Supplementary Information

In situ **nanoformulation of amino acid complexed drug loaded eggshell derived hydroxyapatite: A promising strategy to combat *Staphylococcus aureus* biofilm**

Ranchini Sampath[a], Karthickraja Duraisamy[a,b], Muthu Devaraj[a,c], Monali Priyadarshini[d], Girija Easwaradas Kreedapathy[a*]

[a]Department of Physics, Periyar University, Salem 636 011, Tamil Nadu, India

[b]Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, 2730 SW Moody Avenue, Portland, Oregon, 97201, USA

[c]Environment Research Institute, Shandong University, Qingdao-266237, Shandong Province, China.

[d]Centre for Clean Environment, Vellore Institute of Technology, Vellore 632014, India

[*]Corresponding Author

E-mail Id: girijaeaswaradas@gmail.com

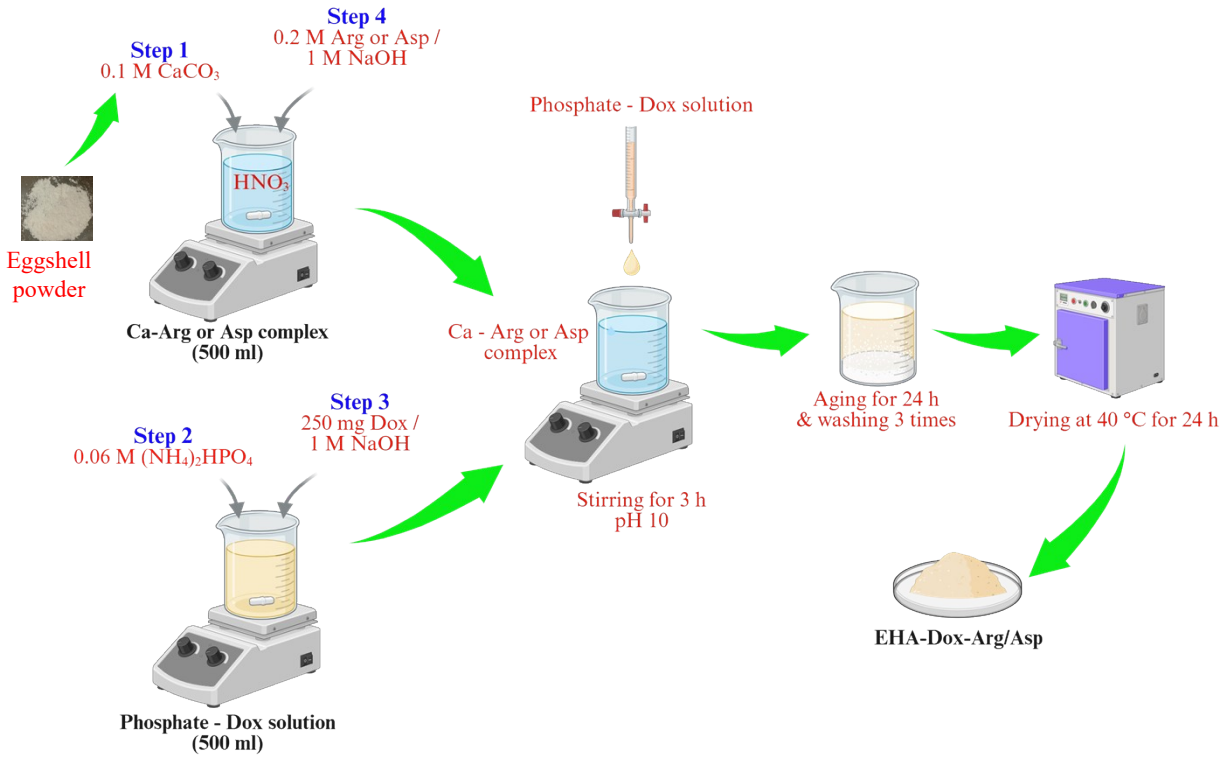

Fig. S1. Schematic illustration of the synthesis of the nanoformulations such as EHA (step 1 &2), EHA-Dox (step 1-3), EHA-Arg (step 1,2 & 4), EHA-Asp (step 1,2 & 4), EHA-Dox-Arg (step 1-4) and EHA-Dox-Asp (step 1-4), prepared by following the necessary steps.

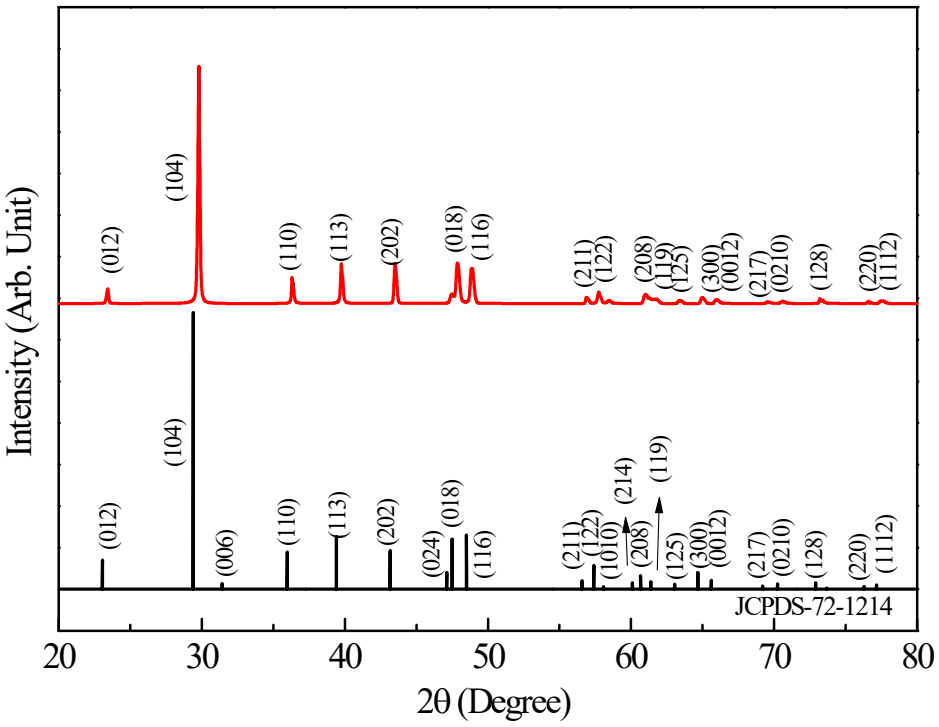

Fig. S2. XRD pattern of eggshell derived $CaCO_3$

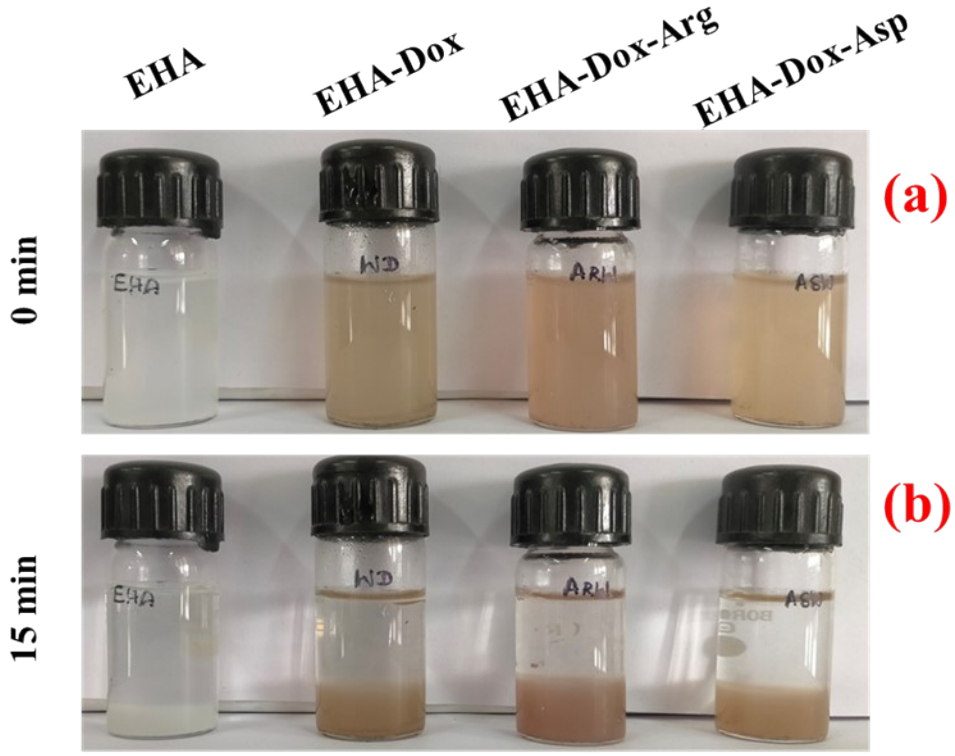

Fig. S3. Colloidal stability of EHA, EHA-Dox, EHA-Dox-Arg and EHA-Dox-Asp samples for (a) 0 min and (b) 15 min

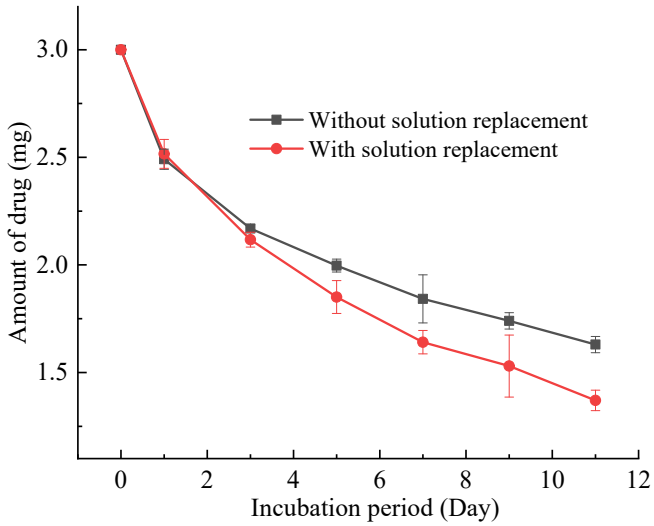

Fig. S4. Degradation of Dox in PBS at pH 7.4

In the drug release profile, the unusual decrease in drug release behavior is observed after reaching the maximum release. This decrease in drug quantity in the medium may be attributed to the partial degradation of Dox in the release medium over time. The periodic removal of aliquot and replacement with fresh solutions may also play a role in the decrease of the drug in the medium (Fig. S4).

In order to understand this phenomenon, the stability of Dox in PBS (pH 7.4) at 37 °C was evaluated for 11 days, as shown in the following figure. For this, 3 mg of Dox was dissolved in 30 ml of PBS and incubated at 37 °C, and 300 µl of the solution was withdrawn to measure OD at 346 nm and replaced with fresh PBS. While the experiment was also conducted without solution replacement. The experiment was conducted in triplicate.

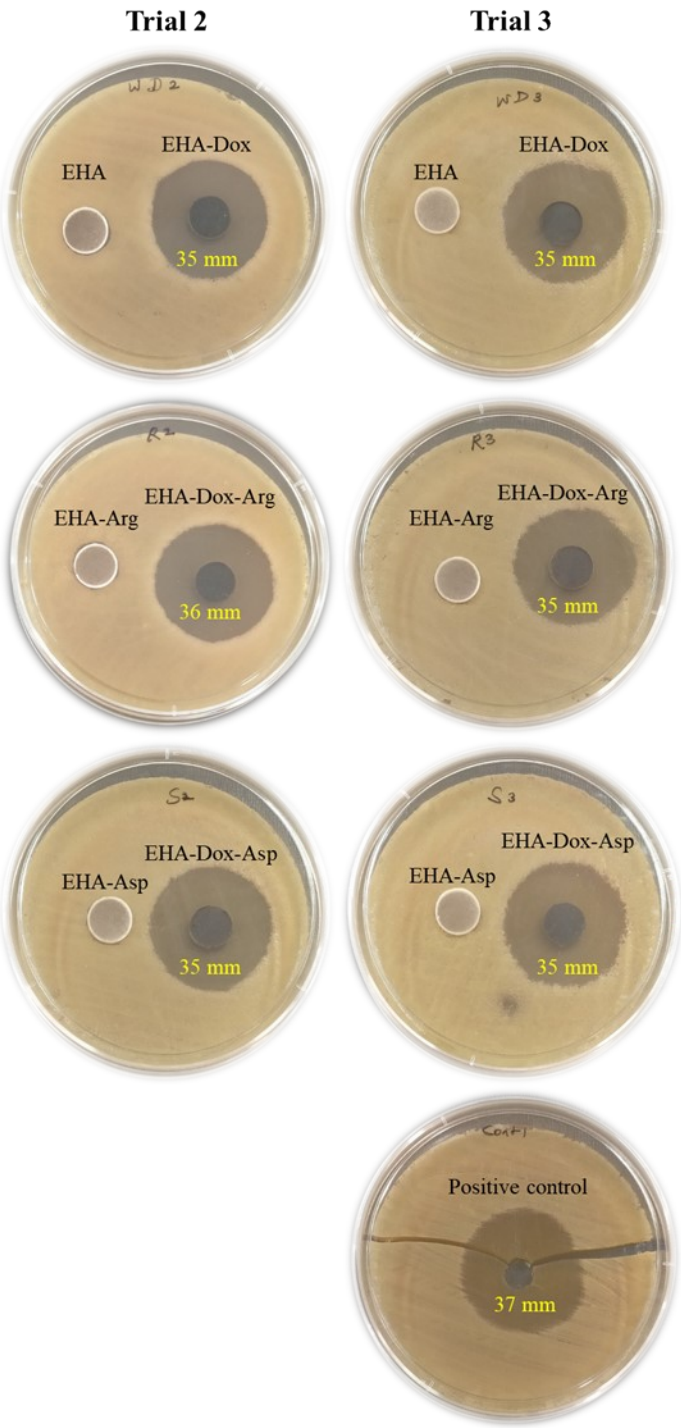

Fig. S5. Antibacterial activity of the prepared samples against *S. aureus* for the replicates

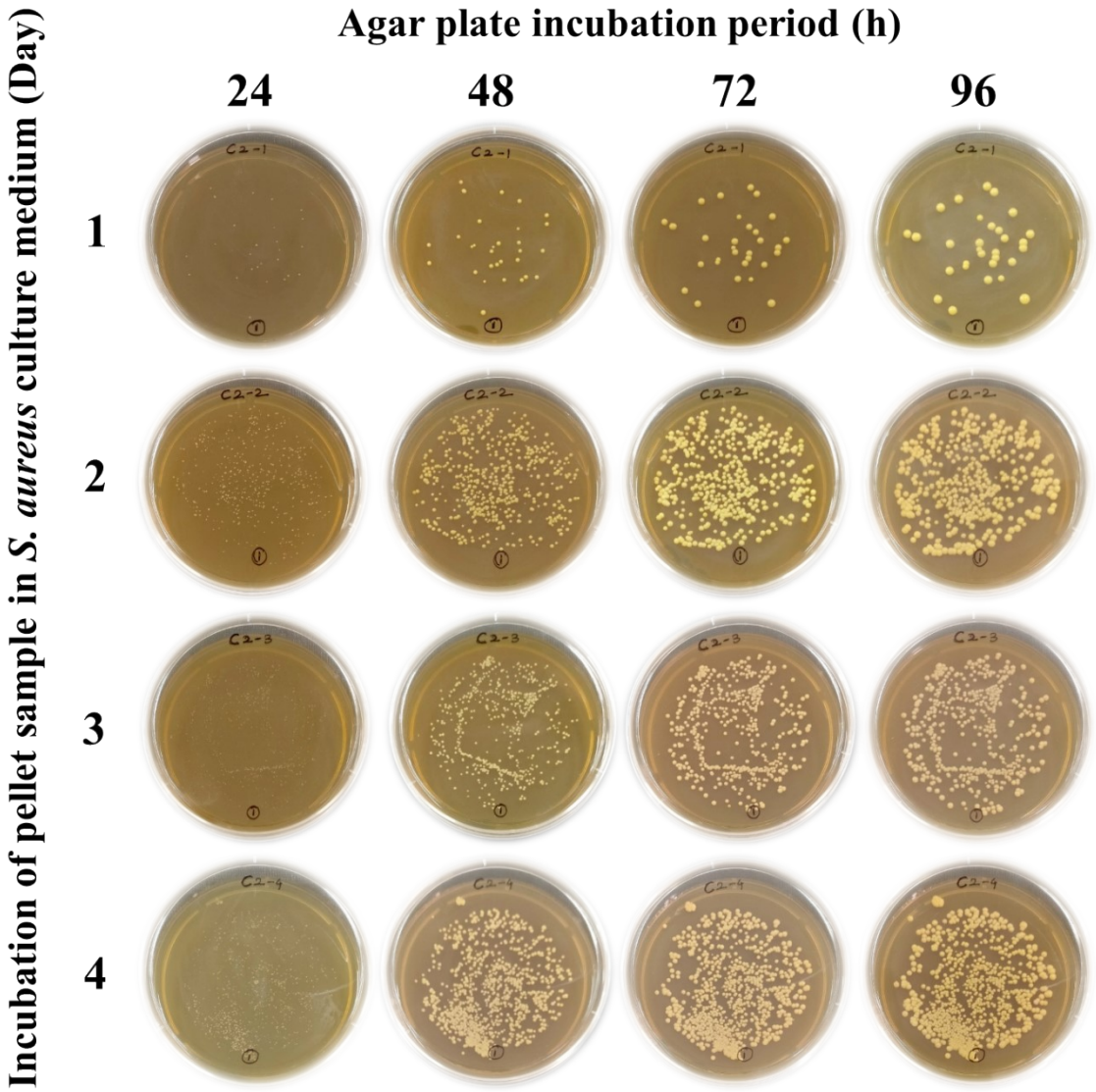

Fig. S6. Agar plates seeded with Day 1 to Day 4 culture media of planktonic *S. aureus* treated with EHA, assessed by the colony counting method after 24 h, 48 h, 72 h, and 96 h of agar plate incubation.

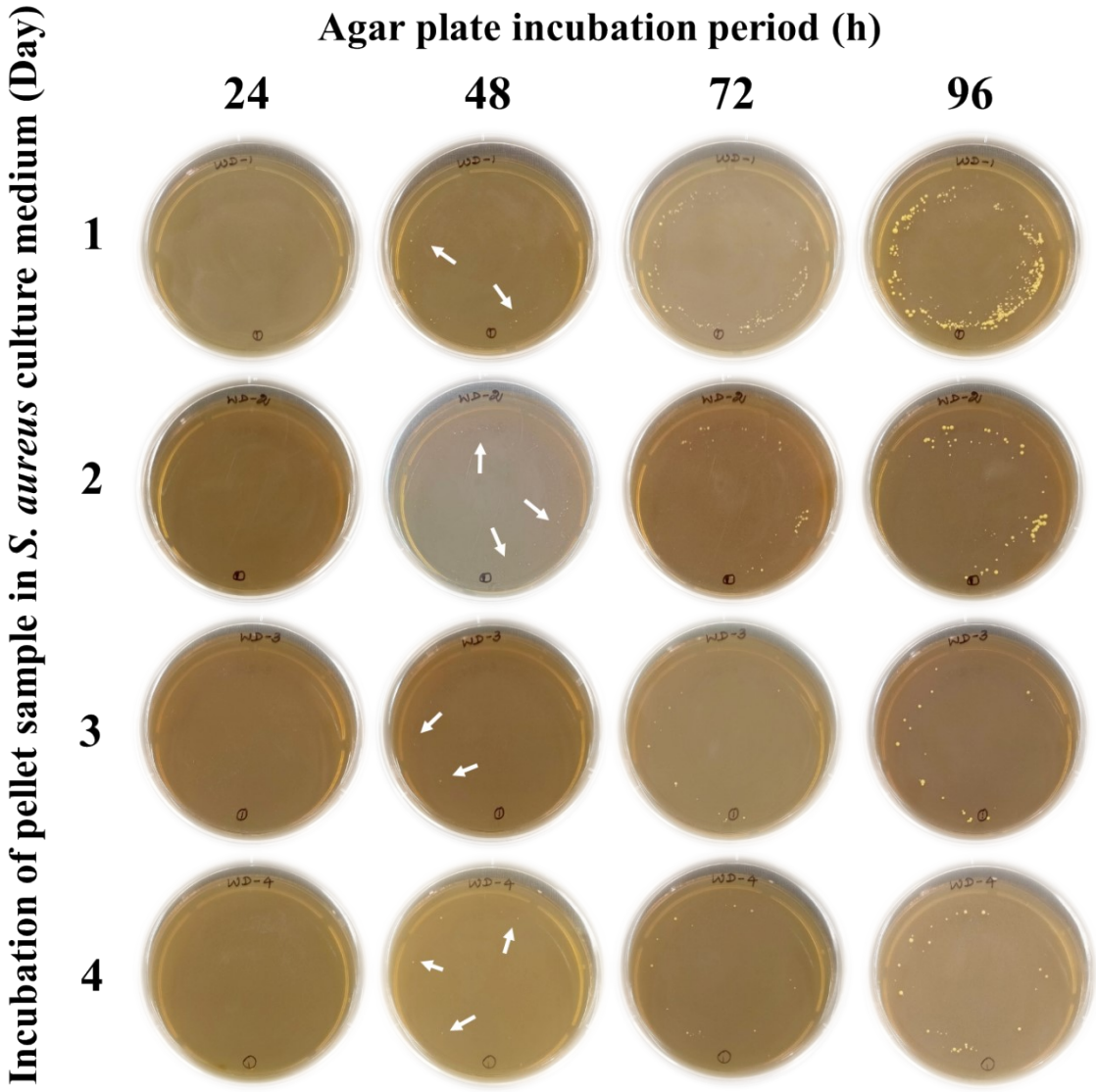

Fig. S7. Agar plates seeded with Day 1 to Day 4 culture media of planktonic *S. aureus* treated with EHA-Dox, assessed by the colony counting method after 24 h, 48 h, 72 h, and 96 h of agar plate incubation.

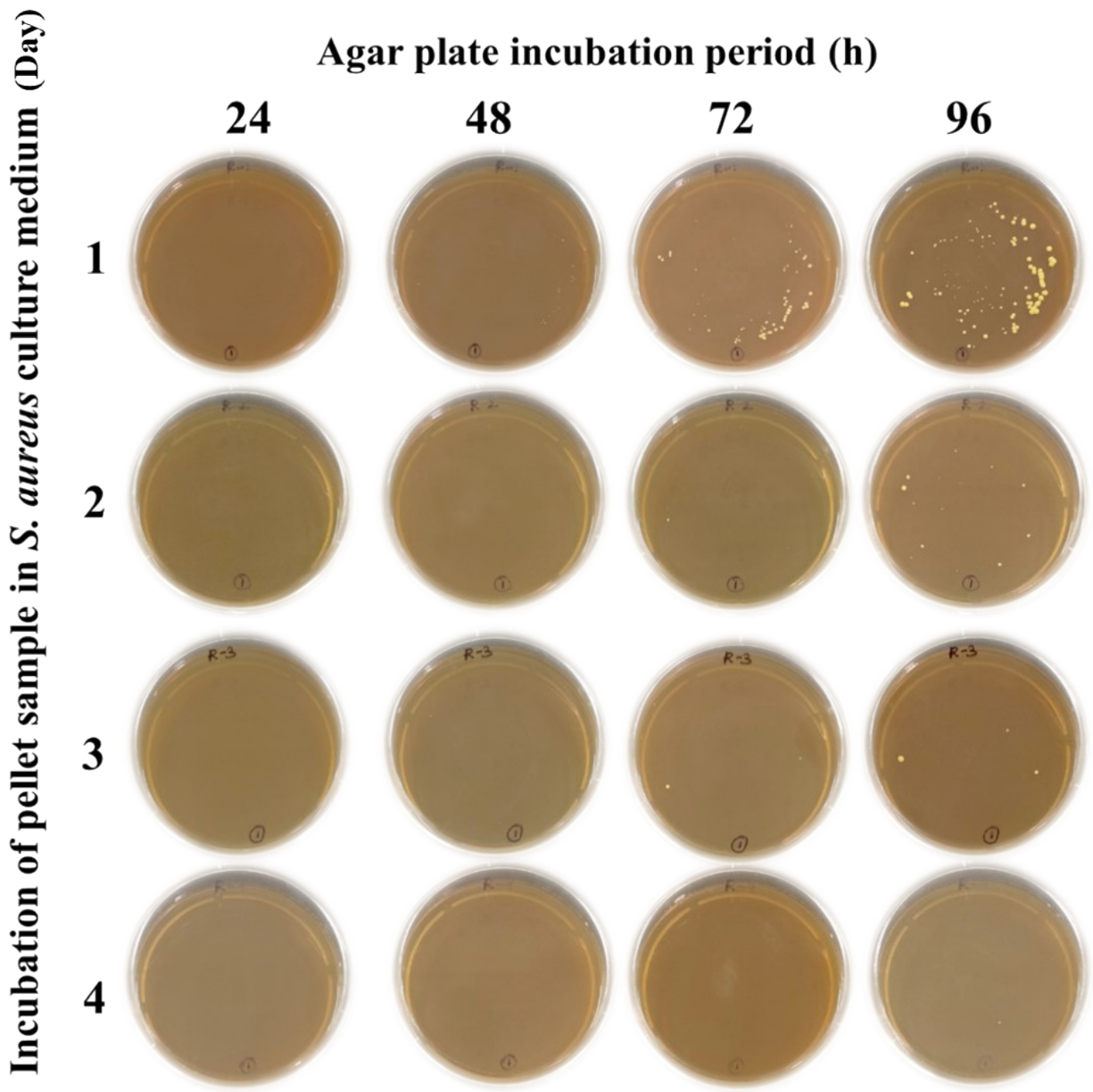

Fig. S8. Agar plates seeded with Day 1 to Day 4 culture media of planktonic *S. aureus* treated with EHA-Dox-Arg, assessed by the colony counting method after 24 h, 48 h, 72 h, and 96 h of agar plate incubation.

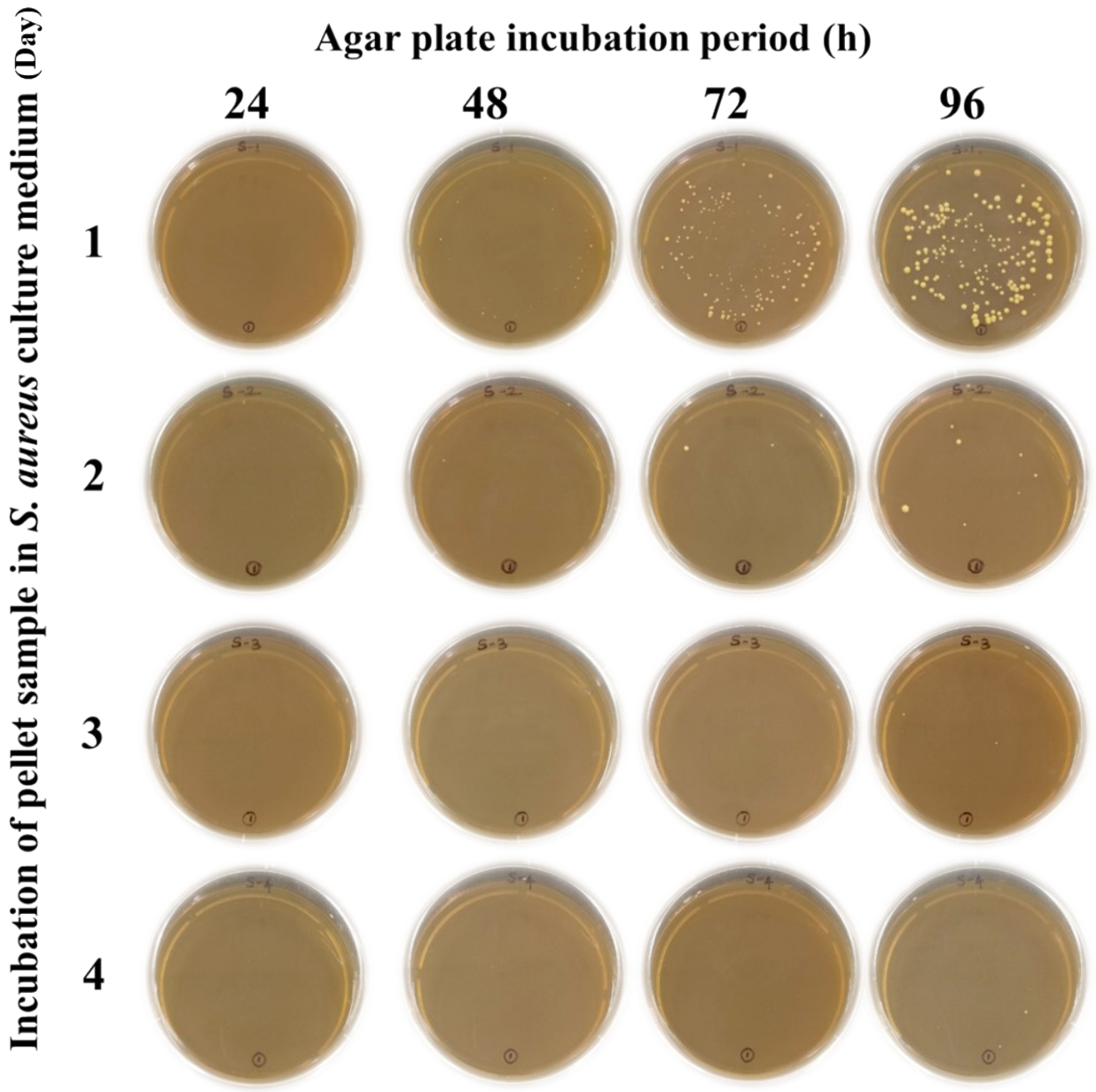

Fig. S9. Agar plates seeded with Day 1 to Day 4 culture media of planktonic *S. aureus* treated with EHA-Dox-Asp, assessed by the colony counting method after 24 h, 48 h, 72 h, and 96 h of agar plate incubation.

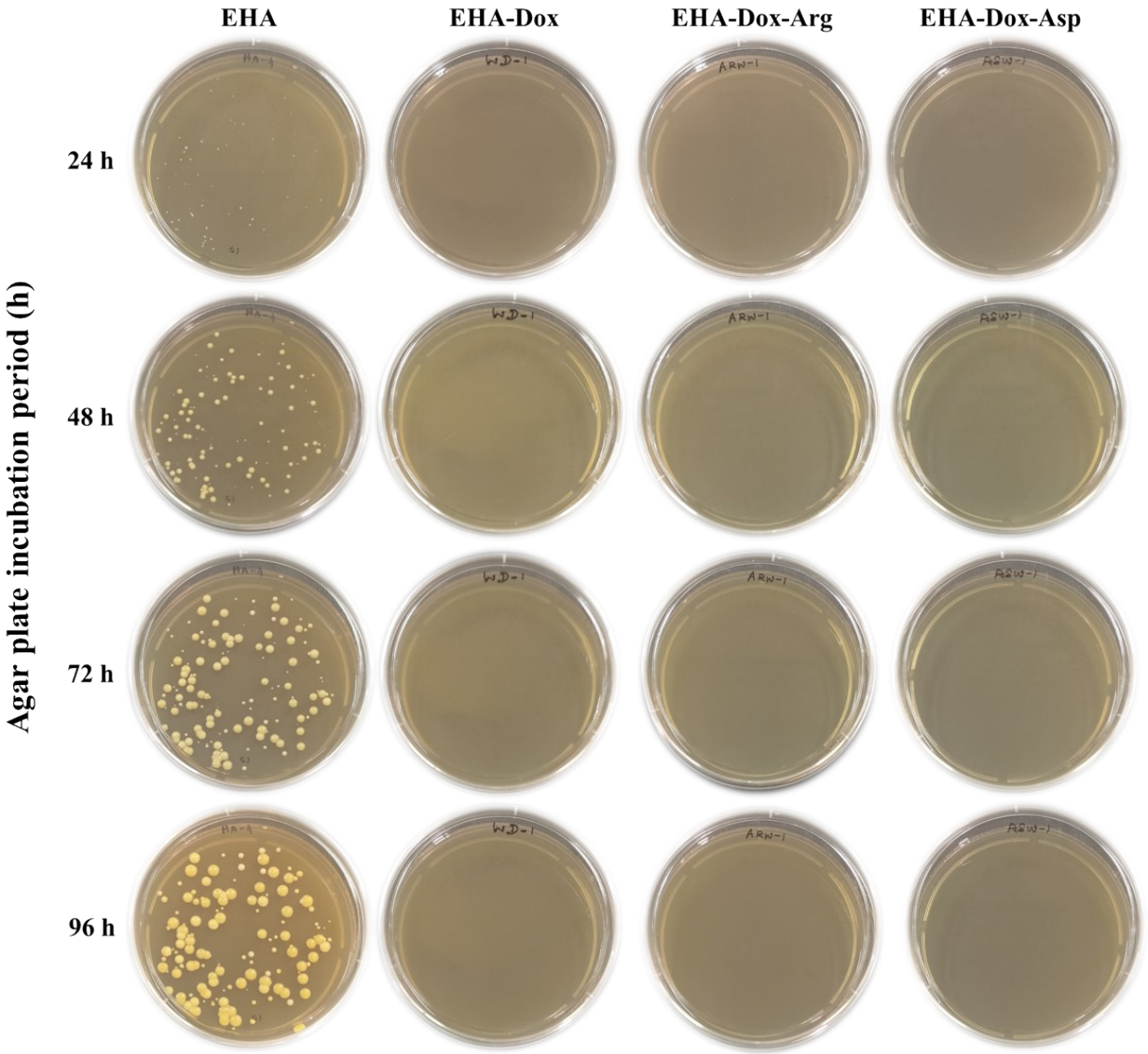

Fig. S10. Agar plates seeded with sessile *S. aureus* treated with EHA, EHA-Dox, EHA-Dox-Arg, and EHA-Dox-Asp samples were assessed by the colony counting method after 14 days of biofilm formation in culture medium for 24 h to 96 h of plate incubation

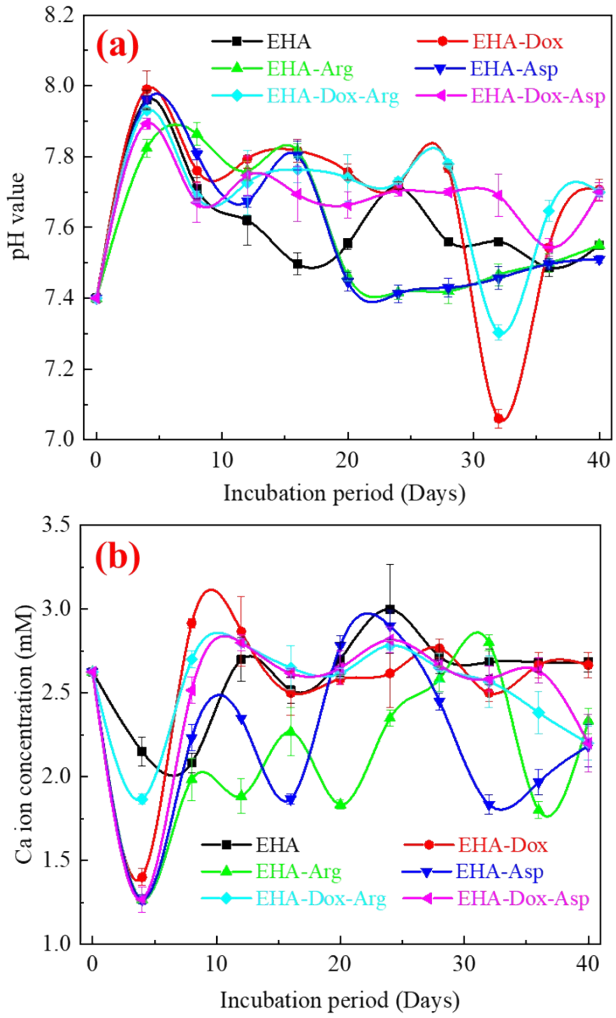

Fig. S11. (a) pH value and (b) Ca^{2+} ion concentration of SBF medium soaked with prepared samples EHA, EHA-Dox, EHA-Arg, EHA-Dox-Arg, EHA-Asp, and EHA-Dox-Asp during bioactivity for different incubation periods

The pH variation of the immersion medium during the incubation of the pellet is shown in Fig. S7(a). Initially, an increase in pH was observed for 4 days and then decreased at Day 8, followed by variation in a random manner, which may be due to the dissolution and reprecipitation of apatite. The dissolution of the sample leads to an increase in pH value due to the release of OH^- ions from the pellet into the immersion medium [1,2]. The precipitation of apatite on the surface of the pellet sample consumes OH^- ions, resulting in a decrease in pH of the medium. The Ca^{2+} ion concentration in the immersion medium during the incubation period is shown in Fig. S7(b). The dissolution of the sample results in the release of Ca^{2+} and PO_4^{3-} ions, which increases Ca^{2+} ion

concentration in the medium [3]. The subsequent reprecipitation of apatite due to supersaturation of Ca^{2+} and PO_4^{3-} ions in the immersion medium led to a reduction in the concentration of Ca^{2+} ions in the medium. The apatite deposition formed on the surface of the pellet due to dissolution and reprecipitation of Ca^{2+} and PO_4^{3-} ions.

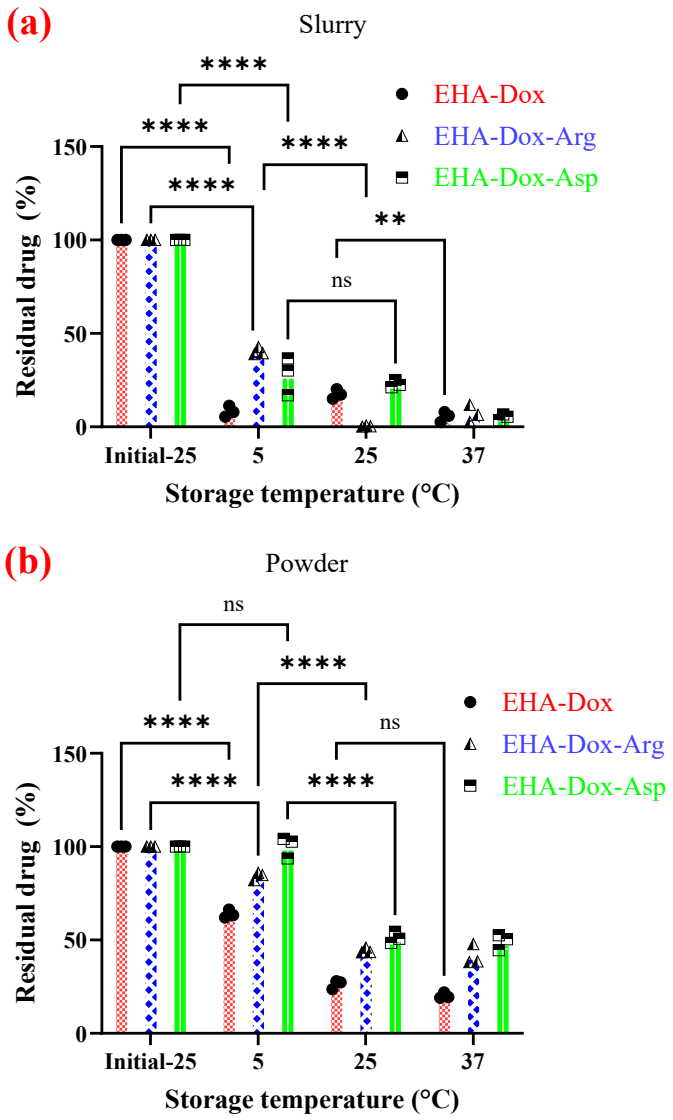

Fig. S12. Residual percentage of drug in EHA-Dox, EHA-Dox-Arg & EHA-Dox-Asp samples after two months of storage at 5, 25, and 37 °C in (a) slurry and (b) powder forms. Data are expressed as mean ± SD ($n = 3$). Statistical significance is indicated by ns – not significant, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

Table S1: Loading efficiency (LE) and Loading content (LC) of drug and amino acid in prepared samples

Sample code	Yield (mg)	Weight loss between 100-600 °C (WL %)	Dox loading by UV		LE_{AA}%	LC_{AA}%	LC_{AA} (µg/mg)	LC_{AA} in total yield (mg)
			LE_{Dox}%	LC_{Dox}%				
EHA	4655	4.8	-	-	-	-	-	-
EHA-Dox	4798	5.9	31.4 ± 6.8	1.8 ± 0.5	-	-	-	-
EHA-Arg	3115	4.9	-	-	0.02	0.1	1	3
EHA-Asp	5156	7.2	-	-	0.9	2.4	24	124
EHA-Dox-Arg	5093	8.7	42.0 ± 2.9	2.2 ± 0.2	0.6	2.0	20	102
EHA-Dox-Asp	5424	10.1	36.5 ± 3.8	1.8 ± 0.3	1.6	3.9	39	212

Estimation of amino acid from TGA analysis

$$Weight\ loss\ between\ 100\ to\ 600°C\ = WL(\%) = \frac{WR_{100} - WR_{600}}{WR_{100}} \times 100 \qquad Eq.S1$$

Where, WR_{100} and WR_{600} are residual weight of the sample at 100°C and 600°C, respectively.

$$Organic\ content\ (\%) = WL_{EHA-AA/EHA-Dox-AA} - WL_{EHA}$$

Where, $WL_{EHA-AA/EHA-Dox-AA}$ is weight loss of EHA-Asp, EHA-Arg, EHA-Dox-Asp or EHA-Dox-Arg, and WL_{EHA} is weight loss of EHA between 100 to 600°C, respectively.

$$The\ amount\ of\ organic\ content\ (mg) = \frac{Yield\ \times Organic\ content\ (\%)}{100} \qquad Eq.S2$$

Loading content of amino acid

$$Quantity\ of\ amino\ acid = The\ amount\ of\ organic\ content\ (mg) - Drug\ LC\ in\ total\ yield\ (mg) \qquad Eq.S3$$

Amino acid loading efficiency (LE_{AA}%)

$$= \frac{Amount\ of\ amino\ acid\ in\ total\ yield\ \ (mg)}{Total\ amount\ of\ amino\ acid\ added\ initially\ (mg)} X\ 100 \qquad Eq.S4$$

Amino acid loading content ($LC_{AA}\%$)

$$= \frac{Amount\ of\ amino\ acid\ encapsulated\ in\ the\ sample\ (mg)}{Sample\ yield\ (mg)} X\ 100 \qquad Eq.S5$$

Table S2: Vibrational assignments of prepared samples

Vibrational assignments	Wavenumber (cm^{-1})					
	EHA	**EHA-Dox**	**EHA-Arg**	**EHA-Asp**	**EHA-Dox-Arg**	**EHA-Dox-Asp**
Structural OH vibration	-	-	3569	-	3572	3572
CH_2 stretching vibration (υ_{as})	-	2961	2962	2962	2967	2966
CH_2 stretching vibration (υ_{as})	-	2927	2926	2926	2927	2926
CH_3 stretching vibration (υ_{sy})	-	2855	2855	2855	2856	2857
Overtone and combination bands of PO_4^{3-} ions	2001, 2077	2001, 2077	-	-	-	-
Overtone and combination bands of PO_4^{3-} ions	2142	2142	-	-	-	-
CO_2 absorption from atmosphere	2331, 2356	-	-	-	-	-
OH stretching of water molecules	1643	1641	1645	1638	1639	1639
υ_3 (CO_3^{2-})	1458	1456	1457	1459	1459	1454
υ_3 (CO_3^{2-})	1421	1421	1420	1421	1420	1420
υ_3 (CO_3^{2-})	-	1385	-	-	1384	1385
Vibration C-N group	-	1321	-	-	1327	1317
υ_3 vibration of phosphate	1099-1030	1093-1035	1091-1036	1099-1041	1095-1039	1094-1039
υ_2 vibration of phosphate	-	961	964	965	963	962
B-type carbonate	873	874	874	874	874	874
Characteristic O-H stretching vibration	675	637	-	-	-	-
Vibration of tetrahedral phosphate υ_4	601	603	603	601	603	603
Vibration of tetrahedral phosphate υ_4	568	564	566	564	564	563
υ_1 vibration of phosphate	476	471	471	472	470	472

Table S3: The lattice parameter and crystallite size of prepared samples

Sample Code	**Lattice parameter (Å)** $a = b \neq c$		**Crystallite size (nm)** $D_{(002)}$
	$a = b$	c	
JCPDS	9.418	6.884	
EHA	9.41	6.87	35 ± 1.4
EHA-Dox	9.45	6.90	39 ± 0.7
EHA-Arg	9.42	6.88	34 ± 2.8
EHA-Asp	9.45	6.86	30 ± 0.7
EHA-Dox-Arg	9.38	6.87	28 ± 0.7
EHA-Dox-Asp	9.44	6.87	34 ± 2.1

Table S4. Length, width, and aspect ratio of prepared NPs calculated from TEM

Sample code	Length (nm)	Width (nm)	Aspect ratio
EHA	35.5 ± 9.4	5.1 ± 0.9	7.0
EHA-Dox	40.2 ± 10.9	5.3 ± 0.7	7.6
EHA-Arg	33.5 ± 5.9	5.8 ± 1.1	5.8
EHA-Asp	30.1 ± 4.8	4.7 ± 0.6	6.4
EHA-Dox-Arg	30.4 ± 6.0	4.5 ± 0.7	6.8
EHA-Dox-Asp	38.5 ± 6.8	4.2 ± 0.6	9.2

Table S5. Drug release amount of EHA-Dox, EHA-Dox-Arg, and EHA-Dox-Asp samples

Days	Amount of drug release (mg)								
	EHA-Dox			EHA-Dox-Arg			EHA-Dox-Asp		
	pH 5.5	pH 6.7	pH 7.4	pH 5.5	pH 6.7	pH 7.4	pH 5.5	pH 6.7	pH 7.4
1	1.05	0.8	0.65	1.22	0.85	0.8	1.28	0.87	0.77
3	1.35	1.17	0.88	1.50	1.38	1.14	1.45	1.40	1.03
5	1.37	1.23	0.92	1.62	1.48	1.45	1.46	1.55	1.24
8	1.26	1.13	0.95	1.52	1.72	1.70	1.40	1.52	1.46
11	1.32	1.17	1.01	1.50	1.54	1.60	1.30	1.46	1.43
14	1.24	1.07	1.00	1.53	1.60	1.64	1.31	1.46	1.43

Table S6. $\log_{10}$(CFU ml^{-1}) of number of colonies formed on agar plate seeded with Day 1 to Day 4 culture medium at increasing incubation period (24 h, 48 h, 72 h, and 96 h)

Sample code	Incubation in culture medium	Plate incubation period (Number of colonies)			
		24 h	48 h	72 h	96 h
EHA	Day 1	6.7 ± 0.2	*		
	Day 2	7.4 ± 0.3			
	Day 3	7.7 ± 0.1			
	Day 4	7.9			
EHA-Dox	Day 1	0	3.1 ± 0.2	3.4 ± 0.2	3.5 ± 0.2
	Day 2	0	1.5 ± 1.3	2.9 ± 0.2	3.1 ± 0.2
	Day 3	0	1.9 ± 0.2	2.2 ± 0.2	2.6 ± 0.3
	Day 4	0	1.0 ± 0.9	1.8 ± 0.5	2.3 ± 0.5
HA-Dox-Arg	Day 1	0	2.2 ± 0.3	2.7 ± 0.3	2.8 ± 0.3
	Day 2	0	0	1.8 ± 0.1	2.2 ± 0.3
	Day 3	0	0.6 ± 1.0	1.7 ± 0.5	1.9 ± 0.5
	Day 4	0	0	0.3 ± 0.6	1.3 ± 0.3
HA-Dox-Asp	Day 1	0	2.4 ± 0.5	2.9 ± 0.4	3.0 ± 0.4
	Day 2	0	1.1 ± 1.2	2.1 ± 0.6	2.3 ± 0.5
	Day 3	0	0	0.9 ± 0.8	1.2 ± 1.0
	Day 4	0	0	0.6 ± 1.0	1.4 ± 0.4

Table S7: Cell viability of EHA-Arg and EHA-Asp at concentrations from 0 to 800 µg

Dose per well (µg)	% of cell viability					
	EHA	**EHA-Dox**	**EHA-Arg**	**EHA-Asp**	**EHA-Dox-Arg**	**EHA-Dox-Asp**
Control	100.0 ± 1	100.0 ± 10.5	100.0 ± 0.5	99.0 ± 0.5	100.1 ± 6.4	100.1 ± 7.9
100	90.1 ± 1.6	85.8 ± 1.8	93.8 ± 3.0	90.4 ± 0.2	61.6 ± 3.7	67.3 ± 12.5
200	86.3 ± 0.9	74.7 ± 2.0	90.7 ± 1.6	88.1 ± 0.9	49.4 ± 3.8	56.0 ± 7.9
300	82.5 ± 1.5	72.0 ± 2.6	89.5 ± 1.3	87.0 ± 0.3	42.3 ± 9.1	48.9 ± 3.4
400	77.9 ± 1.2	70.5 ± 5.6	87.8 ± 2.1	85.6 ± 0.7	37.6 ± 8.7	38.9 ± 7.9
500	71.9 ± 1.5	61.1 ± 1.9	85.2 ± 1.7	82.8 ± 1.1	31.1 ± 10.8	31.9 ± 12.1
600	67.3 ± 1.2	48.7 ± 5.5	83.5 ± 1.7	80.2 ± 0.4	27.9 ± 9.0	29.0 ± 9.0
700	60.3 ± 2.4	44.7 ± 7.1	82.1 ± 2.2	78.5 ± 0.4	22.0 ± 3.1	21.0 ± 3.5
800	53.5 ± 2.5	44.2 ± 10.4	78.0 ± 3.5	74.3 ± 0.9	20.0 ± 1.8	19.1 ± 0.6

References

1 V. Ganesan, M. Devaraj, S. K. Govindan, V. S. Kattimani and G. Easwaradas Kreedapathy, *Int. J. Appl. Ceram. Technol.*, 2019, **16**, 1932–1943.

2 D. Siva Rama Krishna, A. Siddharthan, S. K. Seshadri and T. S. Sampath Kumar, *J. Mater. Sci. Mater. Med.*, 2007, **18**, 1735–1743.

3 S. P. V. and T. S. S. Kumar, *J. Biomed. Nanotechnol.*, 2008, **4**, 203–209.