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

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Humans are increasingly exposed to various types of engineered nanomaterials (ENMs) via dietary ingestion of nano-enabled food products, but the impact of these ENMs 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 to increasing ENMs on the human gut microbiome. In this study, we developed a DNA-based quantification (DBQ) method in a 96-well plate format. A post-spiking method was used to correct the interference from ENMs in the reading. We showed the applicability of this method for several types of ENMs, i.e., cellulose nanofibers (CNFs), 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 over the course of 8 h. We believe that this method has great potential to be used in screening the effect of ENMs on the growth of gut bacteria or any other in vitro models and normalization for metabolite or protein analysis.

1. Introduction

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 2020.1 Dramatic nanotechnology growth has also been noted in the agriculture and food industries 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 into various food products as a 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 be released and migrate to the food. Therefore, human oral exposure to ENMs is unavoidable.8

Emerging nanotoxicology research shows that the nanoscale size of ENMs provides them unique physicochemical properties compared to bulk materials, which makes them more bioactive.9 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 exposure to environmental chemicals and pharmaceuticals can directly alter the composition and functionality of the indigenous gut microbiome.19–22 Clinically relevant outcomes such as colitis, obesity, and immunological changes may ensue.23 There are also some additional studies reporting the effect of ENMs on the bacteria. For example, silver ENMs were proved to have an antibacterial effect,24 while ingestion of cellulose nanofibers (CNFs) at physiologically relevant concentration and exposure to ZnO, CeO2, and TiO2 at environmentally relevant concentrations caused non-lethal yet significant changes to the gut microbial community’s phenotype.25,26

Among all the biological end points, change in the bacterial growth kinetics 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 the 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 the 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 autofluorescence, resulting in unreliable assay results. On the other hand, cultivation-based assays, especially the agar plate-based colony counting assay, are unlikely to suffer from optical interference by ENMs; however, they are hard to adapt to 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-intensive.27 A few recent studies have explored novel methods to quantify the bacterial growth under nanoparticle treatment. In one study, a series of dilutions were conducted to correct the ENM effect on the OD reading.27 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 culture.28 This method was demonstrated to be more accurate and efficient than the colony counting method and optical density method in the quantification of bacterial samples after 1 h 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 the 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 and reduce labor intensity, i.e. high-throughput. The method has been demonstrated to be applicable to the kinetic study of both pure bacteria culture and gut microbiome culture in the presence of four selected model ENMs, chitosan, CNFs, GO and SiO2. We believed that this method can have great potential in the toxicity screening of ENMs in terms of growth kinetics and normalization for metabolite or protein 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. The in vitro human gut microbiome was inoculated with 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 a residence time of 2.8 days at 37 °C. Modified Gifu anaerobic broth (mGAM; HyServe GmbH & Co.KG, Germany) was used as the growth medium. The inoculation process is provided in detail in the ESI† and the reactor set up is shown in ESI† Fig. 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 of 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 (CNFs and chitosan), food-grade inorganic ENMs (SiO$_2$ known as E551), and 2-dimensional ENMs (GO). The ENMs used in this study were synthesized and characterized extensively. This work is part of the Harvard-NIEHS Reference ENM repository established as part of the National Institute of Environmental Health Sciences (NIEHS) Nanotechnology Health Implication Research (NHIR) Consortium. To verify the sterility, ENMs were tested for the endotoxin level using the EndoZyme™ recombinant factor C (rFC) assay (Hyglos, Germany) according to the manufacturer’s instruction as described in detail by the authors. Microbiological sterility was also assessed using the WHO protocol in the International Pharmacopoeia as previously described.

A detailed synthesis method and characterization of cellulose nanofibers (CNFs) were previously described by the authors. Briefly, CNFs were synthesized by mechanical grinding of dried sheets of softwood bleached kraft fiber (St. Felicien Mill, Canada). As measured by scanning electron microscopy (SEM), the length of CNFs 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 m$^2$ 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 cellulose has been designated “Generally Recognized As Safe” (GRAS) and allowed by the United States Department of Agriculture (USDA) to be added into food production at up to 0.25–3.5% (2500–35 000 ppm). However, no regulations were referred to nanocellulose, i.e., CNFs. Meanwhile referring to regulations for other nano-enabled food additives, i.e., Title 21 of the Code of the Federal Regulations (21 CFR §73.575 and §172.480), the allowable maximum concentrations of the color additive TiO$_2$ (E171) and the anticaking agent SiO$_2$ (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, 1000 ppm of CNFs was employed in this study.

Chitosan ENMs were fabricated by electrospray synthesis as previously described. Detailed characterization was also reported by the authors. Briefly, the size measured by the Brunauer-Emmett-Teller (BET) method is 159 ± 2.3 nm and the SSA is 23.66 ± 0.32 m$^2$ 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 absorption and is marketed for fat reduction in the food industry. Based on the existing product for human consumption (3300 ppm as ingested concentration) and a study for a potential therapeutic application (0.03%, 300 ppm), an upper concentration of 2000 ppm was selected.

Food-grade SiO$_2$ (E551) was purchased from Spectrum Chemical MFG. Corp. (New Brunswick, NJ, USA). Detailed physicochemical characterization was reported previously by the authors. In brief, X-ray diffraction (XRD) patterns revealed an amorphous morphology of SiO$_2$ (E551). The mean size measured by the Brunauer-Emmett-Teller (BET) method is 12 nm and the SSA is 190.4 m$^2$ g$^{-1}$. The endotoxin level is less than 0.5 EU mg$^{-1}$ and the microbiological result is 0 CFU g$^{-1}$. SiO$_2$ (E551) is primarily used for direct addition to food as an 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 SiO$_2$ (E551) is 2% w/w (i.e., 20 000 ppm) in food. Considering the viscosity of the 10 times stock solution, 2000 ppm was utilized in this study as the highest dosing level.

Endotoxin-free synthesis of graphene oxide (GO) was performed according to an improved Hummers method previously presented by the authors. Complete characterization data, including atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), and endotoxin and microbiological tests are presented in SI† 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 ratio of GO is 1.53. The endotoxin level is less than 0.5 EU mg$^{-1}$ and the microbiological result is 0 CFU g$^{-1}$. Increasing interest has been shown in exploring the potential application of GO in the food, agriculture, biomedical, and water purification industries. Due to the limited exposure data and available in vitro oral toxicology data, 250 ppm as a relatively high concentration has been decided as an upper testing limit.

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

Step 1: plate layout and design of controls. The first section of the developed method comprised the plate layout design and the preparation of controls as shown in Fig. 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 an ultrasonic bath (Elmasonic E30H; Germany) for 10 min to achieve homogeneous distribution before use in the experiment. In addition, triplicates of the negative control and blank control were prepared in the same plate by adding water instead of the ENM solution and a blank medium instead of the
bacterial culture, respectively (Fig. 1(a)). The negative control was prepared for each “ENM + bacteria” combination while the blank control was prepared for a whole batch and only when a nutrient-rich medium was used for bacterial cultivation. All plates were autoclaved for sterilization prior to the experiment.

**Step 2: sample treatment.** The second section to post-treat the samples is shown in Fig. 1(b). After ENM treatment and incubation of bacterial culture in the deep 96-well plate for the designed exposure/incubation time, the plate was taken out of the incubation chamber. Following that, the respective negative controls were post-spiked with the same amount of ENM solution and mixed by pipetting before the subsequent treatment. The plate was then centrifuged at 4000 rpm (≈3220 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 the 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.** Fig. 1(c) shows the third part of the method that involved partial DNA extraction. Briefly, 200–250 μL lysis buffer per well (0.1 M NaCl, 0.01 M EDTA, 0.1 M 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 for two cycles by soaking the plate in liquid N₂ and boiling water for at least 1 min each. An 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 an 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 TE buffer was added 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 ENMs 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 Fig. 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 μL per well and at least two wells per sample were prepared as technical replicates. For each black 96-well plate, a series of λDNA dilutions at 0–500 ng ml⁻¹ in TE were prepared to construct the fluorescence vs. DNA calibration curve for DNA calculation. The same concentration of lysis buffer as that in the diluted samples was added into the standard DNA solutions to correct the effect of lysis buffer. After all
the samples and λ-DNA standards were transferred to the black 96-well plate with replicates, a PicoGreen working reagent was prepared as 1:200 dilution of PicoGreen (Invitrogen, P11496) in TE buffer and transferred to the samples in the black 96-well plate at 50 μL per well with pipette mixing. After 8–10 min incubation in the dark at room temperature, the samples in the black 96-well plate were measured with a microplate reader (Tecan, MPro1000) at excitation/emission wavelengths of 485/535 nm. To minimize potential interference from the medium components, the measured fluorescence value was corrected against the blank control data, as shown in ESI† Fig. S2. The standard DNA for calibration curve construction was prepared containing the same concentration of lysis buffer as that in the diluted samples. For better accuracy, trial experiments may be required to maintain the measured DNA concentration in the range of 100–500 ng mL⁻¹ by adjusting the dilution number as mentioned earlier in this section.

2.4. Method optimization

Testing on pure DNA adsorption by ENMs. ENM solutions were mixed into TE buffer with λ-DNA at 0, 100, 200, 300, 400 and 500 ng mL⁻¹ in the transparent 96-well plate. The final ENM concentrations were maintained at the level of 1000 ppm, 250 ppm, 2000 ppm and 2000 ppm for CNFs, GO, SiO₂ (ES51) and chitosan, respectively. Three replicate wells of 200 μL each were prepared for every “ENM + λ-DNA concentration” combination. The plate was then centrifuged at 4000 rpm (~3220g) and 100 μL supernatant per well was transferred to a black 96-well plate for the PicoGreen assay as described above (see section 2.3, Step 4).

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

Testing on biomass change after washing. The fresh culture obtained from the 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 the 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 the 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 μL per well. Three replicates were prepared for each concentration. At least 700 μL 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 μL 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 the plate layout effect and DNA extraction process optimization. To test whether there was any effect from different plate layout designs, we compared the readout from replicates at different locations in the plate. Specifically, 200 μL 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 ESI† Fig. S3. The plate was centrifuged for 5 min and the liquid portion was discarded. Subsequently, 200 μL 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 μL 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 μL 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 μL 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 step, and the final 40 μL supernatant was collected. The supernatant collected through three rounds were then diluted 100 times with TE buffer and quantified with the PicoGreen assay.

2.5. Standard curve construction and limit of detection calculation

The fresh culture obtained from the 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 solution or water, which was pre- aliquoted to the deep 96-well plate at 25 μL per well, following the pattern in ESI† Fig. S4a. At least three replicates were tested for each dilution. Then the same procedures as described in the finalized DBQ method 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 equation:⁴²

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

where LOD is the limit of detection, σ is the standard error of the regression, and m is the slope of the 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 \( \lambda \)DNA and produced the most deviated standard curve compared with the non-ENM control in the experiment result. Three chitosan dosages (500, 1000 and 2000 ppm) were adopted in this experiment. Pre-sonicated, ten times concentrated chitosan stock solution (5, 10 and 20 mg mL\(^{-1}\), 25 \( \mu \)L per well) was added into nine wells in each of the three deep 96-well plates. Another nine wells per plate were filled with 25 \( \mu \)L water and served as negative controls. The positions of the total 18 wells on each plate were identical. Since we were more interested in the growth change in the presence of ENMs 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 \( \mu \)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 \( ^{\circ} \)C. The plate with blank controls was taken as 0 h sampling and processed immediately. Specifically, the controls were post-spiked with the respective ten times chitosan stock solution and mixed by pipetting. The plates were then processed following the subsequent steps described in section 2.3 from step 2 onwards. At 4 h and 8 h, the rest two plates were also processed following the same treatment procedures.

3. Results and discussion

3.1. Optical interference of the four ENMs in the bacteria counting

We first evaluated the interference of the selected ENMs in the OD\(_{600}\) reading at different concentrations. Concentration dependent interference in the measured OD\(_{600}\) was observed across the four tested ENMs (Fig. 2). Among them, GO produced the highest interference in the OD\(_{600}\) reading, followed by CNFs and chitosan. Significant interference in the OD\(_{600}\) reading was noted at concentrations as low as 2.5, 10, and 20 ppm for GO, CNFs, and chitosan, respectively. The measured OD\(_{600}\) of GO and chitosan at the highest tested concentrations even reached similar levels to the highest culturable bacterial concentration (OD\(_{600}\) \sim 1.5) as depicted in Fig. 2b and d. 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 an ultrabroad absorption spectrum\(^{43}\) and ultrafast response towards light.\(^{44}\) The least interference was observed for the inorganic ENM SiO\(_2\) (E551). However, the maximum allowable level of SiO\(_2\) (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 the traditional optical method to screen the toxicity of different ENMs.

3.2. Method optimization

DNA adsorption affected by ENMs. Results showed that the tested ENMs had different DNA adsorption capacities (Fig. 3a). Among the four tested ENMs, CNFs and SiO\(_2\) (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 Fig. 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 onto ENMs was highly proportional to the original DNA concentration at the given 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 an additional DNA purification step. Therefore, the lysis buffer residue remained in the assay medium until the final PicoGreen assay. Based on the result shown in Fig. 3b, 1% lysis buffer had a negligible effect on the measured DNA concentration. In contrast, increasing the 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, dilutions 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.** The loss of biomass was almost inevitable when the washing step was performed. However, the loss was no more than OD_{600} of 0.11 for all tested culture densities, which would be insignificant if the original biomass was higher (Fig. 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 the 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 the pure bacteria density (without ENMs) was less than OD_{600} = 0.2.

**Plate layout effect.** While the 96-well plate design greatly improved the efficiency of the quantification process, the effect of the sample position in the plate was also examined. Since different wells of the 96-well plate would experience centrifugal force of different strengths and different temperature gradients during the freeze–thaw cycles, it was suspected that the different positions in the deep 96-well plate could generate a 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 (Fig. 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 ESI† Fig. S3.

**Extraction process optimization.** In our trial experiment, lysed bacterial samples without any sonication produced a 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 the 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 (Fig. 3d). On the other hand, the number of freeze–thaw cycles was positively correlated with the extraction efficiency.
efficiency but did not improve the variation between the different samples (Fig. 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 the DBQ method protocol (depicted in Fig. 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 the DNA standard with its concentration less than 5%; (3) using an appropriate 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 sonication for 15 min to achieve reasonable extraction efficiency and reduce the processing time.

3.3. The detection limit of the method against the four ENMs

Standard curves of the four different testing ENMs were obtained with a series of dilutions of the fresh gut microbiome culture as shown in Fig. 4a. All four standard curves indicated good linearity from 0–100% of the original culture density (OD$_{600}$ = ∼1.5) with SiO$_2$ (E551) yielding the highest regression coefficient of $R^2 > 0.999$. Chitosan showed a reduced nominal DNA concentration and displayed the lowest $R^2$ of 0.986. The coefficients of variation for the control, CNFs, 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 are shown in Fig. 4b. The LOD with no ENMs 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 LOD than the control at 0.05 as OD$_{600}$. This was possibly due to the reason that the addition of the SiO$_2$ ENM reduced the biomass loss during the washing step as hypothesized previously. While the 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. CNFs, GO and chitosan displayed higher LODs of 0.18, 0.19 and 0.24, respectively. It was very interesting to find that dosing of ENMs 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 the adsorption of genomic DNA which was released during cell lysis on the surface of the ENMs, resulting in the decreased amount of DNA detected with the PicoGreen assay. Besides, GO, which was demonstrated to adsorb 66% of DNA molecules in our protocol optimization stage, exhibited a similar standard curve slope (<10% difference) to the control group but did not pass the origin as the other standard curves. It was suspected that the 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 into 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 to be 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 Fig. S6†), the lysing buffer may have
different extraction efficiencies 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 in DNA quantification and the highest detection limit, chitosan was used to monitor the growth kinetics of E. coli from 0 to 8 h at three different concentrations: 500, 1000 and 2000 ppm. Since the samples of different chitosan dosages were extracted with different amounts of chitosan, the data obtained were not directly comparable. Considering that the negative controls for 500, 1000 and 2000 ppm were virtually the same during the incubation phase until they were post-spiked with different concentrations of chitosan stock solution after sampling, the controls at 8 h for different chitosan dosages were set at 100% and the other results at the same chitosan dosage were converted to the percentage value of their respective 8 h-control for comparison (Fig. 5). Based on the result, chitosan had displayed an inhibitory effect at the early log phase (4 h) at all three tested concentrations and the inhibition became stronger as the chitosan dosage increased. However, lower chitosan dosages (500 ppm and 1000 ppm) significantly enhanced the growth of E. coli at 8 h and produced 12% and 28% higher DNA yields 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 a 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 infections. Another study also reported that mice gut microbiota population was significantly reduced with chitosan oligosaccharides at the early stage but returned to normal at 72 h, though the bioactivity and mechanism of chitosan oligosaccharides on gut microbiota were still poorly understood. Future experiments would be required to further investigate this issue.

3.5. Advantages of the DBQ method

The previous section demonstrated that the DBQ method was able to detect the change of growth kinetic information with different chitosan ENM dosages. Compared to other quantification methods for bacteria with ENMs, the 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 methods like CFU are only applicable to pure strains culturable on agar plates. Moreover, the DBQ method produced relatively smaller variation across the biological and technical replicates. Last but not least, the DBQ method could be implemented for high throughput screening assessment on the ENM exposure effect. Fig. 6 shows 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 h
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 shown in ESI† Fig. S4b; the wells for one combination may need to be reserved for blank controls for a batch), the time needed for the DBQ method only increased by 13 min, giving a total processing time of 2 h. If 60 “ENM + culture” combinations were to be screened, the 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 methods 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 types of ENMs, suggesting its applicability to a wide variety of ENMs. The standard curve using the DBQ method at the highest ENM dosing could reach an LOD of as low as OD_{600} of 0.05. The DBQ method was highly advantageous compared to other methods including the turbidity measurement method which was prone to ENM optical interference and the labor- and time-intensive CFU method. The unit time requirement was as low as 3.4 min per assay for a batch run of 60 assays. In conclusion, this method has great potential in the kinetic studies of bacterial growth and other studies with requirements of relative change in bacterial quantities in the presence of ENMs with high throughput and sufficient accuracy.

Conflicts of interest

There are no conflicts to declare.

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Fig. 6 The predicted total time and unit time consumption for the DBQ method. One assay was defined as the experiment for one strain/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.
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