeze-thaw cycles, it was suspected that the different positions in the deep 96-well plate could generate certain pattern of extraction efficiency. After comparing the measured values along the same row and column along the plate edge or center of the plate, we noted no significant difference ($p < 0.05$) in the amount of DNA concentration measured from the different well positions (Figure 3d). Nevertheless, it was still recommended to spread replicates across different positions of the deep 96-well plate when the plate was intensely occupied as indicated in Supplementary Figure S3.

Extraction process optimization.—In our trial experiment, lysed bacterial samples without any sonication produced filamentous material bound with ENMs extending from the

bottom pellet to the upper supernatant. The filamentous material was highly possible to be the intact genomic DNA and its binding with ENMs made it difficult to transfer pure supernatant without ENMs for DNA quantification. Therefore, sonication became a necessary step to improve phase separation possibly by breaking the binding between ENMs and DNA, and also breaking down long DNA into fragments. While sonication reduced the sample variance attributable to sample handling, it did not affect the mean of quantification results, i.e. the total DNA yield or extraction efficiency (Figure 3d). On the other hand, number of freeze-thaw cycles was positively correlated with the extraction efficiency but did not improve the variation between different samples (Figure 3d). The number of freeze-thaw cycles could be a user-defined parameter according to the required extraction efficiency.

After considering the above factors that may affect the extraction efficiency and/or quantification accuracy, the special points of DBQ method protocol (depicted in Figure 1) can be summarized as follows: (1) preparing negative controls by post-spiking the same amount of ENMs as the test samples to eliminate the DNA adsorption effect elicited by these ENMs; (2) introducing the same concentration of lysis buffer when preparing DNA standard with its concentration less than 5%; (3) using proper culture volume to generate enough biomass and positioning the replicates across different spots of the deep 96-well plates; and (4) employing two freeze-thaw cycles and a sonication for 15 min to achieve reasonable extraction efficiency and reduce processing time.

3.3 The detection limit of the method against four ENMs

Standard curves of the four different testing ENMs were obtained with a series of dilution of fresh gut microbiome culture as shown in Figure 4a. All four standard curves indicated good linearity from 0~100% of original culture density ($OD_{600} = \sim 1.5$) with SiO_2 (E551) yielded the highest regression coefficient of $R^2 > 0.999$. Chitosan showed a reduced nominal DNA concentration and displayed the lowest R^2 at 0.986. The coefficients of variation for control, CNF, GO, and SiO_2 (E551) were less than 10% and that for chitosan was less than 16% for data points above 0%. The calculated LODs for each ENM based on the standard curves were shown in Figure 4b. The LOD with no ENM could be considered as the detection limit of the method itself which was approximately 0.15 as OD_{600} . SiO_2 (E551) has shown an even lower LODs than the control at 0.05 as OD_{600} . This was possibly due to the reason that the addition of SiO_2 ENM reduced the biomass loss during washing step as hypothesized previously. While SiO_2 (E551) ENM did not adsorb DNA as GO and chitosan, the overall LOD for SiO_2 (E551) was improved compared with the control group. CNF, GO and chitosan displayed higher LODs of 0.18, 0.19 and 0.24, respectively. It was very interesting to find that dosing of ENM in the testing medium did not greatly alter the LOD of our developed method. The higher LODs of GO and chitosan could be due to adsorption of genomic DNA which was released during cell lysis on the surface of the ENM, resulting in the decreased amount of DNA to be detected with the PicoGreen assay. Besides, GO, which was demonstrated to adsorb 66% of DNA molecules in our protocol optimization stage, exhibited similar standard curve slope (<10% difference) as the control group but did not well passing the origin as other standard curves. It was suspected that sonication step applied in the optimized extraction process (not applied during the DNA adsorption experiment) could break some of the bindings between GO and DNA molecules and thus partially release

the DNA to the liquid phase. At the same time, there could be still an “inert” portion of DNA which was not released, and its quantity was hypothesized not proportional to the original DNA concentration but related to the amount of GO. Based on this hypothesis, this “inert” DNA binding reduced the measured DNA concentration by a similar amount at all original DNA concentrations, and therefore caused the standard curve moving downward and not passing the origin.

In addition, although the standard curve of pure strain gram-positive and gram-negative bacteria also showed good linearity (as shown in Figure S6), the lysing buffer may have different extraction efficiency for different species. For quantifications with the purpose of growth monitoring for mixed culture, the easier-lysed strains may shadow the change of harder-lysed strains when the former is in dominance. Therefore, the community analysis was highly recommended to supplement the quantification result in the toxicity study of ENMs.

3.4 Dose response of *E. coli* growth with chitosan

As the ENM with strong interference on DNA quantification and the highest detection limit, chitosan was used to monitor the growth kinetics of *E. coli* from 0 to 8 hr at three different concentrations: 500, 1000 and 2000 ppm. Since the samples of different chitosan dosage were extracted with different amount of chitosan, the data obtained were not directly comparable. Considering the negative controls for 500, 1000 and 2000 ppm were virtually the same during the incubation phase until they were post-spiked with different chitosan stock solution after sampling, the controls at 8 hr for different chitosan dosage were set at 100% and the other result at the same chitosan dosage were converted to the percentage value of their respective 8 hr-control for comparison (Figure 5). Based on the result, chitosan had displayed inhibitory effect at early log phase (4 hr) at all three tested concentrations and the inhibition became stronger as the chitosan dosage increased. However, lower chitosan dosage (500 ppm and 1000 ppm) significantly enhanced the growth of *E. coli* at 8 hr and produced 12% and 28% higher DNA yield than the control groups although the data was 14% lower at the highest dosage (2000 ppm).

It was very interesting to discover the changing growth behaviour of *E. coli* at increasing chitosan ENM dosage and different growth phases. It was likely that chitosan can supply as a carbon source at lower dosing concentrations when the nutrient was depleted, but induced toxicity at higher dose. The fact that chitosan can affect the bacterial growth was recognized by a previous study, in which dietary chitosan supplementation was shown to have positive effects on the host gut health by increasing microbial diversity and alleviate certain types of infections⁴⁶. Another study also reported that mice gut microbiota population was significantly reduced with chitosan oligosaccharides at early stage but returned to normal at 72 hr⁴⁷, though the bioactivity and mechanism of chitosan oligosaccharides on gut microbiota was still poorly understood. Future experiment would be required to further investigate this issue.

3.5 Advantages of DBQ method

The previous section demonstrated that DBQ method was able to detect the change of growth kinetic information with different chitosan ENM dosages. Compared to other quantification method for bacterial with ENMs, DBQ method has many advantages. For one, it can be applied to quantify all culture types including pure or mixed bacteria culture, while some growth-based method like CFU is only applicable to pure strains culturable on agar plates. Moreover, DBQ method produced relatively smaller variation across the biological and technical replicates. Last but not least, DBQ method could be implemented for high throughput screening assessment on the ENMs exposure effect. Figure 6 depicted the predicted total time and unit time consumption with different numbers of samples in terms of the processing time. When only two assays were run (one assay included three tests and three negative controls), 1.8 hr was needed. However, when the number of assays increased to 15 (the maximum capacity of one 96-well plate was 16 “ENM + culture” combinations as in Supplementary Figure S4b; the wells for one combination may need to be reserved for blank controls for a batch), the time needed for DBQ method only increased by 13 min, giving a total of 2 hr in processing time. If 60 “ENM + culture” combinations were to be screened, DBQ method would provide a much more efficient option, as it would only require 3.4 min per assay. Besides, no extra incubation time was required as other growth-based quantification method did.

4. Conclusions

This study developed a robust high throughput DNA-based quantification method for monitoring the growth kinetics of bacterial culture with ENM dosage. The method was developed and validated based on different influencing factors and the optimized experiment protocol was suggested in detail. The method was demonstrated and validated for four distinct type of ENMs, suggesting its applicability to a wide variety of ENMs. The standard curve using DBQ method at the highest ENM dosing could reach LOD of as low as OD₆₀₀ of 0.05. The DBQ method was highly advantageous compared to other methods including the turbidity measurement method which was prone to ENMs optical interference and the labor- and time- intensive CFU method. The unit time requirement was as low as 3.4 min/assay for a batch run of 60 assays. In conclusion, this method has great potential on the kinetic studies of bacterial growth and other studies with requirement of relative change in bacterial quantities in the presence of ENMs with high throughput and sufficient accuracy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Environmental Significance Statement

Dramatic nanotechnology growth is bringing increasing dietary ingestion of engineered nanomaterials (ENMs) while their health impact remains poorly understood, especially the interaction with gut microbiome. Current efforts in the toxicological assessment of these ENMs on gut bacteria are hampered by their optical interferences in bacterial quantification with ENMs treatment. In this study, we developed a high-throughput DNA-based quantification (DBQ) method in a 96-well plate format. This method had shown its robust applicability for different types of ENMs both in pure bacterial culture and in vitro gut microbiome community. We believe that this method has great potential to be used in high-throughput toxicity screenings of ENMs on the gut bacteria or any other in vitro models.

Figure 1 -

Flow chart of the developed DBQ assay. Four main parts were included: (a) Plate layout and design of controls during sample preparation, (b) Sample treatment, (c) Partial DNA extraction and (d) PicoGreen assay.

Figure 2-

The OD₆₀₀ of (a) CNF, (b) GO, (c) SiO₂, and (d) chitosan at the tested concentrations and after a series of ten-fold dilutions. The data presented are the average and standard deviation of triplicates (n=3). Difference between groups were examined with one-factor analysis of variance (ANOVA) with Duncan's post-hoc test. "*" represents the statistical significance of " $p < 0.05$ ".

Figure 3-

Experimental result for method optimization by (a) DNA adsorption by four different ENMs; (b) lysis buffer interference on PicoGreen assay; (c) effect of washing bacteria in deep 96-well plate; and (d) effect of sample position in the deep 96-well plate, freeze-thaw cycle numbers, and sonication duration on the DNA extraction. CNF, GO, SiO₂ (E551), and chitosan ENMs were dosed at the highest suggested concentration at 1000 ppm, 250 ppm, 2000 ppm, and 2000 ppm respectively in (a). R^2 is the correlation coefficient. The data presented are the average and standard deviation of triplicates (n=3).

Figure 4-

(a) The correlation between measured DNA concentration and bacterial concentration for each ENM. CNF, GO, SiO₂ (E551) and chitosan ENMs were dosed at the highest suggested concentration at 1000, 250, 2000, and 2000 ppm, respectively. R^2 is the correlation coefficient. The data presented are the average and standard deviation

Figure 5.

The growth monitoring of *E. coli* at 0–8 hr with chitosan dosing at 500 ppm, 1000 ppm and 2000 ppm using DBQ method. DNA concentration was converted to the percentage of their respective control at 8 hr. The data presented are the average and standard deviation of triplicates (n=3). Differences between paired groups were examined for statistical significance using Student's t-test. “*” represents the statistical significance of “ $p < 0.05$ ”.

Figure 6-

The predicted total time and unit time consumption for DBQ method. One assay was defined as the experiment for one stain/group of bacterial culture and one ENM at one dosing concentration with and without ENM dosing (test assay + negative controls). Three biological replicates were adopted by default.