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Microbiological quality control of single-walled carbon-nanotubes–coated surfaces experimentally contaminated

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Summary. — The emergence of new nanotechnologies involves the spreading of nanoparticles in various fields of human life. Nanoparticles in general and, more specifically, carbon nanotubes have been adopted for many practical approaches *i.e.*: coatings for medical devices, food process industry and drug delivery. Humans will be increasingly exposed to nanoparticles but the susceptibility of nanostructured materials to microbial colonization in process of manufacturing and storage has not been thoroughly considered. Therefore, the microbiological quality control of nanoparticles plays a pivotal role. Different analytical methods have been attempted for detecting bacterial population contaminating a surface, but no one can be considered fully appropriate. Here, BioTimer Assay (BTA) and conventional sonication followed by colony forming units method (S-CFU) were applied for microbiological quality control of single-walled carbon nanotubes (SWCNTs)-coated surfaces experimentally contaminated with *Streptococcus mutans* and *Pseudomonas aeruginosa*. Our results demonstrated that S-CFU is unreliable to actually determine the number of bacteria, contaminating abiotic surfaces, as it does not detach all adherent bacteria and kills part of the bacterial population. Instead, BTA is a reliable method to enumerate bacteria colonizing SWCNTs-coated surfaces and can be considered a useful tool for microbiological quality control of nanomaterials for human use.

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1. – Introduction

In the past few years, several nanostructured materials have been proposed for a variety of medical protocols, as drug delivery, cancer diagnosis, treatment, and imaging. Among the nanomaterials, quantum dots, dendrimers, gold and silver nanoparticles, micelles, liposomes and carbon nanotubes (CNTs) have been employed [1-4]. CNTs enter the cell via different methods, such as passive diffusion across the lipid bilayer, or endocytosis, whereby the CNT attaches to the surface of the cell and is subsequently engulfed by the cell membrane [4, 5]. Typically, CNTs are classified as single-walled (SWCNT) or multi-walled (MWCNT), according to the number of layers. Different reports have suggested that once the functionalized SWCNT releases the drug into a specific area, it is gradually excreted from the body via the biliary pathway and finally in the feces [6]. This suggests that SWCNTs are suitable candidates for drug delivery and a promising nanotechnological platform for future cancer therapeutics. Despite these advantages of CNTs, there are limitations to their biomedical use. Although many studies have been done on the toxicity of nanoparticles, little is known about their susceptibility to microbial colonization. In particular, the susceptibility of nanostructured materials to microbial colonization and biofilm formation in process of manufacturing and storage of nanostructured medical devices has not been thoroughly considered. As demonstrated by Pantanella *et al.* [7], despite some antibacterial activity on planktonic bacteria [8], SWCNTs-coated surfaces are not suitable to counteract bacterial adhesion and biofilm development. Therefore, the potential risk of contamination by adherent bacteria and/or biofilm formation on nanostructured surfaces can lead to the unwanted onset of bacterial infections. Although CNTs could be sterilized [7] and the antibacterial activity of CNTs could be enhanced by functionalization with specific antibacterial agents [9], the sterility of nanostructured materials during all the manufacturing process cannot be ensured. For this reason, the ability of carrying out the microbiological control quality of nanostructured materials before administration for human use, as food industry or therapeutic treatments is an essential requirement. A fundamental prerequisite in studying bacterial adhesion and biofilm formation on medical devices and biomaterials is the quantitative evaluation of the actual bacterial number. However, the standard method used to evaluate the number of bacteria based on determination of colony forming units (CFUs) can be considered fully appropriate only when bacteria are in planktonic lifestyle but it is unreliable to count bacteria adherent and in biofilm lifestyle. Different analytic procedures have been attempted for detecting bacterial population adherent or organized in biofilm on surface [10-17]. Among these, the most commonly used method is based on detaching the microorganisms from abiotic surfaces by sonication and then counting the detached bacteria using the standardized CFU method [18, 19]. However, this method displays several drawback, because it can affect bacterial viability [20] and does not guarantee that all the microorganisms will be detached from the substrate, resulting in a misevaluation of the adherent bacterial population.

Here, the ability of two bacterial pathogenic species, *i.e.*, *Streptococcus mutans* and *Pseudomonas aeruginosa*, to adhere on surfaces coated with SWCNTs was evaluated by conventional sonication followed by CFU method (S-CFU) and BioTimer Assay (BTA), which allows easily counting bacteria in adherent and biofilm lifestyle without sample manipulation [15, 16, 21, 22]. *S. mutans* and *P. aeruginosa* have been chosen as bacterial models for their well-known ability to adhere and grow in biofilm lifestyle and for their implication in human diseases [23-27].

2. – Materials and methods

2˙1. *Bacterial strains and culture media.* – *S. mutans* ATCC 25175T and *P. aeruginosa* ATCC 15692 (PAO1) were maintained in Trypticase Soy broth (TS; Difco Laboratories, MD, USA) with glycerol (25%) at −80 °C and checked for purity on Columbia CNA agar (Difco) with 5% red sheep cells and TSA, respectively, before use. *S. mutans* and *P. aeruginosa* were grown in 1% sucrose-Brain Hearth Infusion (BHI; Oxoid Ltd., UK) and BHI (Oxoid) broth, respectively, at 37 °C without agitation for 18 to 24 hours.

2˙2. *Single-walled carbon-nanotubes–coated glass surfaces.* – Commercial glass beads (GBs) with 5 mm of diameter were used, either as uncoated or coated with single-walled carbon nanotubes (SWCNTs) films. SWCNTs-coated GBs were produced by first cleaning the purchased GBs for 30 min in a solution composed of one-third of H_2O_2 (30%) and two-thirds H_2SO_4 (18 M). GBs were subsequently washed with distilled water and dried under a N_2 flow. Immediately after cleaning, GBs were coated by drop casting with commercial SWCNTs (Cheap Tubes Inc., purity > 90% and outer diameter 1-2 nm) previously dispersed in a $CHCl_3$ solution. Uncoated and SWCNTs-coated GBs were sterilized by autoclaving at 121 °C for 15 min before use. The sterilization process was efficient and did not alter the overall quality of SWCNTs coating the glass surfaces [7].

2˙3. *Detection of bacterial colonization on uncoated and SWCNTs-coated GBs.* – To obtain bacterial adhesion and biofilm formation, 10^6 CFU/ml of *S. mutans* and *P. aeruginosa* were incubated in sucrose-BHI and BHI, respectively, for 24 hours at 37 °C in the presence of uncoated and SWCNTs-coated GBs. After incubation, uncoated and SWCNTs-coated GBs were washed three times in sterile saline solution and the bacterial number was estimated by S-CFU and BTA methods, as described below.

i) *Sonication and CFU counting method (S-CFU).* The first step of S-CFU method required the detaching of *S. mutans* and *P. aeruginosa* from colonized GBs by sonication. For this purpose, colonized uncoated and SWCNTs-coated GBs were immersed in 1 ml of sterile saline solution and sonicated using Soniprep Model 150MSE at a frequency of 23 kHz for a time of 10 s for *P. aeruginosa* and 30 s for *S. mutans*, such values having been determined as those providing the highest efficacy of detachment in preliminary sonication experiments (data not shown). The sample vials were kept in an ice-water bath to prevent significant heating in the sample during sonication. Thereafter, detached bacteria were vortexed for 30 s to disrupt bacterial aggregates. Bacterial suspensions were properly diluted in sterile saline and 0.01 ml of each dilution was plated on BHI agar plates. After 24-48 hours of incubation at 37 °C, the number of colonies was estimated and the results were referred as CFUs/ml.

ii) *BioTimer Assay.* BTA employs different specific reagents for *Streptococcus* and *Pseudomonas* genera. BioTimer-phenol red reagent (BT-PR) [15] was used to count *S. mutans*, a fermenting bacterium, and BioTimer-resazurin reagent (BT-RZ) [7, 22] was used to count *P. aeruginosa*, a non-fermenting bacterium. BTA measures microbial metabolism: the time required for colour switch of BTA reagents (*i.e.*, BT-PR: red-to-yellow; BT-RZ: blue-to-pink), due to the bacterial metabolism, is correlated to the initial bacterial concentration. Therefore, the time required for colour switch determines the number of bacteria present in a sample at time 0 through a specific correlation line. Correlation lines to count *S. mutans* and *P. aeruginosa* were obtained as described elsewhere [7]. For detection of adherent bacteria, colonized GBs

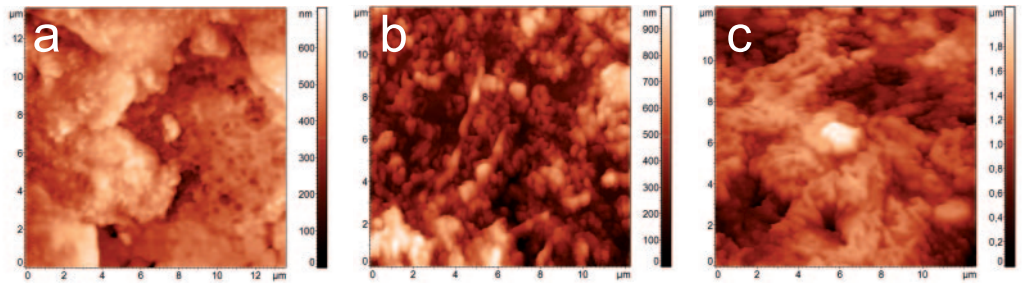

Fig. 1. – AFM images of: (a) uncoated GB; (b) SWCNTs-coated GB and (c) *Streptococcus mutans* colonized SWCNTs-coated GB.

were immersed in 1 ml of the specific BTA reagent and incubated at 37 °C. The time required for colour switching of the inoculated BT-PR and BT-RZ reagents was recorded and used to evaluate the number of *S. mutans* and *P. aeruginosa*, respectively, through the specific correlation line. Moreover, the residual adherent bacteria after sonication were estimated by immersing the colonized GBs, after sonication and vortex, in 1 ml of the specific BTA reagent and recording the time required for colour switching. As the correlation lines correlated the time for colour switch of BTA reagents with the number of planktonic CFUs, the number of adherent bacteria was expressed as planktonic-equivalent CFUs (PE-CFUs) [15].

2˙4. *Microscopy morphological characterization.* – The surface morphology of sterile and colonized SWCNTs-GBs was observed by atomic force microscopy (AFM). AFM morphological characterization has been performed using a standard apparatus (Solver, NT-MDT, Russia) equipped with standard Si cantilevers. Images were collected in standard AFM semicontact mode in air and at room temperature.

2˙5. *Statistics.* – All experiments were repeated at least five times to obtain mean values and standard deviations. Statistical analysis was performed using Student's t-test and P values < 0.05 were considered significant.

3. – Results and discussion

The surface morphology of uncoated GBs (fig. 1a), SWCNTs coated GBs (fig. 1b) and colonized SWCNTs-GBs (fig. 1c) was observed by AFM. The adopted nano-coating methodology was able to ensure a uniform nano-coated surface constituted by randomly entangled SWCNTs bundles. Bacteria in biofilm colonizing the SWCNTs-coated surfaces were clearly observed (fig. 1c).

The enumeration of *S. mutans* and *P. aeruginosa* colonizing uncoated and SWCNTs-coated GBs was performed using both S-CFU and BTA methods (table I). We showed that *S. mutans* and *P. aeruginosa* were able to adhere after 24 hours to uncoated and SWCNTs-coated surfaces with comparable adhesion efficiency. The counts of adherent bacteria on both uncoated and SWCNTs-coated colonized GBs were lower using S-CFU than BTA method. These results indicated that part of the bacterial population was not detached by sonication. To confirm this observation, the residual adherent bacterial population on colonized GBs after sonication was evaluated by BTA (table I) showing that about 10–14% of bacteria were not detached from the colonized GBs by sonication (table II). The results were comparable for both, uncoated and SWCNTs-

TABLE I. – *Enumeration of adherent bacteria and residual adherent bacteria after sonication for Streptococcus mutans and Pseudomonas aeruginosa colonizing uncoated or SWCNTs-coated glass beads (GBs) by the conventional sonication and colony-forming unit method (S-CFU) or BioTimer Assay (BTA). All results are expressed as the mean ± standard deviation of at least three independent experiments. ND: not determined.*

Bacteria	Uncoated GBs				SWCNT-GBs			
	Adherent		Residual		Adherent		Residual	
	S-CFU ($\times 10^8$)	BTA ($\times 10^8$)	S-CFU ($\times 10^8$)	BTA ($\times 10^8$)	S-CFU ($\times 10^8$)	BTA ($\times 10^8$)	S-CFU ($\times 10^8$)	BTA ($\times 10^8$)
S. mutans	1.0 ± 2.3	3.6 ± 0.6	ND	0.51 ± 0.05	1.2 ± 1.7	3.4 ± 0.5	ND	0.35 ± 0.07
P. aeruginosa	1.9 ± 1.8	5.0 ± 0.9	ND	0.48 ± 0.10	2.5 ± 2.4	6.0 ± 1.0	ND	0.70 ± 0.13

TABLE II. – *Percentage of detached bacteria by S-CFU method (calculated as the percentages of adherent bacteria as determined by S-CFU respect to adherent bacteria as determined by BTA), residual adherent bacteria after sonication (calculated as the percentages of residual adherent bacteria after sonication respect to adherent bacteria as determined by BTA) and dead bacteria by sonication (percentage of the difference between the number of adherent bacteria as determined by BTA (*100*%) and the sum of detached and residual adherent bacteria), calculated for Streptococcus mutans and Pseudomonas aeruginosa colonizing uncoated or SWCNTs-coated glass beads (GBs).*

Bacteria	Uncoated GBs			SWCNT-GBs		
	Detached (%)	Residual (%)	Dead (%)	Detached (%)	Residual (%)	Dead (%)
S. mutans	27.8	14.2	58.0	35.2	10.3	54.5
P. aeruginosa	38.0	9.6	52.4	41.7	11.7	46.6

coated, colonized GBs. Moreover, the adherent bacterial population on uncoated and SWCNTs-coated GBs calculated by BTA before sonication was higher than that calculated by the sum of detached and residual adherent bacteria, suggesting that the sonication method killed a fraction of bacterial population (table II). The killing efficiency varied in relation to bacterial species and the results showed elevated variability as indicated by the high standard deviation values. Therefore, unlike the S-CFU method, BTA showed to be more reliable to count the actual number of bacteria colonizing SWCNTs-GBs.

4. – Conclusion

Our results demonstrate that *S. mutans* and *P. aeruginosa* were able to adhere to uncoated and SWCNTs-coated surfaces with comparable adhesion efficiency and moreover that the sonication procedure is unreliable to actually determine the number of adherent bacteria as it kills part of the bacterial population and provides unreliable results with high standard deviation values. On the other hand, the advantages of BTA are represented by the reliability and versatility of a non-invasive method of investigation, which does not require any manipulation of the samples. For these reasons, BTA can be useful employed to evaluate the sterility of the nanostructured materials as well as their susceptibility to bacterial adhesion to perform microbiological quality control of nanostructured materials for human use.

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