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Supporting information for

Antifungal Vanillin Imino-Chitosan Biodynameric Films

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Materials and Methods: All reagents were obtained from Aldrich and used without further purification. The molecular weight of chitosan dried overnight at 50 ^{o}C has been calculated to be 125 kDa by measuring the intrinsic viscosity of chitosan solved in 0.3 M CH_3COOH – 0.2 M CH_3COONa (1:1, v/v), at 25 ± 0.1 ^{o}C. The viscometric average molar mass was estimated using the eq. $[\eta] = 1.38 \; x \; 10^{-4} \; M_v^{0.85}$.[1S] The degree of acetylation DA = 15 % of chitosan (125 kDa) was evaluated from infrared transmission spectra registered on KBr pellets. Eq. $A_{1320}/A_{1420} = 0.3822 + 0.03133 \; DA$ was used for DA determination, considering the 1420 cm^{-1} band as reference and the band located at 1320 cm^{-1} as characteristic for N-acetylglucosamine. [2S]

FTIR spectroscopy: Comparing the FTIR spectrum of imino-chitosan biopolymers **CV** with those of chitosan, **C** and vanillin, **V**, the formation of imino bonds is evident (Figure 1S, see main text). FTIR spectra measured on different areas of the same film sample exhibit different values of the CH=N absorption band in the range 1633 – 1641 cm^{-1}, while Schiff base biopolymer obtained in toluene shows the same value at 1631 cm^{-1}, and CVM biopolymer shows a shoulder at about 1630 cm^{-1}. This displacement of the imine band could represent the evidence of intramolecular hydrogen bonding between imine nitrogen of the Schiff bases and OH groups on chitosan, due to the closer contact reached by the functional groups in solution. The displacement of c.a. 20 cm^{-1} of the ether/phenolic band on vanillin ring also indicates their participation in hydrogen bonds. Additionally, all the other characteristic bands for the imine derivatives are present in the spectra, confirming their structure, as follows. The strong absorption peaks within 1600 – 1517 cm^{-1} and 820 – 754 cm^{-1} have been ascribed to ν C=C and γ C-H of 1,4 substituted phenylene ring, while the weak absorption peaks at 2876 – 2923 cm^{-1} were attributed to ν C-H in methyl and methylene groups. Strong absorption bands were observed around 1288 cm^{-1} due to the presence of ether/phenolic linkages on vanillin ring and a broad band around 3435 cm^{-1}, characteristic of water clustered in the sample or of H bonds.[3S] Despite the fact that infrared spectroscopy has been used to characterize the design of acetylation of chitin and/or chitosan,[2S] our approach in comparatively evaluating the degree of conversion of amino groups into imine linkages from the new formed imine band (counting band) to N-H bond into chitosan band (as reference band) absorbance ratio failed, the ratios having close values for all compounds, even for different molar ratios of the same compound. Thus, while FTIR spectroscopy is a very sensitive method, its utilization in chitosan Schiff base derivatives characterization doesn't seem to be a very unequivocal one.

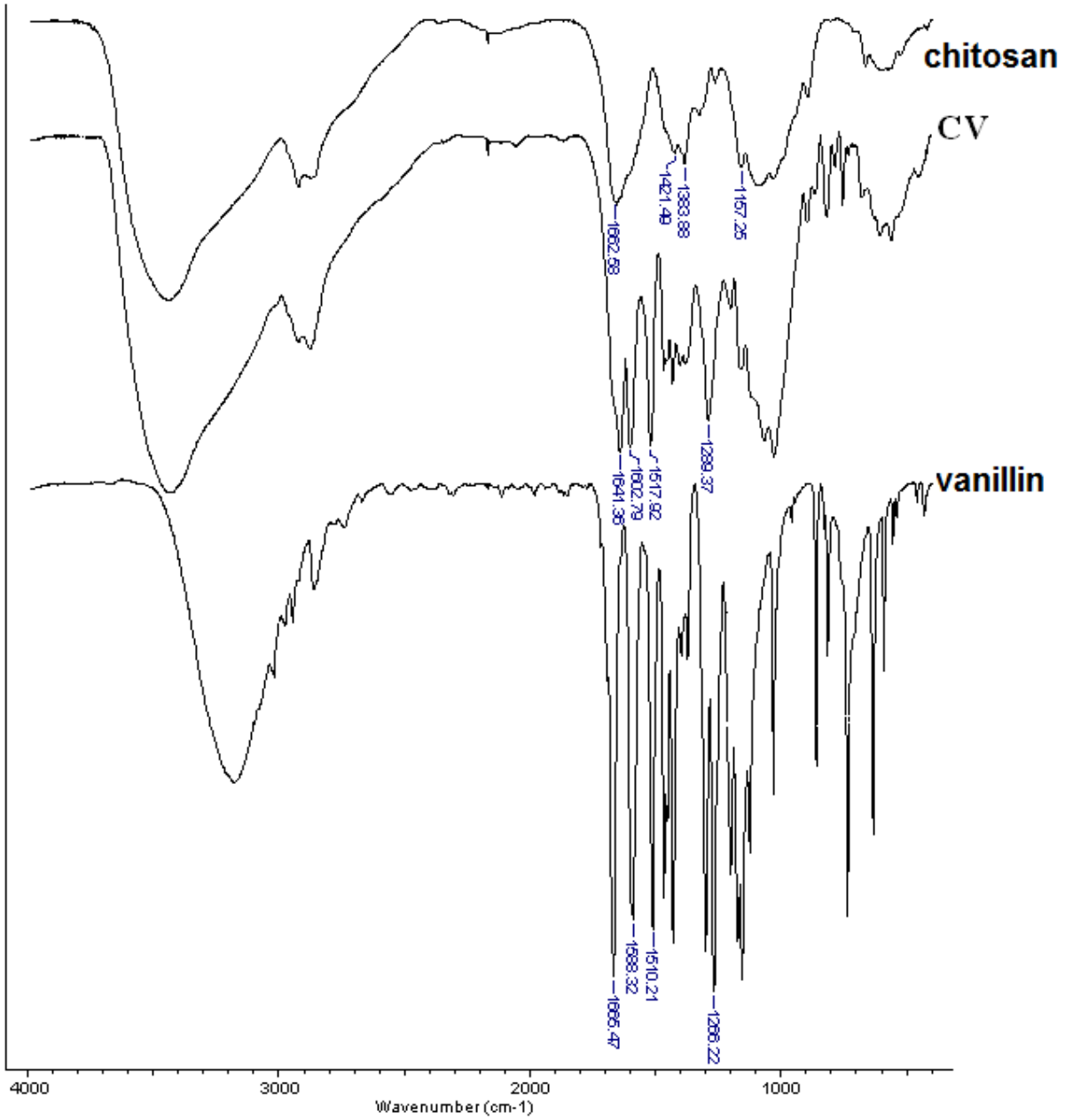


Figure 1S. FTIR spectra on powder samples of chitosan, vanillin and chitosan/vanillin biopolymer **CV**– see text for details

NMR spectroscopy: The ^{1}H-NMR spectra show intense peaks in the aliphatic region (1 - 5 ppm) due to chitosan hydrogens and phenol and methoxy hydrogens on the vanillin ring, while in the aromatic region weak peaks are present, due to the aromatic hydrogens of vanillin ring (6.7 -

7.2 ppm) and aldehyde (9.4 ppm). The rectangular zoom of spectra shows that the peak due to the azomethine hydrogen appears at 8.05 ppm (Figure 2S). As compared to aromatic Schiff bases, the CH=N peak is shifted to higher field due to lower conjugation of imine linkage induced by the aliphatic nature of chitosan. This is contrary to other chitosan Schiff base studies, which ascribe the peak around 9.8 ppm (usually belonging to CHO) to CH=N.[4S]

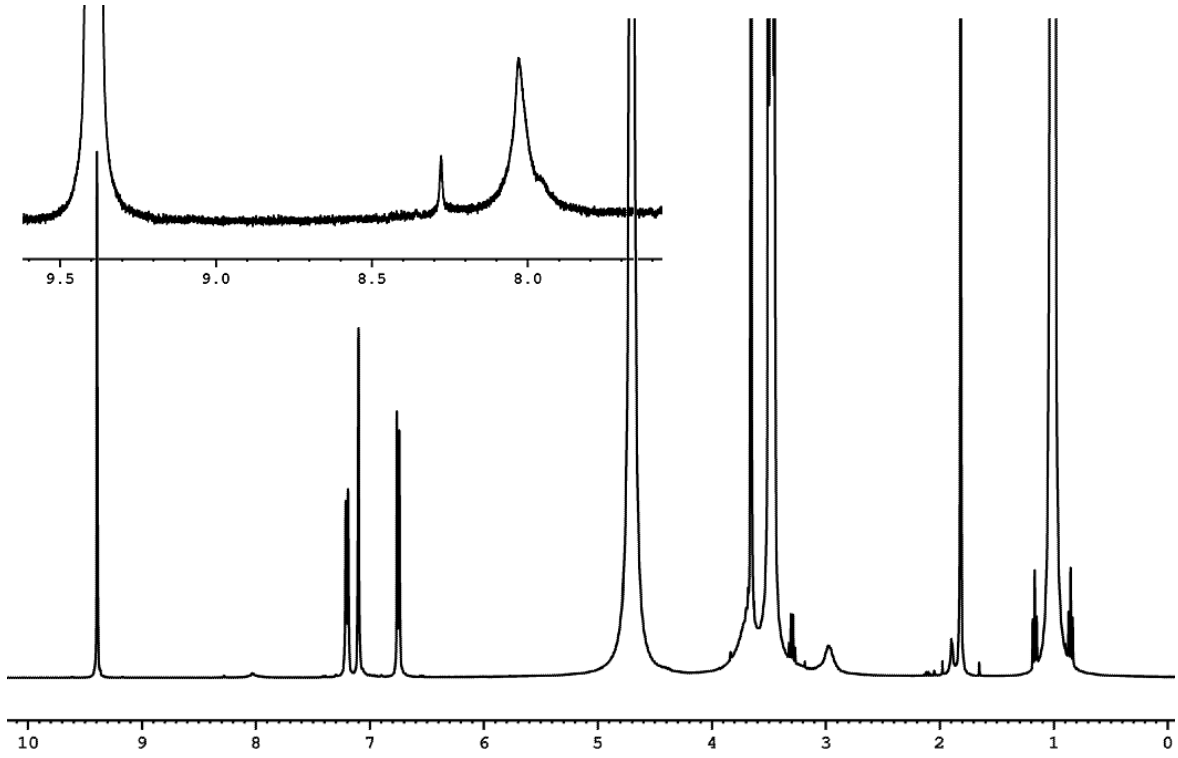


Figure 2S. ^{1}H-NMR of **CV** in acidic $D_2O/(CD_3)_2C$

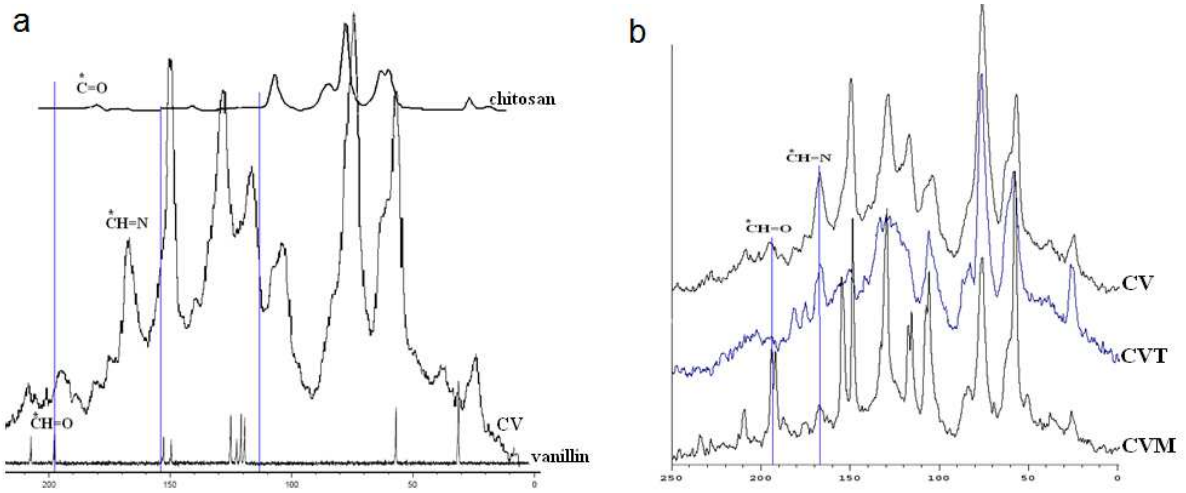


Figure 3S. ^{13}C-NMR of chitosan, vanillin and chitosan/vanillin samples

As for the solid state ^{13}C-NMR spectra, taking into consideration that the chitosan used for this study has a deacetylation degree of 85 %, the conversion degree (η_{solid}) of amino groups into imine linkages has been calculated with $\eta_{solid} = (A_{CH=N}/0.85xA_{C1})x100$, where $A_{CH=N}$ and A_{C1} represent the area of the integrated peaks CH=N and C1, respectively.

Morphological characterization by AFM. The morphology of film surfaces was analyzed by AFM topographic and phase contrast images. Being a statistic investigation, the different areas were not "chosen"; the cantilever landed on film surface and the data were registered for squares beginning with a scan size of 20 μm ($20x20\mu m^2$) up to 0.5 μm ($0.5x0.5\mu m^2$). The arithmetic average roughness (Ra) was measured for all explored areas. For an accurate comparison of the surface characteristics of all films, the roughness exponent (RE) was calculated as the slope of roughness *versus* scan size[3S], in a double log plot. The chitosan film surface displays granular morphology with grain diameters in the 20-30 nm range. The grains have a quite uniform size-dispersity and distribution giving a roughness exponent of 0.169. The chitosan/vanillin derivatives also show a granular morphology, their roughness exponent and grain diameter reflecting their content in imine linkages. **C_5V** film surface shows larger grain diameters with large size-dispersity comprised in the 90-150 nm range for the small scanned areas, while for larger scanned areas (20 μm) one can observe even bigger grains (around 1 μm). This larger dispersity of grain sizes is reflected in a higher roughness exponent of 0.478. As for **C_3V** film surface, one can observe big grains with diameters in the 80-120 nm range and relative uniform grain size dispersion. This is why the roughness exponent is quite small (0.062). Interesting enough, the **C_2V** film presents the most ordered surface. The grains, in a similar way with chitosan film, have the diameter in the 20-30 nm range and are disposed in parallel rows having the inter-row distance around 140 nm. The roughness exponent is low, 0.1082, the surface being very smooth. This film surface feature could be a consequence of the 2/1 molar ratio between amino and aldehyde groups which leads to a quite uniform disposition of the imine units on the chitosan chains, allowing better packing of the resulted biopolymer. The film surface of **CV**, the chitosan derivative containing the highest amount of imine linkages, shows the biggest grains, with diameters around 120-150 nm and with a roughness exponent of 0.32. All films have small values of the phase contrast shift of the contrast phase images, indicating relief differences but not chemical composition variation. Two representative images are shown in Figure 4S.

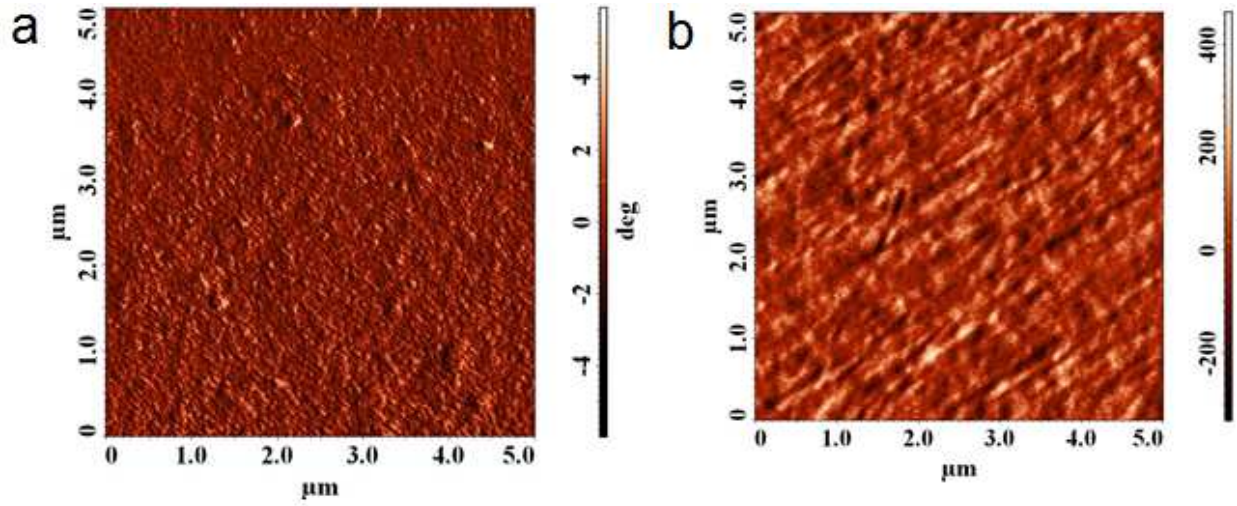


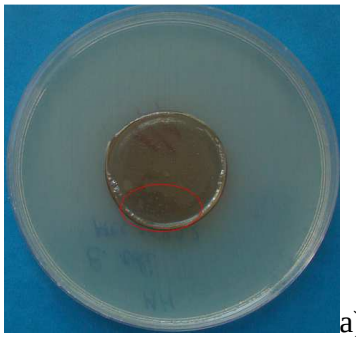
Figure 4S: AFM phase contrast images (Table 1S), indicating relief differences but not chemical composition variation

X-ray diffraction of C and CV films. The diffraction patterns of chitosan and chitosan/vanillin derivative films have been measured at deflection angles ranging from 2 to 40, 2θ degree, using step 0.1^{o} and impulse counting time 15 s.

Table 2S. The XRD parameters of the studied chitosan Schiff base derivatives, at rt

Code	reflection angle (***d-spacing/Å***, relative intensity)
Chitosan	9.04 (***9.8***, 23), 12.04(***7.3***, 24), 19.45(***4.6***, 19)
C_5V	7.24(***12.2***, 22), 20.5(***4.4***, 26)
C_3V	7.10(***12.4***, 16), 20.4(***4.4***, 14)
C_2V	7.03(***12.6***, 29), 19.8(***4.5***, 10)
CV	6.68(***13.2***, 63), 13(***6.85***, 26),13.5(***6.6***, 27) 20.2(***4.4***, 38)

Culture media and inoculation.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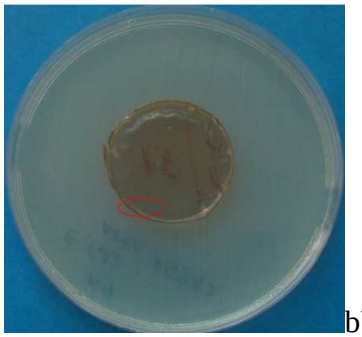
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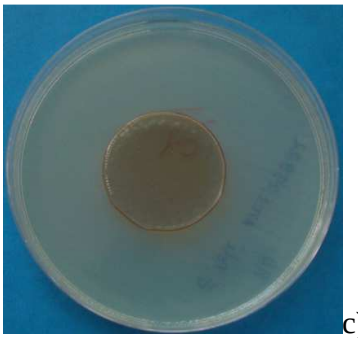
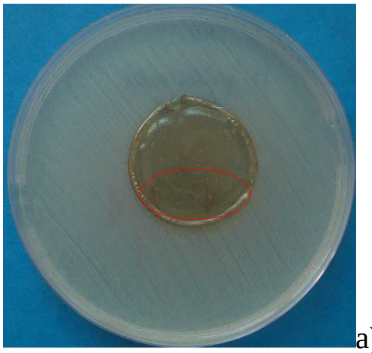

c)

Escherichia coli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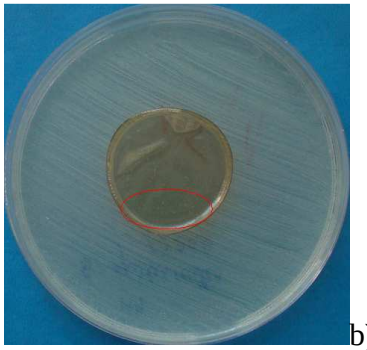
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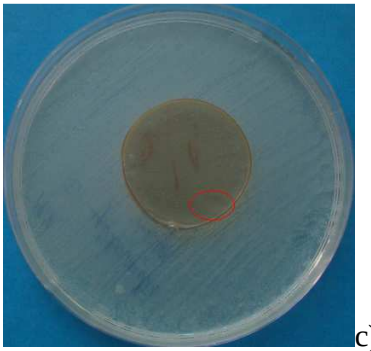
c)

Staphylococcus epidermidis

Figure 5S. The minor inhibitory effect of chitosan/vanillin films **CV** a), $\mathbf{C_{1.5}V}$ b), $\mathbf{C_2V}$ c) on *Escherichia coli* and *Staphylococcus epidermidis*

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