Supplementary Information

Antibiofilm Coatings based on Protein-Engineered Polymers and Antimicrobial Peptides for Preventing Implant-Associated Infections

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Materials: Glassware was soaked in acid water (1% hydrochloric acid), washed with Alconox®, rinsed with distilled water and dried at 80 °C overnight prior to use. All chemicals were purchased from Sigma-Aldrich (Merck Group, Germany) and used as received unless otherwise mentioned. Ultrapure water (15 MΩ·cm) was used from a Milli-Q® Integral water purification system (Merck Group, Germany).

Methods:

Recombinant synthesis of the ELR and L-AMP-ELR

Construction of the encoding genes was performed using previously described procedures.[1] Briefly, encoding genes for the monomeric units of the final construction were purchased from NZYTech, Lda. (Portugal) and cloned into a modified pDrive plasmid flanked by two *Ear*I restriction sites. The final genetic constructions were then completed through the iterative recursive method using *E. coli* XL-1 blue (Agilent, USA) as cloning strain. The hybrid L-AMP-ELR (VCL) was expressed as a pro-polypeptide incorporating a sacrificial block on its N-terminal end.[2]

The resulting genes were cloned into pET-25b (+) expression vectors and transformed into *E. coli* BLR (DE3) for heterologous expression. After overnight fermentation in a 15 L bioreactor (Applikon Biotechnology, the Netherlands), the biopolymers were purified taking advantage of the LCST phase behavior of the ELRs by inverse transition cycling

(ITC), adding 1.5 M NaCl for warm precipitation (40 °C). After four cycles, the biopolymers were then dialyzed against ultrapure water, lyophilized and stored at -20 °C. The yields observed ranged from 190 to 270 mg of purified protein polymer per liter of bacterial culture.

Sacrificial block cleavage and purification

After recombinant expression and purification, the sacrificial block was cleaved using previously procedures.[2] Briefly, pro-polypeptide solution was treated with CNBr under acidic conditions (70% formic acid) in a molar ratio Met:CNBr 1:200 for 20 h at room temperature to release the VCL polymer. CNBr was then eliminated on a rotary evaporator. VCL polymer was resuspended in ultrapure water and dialyzed. After a single warm centrifugation step (40 °C, pH < 4 and NaCl 0.5 M), VCL was completely purified. Finally, the polypeptide solutions were dialyzed, filtered (0.22 μm Nalgene™, ThermoFisher Scientific, USA), lyophilized and stored at -20 °C until further use.

Synthesis of the D-AMP-ELR

D-AMP-ELR hybrid polymer was produced through chemical derivatization.[3] First, free amines of the VC ELR were chemically modified with cyclooctyne groups. To that end, a solution of (1R,8S,9S)- bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate in dimethylformamide (DMF) was added to a solution of the VC ELR in DMF and incubated at room temperature with stirring for 48 h. The ELR was then precipitated by the addition of diethyl ether. The supernatant was discarded and the precipitate was washed three times with acetone, dried under in a rotary evaporator, dissolved with pre-chilled ultrapure water (4 °C), dialyzed, filtered (0.22 μm Nalgene™, ThermoFisher Scientific, USA), lyophilized and stored at -20 °C until further use.

Second, the cyclooctyne groups were modified via a strain-promoted azide-alkyne cycloaddition (SPAAC) with the N_3-D-GL13K peptides. To that end, cyclooctyne-modified VC (cyclo-VC) was dissolved in dimethylsulfoxide (DMSO) at 10 mg mL^{-1} and N_3-D-GL13K peptide was added at a molar ratio cyclooctyne groups:peptide 1:1.5. Finally, the resulting mixture was incubated at 4 °C for 24 h, dialyzed against ultrapure water, filtered, lyophilized and stored at -20 °C.

Characterization of the ELRs

Molecular weights and monodispersity of the ELRs were determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The measurements were carried out by the "Laboratorio de Técnicas Instrumentales (LTI)" of the University of Valladolid (Spain). All the measurements were analyzed at least 3 times.

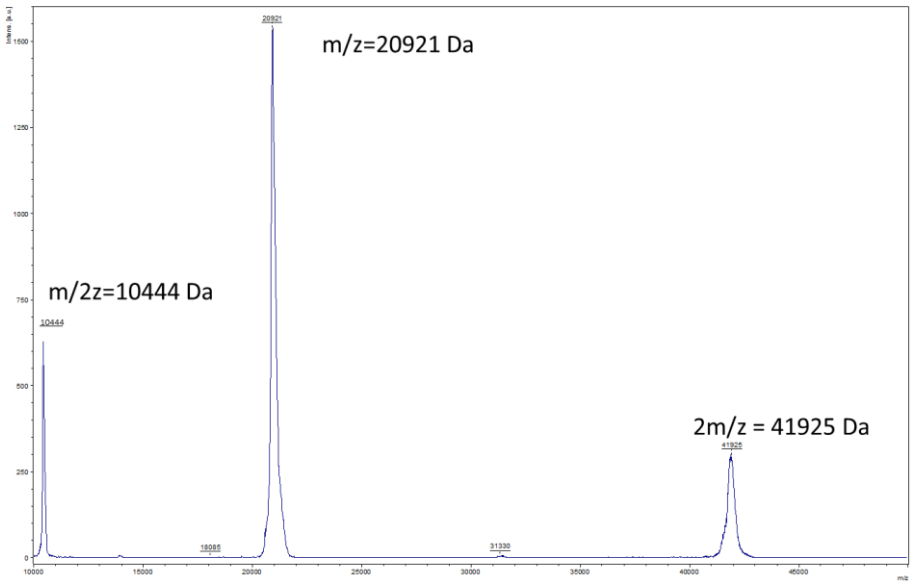


Figure S1. MALDI-TOF spectrum of the VC protein polymer.

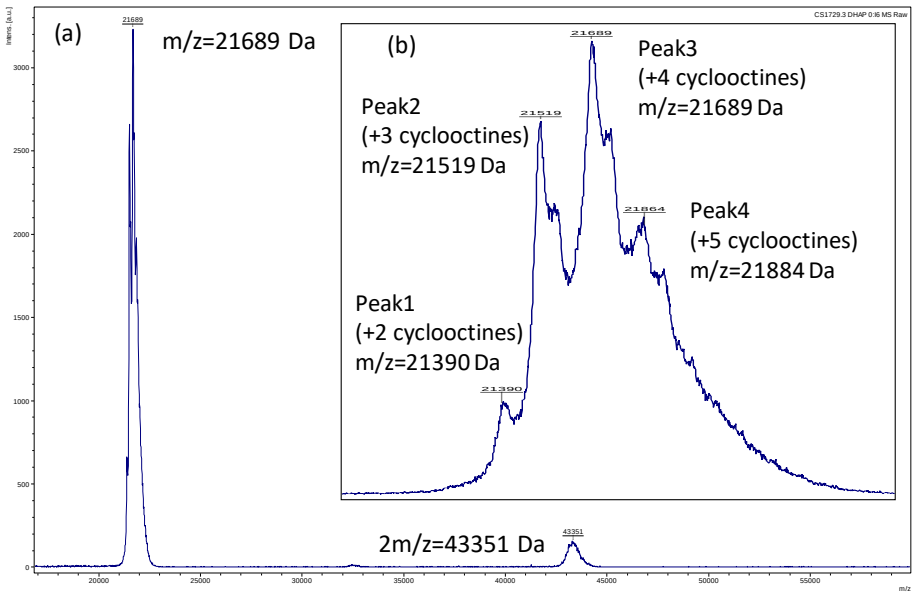


Figure S2. (a) MALDI-TOF spectrum of VC after the modification of the lysine side-chains with cyclooctyne groups (VC-cyclo). (b) showed the magnification of the m/z peak.

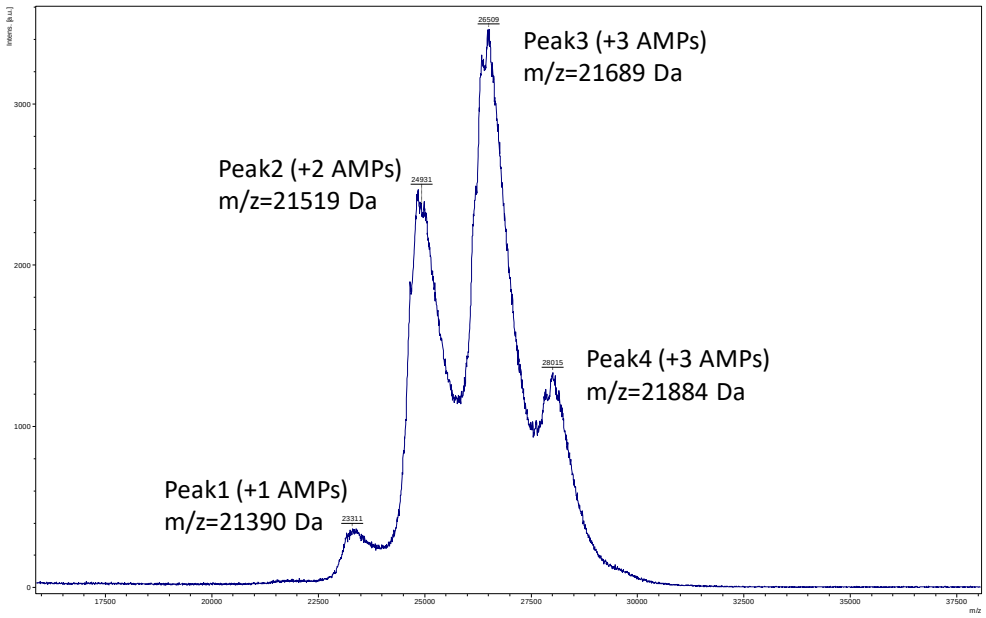
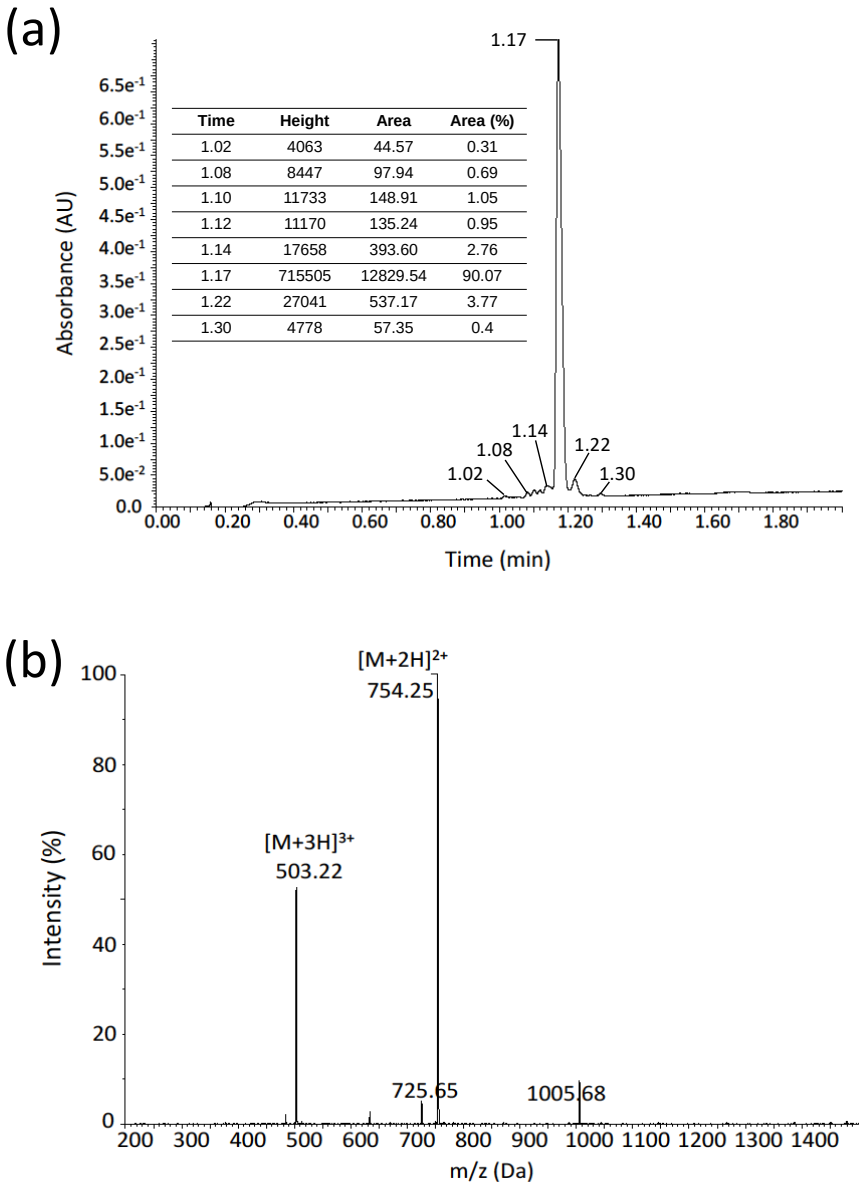


Figure S3. MALDI-TOF spectrum of the VC-cyclo after the addition of D-GL13K-N_3 (VCD).
(MW of N_3-D-GL13K =1507.1 Da)

Table S1. Molecular weight of the VC, VC-cyclo and VCD calculated by MALDI-TOF after the different modification steps. It is represented the theoretical composition comparing to the experimental values obtained. Results are shown as mean ± standard deviation (SD) ($n \geq 3$).
MALDI-TOF analysis revealed that an average of 4 amine groups per VC molecule were modified with cyclooctyne groups. The cyclooctyne groups served, then, to covalently incorporate an average of three D-GL13K peptides per molecule.

	Theoretical		Experimental	
ELR	**Molecules Introduced**	**MW (Da)**	**Peak (m/z)**	**MW (Da)**
VC	-	21038.1	1	20819.8±1.2
VC-cyclo	2 cyclooctyne groups	21342.1	1	21396.7±6
	3 cyclooctyne groups	21494.1	2	21517.3±5.1
	4 cyclooctyne groups	21646.1	3	21690.7±2.1
	5 cyclooctyne groups	21798.1	4	21853.2±6.2
VCD	1 AMP	23305.2	1	23341.6±18.7
	2 AMPs	24812.3	2	24950.4±16
	3 AMPs	26319.4	3	26517.8±7.8
	4 AMPs	27826.5	4	28015.6±8



Time	Height	Area	Area (%)
1.02	4063	44.57	0.31
1.08	8447	97.94	0.69
1.10	11733	148.91	1.05
1.12	11170	135.24	0.95
1.14	17658	393.60	2.76
1.17	715505	12829.54	90.07
1.22	27041	537.17	3.77
1.30	4778	57.35	0.4

Figure S4. (a) HPLC analysis and (b) mass spectroscopy spectrum of the designer peptide N_3-D-GL13K (MW=1507.1 Da).

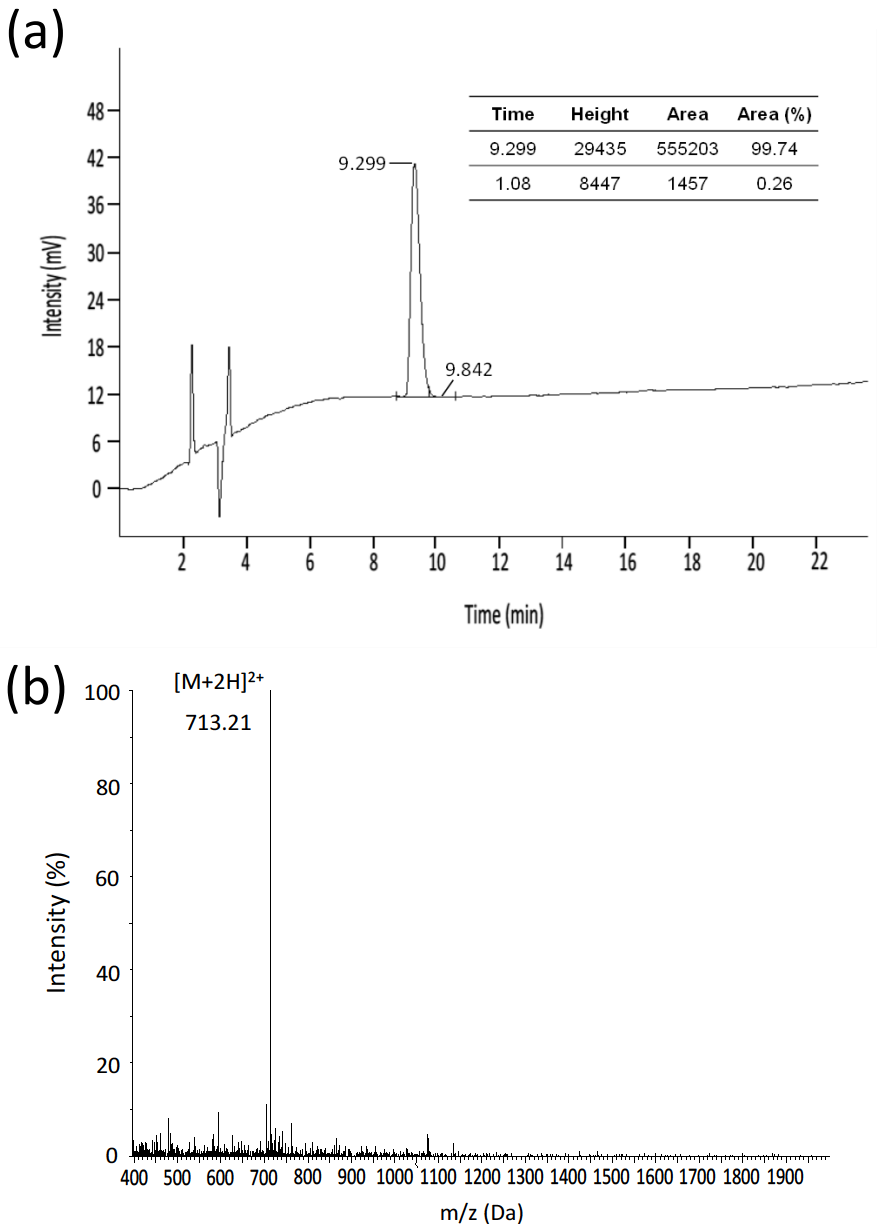


Figure S5. (a) HPLC analysis and (b) mass spectroscopy spectrum of the designer peptide D-GL13K (MW=1423.9 Da).

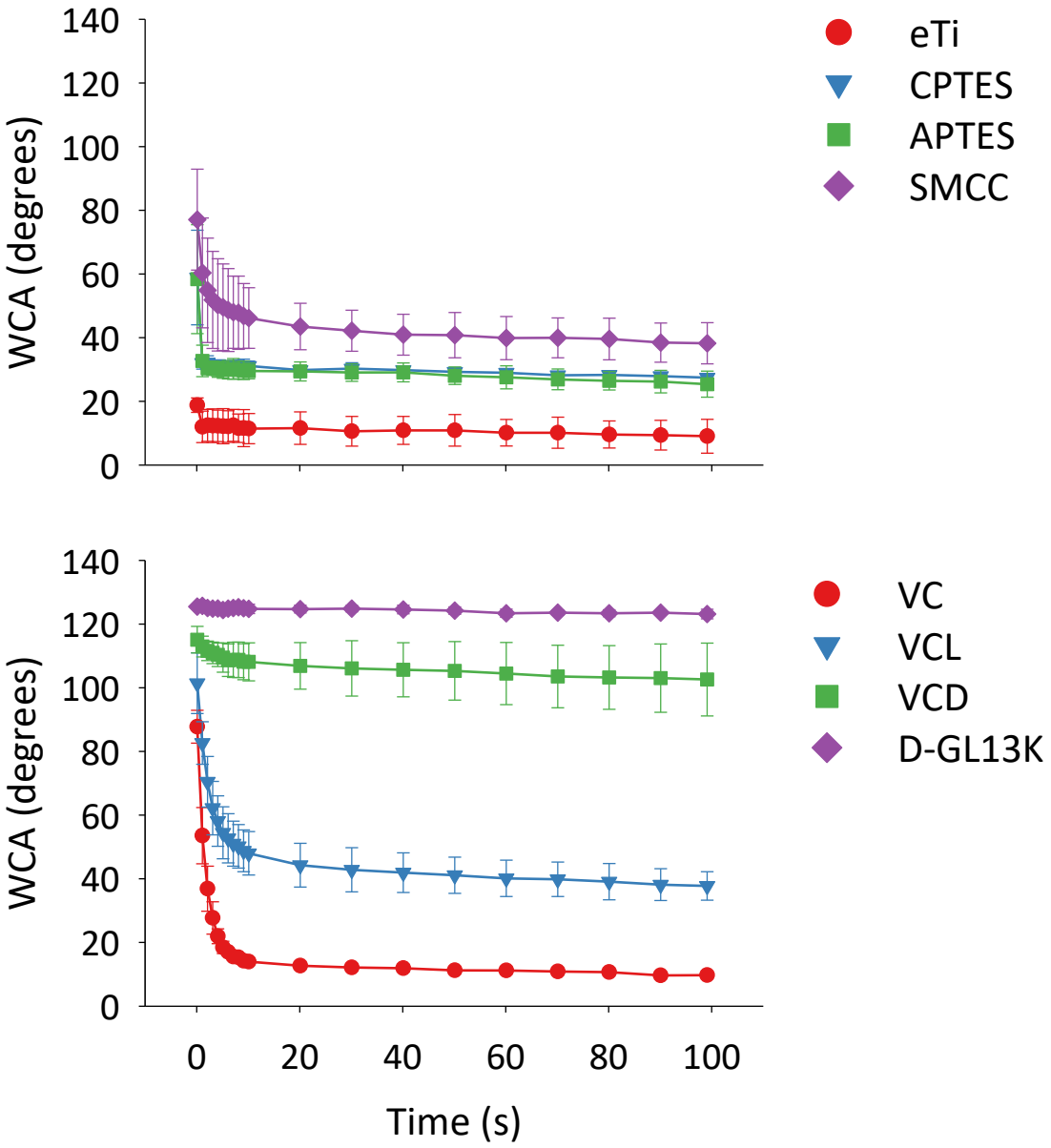


Figure S6. Dynamic WCA profile of the Ti surfaces after the different modification steps during the AMP/ELR/AMP-ELR coating fabrication

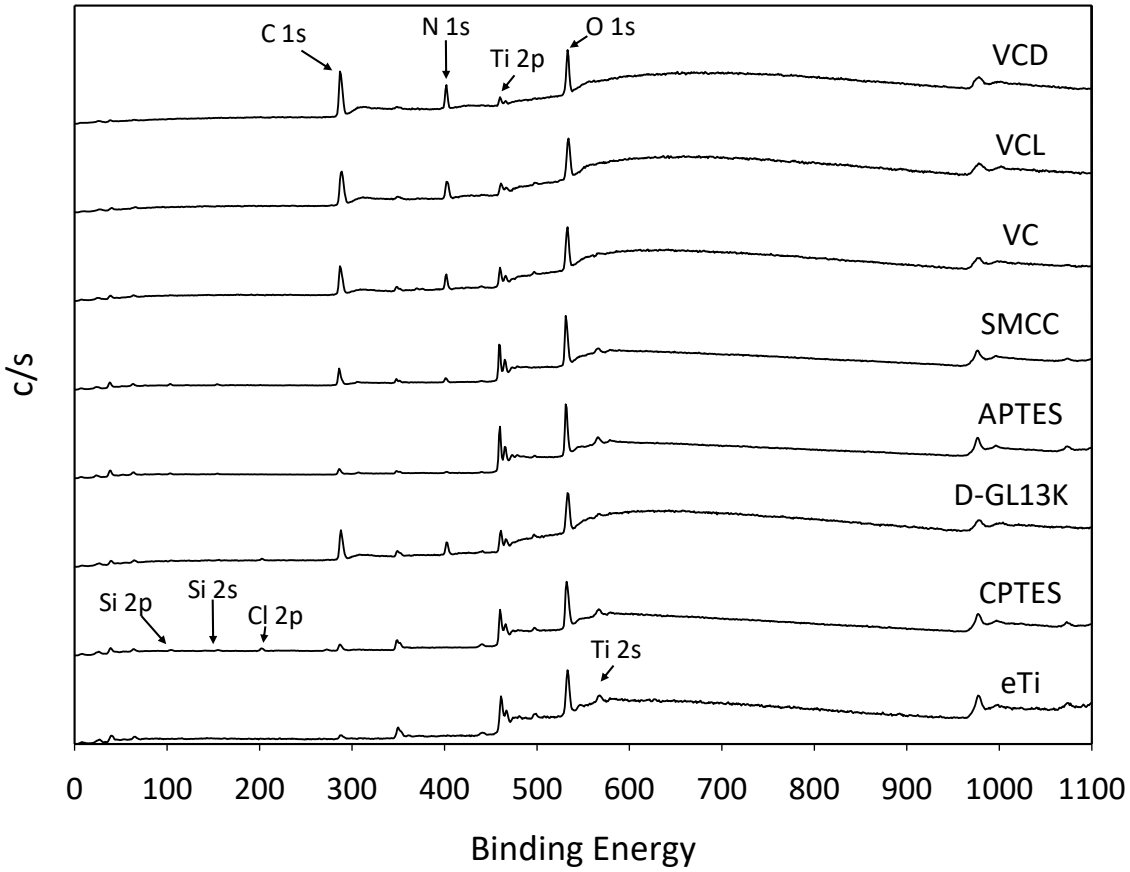


Figure S7. Representative XPS spectra of the surfaces after the diverse modification steps during the AMP/ELR/AMP-ELR coatings fabrication.

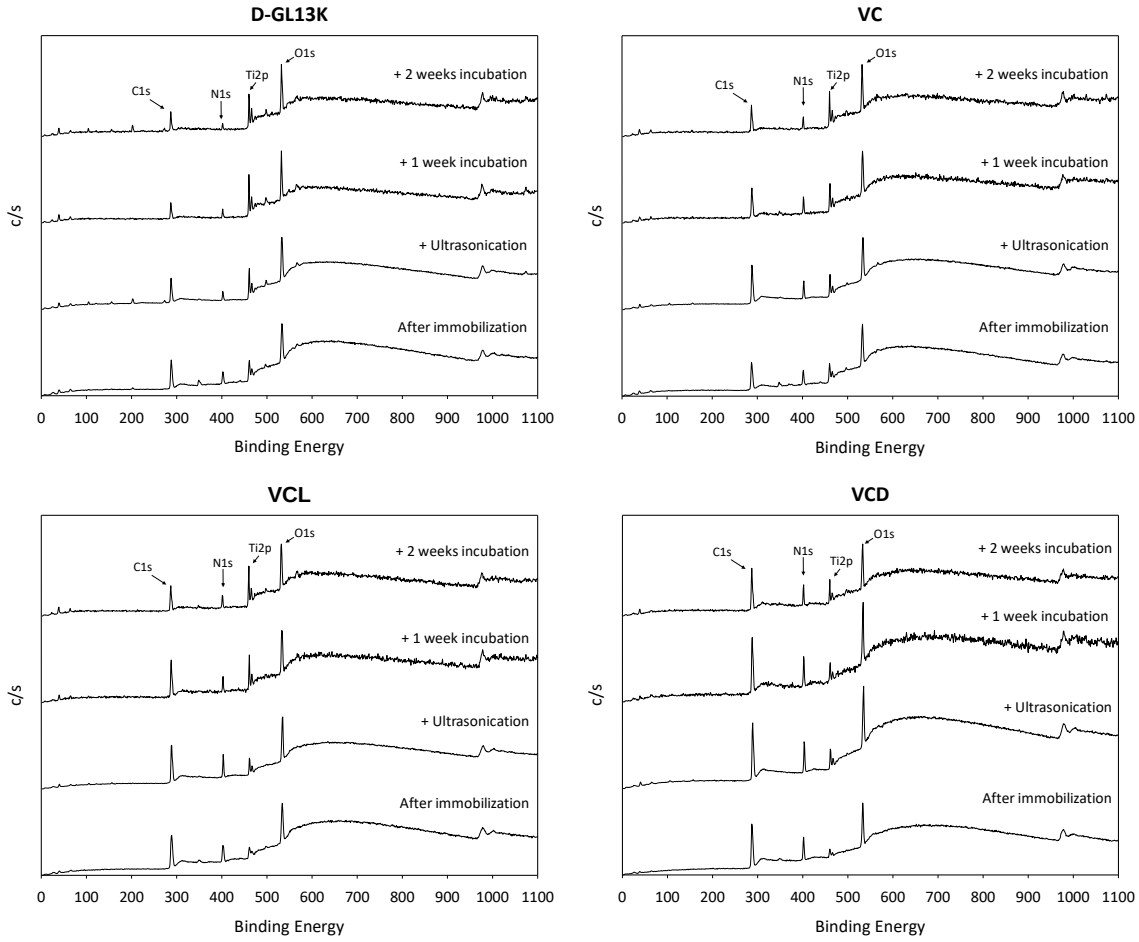


Figure S8. Representative XPS spectra of the Ti surfaces after the AMP/ELR/AMP-ELR immobilization and after the stability tests: ultrasonication in ultrapure water for 2 h and incubation in PBS at 37 °C for 1 and 2 weeks.

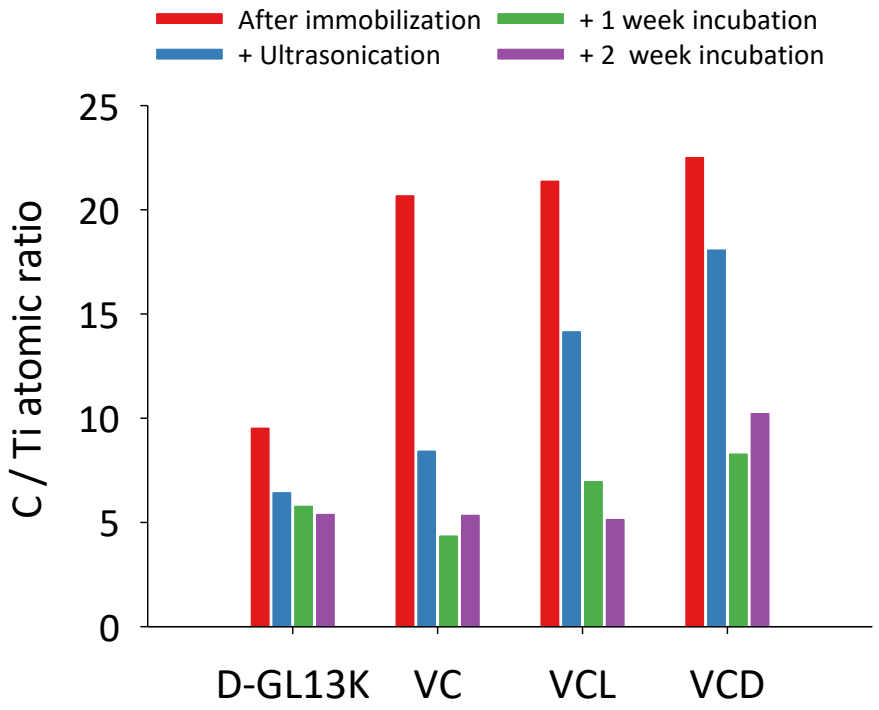


Figure S9. Evolution of the C/Ti atomic ratio of the AMP/ELR/AMP-ELR coatings after the stability tests: ultrasonication in ultrapure water for 2 h and incubation in PBS at 37 °C for 1 and 2 weeks.

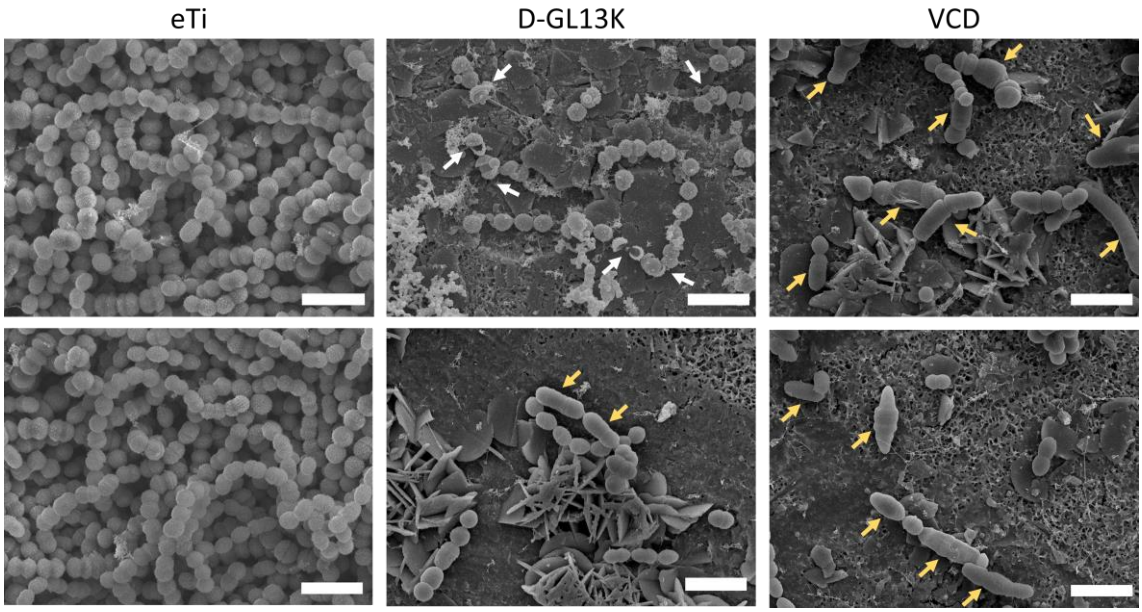


Figure S10. SEM micrographs of the morphology of the streptococci that remained on the D-GL13K (DAMP) and VCD (D-AMP-ELR) coatings after the incubation in the DFBR, compared with the eTi. Scale bar = 2 µm. White arrows indicate cracked bacteria while elongated bacteria are indicated by yellow arrows.

References:

1 J. C. Rodríguez-Cabello, A. Girotti, A. Ribeiro and F. J. Arias, in *Methods in molecular biology (Clifton, N.J.)*, 2012, vol. 811, pp. 17–38.

2 S. Acosta, L. Quintanilla, M. Alonso, C. Aparicio and J. C. Rodríguez-Cabello, *ACS Biomater. Sci. Eng.*, 2019, **5**, 4708–4716.

3 I. González De Torre, M. Santos, L. Quintanilla, A. Testera, M. Alonso and J. C. Rodríguez Cabello, *Acta Biomater.*, 2014, **10**, 2495–2505.