Activating a silver lipoate nanocluster with a penicillin backbone induces a synergistic effect against S. aureus biofilm.

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Summary of Conjugation Reaction & Reaction Workup

Key mechanistic steps are summarized in Scheme S1.

Scheme S1: Key mechanistic steps of conjugation. The presence of the cluster has been omitted for clarity.
In the first step, carried out in pH 6 buffer, an EDC ester intermediate is formed but not isolated. Simultaneous addition of sNHS results in the formation of a sNHS ester that precipitates out of solution. The sNHS ester is cleaned and then allowed to react with 6-APA in pH 7 buffer, resulting in the formation of the conjugated cluster. Scheme S2, summarizes various reaction steps performed in a 2 mL microcentrifugation tube.

Scheme S2: Key steps in cluster conjugation.

Step 1. Start with 1.0 mL of 500 mM MES buffer solution.

Step 2: Add
a) 50 µL of cluster solution (1.4 mg Ag/mL concentration estimated to contain a maximum of 3 µmol RALA in total)  
b) 20 mg free-base EDC (130 µmol)  
c) 2 mg sNHS (13 µmol)

Step 3: React for 60 minutes

Step 4: Centrifuge at 2000 rpm for five minutes to precipitate less soluble sNHS-cluster. Discard supernatant.

Step 5: Wash precipitate twice with 500 µL of distilled water.

Step 6: Dissolve precipitate in 1 mL of 1 M TEAA solution or 250 mM MES buffer

Step 7: React for 60 minutes.

Step 8: Centrifuge at 2000 rpm for five minutes to precipitate less soluble conjugated cluster. Discard supernatant. Wash precipitate four times with 500 µL of distilled water.

Step 9: Dissolve conjugated product in 500 µL 50 mM TEA solution for ESI-MS analysis or suitable buffer for antibiotic testing.
Ampicillin Conjugation

Results of conjugating the \((Ag_{29}LA_{12})^{3+}\) cluster to ampicillin are summarized in Figure S1. The conjugate was obtained by substituting molar equivalents of ampicillin for 6-APA in the synthetic protocol.

Figure S1: ESI-MS evidence of conjugation of Ampicillin to the \((Ag_{29}LA_{12})^{3+}\) Cluster. The spectrometer was operated in negative mode. In addition to the triply charged signals, fragmentation products (*) of the electro-spray ionization process are apparent.
Glycine ethyl ester Conjugation

Liquid chromatography (LC) experiments were performed on an Eksigent nanoLC 2D system coupled to a Bruker microTOF time-of-flight mass spectrometer (MS). All separations were carried out using an Ace 300Å C18 HPLC column (0.5 mm x 150 mm, 3 µm particle size) (Advanced Chromatography Technologies Limited, Aberdeen, UK) maintained at ambient laboratory temperature. Mobile phases were prepared 400 mM hexfluoroisopropanol (HFIP) - 15 mM triethylamine (TEA) in ddH2O (mobile phase A) and neat methanol (mobile phase B). All solvents for direct infusion and LC-MS were obtained from Fisher Scientific (Fairlawn, NJ). The flow rate used for all experiments was ten microliters per minute (µL/min). Injections – 5.0 µL – were carried out by an Eksigent AS-1 autosampler configured with a 20-µL sample loop.

All reaction mixture samples were diluted 20x in mobile phase A. Direct infusion was carried out by loop injection (i.e., no column between autosampler and mass spectrometer) using a mobile phase composition of 95% MP A: 5% MP B. HPLC experiments were carried out using twenty-minute linear gradient methods with varied starting and ending mobile phase conditions.

After completion of the twenty-minute gradient, 100% methanol was rinsed through the column to remove any non-polar components for five minutes. This was then followed by a twenty minute re-equilibration at initial method conditions. Mass spectrometer acquisition settings were identical for both direct infusion and LC-MS experiments. Data was acquired from m/z 100 - 6,000. Ten-thousand spectra were summed per spectrum acquired. Nebulizer pressure was set to 4.0 bar. Nitrogen sheath gas was set to zero L/min. The endplate offset and capillary potentials were held at -1000 V and 3500 V, respectively. Capillary exit and skimmer voltage settings were -100 V and -33 V respectively. Lens 1 pre-pulse storage and transfer times were 35 µs and 140 µs, respectively. MCP detector voltage was increased to 2350 V (from 2100 V standard) for improved detection.

Figure S2: Overlaid LC-MS Base Peak Chromatogram Traces – Analysis of the Ag29LA12 reaction mixture. Red Trace= Ag29(LA)12. Blue Trace = Ag29(Lipoic Acid)11(Lipoic Acid-Glycine)1. Black Trace = Ag29(Lipoic Acid)10(Lipoic Acid-Glycine)2
Figure S3: Averaged mass spectrum from under Ag29(Lipoic Acid)10(Lipoic Acid-Glycine)2 chromatographic peak (black trace in Figure S2)

Figure S4: Averaged mass spectrum from under Ag29(Lipoic Acid)11(Lipoic Acid-Glycine)1 chromatographic peak (blue trace in Figure S2)

Figure S5: Averaged mass spectrum from under Ag29(LA)12 chromatographic peak (red trace in Figure S2)
Summary of Synergy Evaluation

Table S1. Synergistic or antagonistic effect based on the dose effect curves.

| Dose  | Biofilm Inhibition (6-APA) | Dose | Biofilm Inhibition (Ag29) | Dose | Biofilm Inhibition (6-APA + Ag29 = Conjugate) | Biofilm Inhibition (Conjugate) | Fa   | CI\textsuperscript{b} |
|-------|---------------------------|------|--------------------------|------|---------------------------------------------|-------------------------------|------|-----------------|
| µM    | (%)\textsuperscript{a}   | µM   | (%)\textsuperscript{a}   | µM   | (%)\textsuperscript{a}                     |                               |      |                 |
| 93    | 0                         | 0.6  | 0                        | 0.023 + 0.0086 = 0.032 | 3          | 0.03 | 4.43E-04 |
| 185   | 0.1                       | 1.6  | 0                        | 0.047 + 0.0173 = 0.064 | 6          | 0.06 | 5.89E-04 |
| 370   | 2                         | 3.2  | 0                        | 0.117 + 0.0432 = 0.160 | 13         | 0.13 | 9.15E-04 |
| 741   | 9                         | 6.4  | 1                        | 0.233 + 0.0864 = 0.320 | 17         | 0.17 | 0.00153 |
| 1481  | 11                        | 12.8 | 1                        | 0.466 + 0.1727 = 0.639 | 22         | 0.22 | 0.00257 |
| 3009  | 16                        | 25.6 | 4                        | 0.933 + 0.3455 = 1.278 | 33         | 0.33 | 0.00376 |
| 6019  | 28                        | 51.1 | 15                       | 1.866 + 0.6910 = 2.557 | 40         | 0.40 | 0.00635 |
| 12037 | 54                        | 102.3| 43                       | 3.731 + 1.3820 = 5.113 | 61         | 0.61 | 0.00788 |
| 24074 | 75                        | 207.7| 74                       | 7.463 + 2.7639 = 10.227 | 81         | 0.81 | 0.00904 |
| 48148 | 87                        | 415.5| 81                       | 15.158 + 5.6143 = 20.773 | 90         | 0.90 | 0.01213 |

\textsuperscript{a} The data of the dose–effect inhibition (%) was obtained from a phenotypic luciferase assay and were generated by the Origin software. Data are presented as the mean of three independent assays in duplicates. \textsuperscript{b} Fraction affected CI (Fa-CI) plots was generated using CompuSyn software. CI = 1 means additive effect, CI > 1 is antagonistic effect, and CI < 1 means synergy (red numbers).
Table S2. Median dose (IC50) and other parameters reported by curve-fitting the phenotypic assay results (by the CompuSyn program)

| Drug/Combo         | Median Dose µM | Kinetic Parameter, m | Quality of Fit R² |
|--------------------|----------------|----------------------|-------------------|
| 6-APA              | 9989           | 1.8                  | 0.99              |
| Ag29               | 142            | 1.4                  | 0.96              |
| Conjugated Cluster | 2.31           | 0.81                 | 0.99              |