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Electronic Supplementary Information (ESI)

A Combination Therapy Strategy for Treating Antibiotic Resistant Biofilm Infection Using Guanidinium Derivative and Nanoparticulate Ag(0) Derived Hybrid Gel Conjugate

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Table-S1 CFU count in *in-vivo* model

Group	Infection and treatment type	Number of Animal	DAY0	DAY1	DAY2	DAY3	DAY4
A	Surgical control	(Animal- A1, A2)	No microbial infection and treatment	50µl-2xyt Agar A1-0 Colony A2-0 Colony	(100µl-2xyt Agar) A1-0 Colony A2-0 Colony	100µl-2xyt Agar A1-0 Colony A2-4 Colony	100µl-2xyt Agar A1-0 Colony A2-4 Colony
B	*Pseudomonas aeruginosa* (PA)	(Animal- B1, B2, B3)	Bac. Suspension 5µl(10^3Cell/ml)	B1 - 2	B1-6	B1-17	B1-31
				B2 - 3	B2-7	B2-16	B2-28
				B3- 2	B3-7	B3-13	B3-30
C	*Pseudomonas aeruginosa* (PA)+ AD-L@Ag(0)	(Animal- E1, E2, E3)	Bac. Suspension 5µl (10^3Cell/ml) + AD-L@Ag(0)	E1-1	E1-1	E1-2	E1-2
				E2-1	E2-2	E2-2	E2-3
				E3-0	E3-0	E3-1	E3-2
E	*Klebsiella pneumoniae* (KP)	(Animal- C1, C2, C3)	Bac. Suspension 5µl (10^3Cell/ml)	C1-3	C1-11	C1-17	C1-35
				C2-4	C2-10	C2-12	C2-31
				C3-1	C3-8	C3-11	C3-29
G	*Klebsiella pneumoniae*+ AD-@Ag(0)	(Animal- G1, G2, G3)	Bac. Suspension 5µl (10^3Cell/ml) + AD-L@Ag(0)	G1-0	G1-0	G1-0	G1-1
				G2-1	G2-1	G2-2	G2-1
				G3-1	G3-1	G3-1	G3-2
J	*Staphylococcus aureus* (SA)	(Animal- J1, J2, J3)	Bac.Suspension5µl(10^3Cell/ml)	J1-32	J1-59	J1-418	J1-548
				J2-23	J2-54	J2-456	J2-578
				J3-19	J3-48	J3-430	J3-557
M	*Staphylococcus aureus* + AD-@Ag(0)	(Animal- M1, M2, M3)	Bac. Suspension 5µl (10^3Cell/ml) + AD-L@Ag(0)	M1-2	M1-8	M1-11	M1-27
				M2-5	M2-5	M2-7	M2-14
				M3-2	M3-7	M3-9	M3- 24

Table S1- Table represents the type of infection, treatment, different groups of BALB/C mice and colony forming unit (CFU) generated by skin patch lysate spread on the agar plate.

S2: Supplementary information on detailed methods

Determination of minimum inhibition concentration (MIC)

Minimum inhibitory concentration (MIC) is considered as the lowest concentration of antimicrobial agents that will suppress the visible growth of microorganisms after a certain incubation period. As per the Clinical and Laboratory Standards Institute (CLSI) guidelines, the minimum bactericidal concentration (MBC) determination is also equally important. [1-4] MBC assay is used for evaluating any new antimicrobial agent/ drug for predicting its efficacy towards eradication (killing of 99.9% a specific bacterial species) of a particular bacterium. MBC was estimated for each isolate that we have used for the present study. Respective MBC value for *Klebsiella pneumoniae* (Clinical Isolate 16280), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 33592) *Acinetobacter baumannii* (ATCC BAA-2800),

Streptococcus pneumoniae (ATCC 49619), *Shigella flexneri* (Clinical isolate 1a) and *Escherichia coli* (Clinical P-192) is provided in Table S1.

The antibacterial activity of the AD-L@Ag(0) was determined using sterile round-bottom 96-well plates. Wells of each row on a 96-well plate were filled with 100µL of sterilized Mueller Hinton Broth. Specific wells designated for the formulation received an additional 100µl of a mixture of culture medium and AD-L@Ag(0). The AD-L@Ag(0) was serially diluted to create a concentration gradient from 200 µg/ml to 0.02 µg/ml. Each dilution had three replicates. Six wells each were used as a no-treatment growth control and negative control. The microtiter wells were incubated for 24h at 37°C. Quantification of the bacterial growth is done by measuring the optical density at 600 nm with a spectrophotometer, where turbidity attributes to the light scattering by bacteria. MIC for AD-L@Ag(0) was determined after 24h where growth was no longer visible by assessment of turbidity at 600 nm. Three biological and two experimental repetitions were run for each assay. The MBC is evaluated by subculturing the broths used for MIC determination onto fresh agar plates. ATP can be used as a marker for evaluating the cell viability for prokaryotes and eukaryotes. After 24 hours incubation of MIC plate, 10ul culture from the wells was spotted on agar containing plate for further colony count. The determination of ATP via the BacTiter-Glo™ (G8230, Promega) assay is discussed in the BacTiter-Glo™ Microbial Cell Viability Assay Technical Bulletin #TB337. [5] This assay procedure provides a method for determining the number of viable microbial cells in culture based on quantitation of the ATP present. The homogeneous assay involves adding a single reagent (BacTiter-Glo™ Reagent) directly to bacterial cells cultured in medium and measuring luminescence. Following the standard protocol, an equal volume of culture and Baciter Glo was incubated at 37°C for 5 minutes. The relative unit (RLU) was determined by using BioTek Synergy HTX Multimode Reader (Agilent, US). The luminescence value for media only (control-without cells) wells was used to obtain a value for background luminescence and was subtracted to get the actual RLU reading. No colony and less than 500 RLU concentration readings were considered as MBC concentration for a particular bacterium. The experiment was performed in triplicate. The difference in proliferation level of treatment and control group was compared by analysing the using GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA.

Time kill assay

Time kill assay is used to study the activity of AD-L@Ag(0) against a bacterial strain for determining the bacteriostatic and/or bactericidal activity(ies) over timeTime-kill kinetics. Time kill assays were performed for *Klebsiella pneumoniae* (Clinical Isolates, 16280) and *Staphylococcus aureus* (ATCC 33592) following the literature procedure. Concentrations equal to half of MIC, MIC, twice the MIC, and four times the MIC of the AD-L@Ag(0) nano-formulation were prepared. An inoculum size of 5.0×10^5 CFU/mL was added to each of these four solutions and incubated at 37°C with agitation. Aliquots of 200μL of the medium were taken at time intervals of 0, 2, 4, 8,16, and 24 h for measurement of optical density and 50 μL of samples along with positive control were plated by spread plate method on Mueller Hinton agar plate and incubated at 37°C for 24 h. A positive control test was performed for the organisms without the nano-formulation or reference antibiotic. The colony forming unit (CFU) of the organisms was determined. The procedure was performed in triplicate (three independent experiments) and a graph of the log CFU/mL was plotted against time. For all the individual experiments, at least three biological and two experimental repetitions were done.

Crystal violet (CV) for MBIC (Minimum biofilm inhibitory concentration) Determination

Crystal Violet (CV) is a basic dye that binds non-specifically to negatively charged surface molecules such as polysaccharides and eDNA in the extracellular matrix and is generally used to evaluate biofilm biomass. Isolates *Klebsiella pneumoniae* (Clinical Isolate 16280), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 33592) *Acinetobacter baumannii* (ATCC BAA-2800), *Streptococcus pneumoniae* (ATCC strain 49619), *Shigella flexneri* (Clinical isolate 1a) and *Escherichia coli* (Clinical P-192) were used for this assay. To do crystal staining, the 48 hours old biofilm was washed twice with sterile PBS to remove planktonic cells. Then biofilm was stained with an equal volume of 0.5% CV and incubated for 45 minutes at room temp. Excess CV was then removed by washing twice with sterile PBS. The biofilm is then detached by 95% ethanol and quantified on the basis of turbidity. Turbidity was measured by taking OD_{595} in a microplate ELISA reader (Bio-Rad). The experiment was performed in triplicate. The proliferation difference between nanocomposite coated and the uncoated surface was compared by analysing the proliferation using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA.

EPS Quantification for MBEC (Minimum Biofilm Eradication Concentration) Determination

Exopolysaccharide (EPS) quantification was carried out to measure the biofilm eradication effect of AD-L@Ag(0) (0) in *Klebsiella pneumoniae* (Clinical Isolate 16280), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 33592) *Acinetobacter baumannii* (ATCC BAA-2800), *Streptococcus pneumoniae* (ATCC strain 49619), *Shigella flexneri* (Clinical isolate 1a) and *Escherichia coli* (Clinical P-192). Sulfuric acid (ACS reagent, 95.0-98.0%, Cat. No. 258105, Sigma) and phenol (Sigma-Aldrich), 96-well Flat-Bottomed (Polystyrene) microplate (Corning, Cat. No. 3595) were used. EPS quantification is done by Phenol-sulfuric assay.[6] For EPS quantification, 2X MBIC, MBIC and ½ MBIC concentration of AD-L@Ag(0) (0) are used in triplicates along with control i.e. without any formulation. For this, 1×10^6 bacteria were cultured in a 96-well plate for 40 hrs for the formation of a mature biofilm. Media is removed carefully followed by PBS wash to remove any planktonic cells present on the biofilm surface. Then the biofilm was suspended in 50 µl of sterile PBS Buffer. 150 µl of conc. sulfuric acid is added directly to the sample without touching the walls of the plate. This was incubated for 30 minutes at RT, followed by slow addition of 30 µl of 5% phenol to the wells and was allowed to incubate farther at 90ºC for 5-10 minutes. After cooling at room temperature, the microplate was wiped dry and absorbance (λ_{490nm}) was measured by a microplate reader (xMark™ Microplate Absorbance Spectrophotometer, Bio-Rad). The experiment was performed in triplicate. The proliferation difference between nanocomposite coated and the uncoated surface was compared by analysing the proliferation using Graph Pad Prism version 8.0.0 for Windows, Graph Pad Software, San Diego, California USA.

Cytotoxicity analysis of AD-L@Ag(0)

Cytotoxicity of the AD-L@Ag(0) formulation was evaluated against the commonly used epithelial cell line (Vero, E6) using the methyl thiazolyl tetrazolium (MTT) method. MTT method was performed as per previously published standard protocol by ATCC.[7] MTT assay detects reduction of yellow tetrazolium (MTT) by metabolically active cells. The resultant purple formazan is measured using spectrophotometry. Cells were seeded into a 96-well plate in Dulbecco's Modified Eagle's medium (D-MEM) with 10% fetal bovine serum (FBS), 5µl penstrep (penicillin + streptomycin). Cells were incubated at 37 ºC in 5% CO_2 for 4h and 24h with AD-L@Ag (0). After that, MTT reagent was added to each well, further incubation in a CO_2 incubator at 37 ◦C for 1h was done, which resulted a purple precipitate.

Formazan crystals were dissolved using 100µl DMSO, then plates were placed on the shaker for 1hr at RT. Absorbances at 570 nm (reference filter setting was 630 nm) was measured using a spectrophotometer (Bio-Rad). The experiment was performed in triplicate. The cytotoxic effect of AD-L@Ag(0) was checked by comparing control i.e. untreated Vero E6 Cells and treated Vero E6 Cells by different AD-L@Ag(0) concentrations using GraphPad Prism version 8.4.3 for Windows, GraphPad Software, San Diego, California USA.

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