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Simultaneous determination of the ratio of live, dead and adherent bacteria in the presence of various single-walled carbon nanotubes

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Abstract

Objectives: A decrease in bacterial counts as a consequence of their interactions with various carbon nanotubes are usually interpreted as the destruction of viable bacteria. However, changes in adherent properties can also result in a decrease of the detectable bacterial counts.

Methods: This paper provides a simple experimental setup and a mathematical formulation for simultaneous determination of ratios of the live/dead and adherent/non-adherent bacteria based on impedance changes. This method is especially useful in case of colored samples where traditional optical methods fail. The usefulness of our method is demonstrated by analyzing the effect of single-walled carbon nanotubes bearing various functional groups exerted on *Staphylococcus aureus* bacteria.

Results: According to our results, pristine single-walled carbon nanotubes destroy *Staphylococcus aureus* resulting in a decrease in the bacterial counts and do not change the adherent properties of the bacteria. At the same time, amidated single-walled carbon nanotubes have no killing effect and they inhibit the adherence of bacteria to the wall of the sample container. Presence of carboxylated single-walled carbon nanotube neither influences the viability nor the adherent properties of *Staphylococcus aureus.*

Discussion: Our data reveals that nanotubes do not only have the ability to kill bacteria, reducing their viability, but may also affect other deeper details of bacterial life, for example, may change their adhesion ability.

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Introduction

In the last two decades, the continuously increasing amount of various nanomaterials in our environment has raised the question of how they interact with the microenvironment i.e. with various bacteria [1,2]. Interactions of bacteria with various nanomaterials can result in changing the bacterial counts that might be due to their killing or due to their altered adherent properties. Several methods are available to distinguish live from dead bacteria or to separate adherent from non-adherent species e.g. [3,4,5,6]. A method which would simultaneously determine both of the above parameters would be beneficial especially in the case of black colored additives when traditional optical methods [7] for counting bacteria are very tedious.

Impedance changes are not sensitive towards the color of samples as they are originated from soluble metabolites of bacteria which are released from both adherent and non-adherent species.

Here we provide a simple experimental setup and a mathematical formulation for simultaneous determination of ratios of the live/dead and adherent/non-adherent bacteria based on impedance changes. Furthermore, we present its usefulness in case of the interaction between *Staphylococcus aureus* bacteria and single-walled carbon nanotubes bearing various functional groups.

Materials and methods

Chemicals

10x PBS was purchased from Sigma. Physiological salt solution (0.9%) was prepared from NaCl (Reanal, Hungary) and sterilized by autoclave. Whitley impedance broth (WIB) was used as culture medium [8] with slight modification: 11.5 g/L Tryptone (Bio-Lab), 5g/L meat peptone(Merck), 3 g/L yeast extract (Merck), 0.5 g/L $MgSO_4$ (Spectrum-3D), 0.5 g/L $CaCl_2$ (Reanal), pH 7.2-7.4. All chemicals were dissolved in deionized water.

Single-walled carbon nanotubes

SWCNT and its various surface modified forms were purchased from the following companies with characteristics as indicated on the technical sheets attached: Pristine SWCNT (p-SWCNTs, cat. # 704121, outer diameter 0.7-1.1 nm, purity: >90% CNTs and >77% SWCNTs) and amidated SWCNT ($CONH_2$–SWCNTs, cat.# 685380, diameter 4–6 nm, length 0.7–1.0 µm, purity: >90% carbon basis, 5–8% trace metals) – Sigma–Aldrich (USA); carboxylated SWCNTs (COOH–SWCNTs, cat.# 1288YJF, average diameter 1–2 nm, length 5–30 µm, purity: >95% CNTs, >90% SWCNTs, –COOH: 2.59–2.87 wt%) – Nano Amor (Los Alamos, USA).

Morphology of nanotubes was characterized by electron microscopy, and elementary compositions were analyzed by Energy-Dispersive X-ray Spectroscopy (EDS) as described elsewhere [9].

Stock (2 mg/ml) solutions of all the SWCNTs samples were prepared in distilled water using sonication with 4.2x 10^5 kJ/m^3 specific energy in three consecutive steps for 2 min each.

Bacterium

Staphylococcus aureus ssp. aureus (HNCMB, # 110003) which is equivalent to the strain registered as Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ #6247) was purchased from Hungarian National Collection of Medical Bacteria. Bacteria were grown overnight at 37 C on blood agar plate (Columbia agar base: VWR; supplemented with sterile calf defibrinated blood: Culex, Hungary). Cells were suspended in sterile physiological salt solution at room temperature. Cell numbers were adjusted to 0.1 optical density measured at 600 nm (which was estimated to be 2.4x10^8 CFU/ml, [10,11,12] and further diluted 4-fold by physiological salt solution.

Single-walled carbon nanotubes treatment to *S. aureus*

800 µl of 6x10^7 CFU/ml *S. aureus* bacterium suspensions in physiological salt solution were incubated in BacTrac single-use measuring cells (#41-440003, SY-Lab, Austria) with 40 µl (0,1 mg/ml final concentration) of various (pristine, amidated, carboxylated) carbon nanotubes on an orbital shaker (Labinco LD-45, 700 rpm) at room temperature for 1 hour. A *S. aureus* sample prepared the same way with deionized water served as a control.

Impedance and optical density measurements of the bacterial growth curve

420 µl out of 840 µl incubated samples were transferred into a new impedance measuring cell ("supernatant tube"). 9.6 ml of WIB culture medium was added to each cell and run on BacTrac 4100 (SY-Lab, Austria) in M value analysis mode. Bacterial growth was characterized by the detection time when threshold adjusted to 5% of relative conductivity change of the medium was attained.

10 µl of each sample with 190 µl of WIB was run on an Elisa plate reader (Multiskan Spectrum, Thermo Labsystems, USA). Absorptions at 600 nm were detected in every 15 min for at least 15 hours and their time dependences were fitted with 5 parameter sigmoidal curves (Sigma Plot for Windows, v. 12.3. Systat Inc.) and characterized by the Crossing Point (CP) between baseline and the asymptote at the inflection point of the sigmoidal curve.

Detection times obtained from BacTrac measurements as well as CP values were converted into cell densities (CFU/ml) based upon calibrations. For comparability, all data are expressed as the ratio of the initial/inoculated bacterium densities.

Statistics

Data are given as mean values with standard error of mean (SEM). Significant differences relative to the initial/inoculated bacterium densities were tested either by one-sample t-test or by one-sample signed rank test if the normality test (Shapiro-Wilk) failed (Sigma Plot for Windows, v. 12.3. Systat Inc.). A p-value < 0.05 was considered significant.

Results and discussion

Figure 1 represents changes in bacterial counts of *S. aureus* in the presence of various SWCNTs after a 1-hour incubation (room temperature, 700 RPM) as measured by changes in optical densities. The decrease in bacterial concentration was detected in the presence of pristine and carboxylated SWCNT as well as in the control sample. Amidated SWCNT had no effect in the same circumstances. During the treatment procedure, the number of bacteria may decrease for the following reasons: they become injured, adhere to the wall of the sample container, adhere to the nanotube or because of any combinations of the above.

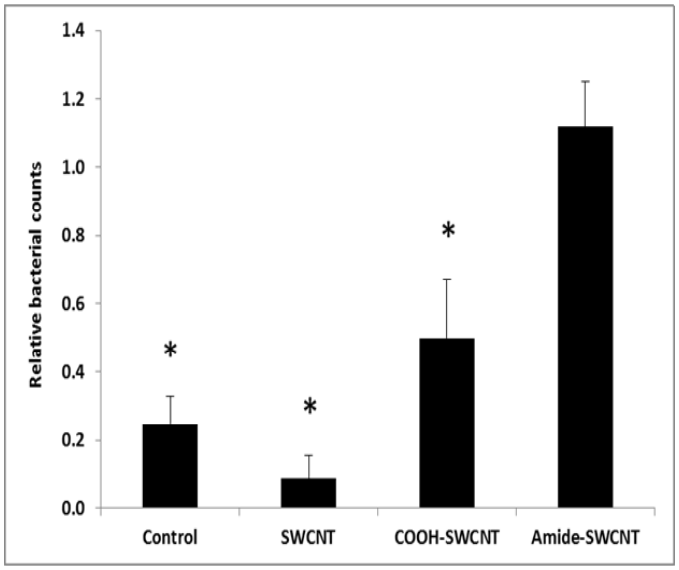
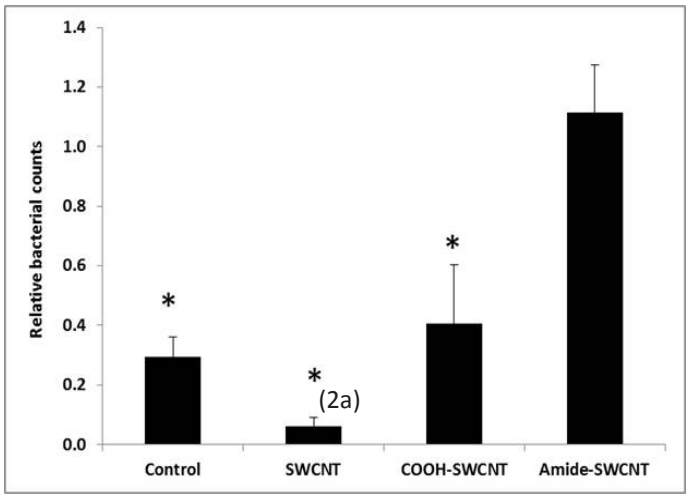


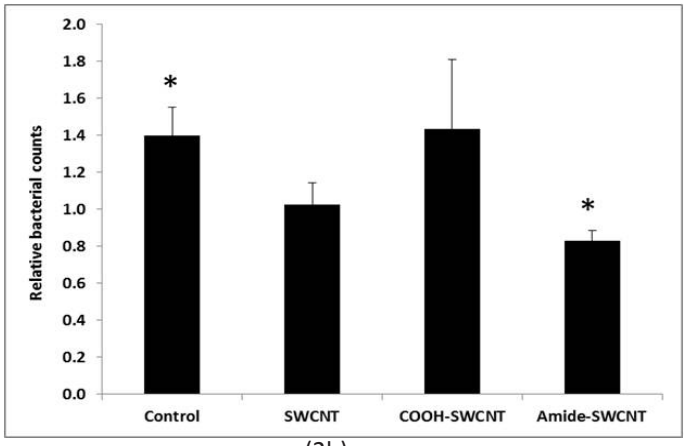
Figure 1: Changes in bacterial counts of *S. aureus* relative to the initial/inoculated bacterium counts in the presence of various SWCNTs after a 1-hour incubation (room temperature, 700 RPM) as measured by changes in optical densities (n=6). SWCNT: pristine single-walled carbon nanotube, COOH-SWCNT: carboxylated SWCNT, amide-SWCNT: amidated SWCNT; * denotes significant difference from value 1 ($p<0.05$)

To reveal the reason behind the decrease of bacterial counts incubated samples were divided into two parts as described in Materials and methods. Impedance changes were measured for both parts. Figure 2a represents changes in the bacterial counts in half of the incubated sample which was transferred into a new sample container. Since the transferred sample is basically the same as that of the one used for the optical density measurement, therefore the data of Figure 1 and Figure 2a reflect the same effect of various SWCNTs exerted on *S. aureus* independently of which method was used.

Figure 2b represents the bacterial counts in half of the sample which was left in the original sample container used for incubation. Although, the data of Figures 2a and 2b belong apparently to the same sample, they proved to be different. This difference can be explained as follows: (1) in case of transferred sample portion only alive dispersed bacteria produce conductivity changes and (2) in case of the sample which was left in the original sample container, conductivity changes are originated both from dispersed and adherent alive bacteria.



(2a)



(2b)

Figure 2: Changes in bacterial counts of *S. aureus* relative to the initial/inoculated bacterium counts in the presence of various SWCNTs after a 1-hour incubation (room temperature, 700 RPM) as measured by impedance changes (n=6). SWCNT: pristine single-walled carbon nanotube, COOH-SWCNT: carboxylated SWCNT, amide-SWCNT: amidated SWCNT; *denotes significant difference from value 1 ($p<0.05$). a: data for half of the incubated sample transferred into a new BacTrac cell; b: data for half of the sample remaining in the BacTrac cell used for incubation

Measuring the impedance changes in the above experimental set up may provide information not only on the changes of the adhesive capabilities of the bacteria upon nanotube treatment but also on the live/dead ratio as well. We formulated a simple mathematical formula to estimate these two effects separately.

The transferred number of alive bacteria (N_S) introduced into the "supernatant" tube can be calculated according to the following equation:

$$N_S = N*\omega*(1-\alpha)*\gamma \qquad (1)$$

Where N is the initial number of bacteria, α and ω denote the ratios of adherent and surviving bacteria upon treatment, and γ is the fraction of sample volume introduced into "supernatant tube" after incubation.

Using the same factors, the number of alive bacteria remaining in the originally incubated tubes can be given as a sum of adherent and non-adherent alive bacteriaafter removing a fraction of "supernatant" (N_R).

$$N_R = N*\omega*\alpha + N*\omega*(1-\alpha)*(1-\gamma) \qquad (2)$$

The ratios of adherent (α) and surviving (ω) bacteria can be calculated by solving eqs. 1 and 2:

$$\alpha = 1 - \frac{N_S}{\gamma*(N_R + N_S)} \qquad (3)$$

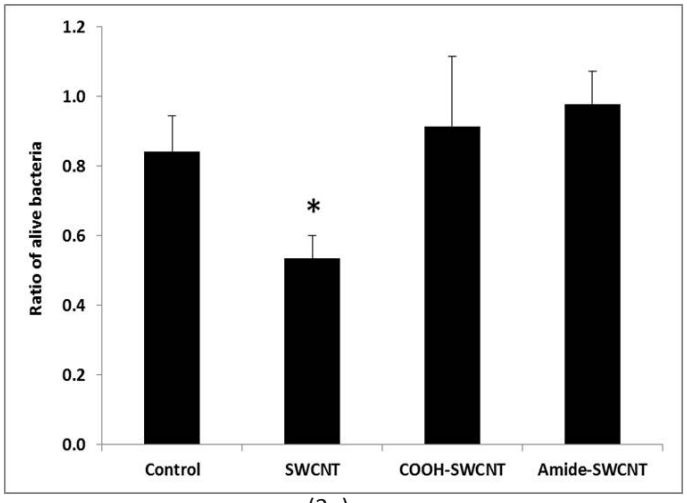
$$\omega = \frac{N_R + N_S}{N} \qquad (4)$$

If exactly half of the sample volume is introduced into the "supernatant tube", i.e. γ=1/2, equ. 3 is simplified:

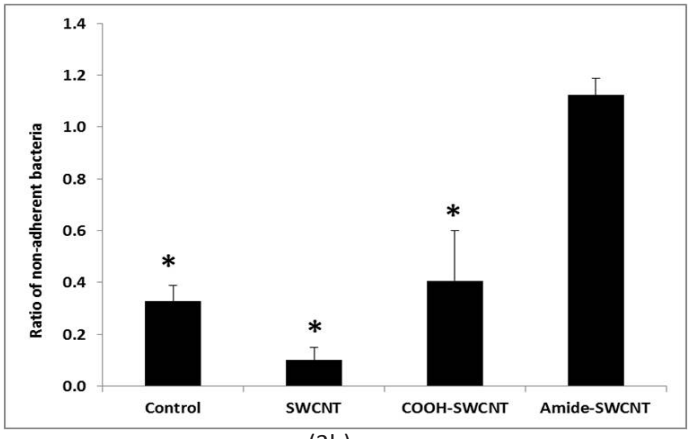
$$\propto = \frac{N_R - N_S}{N_R + N_S} \qquad (3a)$$

$$\propto = \frac{N_R - N_S}{\omega * N} \qquad (3b)$$

Figures 3a and 3b represent the survival and adherent proportion of the of *S. aureus* in the presence of various SWCNTs as calculated by using the above equations. The survival rate of *S. aureus* decreases substantially only upon pristine SWCNT treatment. Practically no change can be detected in the control, in the presence of carboxylated, or in the presence of amidated SWCNTs (Figure 3a). A significant amount of adherent *S. aureus* bacteria can be detected in the presence of pristine and carboxylated single-walled carbon nanotube (Figure 3b). No adherent bacteria can be detected in the presence of amide modified single-walled carbon nanotube. It is very interesting that a similar ratio of adherent bacteria can be detected in the control sample as in the presence of pristine or carboxylated SWCNTs.



(3a)



(3b)

Figure 3: Ratio of alive (a) and non-adherent (b) bacteria in the presence of various single-walled carbon nanotubes (n=6). SWCNT: pristine single-walled carbon nanotube, COOH-SWCNT: carboxylated SWCNT, amide-SWCNT: amidated SWCNT; * denotes significant difference from value 1 ($p<0.05$)

In this study, interactions between *S. aureus* bacteria and three various SWCNTs were investigated. Bacteria behaved differently in the presence of SWCNTs bearing different chemical groups.

In accordance with the literature data [13,14] pristine SWCNT changes the surviving rate of *S. aureus*. The decrease in the bacterial number experienced in the absence of nanotubes or in the presence of carboxylated SWCNT can be attributed only to the extent of adherence of the *S. aureus* to the wall of the container. One can speculate that the presence of negatively charged carboxylated SWCNT is indifferent towards negatively charged *S. aureus*. At the same time, amidated SWCNT does not affect the surviving rate of *S. aureus* and even prevents the adherence of negatively charged bacteria to the wall of the container due to its positive charges.

Our data partly agree with those of Arias et al [15] who just like us did not detect any bactericidal effect of the positively charged NH_2 group bearing single-walled carbon nanotube. They detected the bactericidal effect of carboxylated SWCNT exerted on *S. aureus* in physiological salt solution and in distilled water which effect disappeared in PBS. In our case, presence of carboxylated SWCNT seems to be indifferent towards *S. aureus*. One possible explanation of this discrepancy could be that Arias et al [15] did not separate adhesive and the real bactericidal effect of carbon nanotubes exerted on *S. aureus*. Furthermore, one can suppose that their carboxylated SWCNT might have contained some impurity responsible for the bactericidal effect and that impurity might have been removed by PBS.

In this study three SWCNTs bearing different chemical groups purchased from different companies were used. These SWCNTs affected *S. aureus* bacteria differently in terms of changing their adhesive properties as well as their viability. One cannot exclude that these behavior changes of *S. aureus* bacteria could partly be originated from different physical parameters of SWCNTs. However, our findings strongly suggest that various chemical groups on the surface of the single-walled carbon nanotubes with different charges and structures have a high impact on the adhesive properties and the viability of *S. aureus*.

Summary

Data obtained by our analysis presented here supports not only the literature data as far as the antibacterial effect of single-walled carbon nanotubes are concerned, but also contributes to a better understanding of the interaction of bacteria with various additives by revealing changes in their adherence upon treatment.

Table 1: Overview of the interaction of various SWCNTs and *S. aureus* bacteria

	Decrease of bacterial count	Killing	Adherence
Control	yes	no	yes
SWCNT	yes	yes	same as the control
COOH-SWCNT	yes	no	same as the control
Amide-SWCNT	no	no	inhibited

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