Supplementary information

Thiol-norbornene Photoclick Chemistry for Grafting Antimicrobial Peptides onto Chitosan to Create Antibacterial Biomaterials

Pedro M. Alves[a,b,c,d], Rúben F. Pereira[a,b,e], Beatriz Costa[a,b,f], Natália Tassi[d], Cátia Teixeira[d], Victoria Leiro[a,b], Cláudia Monteiro[a,b], Paula Gomes[d], Fabíola Costa [a,b,‡], M. Cristina L. Martins[a,b,e,,‡]*

[a]i3s, Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal

[b]INEB, Instituto de Engenharia Biomédica, Universidade do Porto, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal

[c]Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

[d]LAQV-REQUIMTE, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre 687, 4169-007 Porto, Portugal

[e]ICBAS - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 4050-313 Porto, Portugal

[f]Faculdade de Ciências e Tecnologia, FCT, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

‡These authors contributed equally.

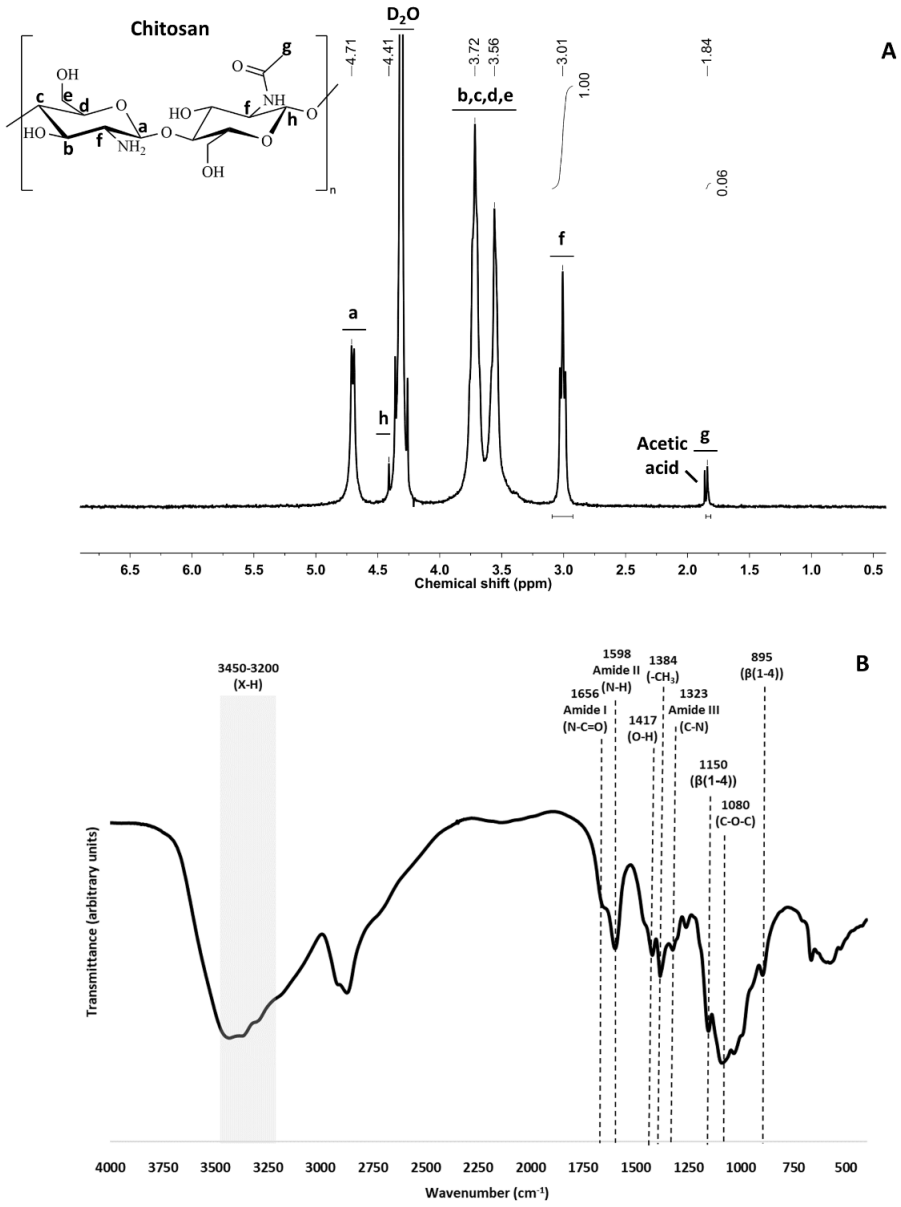

Figure S1. A. Spectrum of unmodified chitosan (Chit) obtained by ^{1}H NMR (400 MHz, 0.5 M DCl in D_2O, 70 ºC). δH: 1.84 (g, 3H, br s, CH_3), 3.01 (f, 1H, br t, H-2 from $GlcNH_2$ and $GlcNHCOCH_3$), 3.56-3.72 (b,c,d and e, 5H, m, H-3,4,5,6), 4.41 (h, 1H, br d, H-1 $GlcNHCOCH_3$) and 4.69 (a, 1H, br d, H-1 $GlcNH_2$) [1,2]. **B.** Spectra of unmodified chitosan (Chit) obtained by FTIR (transmission mode): 3450-3200 cm^{-1} (hydrogen-bonded stretching vibrations), 1656 cm^{-1} (amide I, C=O stretching from secondary amides), 1598 cm^{-1} (amide II, N-H deformation combined with C-N stretching from secondary amides and also N-H bending from primary amines), 1417 cm^{-1} (O-H plane deformation), 1384 cm^{-1} (CH_3 deformation), 1323 cm^{-1} (amide III, C-N stretching), 1150 cm^{-1} (β(1-4) glycoside bridge), 1080 cm^{-1} (C-O-C stretching vibration), 895 cm^{-1} (β(1-4) glycoside bridge) [3].

$$DA = \frac{\mathbf{1}}{\mathbf{3}} \times \frac{\int \mathbf{1.81}\ ppm\ (CH_3\ protons)}{\int \mathbf{3.01}\ ppm\ (H-2\ from\ GlcNH_2\ and\ GlcNHCOCH_3)} \quad \text{(Equation S1)}$$

DA was determined by ^{1}H NMR (400 MHz, 0.5 M DCl in D_2O, 70 ºC) according to Equation S1 [1,4,5]. Briefly, the integral of the peak at 1.81 ppm (from the 3 protons of the acetyl group, g) was compared to that of the peak at 3.01 ppm (H-2 proton from $GlcNH_2$ and $GLcNHCOCH_3$, f). A factor of 1/3 was applied to the equation to correct for the number of protons contributing to each peak.

Table S1. Experimental conditions for optimizing the functionalization of chitosan (Chit) with norbornene groups regarding carbic anhydride (CA) molar excess (1F vs 2F).

	CA:Chit molar ratio	**Chit mass (mg)**	**Chit monomers (mmol)***	**CA added (mmol)**
1-fold	0.9:1	30	0.185	0.163
2-fold	1.8:1	30	0.185	0.327

* - theoretical values

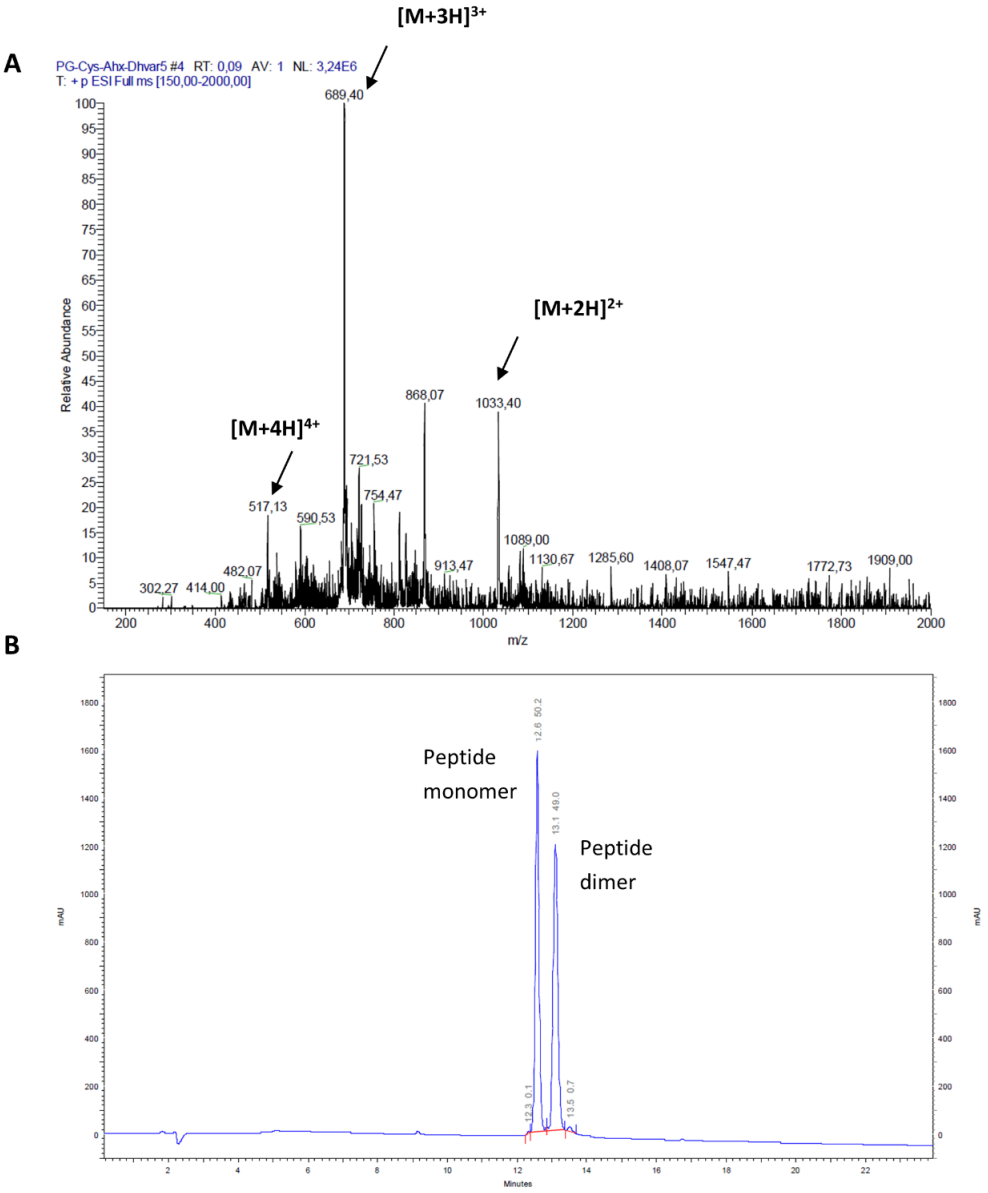

Figure S2. ESI-IT MS (positive mode) spectra (A) and HPLC chromatogram (B) of Cys-Ahx-Dhvar5.

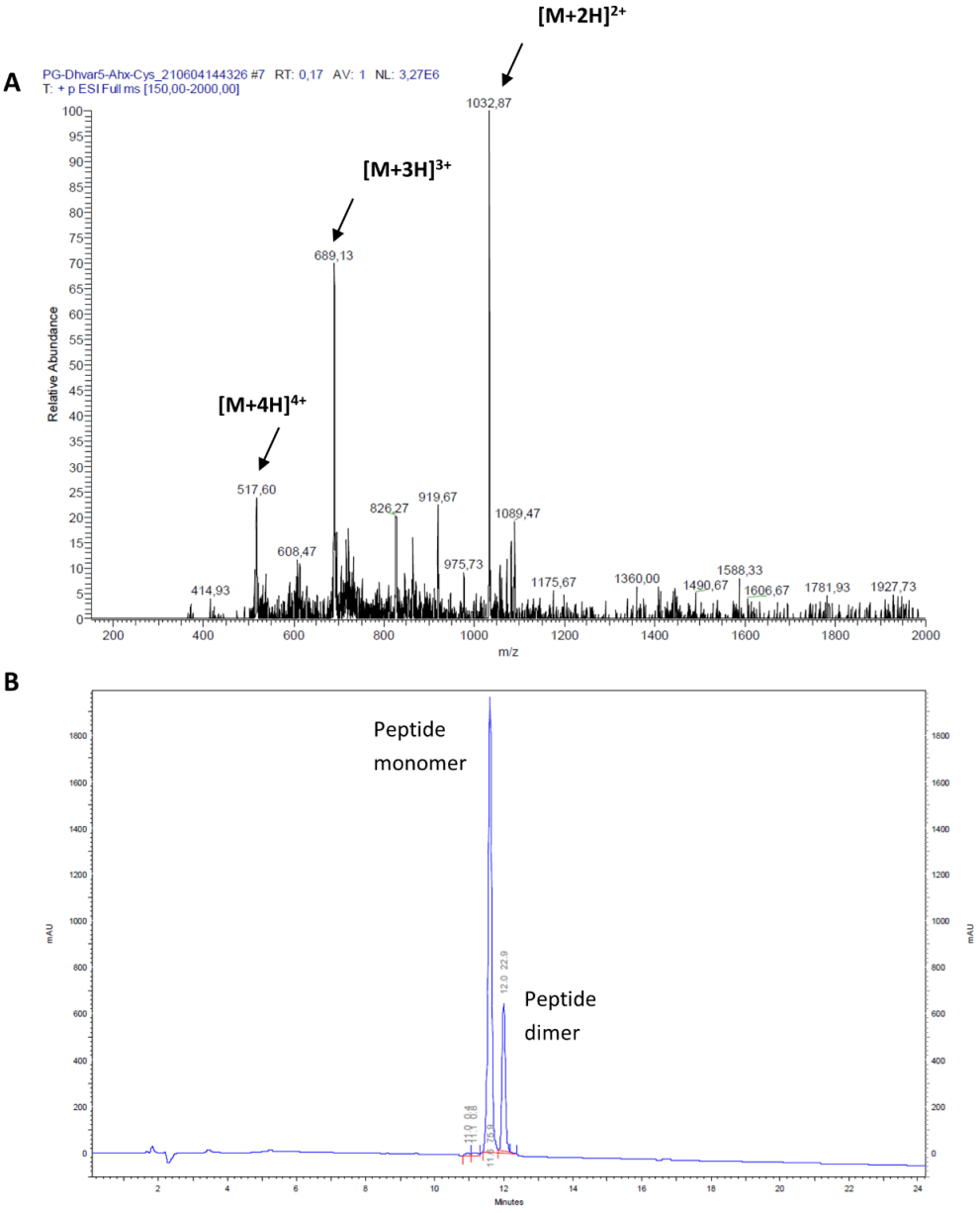

Figure S3. ESI-IT MS (positive mode) spectra (A) and HPLC chromatogram (B) of Dhvar5-Ahx-Cys.

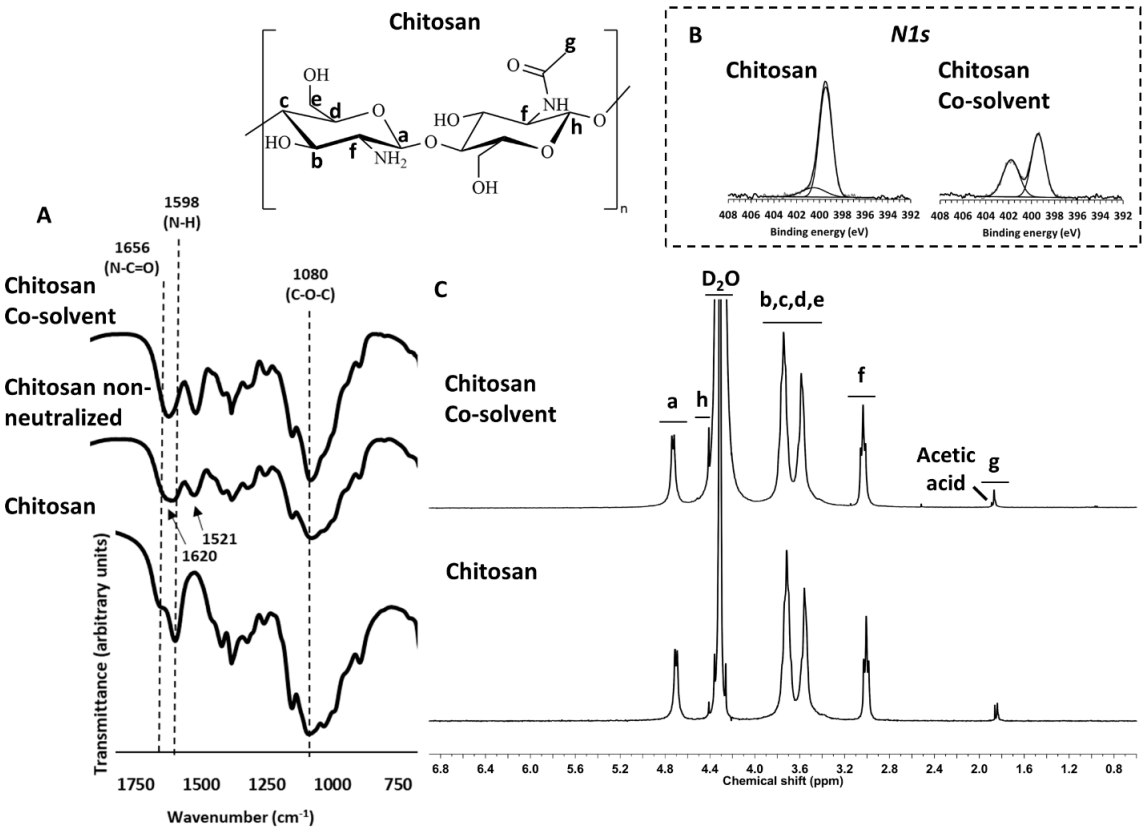

Figure S4. FTIR (A), high-resolution XPS spectra of *N1s* (B) and ^{1}H NMR (400 MHz, 0.5 M DCl in D_2O, 70 ºC) (C) showing no significant impact of the co-solvent system on chitosan structure and composition. No major changes were observed by FTIR for chitosan after solubilization in the co-solvent system (A). The observed shift of the characteristic chit peaks at 1656 cm^{-1} and 1598 cm^{-1} to 1620 cm^{-1} and 1521 cm^{-1}, respectively, in chitosan co-solvent spectrum in comparison with chitosan spectrum is explained by the protonation of the amine groups (NH_3^+), since chitosan with 2% DA was used (98% of deacetylated amines). These groups remained protonated even after solubilization in the co-solvent system and dialysis [6], as shown by the non-neutralized chitosan FTIR spectrum (chitosan non-neutralized). This fact was confirmed by XPS where the only difference between Chit and Chit Co-solvent was in *N1s* spectra that revealed an extra peak at 401.7 eV, assigned to protonated amines [7], in chitosan co-solvent spectrum (B). ^{1}H NMR spectra (C) also demonstrated no significant alterations in chitosan characteristic peaks regarding to chitosan co-solvent.

Table S2. Elemental analysis of chitosan without (Chit) or with exposure (Chit co-solvent) to the co-solvent system. Relative atomic percentages of carbon in the samples was slightly higher than expected, which can be due to adventitious carbon from the atmosphere that naturally deposits on the sample surface [8], as previously observed [3,9]. Residual oxidized sulfur was also detected in the XPS spectrum of chitosan, probably due to an environmental contaminant, as previously hypothesized [7], but it fully disappeared in Chit co-solvent, further validating dialysis as an effective purification step. When comparing the atomic composition before and after co-solvent solubilization, only minor alterations were observed, suggesting no DMF interference in the overall composition of chitosan.

Element	Atomic % (theoretical)	Atomic % (experimental)	
	Chit	Chit	Chit Co-solvent
C1s	54.6	62.5	62.2
N1s	9.0	7.8	7.7
O1s	36.4	29.6	30.1
S2p	0	0.1	N.D.

N.D. – not detected

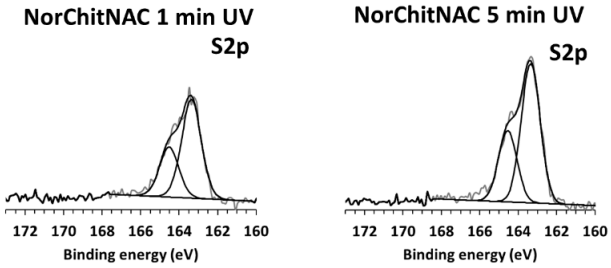

Figure S5. Sulfur high resolution XPS spectra depicting *N*-acetyl-*L*-cysteine (NAC) conjugation onto NorChit (NorChitNAC) and the effect of increasing time of exposure to UV light (*S2p* atomic %: 1% (1 min); 1.2% (5 min)). Peak intensities were normalized using *C1s* peak as reference. NAC, an amino acid derivative with a free sulfhydryl moiety, was applied as a model to optimize the thiol-norbornene photoclick chemistry (TNPC) reaction conditions to be subsequently applied for peptide conjugation. To this end, NorChit was hydrated and solubilized in 0.1 M aq. acetic acid and mixed with 0.63 M NAC in 0.1 M aq. acetic acid at a 2:1 ratio relative to norbornene groups. Photoinitiator VA-086 0.4 wt% (Wako Chemicals) in 0.1 M aq. acetic acid was also added. The reaction mixture was irradiated with UV light (365 nm, RoHS, Hamamatsu) at 10 mW cm-2 for 1 min or 5 min, under magnetic stirring. Finally, the solution containing the target NorChitNAC product was filtered and washed using Amicon® Ultra-15 Centrifugal Filter (Millipore) with 100 kDa cut-off. Final solutions were subsequently frozen, lyophilized and stored in a desiccator until further use.

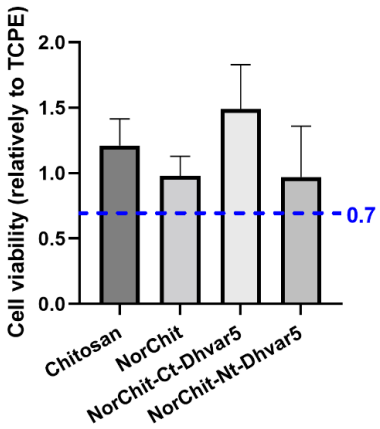

Figure S6. Evaluation of metabolic activity after resazurin assay, normalized by number of cells and relatively to control TCPE. Results are presented as mean with standard deviation for 3 replicates of one representative assay (from a total of two independent assays). The blue dashed line represents the threshold for cytotoxicity according to ISO 10993-1.

Supporting information references

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