ORIGINAL ARTICLE

Resistance to Biofilm Formation by Synergistic Combination of Cerium Oxide Nanoparticles with antibiotics against wound/ urinary catheter isolated bacteria

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ABSTRACT

Biofilm forming multidrug resistant bacteria has become a threat to therapy. In this work we aim to potentiate first line antibiotics by the synergistic combination with cerium oxide nanoparticles. Bacterial specimens collected from burn wounds and urinary catheters are diagnosed by culturing and biochemical tests. CeO_2 NPs are analyzed for fcc structure and nanoparticles size by X-Ray diffraction. Antibacterial activity and inhibition against bacterial isolates for CeO_2 NPs and respective antibiotics are tested by agar diffusion method and checkerboard assay. In the synergistic combination of CeO_2 NPs with Ciprofloxacin, Levofloxacin and Rifampicin against *Proteus mirabilis*, *Escherichia coli* and *Staphylococcus aureus* the minimum inhibitory concentrations for the three antibiotics are reduced by 16- fold from their individual MIC and their inhibition are raised to the edge of 90 %. Above a concentration of 24 µg/ml Ciprofloxacin, Levofloxacin and Rifampicin showed additive interaction judging from their fractional inhibitory concentration values. The study demonstrates the efficacy of cerium oxide nanoparticles in potentiating the antibiotic mechanism of interaction to circumvent bacterial resistance.
Keywords: wounds, urinary catheter, synergy, cerium oxide, antibiotic

INTRODUCTION

Health care-associated infections resulting from urinary tract infections of indwelling catheter and from chronic wounds have high prevalence[1]. *E.coli* is one of the common colonizers of catheter surfaces both external and internal[2]. Biofilm-forming bacteria are encapsulated with viscous layer and become resistant to external stress thereby avoiding the host immune system[3]. The evolution of pathogens resistant to antibiotic treatment turned into a sophisticated global therapy[4]. Even with the use of broad-spectrum antibiotics of the first choice to ward off infections, bacteria still develop resistance to antibiotic due to improper use[5]. One way to circumvent bacterial resistance is to potentiate the antibiotics by synergistic combinations with natural phytochemicals[6] or with other antibiotics[7]. Recently, several non-antibiotic agents have been highlighted as candidates to be used in conjugation with antibiotics to inhibit bacterial resistance[8]. Metal oxide nano-particles in their capacity of high surface area have potential activity to interact with pathogenic bacteria and deactivate their influence[9]. Antibacterial activity of nano particles depend on their size, stability, concentration[10] and minimal toxicity[11]. Bacterial growth inhibition of *E.coli* has been reported to be inversely proportional to nanoparticle size[12]. Among nanoparticles of interest, cerium oxide (CeO_2) - a rare earth metal oxide that belong to the cubic fluorite type structure[13] and of extensive applications in medicine and catalysis[14]. Antimicrobial activity of CeO_2 NPs on opportunistic microorganisms has been systematically reviewed[15]. The mechanism of action of CeO_2 NPs showed variant activity in gram-positive/negative bacteria depending on the peptidoglycan layer thickness that protects the cytoplasmic membrane from external chemical agents[16].

The goal of this study is to explore the synergistic combination between CeO_2 NPs and various first line antibiotics that can inhibit biofilm formation on wound/urinary catheter by bacterial invasion.

MATERIAL AND METHODS

Collection, isolation and identification of bacteria: For wound: Specimens of bacteria were isolated by swabbing wounds with a sterile swab and at once taken to the laboratory in icebox container for isolation. The swabs were moved to a blood agar medium. For urinary catheter: The surface of the catheter was washed with sterile de-ionized water (SDW) and was cut at cross-sections. The cut specimens placed in 0.9% buffered saline, incubated for 1h and the bacteria were isolated by the pour plate technique. Bacterial growth samples from both sources were moved via sterile loop into nutrient broth media and incubated for 24 h at 37°C. Biochemical characterization tests were performed to diagnose and verify the bacterial strains[17].

NPs identification and dilution preparation: Nano cerium oxide powder of purity 99.97% were obtained from the Nanografi Nanotechnology, Ankara, Turkey. In order to confirm the identity of the nano powder, X-ray diffraction techniques were utilized in conjunction with the peak profile analysis for identifying the crystalline structure and measuring the nano-particle size.

Following the same procedure reported earlier in[10], the CeO_2 nanoparticles of 10 mg was suspended in 10 ml Dimethyl sulfoxide (DMSO) to prepare a stock solution of 1mg/ml. Sequential lower dilutions were made by the addition of 10 ml DMSO to the precursor stock solution.

Bacterial susceptibility to antibiotics: Susceptibility test was done for the bacterial isolates cultured to (10^8 CFU/ml) by using the disc diffusion test in accordance with the CLSI document M02-A12[18] in Mueller-Hinton agar plate (Himedia, India). Discs of ampicillin (30 µg), ciprofloxacin (30 µg), erythromycin (15 µg), levofloxacin (30 µg), rifampicin (30 µg) and tetracycline (30 µg) were used. The plates were cultured for 12 h at 37°C, followed by

measuring the diameter of inhibition zone around the well. The inhibition zone was interpreted by the Clinical and Laboratory Standards Institute[19].

Determination of MIC of CeO_2 NPs and antibiotics: The antibacterial activity of CeO_2 NPs and the selected antibiotics were performed by well diffusion in broth against the 23 selected clinical bacterial isolates. In the 1st step, we added 0.2 ml of antibiotics (concentration 384 µg/ml) in one well; in the 2st step, we added 0.2 ml of CeO_2 NPs (concentration 192 µg/ml) in a separate well. From these wells 2-fold serial dilutions were added in successive wells. In the 3st step, we added 0.2 ml of the bacterial isolates (10^8 CFU/ml) to the respective wells. The obtained serial dilutions of CeO_2 NPs and antibiotics with the bacterial inoculums were incubated at 37°C for 24 hours. The concentration where no visible growth was seen was identified as MIC.

Synergic interaction of CeO_2 NPs and antibiotics: Estimation of synergy between CeO_2 NPs and antibiotics was done by the broth dilution checkerboard assay[20].

The FIC of CeO_2 NPs and antibiotics were evaluated by 2-fold serial dilution from 48 µg/ml down to 0.75 µg/ml. The broth and the growth control in the plates were kept with the culture alone without antimicrobial agent for comparison. The wells were inoculated with 20 µl of 10^6 CFU/ml of bacterial cultures and the plates were incubated for 24h at 37°C. The FIC index was calculated for CeO_2 NPs and antibiotics as in the following:

FIC index = $FIC_A + FIC_B$

Where $FIC_A = MIC_{A\ in\ combination} / MIC_{A\ alone}$

$FIC_B = MIC_{B\ in\ combination} / MIC_{B\ alone}$

The indices of FIC were interpreted as follows:- FIC ≤ 0.5 synergism ; FIC 0.5 - 1.0 additive ; FIC 1.0 – 4.0 indifference and FIC > 4.0 antagonism in accordance to the European Committee on Antimicrobial Susceptibility Testing[21].

f. Activity of CeO_2 NPs and antibiotics on biofilm formation

The clinical isolates (1x 10^6 CFU/ml) were incubated in LB broth for 24 hours at 37°C, then washed three times with PBS (pH= 7.4) in order to remove planktonic cells. In the next step, 0.2 ml of 2% crystal violet was added to the wells and kept stationary for 20 min., followed by the washing off excess crystal violet stain with SDW. Samples prepared in triplicates were introduced to a spectrophotometer set at 570 nm to measure Optical Density from which a mean value of each set was calculated.

Inhibition of biofilm formation measurements: Biofilm inhibition was done in a microtiter plate assay in accordance with the biofilm formation method[22]. For this, 20 µl each of 10^6 CFU/ml of *P. mirabilis*, *E. coli* and *S. aureus* was inoculated onto 18 µl of LB, then 20 µl of CeO_2 NPs (24 µg/ml) and ciprofloxacin, levofloxacin, rifampicin each (48 µg/ml) were added to the wells, and incubated at 37°C for 24 h. Optical densities were obtained as described earlier for CeO_2 NPs, each respective antibiotic and in combination against *P. mirabilis*, *E. coli* and *S. aureus* and the inhibition (%) was calculated by the formula[7].

Inhibition (%) = $OD_{control} - OD_{treated} / OD_{control} \times 100$

Experiments designed in three replications and statistical calculations were performed by the analysis of variance (ANOVA) SAS[23] with a significance of $P \leq 0.05$.

RESULTS

Physiomorphological identification of CeO_2 NPs: The X-ray diffraction pattern of CeO_2 NPs obtained by Cu Kα radiation is shown in Figure 1. The peaks of the diffraction profile are indexed by the PDF2-ICDD files to belong to the fcc structure of CeO_2. No additional peaks are observed in the diffraction pattern which reflects one pure phase of the CeO_2 NPs.

The width of the diffraction peaks at half-maximum were measured and used in the Debeye Scherer's formula to determine the crystallite size. To achieve accurate size analysis of the crystallites, broadened width of the diffraction peaks due to instrumental factors were subtracted from the actual peaks widths. The average size and standard error calculated for the six peaks in the X-ray pattern was found to be 28 ± 6.8 nm. Such a scale of particle size is preferable for antimicrobial activity as highlighted in[24].

In addition, significant feature observed in the diffraction pattern of the fcc structure of CeO_2 NPs is the diminishing of intensities of the even indices namely (200, 222) on the account of the increased intensity of the odd indices namely (111) which indicate a phenomena of preferred orientation of the crystallites and in-turn suggest ellipsoidal morphology of the nano-particles. These findings are in agreement with the literature describing morphology of CeO_2 NPs obtained by different methodology[13,25,26]. This renders the usefulness of X-ray diffraction method as a multi-purpose informative technique.

Susceptibility of isolates to antibiotics: Different classes of antibiotics were used to assess the susceptibility of the isolated bacteria, and the results are shown in Table 1.

Ampicillin, erythromycin and tetracycline reveal very low susceptibility to *P. mirabilis*, *E. coli* and *S. aureus* and are considered to be within the resistance zone according to the CLSI[18].

The highest inhibition zone was revealed by levofloxacin as ranging 26, 28, 24 mm for *P. mirabilis*, *E. coli, S. aureus* respectively. The results show that with the guidelines in[19] the bacteria are resistant to more than one class of antibiotic and are designated as multidrug resistant (MDR) which necessitate the demand of target strategies to confine MDR in bacteria.

Minimum Inhibitory Concentration: As the bacterial activity was assessed by well diffusion, the minimum concentration required to inhibit bacterial growth was done by determining MIC for CeO_2 NPs and for each of the six antibiotics and the results are given in Table 2.

Synergism between CeO_2 NPs and antibiotics against bacteria: The impact of the combination of CeO_2 NPs and antibiotics in different ratios on FIC index are shown in Table 3. The antibiotics considered at this stage of evaluation were ciprofloxacin, levofloxacin and rifampicin by referring to their lower MIC's in comparison with ampicillin, erythromycin and tetracycline (see Table 2). It can be noted that the combination of CeO_2 NPs and ciprofloxacin, levofloxacin and rifampicin antibiotics produce synergistic interaction whereby MIC is reduced by about 16-fold for *P. mirabilis*, *E. coli* and *S. aureus.*

The results obtained from dilution checkerboard assay confirmed the synergistic activity of CeO_2 NPs and

levofloxacin through a representative isobologram shown in Figure 2. MIC_A and MIC_B are the MIC of CeO_2 NPs and levofloxacin respectively. MIC_{AX} and MIC_{BX} are the MIC concentration of CeO_2 NPs and levofloxacin in the synergistic combination respectively.

The FIC index at the synergistic interaction for ciprofloxacin, levofloxacin and rifampicin with CeO2 NPs are shown as mean values and standard error in Table 4.

The influence of the combination of CeO_2 NPs and different antibiotics is assessed for their inhibitory effects on biofilm formation by *P. mirabilis*, *E. coli* and *S. aureus* and the results are given in Figure 3. Ciprofloxacin inhibited 19, 22, 25% , levofloxacin 25, 28, 32% and rifampicin 21, 23, 20% of *P.mirabilis*, *E. coli* and *S. aureus* biofilm respectively indicating low effectiveness of biofilm suppression and may be due to MDR resistance bacteria. However, the synergistic combination of CeO_2 NPs with ciprofloxacin, levofloxacin and rifampicin have significantly inhibited biofilm formation by three times as much as their inhibition alone for *P. mirabilis*, *E. coli* and *S. aureus* bacteria thereby showing that biofilm and planktonic cells of these bacteria having higher susceptibility to CeO_2 NPs and antibiotics in the synergistic combination.

Figure 1: X-ray diffraction of CeO2 NPs powder

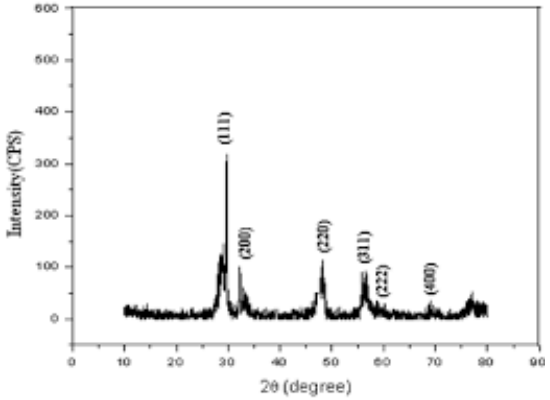


Table 1: Antibiotic sensitivity profile of bacteria

Bacteria	Diameter of inhibition zone (mm)					
	Amp	**Cipr**	**Eryth**	**Levo**	**Rifa**	**Tetra**
P. mirabilis	5	15	8	26±1	13	8
E. coli	3	13	7	28±1	11	10
S. aureus	4	10	9	24±1	14	7

Table 2: Antibacterial activity of CeO_2 NPs and antibiotics

	MIC (µg/ml)		
	P. mirabilis	*E. coli*	*S. aureus*
CeO_2 NPs	64	66	70
Ampicillin	180	175	169
Ciprofloxacin	95	98	99
Erythromycin	160	155	149
Levofloxacin	96	98	99
Rifampicin	97	94	96
Tetracycline	120	131	125

Table 3: FIC index of CeO_2 NPs and Levofloxacin

	CeO_2 NPs µg/ml	**Lev g/µml**	**FICA**	**FIC B**	**FIC Index**	**Inter-action**
P. mirabilis	24	6	0.38	0.06	0.44	Synergy
	12	12	0.19	0.12	0.31	Synergy
	6	24	0.09	0.25	0.34	Synergy
	3	48	0.05	0.5	0.55	Additive
E. coli	24	6	0.36	0.06	0.42	Synergy
	12	12	0.18	0.12	0.30	Synergy
	6	24	0.09	0.24	0.33	Synergy
	3	48	0.04	0.49	0.53	Additive
S. aureus	24	6	0.34	0.06	0.40	Synergy
	12	12	0.17	0.12	0.29	Synergy
	6	24	0.08	0.24	0.32	Synergy
	3	48	0.04	0.48	0.52	Additive

Figure 2: Isobolograms of potentiation between CeO_2 NPs and Levofloxacin for *P. mirabilis*, *E. coli* and *S. aureus.*

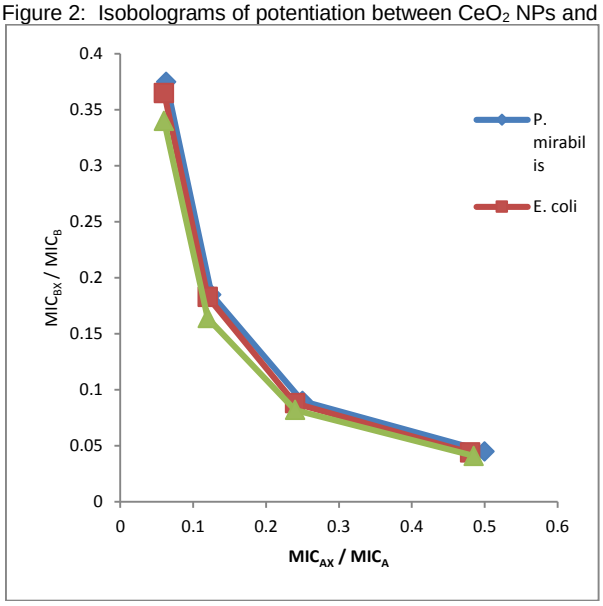
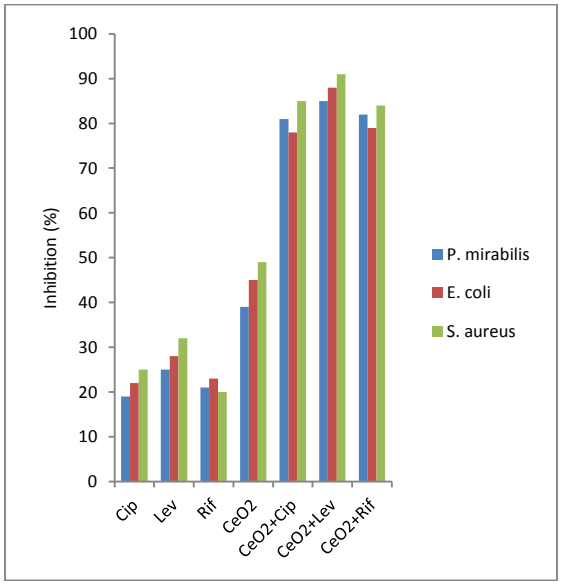


Table 4: Mean and SE of FIC index at synergistic interaction

Combination	FIC ± SE		
	P. mirabilis	*E. coli*	*S. aureus*
CeO_2NPs + Cip	0.38±0.04	0.36±0.03	0.35±0.04
CeO_2NPs + Lev	0.38±0.04	0.35±0.04	0.34±0.04
CeO_2NPs + Rif	0.39±0.03	0.38±0.04	0.36±0.03

e. Effect of CeO_2 NPs and antibiotics on biofilm formation

Figure 3: Inhibition of biofilm by CeO_2 NPs, Ciprofloxacin, Levofloxacin, Rifampicin and at synergic combinations for *P. mirabilis*, *E. coli* and *S. aureus*.



DISCUSSION

The minimum concentrations of CeO_2 NPs and ciprofloxacin, levofloxacin and rifampicin required in the synergistic combination is due to the physiochemical action against bacteria. CeO_2 NPs interact with the negatively charged membrane of bacteria by electrostatic attraction resulting in the confinement of the bacteria[27]. Furthermore, CeO_2 NPs induce oxidative stress by forming free radicals, which penetrate freely through bacterial cell membrane causing disruption effect[28]. The bactericidal activity of CeO_2 NPs may be envisaged in releasing cerium ions which make their way in the disruption of membrane integrity and the inhibition of metabolic activity. The efficacy of CeO_2 NPs for *P. mirabilis*, *E. coli* and *S. aureus* was identified as approximately equivalent to a value of 66.6±2.5 µg/ml, but in the synergistic combination with the ciprofloxacin,

levofloxacin and rifampicin, the FIC was a maximum at 24 µg/ml.

CONCLUSION

In the synergistic interaction between CeO_2 NPs and first line antibiotics in the fight against biofilm forming bacteria, we find a pronounced activity of inhibition of bacterial growth coupled with the concentration reduction of minimum inhibition by 16-fold. This renders synergism a frontier procedure to target multidrug-resistant and forming biofilm bacteria.

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Conflict of Interest: None.

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