CHARACTERISATION OF ANTI-MICROBIAL ACTIVITY OF THE NANOCOMPOSITE AgNPS-CNC-GO FOR BILIARY STENTS MANUFACTURING

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INTRODUCTION

Endoscopic biliary stenting is a well-established therapeutic approach in biliary obstructive diseases. Common complication in this practice is the reiterated occlusion, caused by biofilm formation inside the stent and is associated with recurrent jaundice, with or without cholangitis. Stent removal and replacement with a new one is often needed incurring additional health care costs and worsening the patient's quality of life. Thus, prevention of bacterial infection before the formation of bacterial biofilms on the surface of the stent is critical to achieve long- term stent patency.

The aim of this study is the characterization of the anti-planktonic and anti-biofilm activity of AgNPs-CNC-GO nanocomposite against *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Candida albicans* to be used in biliary stents manufacturing.

MATERIAL AND METHODS

AgNPs-CNC-GO (graphene oxide conjugated with silver nanoparticles and nanocrystallized cellulose) was synthetized in two formulations: solid soluble in water and as a surface coating developed with quartz crystal microbalance with dissipation monitoring. Antimicrobial activity was tested against three microorganism isolated from occluded stents collected during stent replacement: *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Candida albicans.* Anti-planktonic and anti-biofilm activity characterization was performed by MIC, MBC and MFC determination, viability curves, Radical Oxigen Species (ROS) quantification and Lactate dehydrogenase activity quantification assay (LDH assays), live and dead fluorescent assays and SEM imaging. Mammalian cytotoxicity was tested with MTT assay.

RESULTS

ANTI-PLANKTONIC ACTIVITY CHARACTERISATION of AgNPs-CNC-GO

1. MIC DETERMINATION

The effect of the nanocomposite against bacteria and yeast isolated from occluded stents was firstly investigated by MIC determination. Table 1 shows MIC values for *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Candida albicans,* respectively.

Tested isolates	MIC (µg/ml)		
	AgNPs-CNC-GO	GO	CNC
K. pneumoniae	3,12	>32	>128
E. faecalis	6,25	>32	>128
C. albicans	3,12	>32	>128

Table 1. MIC values of the isolates tested in the study

2. GROWTH CURVES

Klebsiella pneumoniae, *Enterococcus faecalis* and *Candida albicans* were incubated alone and with MIC/2, MIC e 2XMIC concentrations of AgNPs-CNC-GO for 18 hours at 37°C. Optical density (O.D.) was measured every hour. As shown in figure 1, AgNPs-CNC-GO at 2X concentration completely inhibits the grow of the microorganisms tested, while at MIC and MIC/2 the growth is significantly delayed compared to the control and reaches lower OD values after 18 hours compared to the growth of the untreated cells. The greater effect has been detected for *C. albicans* and *K. pneumoniae*.

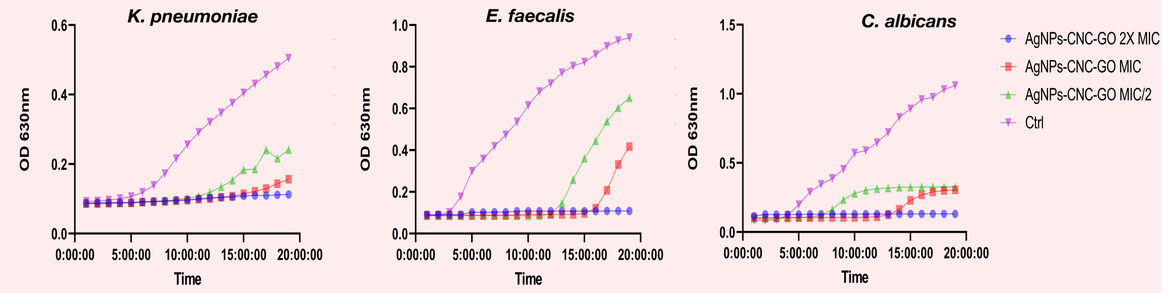


Figure 1. Viability curves of *K. pneumoniae, E. faecalis* and *C. albicans.* $O.D._{630}$ was monitored every hour for 18h with Cytation 5 Cell Imaging Multi-Mode Reader.

3. ROS and LDH assays

K. pneumoniae, *E. faecalis* and *C. albicans* were incubated alone and with MIC concentration of AgNPs-CNC-GO for 4 hours. ROS was measured using DCFDA (Fig. 3). After 4 h incubation, significantly increased ROS was detected in AgNPs-CNC-GO treated group of the three microorganisms tested compared to control, indicating the the induction of oxidative stress by AgNPs-CNC-GO. To determine oxidative stress-induced damage of the respiratory system of the cells, LDH activity was measured. LDH activity in treated cells group was significantly lower than that of the untreated control group (Fig 4). These results indicate that either ROS inhibits LDH, an important enzyme in cellular respiration. As a result, AgNPs-CNC-GO cause inhibition of bacterial growth and reproduction.

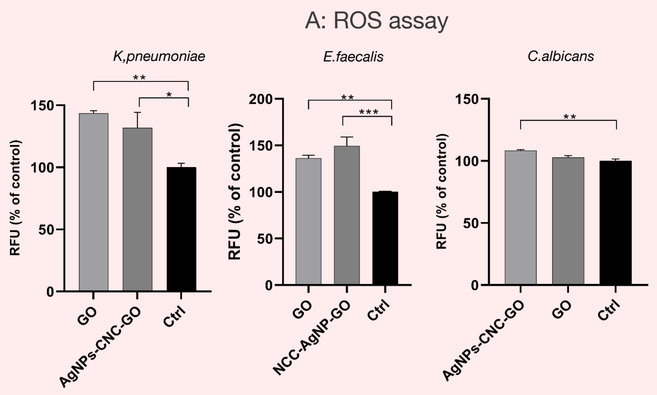


Figure 2. ROS assay. Intracellular ROS generation quantification was performed through the DCFH-DA assay. Results are indicated as % of control (untreated cells).

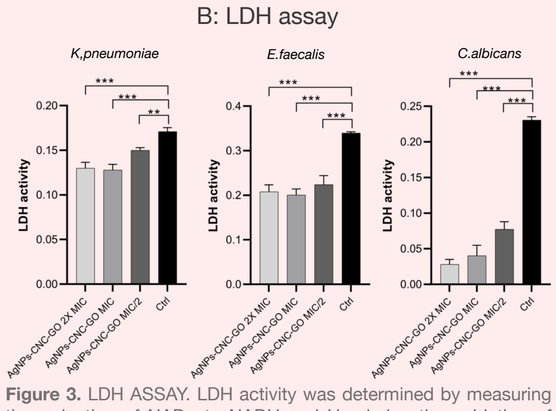


Figure 3. LDH ASSAY. LDH activity was determined by measuring the reduction of NAD+ to NADH and H+ during the oxidation of lactate to pyruvate. LDH the activity was calculated by subtracting the absorbance at 630nm and that at 450nm, as indicated by the manufacture's instruction.

4. CYTOXICITY assay

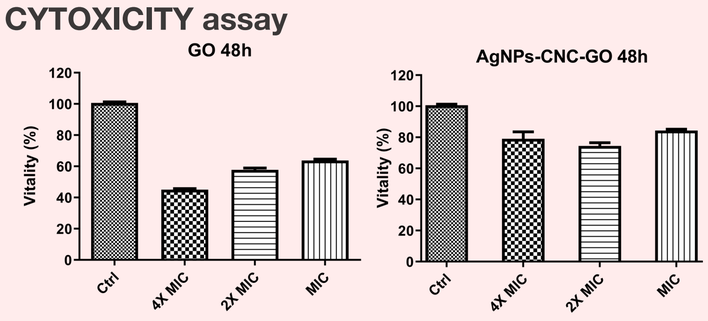


Figure 4. MTT cytotoxicity assay on MT3 mouse fibroblast of GO alone and of AgNPs-CNC-GO after 48 hours of incubation with different concentrations of the compounds. Results are reported as % of vitality compared to the untreated cells.

The biocompatibility of the nanocomposite was evaluated on MT3 mouse fibroblasts. GO alone was also tested as control. AgNPs-CNC-GO resulted to be biocompatible, with a good vitality rate from 77% to 92%, demonstrating that the functionalisation of GO decreases its toxicity.

ANTI-BIOFILM ACTIVITY CHARACTERISATION of AgNPs-CNC-GO

5. AgNPs-CNC-GO COATED SURFACE PREPARATION

Two bilayers of chitosan/graphene oxide/chitosan/AgNPs-CNC were assembled following the layer by layers procedure; the procedure was monitored using a Quartz Crystal Microbalance with dissipation monitoring (QCM-D). The coated surface was then analysed by AFM (Atomic Force Microscopy), showing a uniform distribution of the nanocomposite on plastic disk.

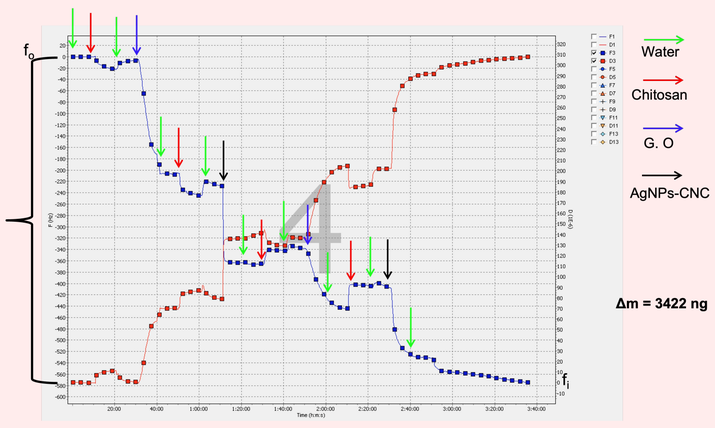


Figure 5. Quartz Crystal Microbalance with dissipation monitoring (QCM-D. The coated surface was assembled using chitosan as assembling agent.

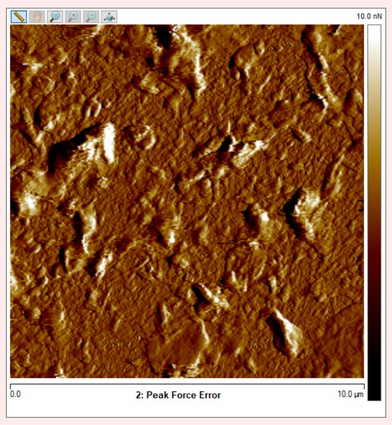
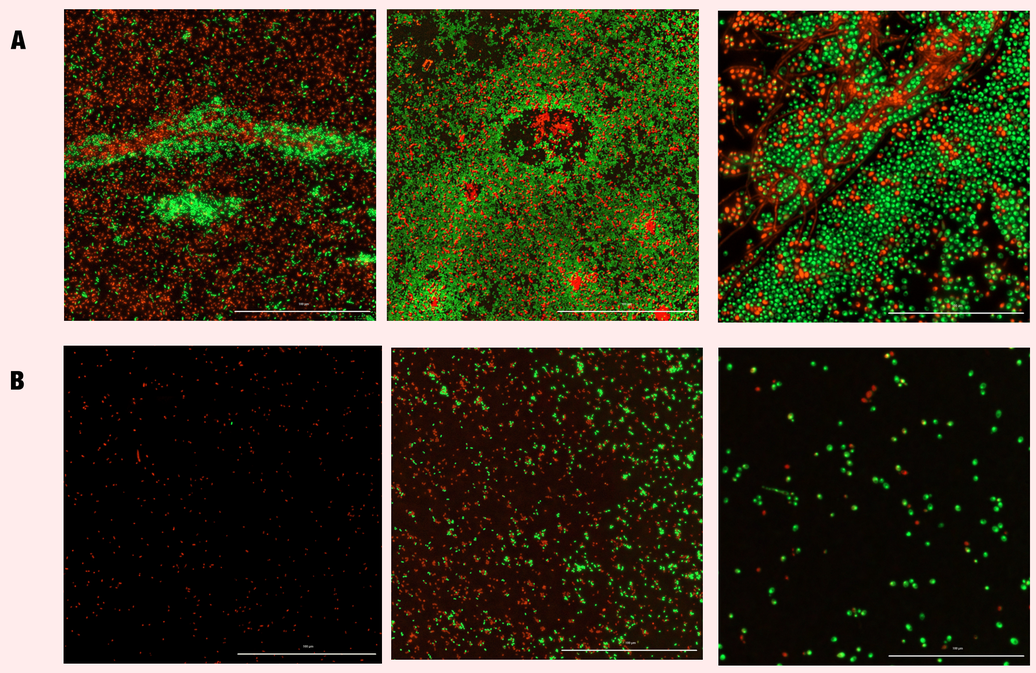


Figure 6. Atomic Force Microscopy (AFM) of AgNPs-CNC-GO coated surface.

6. BIOFILM LIVE AND DEAD assay



7. BIOFILM MICROSCOPY: SEM

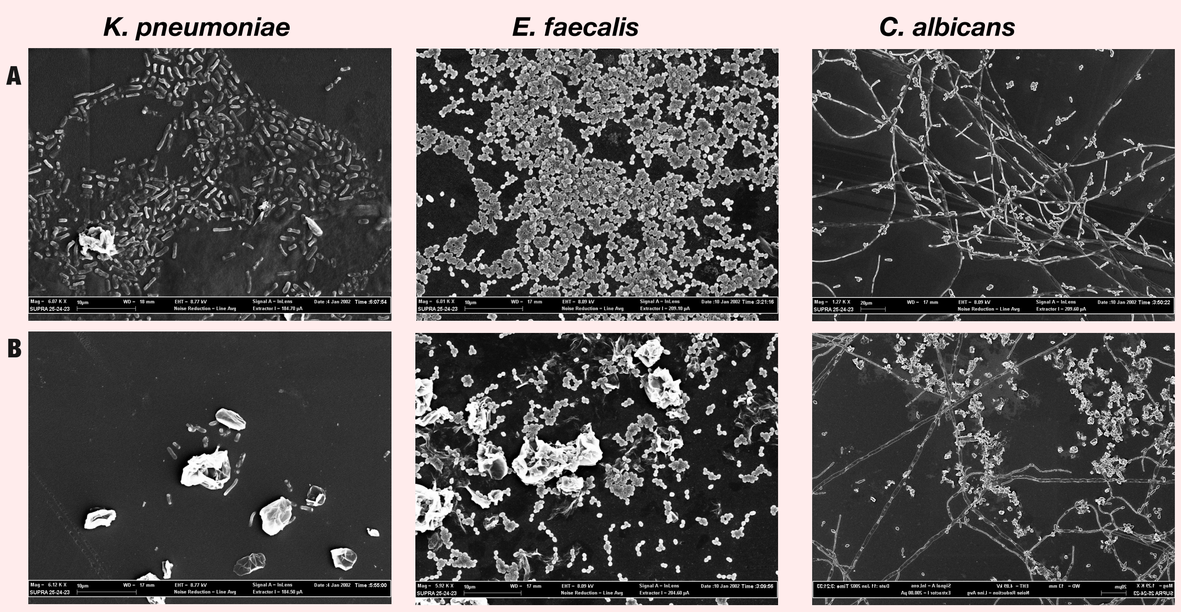


Figure 8. Representative images of SEM morphology of *K. pneumoniae*, *E .faecalis* and *C. albicans* grown on plastic (A) and on AgNPs-CNC-GO coated surface (B) for 72 hours.

Figure 7. Representative images of LIVE and DEAD assay of *K. pneumoniae*, *E. faecalis* and *C. albicans* grown on plastic (A) and on AgNPs-CNC-GO coated surface (B). Live cells are stained with Syto9 (green), dead cells are stained with propidium iodine (red). Images were taken at 40X magnification with Cytation 5 Cell Imaging Multi-Mode Reader and merged with ImeJ software.

The antibiofilm properties of Ag-NPs-CNC-GO coated surfaces were investigated incubating the three microorganisms with the coated surface and with plastic disks, as control for 72 hours at 37°C. Then, live and dead fluorescent assay and SEM microscopy were performed to analyse the possible inhibition of biofilm formation on coated surfaces. Live and dead assay allows the visualisation of live cells, stained in green, and dead cells, stained in red, respectively. As shown in figure 7, *K. pneumoniae*, *E. faecalis* and *C. albicans* grown on plastic disks shown numerous cells adherent, with high proportion of live cells while cells incubated with coated surfaces show lower adherent cells with higher proportion of dead cells. The morphology of the biofilm formed either on plastic and coated-surfaces was investigated by SEM microscopy (figure 8). Biofilm formed on plastic disks showed the typical architecture of mature biofilm with numerus cells organised in micro and macro-colonies surrounded by extra-cellular polymeric substances (EPS), while biofilm grown on Ag-NPs-CNC-GO surface present morphological alterations. Imaged showed few adherent cells and a complete inhibition of the secretion of EPS compared to the control, confirming the live and dead assay.

CONCLUSION

AgNPs-CNC-GO showed a strong anti-planktonic and anti-biofilm activity against the main microorganisms isolated from occluded stents: *K. pneumoniae*, *E. faecalis* and *C. albicans*, demonstrating a broad-activity against gram positive and gram negative bacteria and yeast. Moreover, it appears to be biocompatible. These data suggest that the nanocomposite Ag-NPs-GO is a promising material for biliary stent manufacturing. It is now in progress the evaluation of the efficacy in a dynamic model to reproduce the biliary system.

REFERENCES: Lübbert C, Wendt K, Feisthammel J, Moter A, Lippmann N, Busch T, Mössner J, Hoffmeister A, Rodloff AC. Epidemiology and Resistance Patterns of Bacterial and Fungal Colonization of Biliary Plastic Stents: A Prospective Cohort Study. PLoS One. 2016 May 12;11(5):e0155479. doi: 10.1371/journal.pone.0155479. PMID: 27171497; PMCID: PMC4865241.