

Supplementary Information for:
**Surface Functionalized Binary Antibiotic Nanoparticles
of Enhanced Antimicrobial Action**

**Nuriya Nurlankyzy,^a Yersin Kalmagambet,^b Arailym Kadir,^b Amro K.F. Dyab,^b
and Vesselin N. Paunov^{*,b}**

^a Department of Biology, School of Sciences and Humanities, Nazarbayev University, Kabanbay Batyr Avenue 53, Astana, 010000, Kazakhstan.

^b Department of Chemistry, School of Sciences and Humanities, Nazarbayev University, Kabanbay Batyr Avenue 53, Astana, 010000, Kazakhstan

*Correspondence: vesselin.paunov@nu.edu.kz

(*Mater. Adv.* 2026)

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Table S1. Method conditions for HPLC analysis of Cefoperazone and Tetracycline

Mobile phase	30% acetonitrile and 70% (DI water with 0.1 % vol formic acid)
Stationary phase	Hypersil Gold C18 (dimensions: length 150 mm, width 2.1 mm, particle size 1.9 micron) (Dim.: 150 mm x 2.1mm; Particle size: 1.9 μ m)
Temperature	25 $^{\circ}$ C
Wavelength	250-300nm
Flow	0.15 mL/min
Injection volume	2 μ L
Tetracycline t_r	3.4 min
Cefoperazone t_r	4.7 min

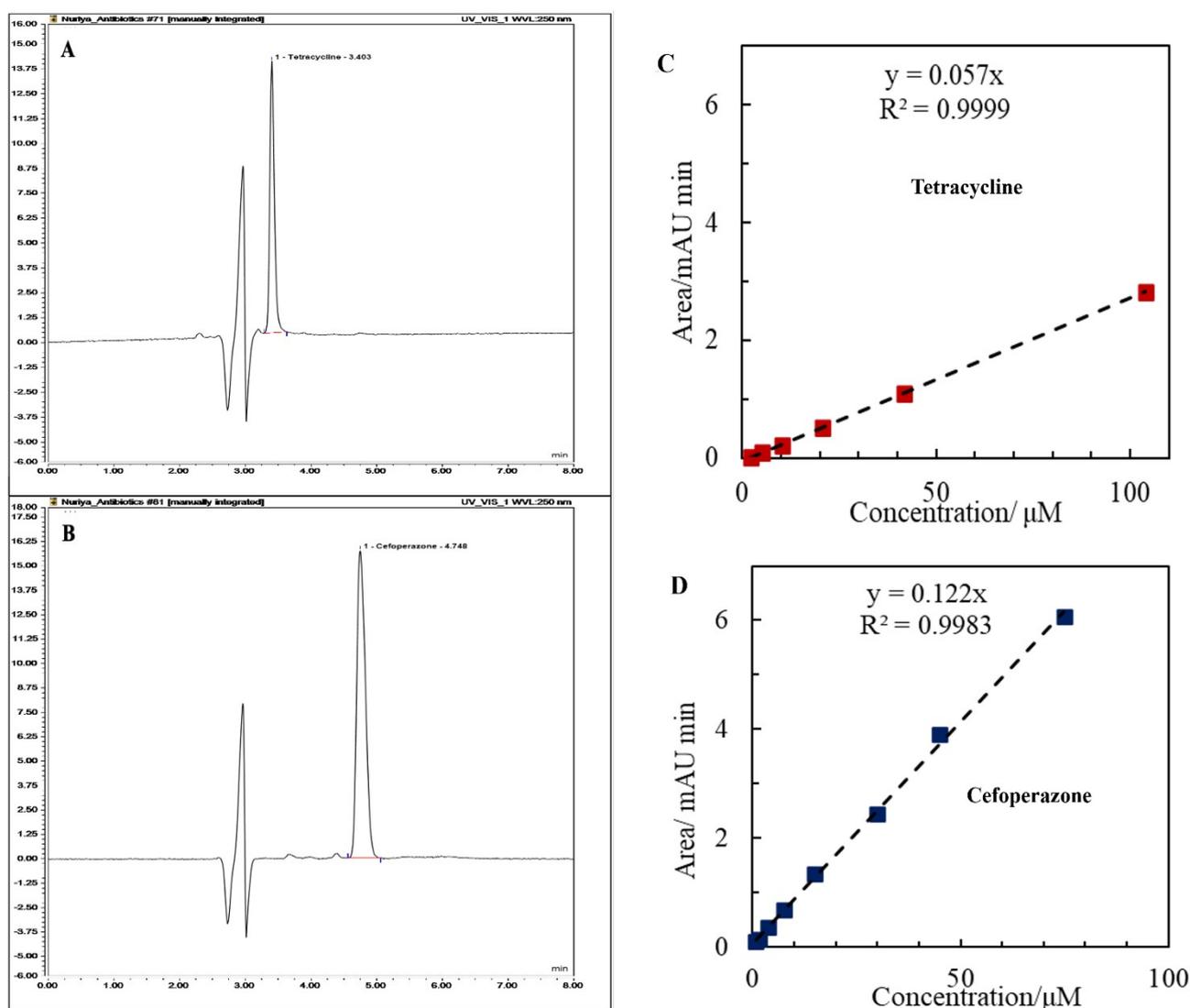


Figure S1. HPLC chromatograms of (A) tetracycline hydrochloride and (B) sodium cefoperazone at 2.5 ppm concentration with retention times of 3.403 and 4.748 min, respectively. Calibration curves for Tetracycline (C) and sodium cefoperazone (D) obtained via HPLC.

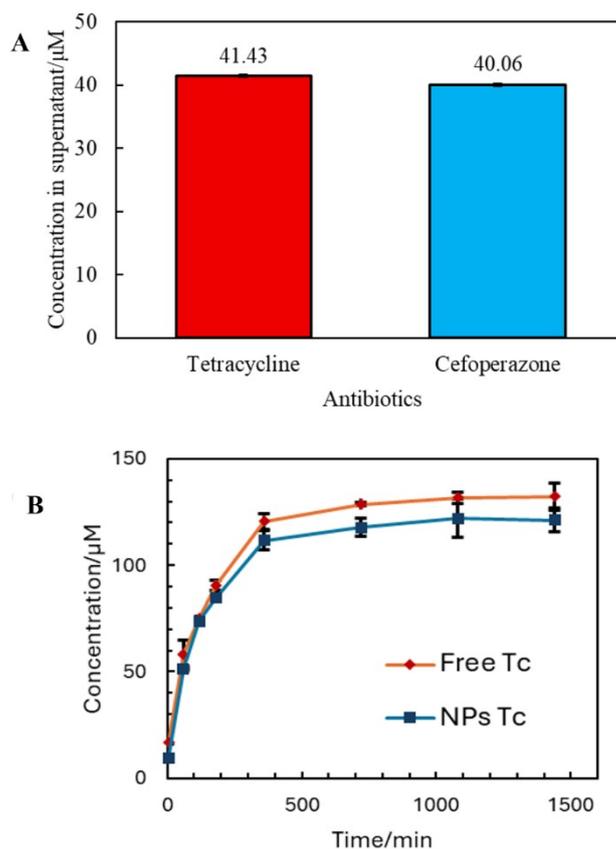


Figure S2. (A) The mean concentrations of the soluble antibiotics in the binary antibiotic particle suspension are found via HPLC to be nearly the same, $41.43 \pm 0.36 \mu\text{M}$ and $40.06 \mu\text{M} \pm 0.33 \text{ mM}$ for sodium cefoperazone and tetracycline hydrochloride, respectively after precipitating 4 mM sodium cefoperazone (aq) and 4mM tetracycline hydrochloride (aq) – see Table S3. The equality of the two antibiotics’ concentrations in the supernatant indicates that that in the precipitate they are in the same 1:1 ratio. (B) Release kinetics of free tetracycline (TC) and AB NPs loaded TC at the same initial concentrations (2 mM) a from a dialysis bag to a beaker with DI water over the course of 1500 min.

Table S2. Tetracycline and Cefoperazone solubilities determined via the regression equation.

#	Tetracycline/ μM	Cefoperazone/ μM
1	41.26	40.06
2	41.70	40.18
3	41.33	39.96
Mean	41.43 ± 0.19	40.06 ± 0.40

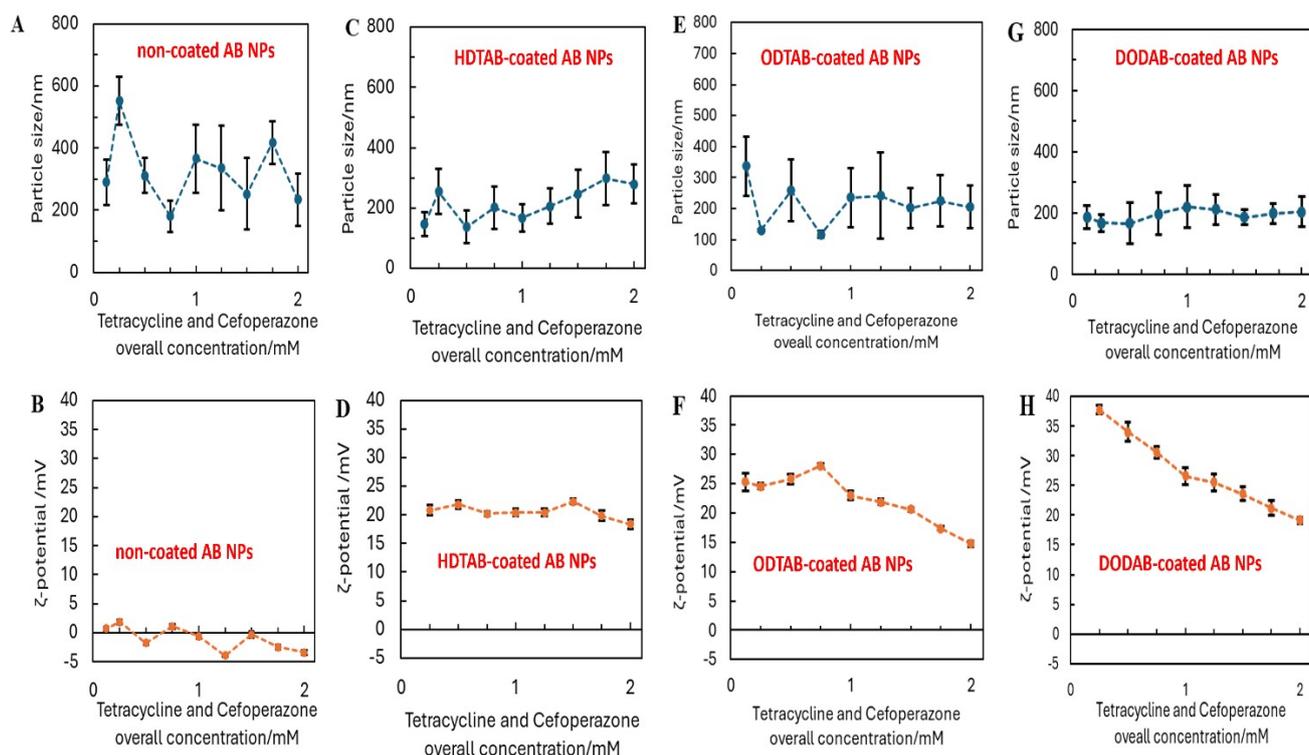


Figure S3. The effect of the dilution of the binary antibiotic particle formulation obtained by coprecipitating 4 mM Cefoperazone and 4 mM Tetracycline sterically stabilised by 0.05 wt% P407 on the (A,C) mean particle diameter and (B,D) their ζ -potential. The dilution effect of the binary antibiotic particle formulation obtained by coprecipitating 4 mM Sodium Cefoperazone and 4 mM Tetracycline Hydrochloride sterically stabilised by 0.05 wt% P407 and coated with 0.0075 wt% (C,D) HDTAB, (E,F) ODTAB and (G,H) DODAB on the mean particle diameter (C,E,G) and (D,F,H) their ζ -potential. The data points represent the mean, and the error bars show the standard deviation from three independent measurements.

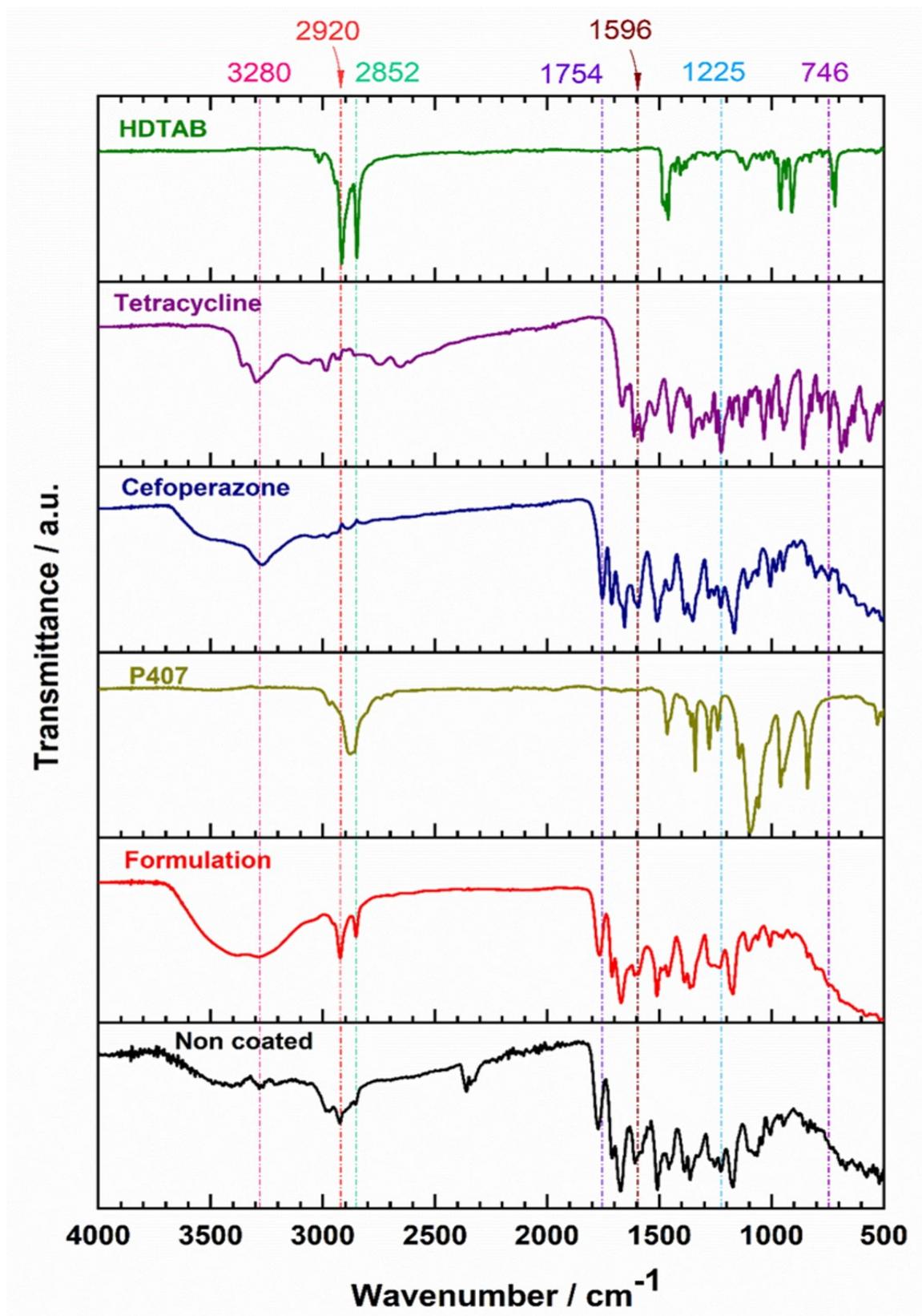


Figure S4. FTIR spectra for powders of tetracycline hydrochloride, sodium cefoperazone, Poloxamer 407, HDTAB, as well as the HDTAB-coated binary antibiotic particle formulation obtained by co-precipitating 2 mM sodium cefoperazone and 2 mM tetracycline hydrochloride stabilised by 0.05 wt% P407 followed by coating with 0.0075 wt% HDTAB.

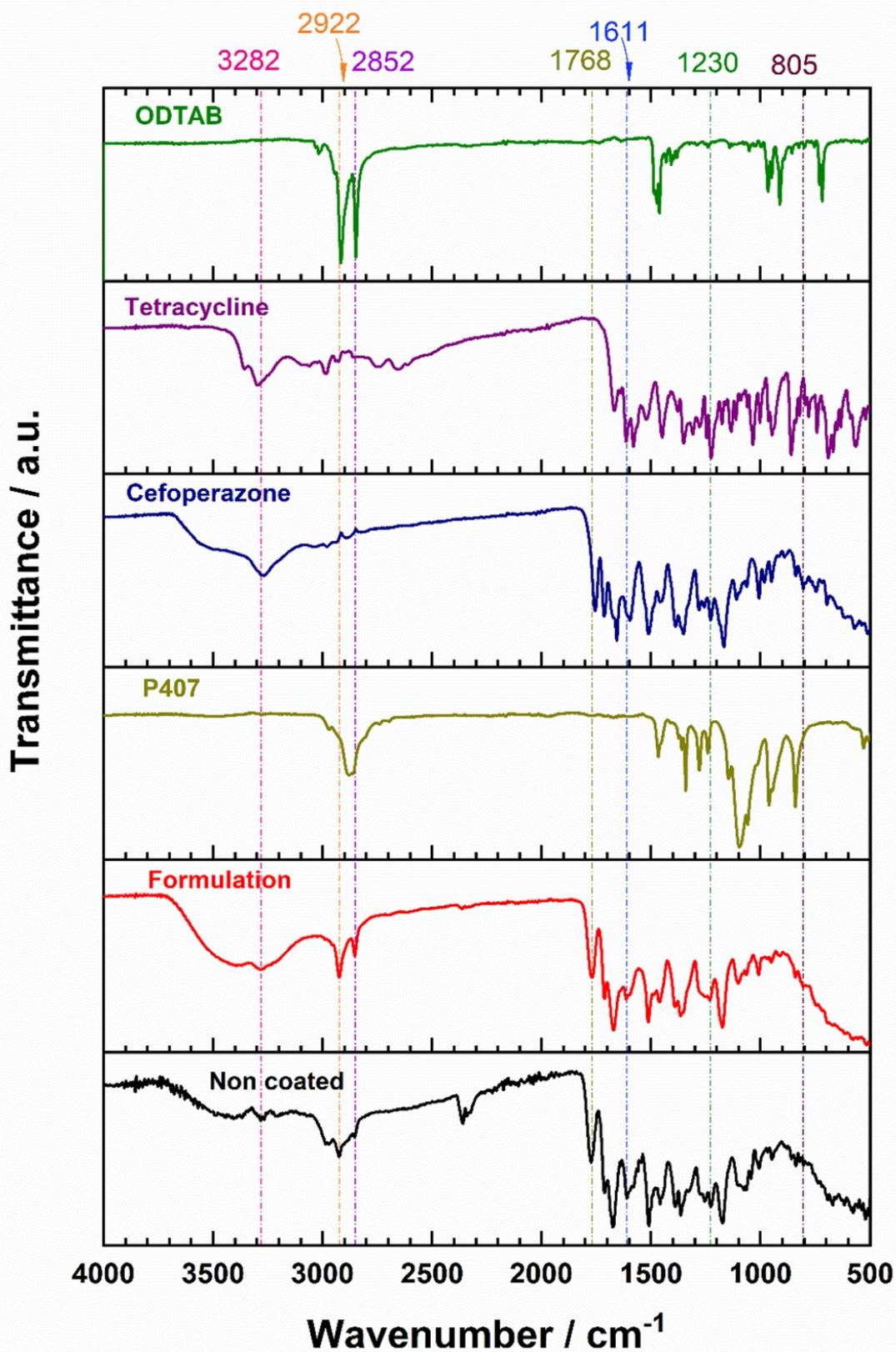


Figure S5. FTIR spectra for powders of tetracycline hydrochloride, sodium cefoperazone, Poloxamer 407, ODTAB, as well as the coated binary antibiotic particle formulation obtained by coprecipitating 4 mM sodium cefoperazone and 4 mM tetracycline hydrochloride stabilised by 0.05 wt% P407 followed by coating with 0.0075 wt% ODTAB.

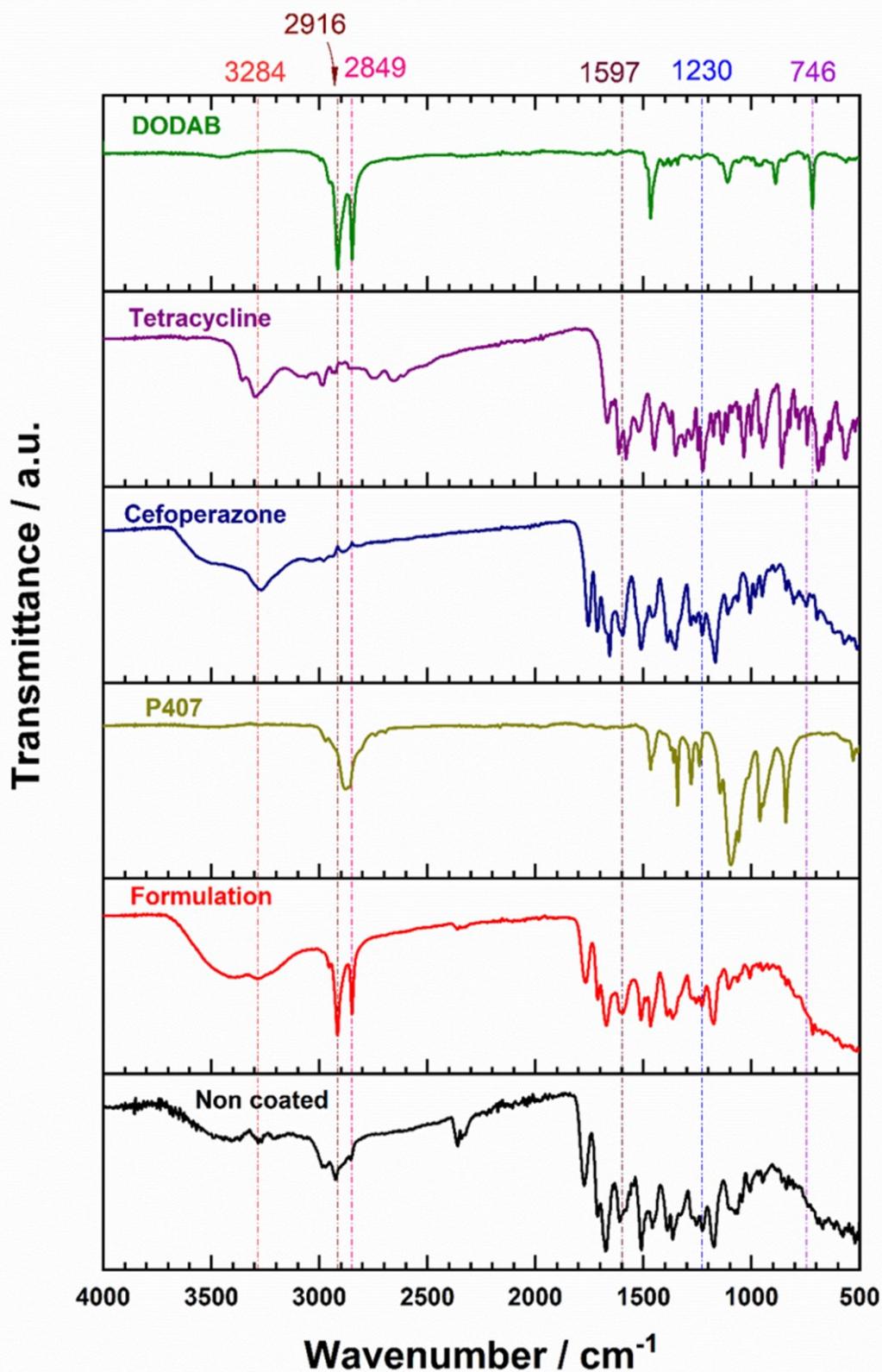


Figure S6. FTIR spectra for powders of tetracycline hydrochloride, sodium cefoperazone, Poloxamer 407, DODAB, as well as the coated binary antibiotic particle formulation obtained by co-precipitating 4 mM sodium cefoperazone and 4 mM tetracycline hydrochloride stabilised by 0.05 wt% P407 followed by coating with 0.0075 wt% DODAB.

Table S3. HDTAB-coated AB NPs FTIR peaks analysis.

Peak (cm ⁻¹)	Functional Group	Assignment / Description
3280	O–H / N–H stretching (broad)	Tetracycline hydrochloride, Cefoperazone sodium salt, Stretching of phenolic –OH and amide –NH groups.
2920	C–H stretching (aliphatic)	HDTAB, P407, Aliphatic C–H stretching. Typical for aliphatic chains in surfactants/polymers.
2852	C–H stretching (aliphatic)	HDTAB, P407, Aliphatic C–H stretching
1754	β -Lactam C=O	– C=O stretch from amide groups, Cefoperazone, Tetracycline
1596	C=C Aromatic + N=N (azo)	Tetracycline – Aromatic ring and amide N–H deformation
1225	C–N or phenolic C–O	Cefoperazone, Tetracycline – C–N stretching from the amine group
746	C-S (aromatic)	Cefoperazone C-S aromatic stretching

Table S4. ODTAB-coated NPs FTIR peaks analysis.

Peak (cm ⁻¹)	Functional Group	Assignment / Description
3282	O–H / N–H stretching (broad)	Tetracycline hydrochloride, Cefoperazone sodium salt
2922	C–H stretching (aliphatic)	ODTAB, P407 – Aliphatic C–H stretching
2852	C–H stretching (aliphatic)	ODTAB, P407 – Aliphatic C–H stretching
1768	β -Lactam C=O	Cefoperazone, Tetracycline – C=O stretch from amide groups
1611	C=C aromatic + N–H bending	Tetracycline – Aromatic ring and amide N–H deformation
1230	C–N stretching (amine)	Cefoperazone, Tetracycline – C–N stretching from the amine group in Cefoperazone
805	C–S stretching	Cefoperazone C-S stretching

Table S5. DODAB-coated NPs FTIR peaks analysis.

Peak (cm ⁻¹)	Functional Group	Assignment / Description
3284	N–H stretching	Cefoperazone – Amide N–H stretching
2916	C–H stretching (aliphatic)	DODAB, P407 – Aliphatic C–H stretching
2849	C–H stretching (aliphatic)	DODAB, P407 – Aliphatic C–H stretching
1597	C=C aromatic + N–H bending	Tetracycline – Aromatic ring and amide N–H deformation
1230	C–N stretching (amine)	Cefoperazone, Tetracycline – C–N stretching from the amine group in Cefoperazone
746	C–S stretching	Cefoperazone C-S stretching

Table S6. Tabulated EDS analysis results of HDTAB-coated and non-coated A⁺B-NPs.

Element	2 mM Cefoperazone and 2 mM Tetracycline in 0.05 wt% P407		2 mM Cefoperazone and 2 mM Tetracycline in 0.05 wt% P407 coated with 0.0075 % HDTAB	
	Mass%	Atomic%	Mass%	Atomic%
C	57.40±0.07	65.30±0.08	55.96±0.08	63.82±0.09
N	4.30±0.10	4.19±0.10	6.40±0.14	6.26±0.13
O	35.21±0.14	30.07±0.12	34.41±0.16	29.46±0.14
Na	0.12±0.01	0.07±0.01	0.12±0.01	0.07±0.01
S	0.45±0.01	0.19±0.00	0.49±0.01	0.21±0.01
Au	2.52±0.04	0.17±0.00	2.62±0.05	0.18±0.00

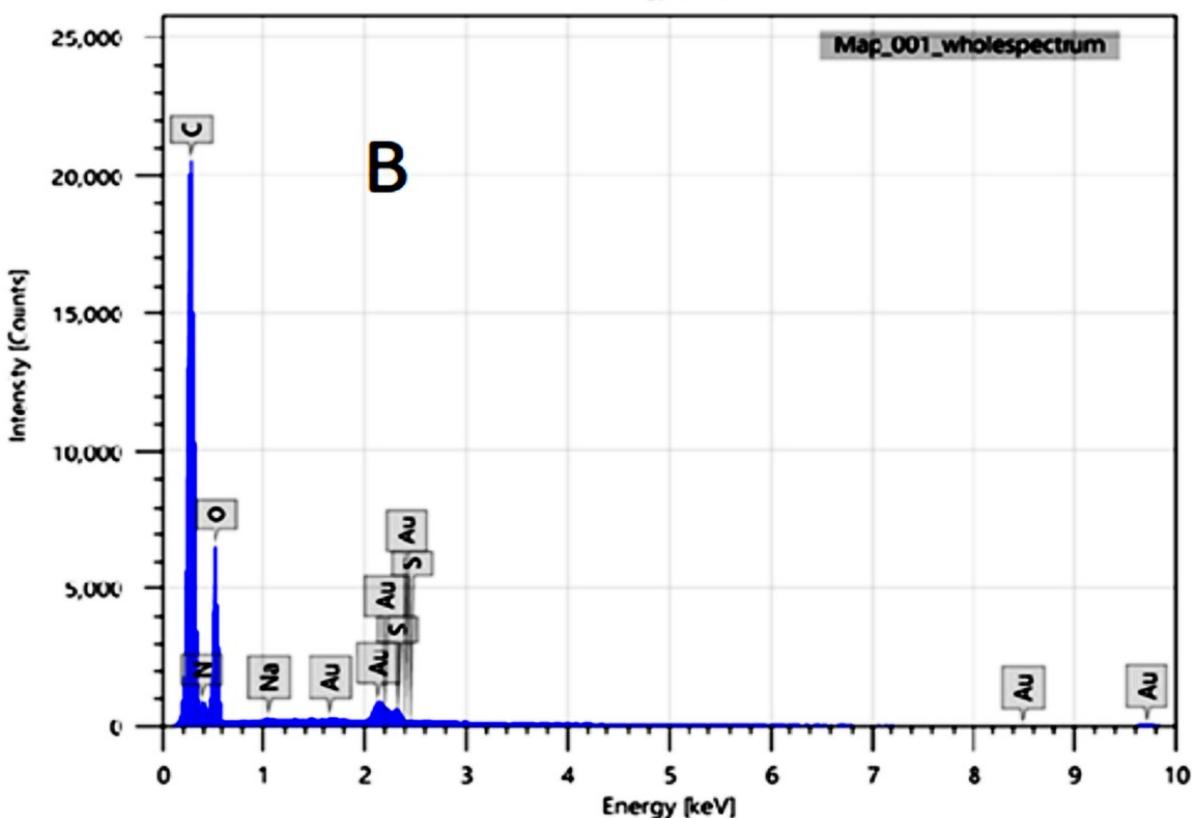
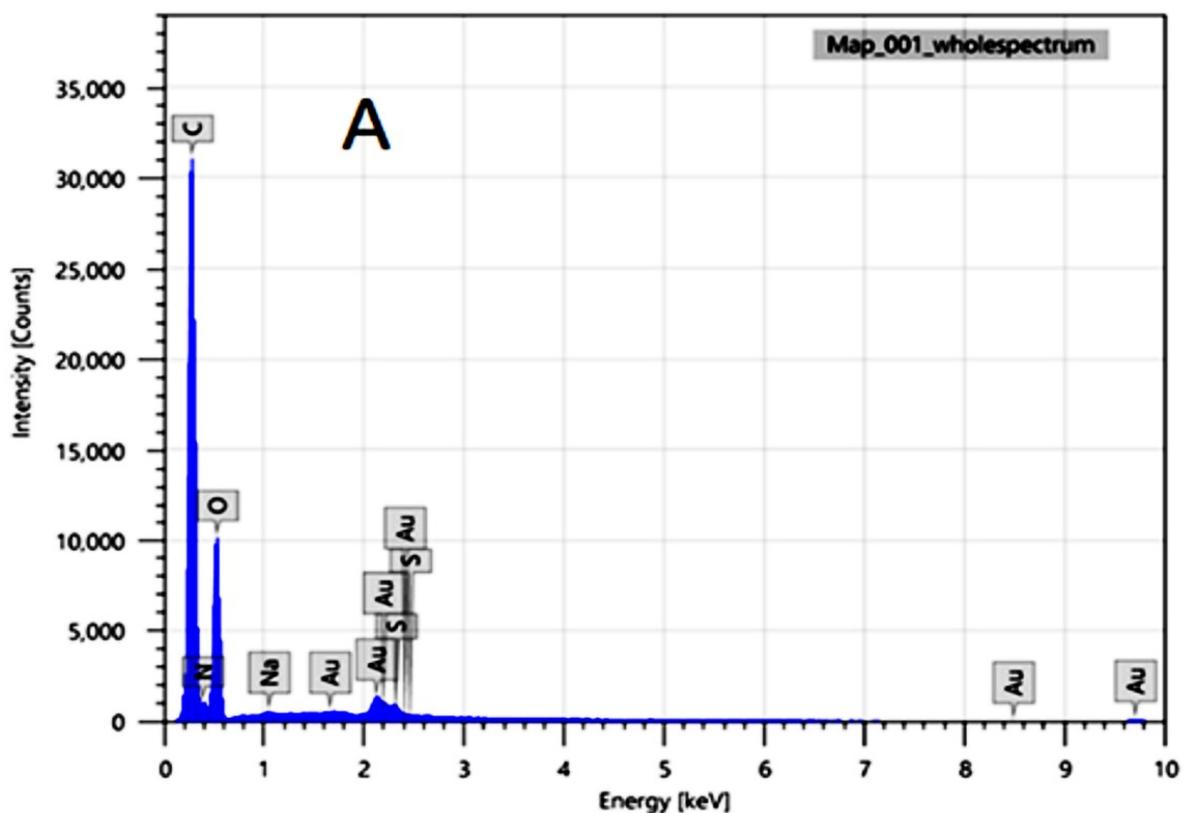


Figure S7. EDS spectra of: (A) non-coated particle formulation produced by co-precipitating 2 mM sodium cefoperazone and 2 mM tetracycline hydrochloride sterically stabilised by 0.05 wt% P407; (B) the same binary antibiotic particle formulation as in (A) coated with 0.0075 wt% HDTAB.

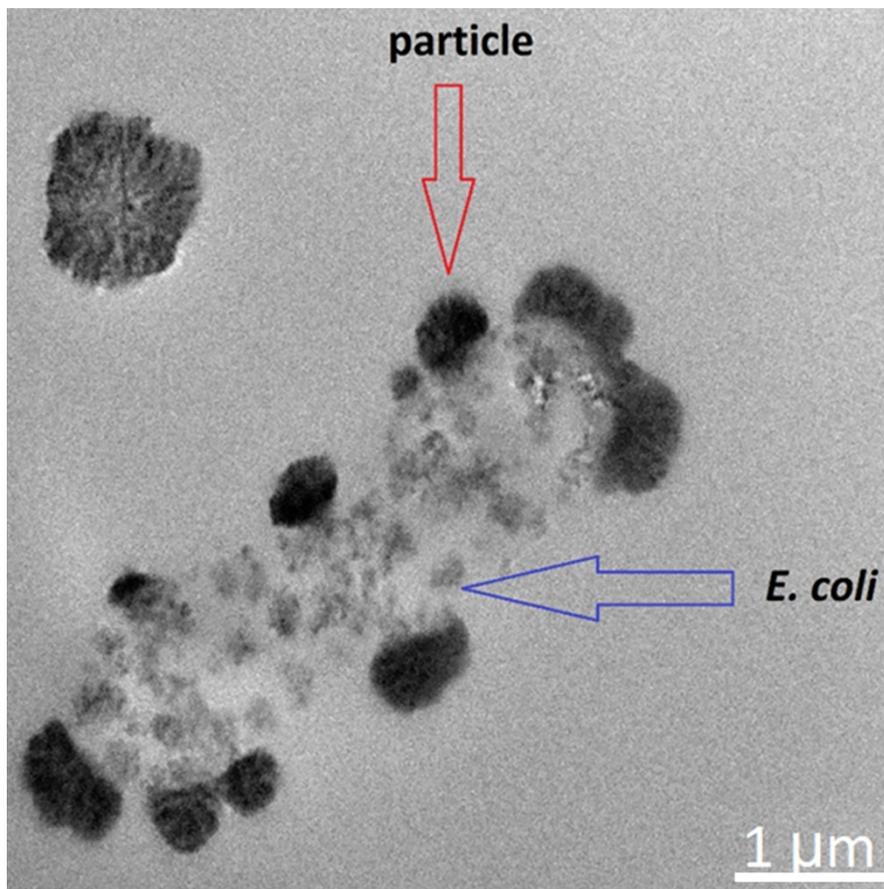
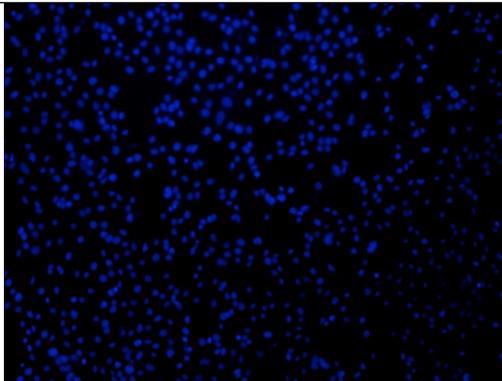
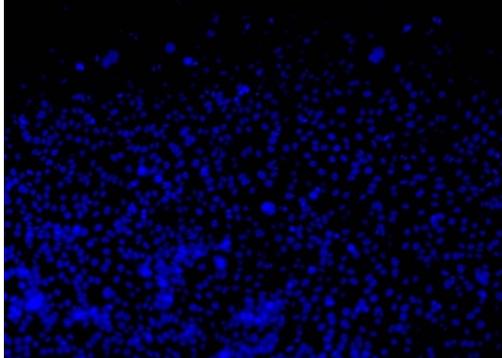
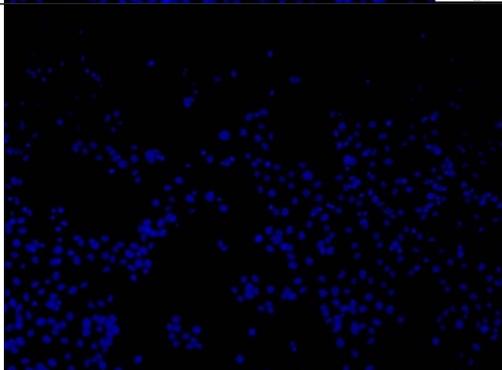
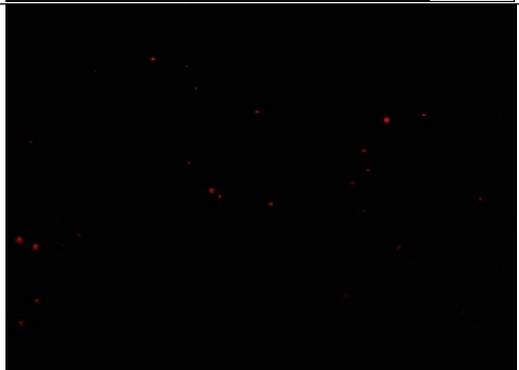
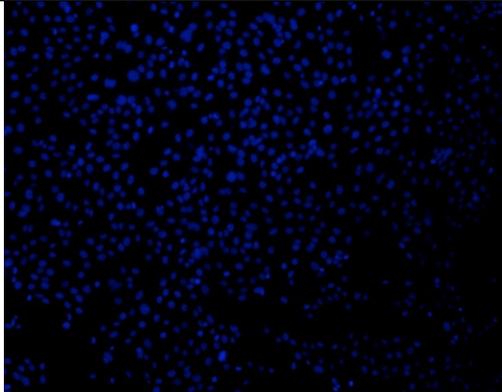


Figure S8. TEM image of *E. coli* after 12 h of incubation with binary antibiotic particles prepared by coprecipitation of 4 mM Cefoperazone and 4 mM Tetracycline stabilised with 0.05 wt% P407 and coated with 0.0075 wt% HDTAB.

Condition	Hoechst staining	PI staining
Growth Control		
Poloxamer 407		
Free Tetracycline		
Free Cefoperazone		

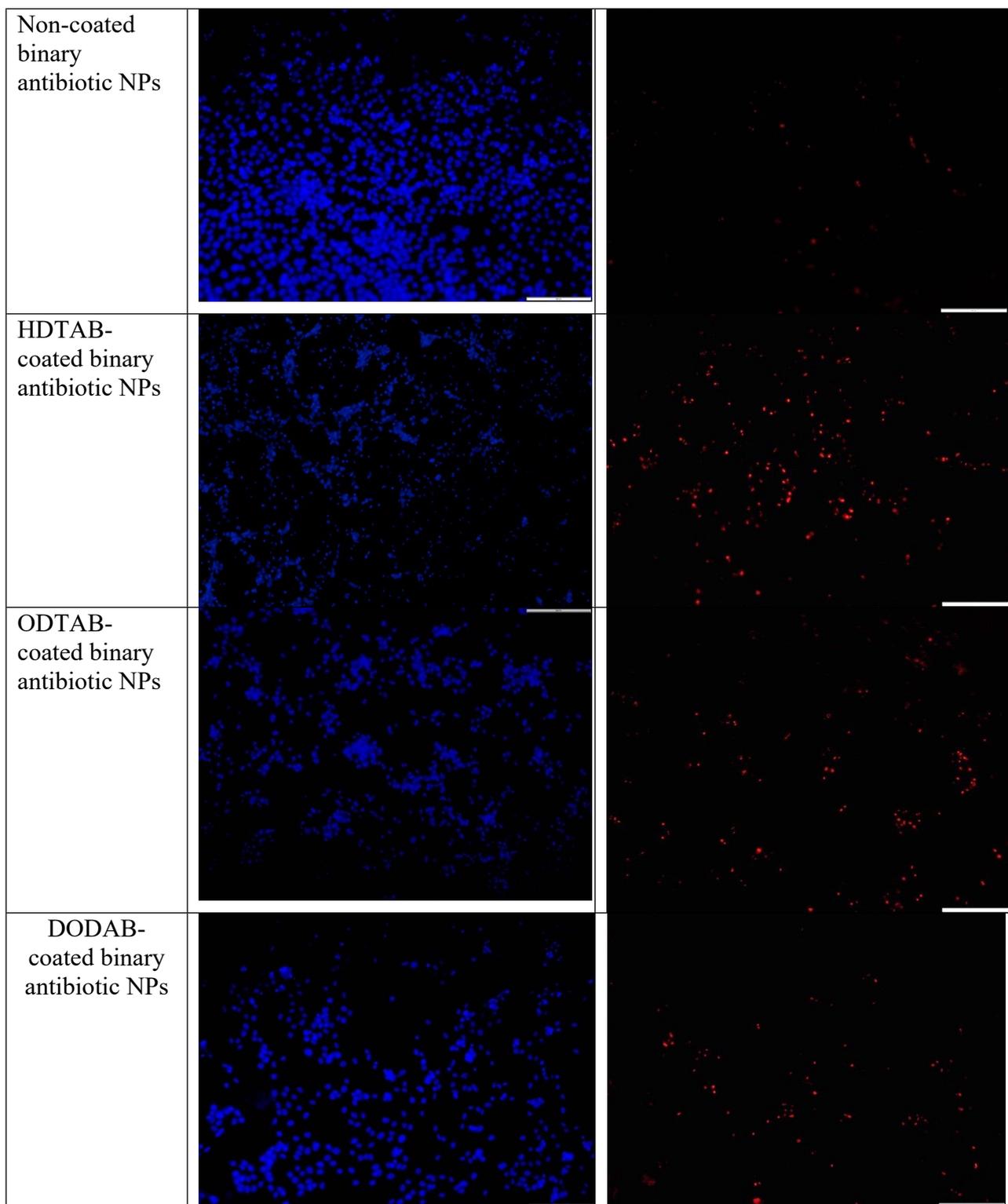


Figure S9. Hoescht/PI live/dead assay representative images of HaCaT obtained by using fluorescence microscopy with DAPI filter set and TRITC filter set for Hoescht and PI, respectively, after 24 hours of incubation with free antibiotics, non-coated binary antibiotic NPs and coated binary antibiotic NPs. The binary antibiotic particles are prepared by mixing 1:1 4mM cefoperazone and 4mM tetracycline hydrochloride in 0.05 wt% P407. The suspension (after coating with 0.0075% HDTAB, ODTAB or DODAB) was mixed in 1:1 with DMEM media. Images are taken by fluorescence microscopy after 24 hours of incubation in 5 vol% CO₂ atmosphere in an incubator at 37 °C. Scale bar is 200μm.