

Supporting Information

Supramolecular Antimicrobial Capsules Assembled from Polyoxometalates and Chitosan

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1. Experimental details

Starting materials: All chemicals and solvents were of analytical grade purchased from Sigma Aldrich and used as supplied, without further purification. Pure water was used throughout by passing water through a Millipore-Q Academic purification set. Polyoxometalates $H_3PW_{12}O_{40}$ (phosphotungstic acid, PTA) and $H_3PMo_{12}O_{40}$ (phosphomolybdic acid, PMA) were purchased from Sigma-Aldrich and $(NH_4)_{15}\{Na[(Mo_2O_4)_6(\mu_2-SO_3)_3(\mu_2-SO_3)]_2\} \cdot 5H_2O$ (Kabanos) was prepared following the procedure described in M. J. Manos, J. D. Woollins, A. M. Z. Slawin and T. A. Kabanos, *Angew Chem Int. Ed.*, 2002, **41**, 2801.

The authors would like to acknowledge the regular use of *The Advanced Microscopy Laboratory (LMA)*, Universidad de Zaragoza and its technicians Dr. Carlos Cuestas Ayllón (LMA-UNIZAR), Dr. Rodrigo Fernández-Pacheco (LMA-UNIZAR), both of whom provided invaluable advice and assistance with the electron microscopy data collection.

Scanning Electron Microscopy (SEM): SEM images and energy dispersive X-ray spectroscopy (EDX) spectra were acquired using a field emission SEM Inspect F50 with an EDX system INCA PentaFETx3 (FEI Company, Eindhoven, The Netherlands) in the energy range 0-30 keV.

Environmental Scanning Transmission Electron Microscopy (ESTEM): Data were collected on a Quanta FE6-250 (FEI Company) field emission SEM for high-resolution imaging working at low vacuum mode using a STEM detector.

Transmission Electron Microscopy (TEM): TEM images collected using a Cs-probe-corrected Titan (FEI) operated at 300 kV.

Fourier-transform infrared (FT-IR) spectroscopy: All FTIR spectra were recorded on a JASCO FT/IR – 4100 Fourier transform infrared spectrometer in a frequency range of 600-4000 cm^{-1} with a resolution of 2 cm^{-1} and a scanning number of 32. Wavenumbers ($\tilde{\nu}$) are given in cm^{-1} .

Thermogravimetric analysis (TGA): Thermal stability data were collected on TA Q500 instrument under a nitrogen atmosphere. The heating range was from RT to 800 ° C at 5.00 ° C per min.

Bacterial growth curves: Bacterial growth was recorded measuring the optical density (OD) of the samples at 600 nm every one hour (0-8 hours) using a microplate reader (Thermo Scientific MULTISKAN GO). Results were compared with the OD variation of a control culture containing only *E. coli*. All controls and antibacterial assays were repeated a total of six times to verify the reproducibility of the results and to calculate the mean values and the standard deviation error bars that are shown on the graphed results of these assays.

2. Synthesis of POM-chitosan hybrid capsules

In a glass vial 100 mL of CTAB 0.2 M in EtOH 96% was added under ultrasonication to a 1 mL aqueous solution of the POM (3.8×10^{-6} moles) which resulted in the formation of POM-decorated micelles (the micelle-core of the capsule). Then 1 mL of 5 mg/mL chitosan is added under a short sonication (2 minutes) producing the intimate mix of the polymer with POM-decorated micelles. Finally, complex coacervation of the suspension of capsules was carried out by adding them to a solution of 50 mM Na_2SO_4 (aq) under ultrasonication. The suspension is precipitated by centrifugation (5000 rpm, 10 min), resuspended in ddH₂O. This centrifugation/resuspension process was repeated twice to clean the capsules. Finally the obtained capsules are resuspended in a minimum volume of 2 mL ddH₂O. As the POMs selected for this study each possess distinctive colours (Kabanos=orange, PMA=green and PTA=white), their incorporation into the polymer matrix could be verified by eye in the first instance since the colours of the colloidal suspensions were commensurate with the corresponding POMs employed in the synthesis, whereby no colour change was observed following interaction with CTAB and subsequent encapsulation with chitosan (See Figure S1).

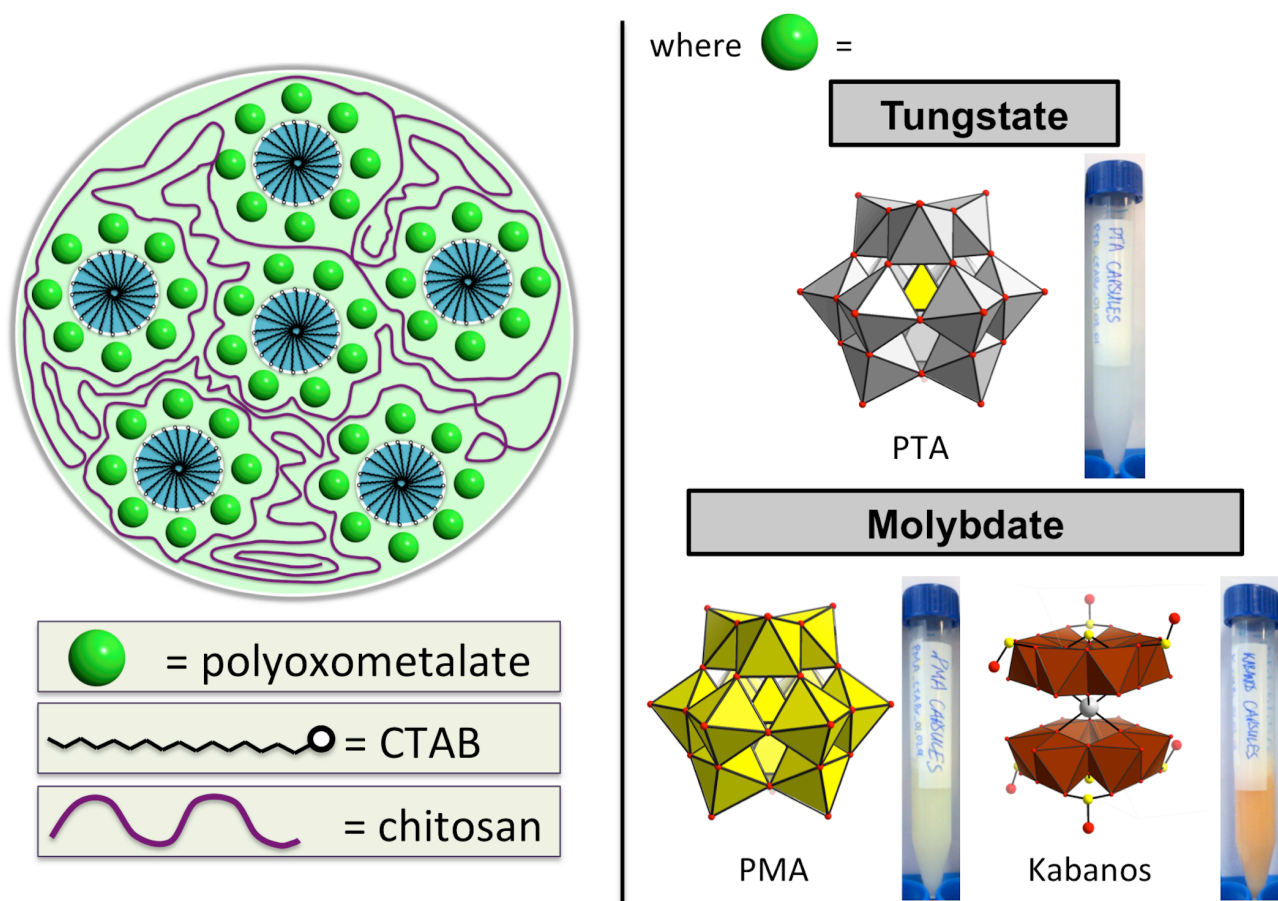


Figure S1. Schematic of the composition of Chitosan/CTAB capsules with POMs. The incorporation of POMs into the polymer matrix can be verified by eye due to the distinctive colour of each type of POM employed in this study: Kabanos (orange), PMA (green) and PTA (milky white).

3. Electron microscopy analysis of capsules

Sample preparation for electron microscopy analysis:

Capsule deposition for SEM analysis

Freshly synthesised capsules suspended in ddH₂O were diluted with ddH₂O to an appropriate volume and a 3 μ L aliquot was deposited on a silicon wafer and allowed to evaporate in a fumehood.

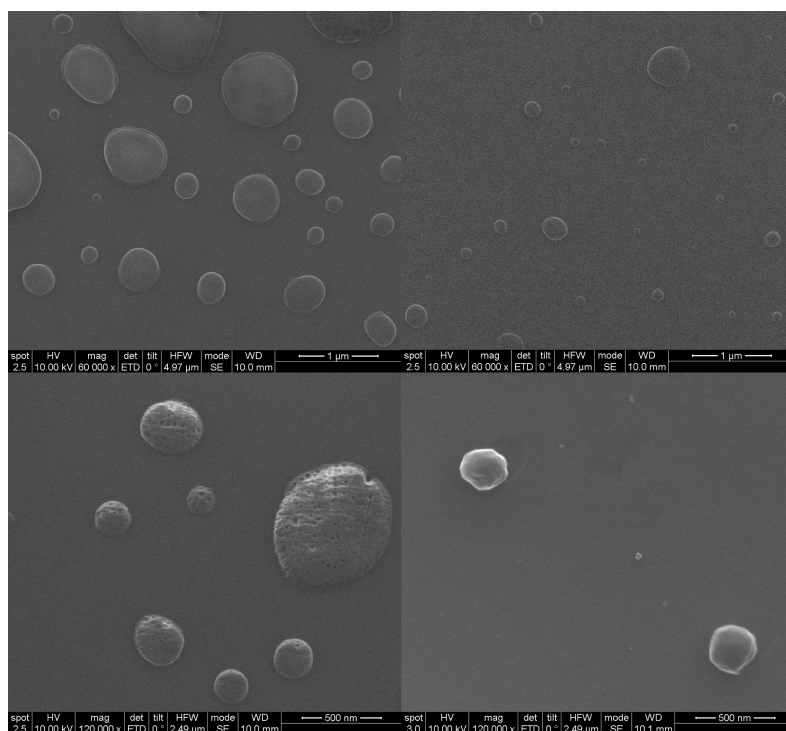


Figure S2. SEM images of Kabanos capsules deposited on Si-wafer.

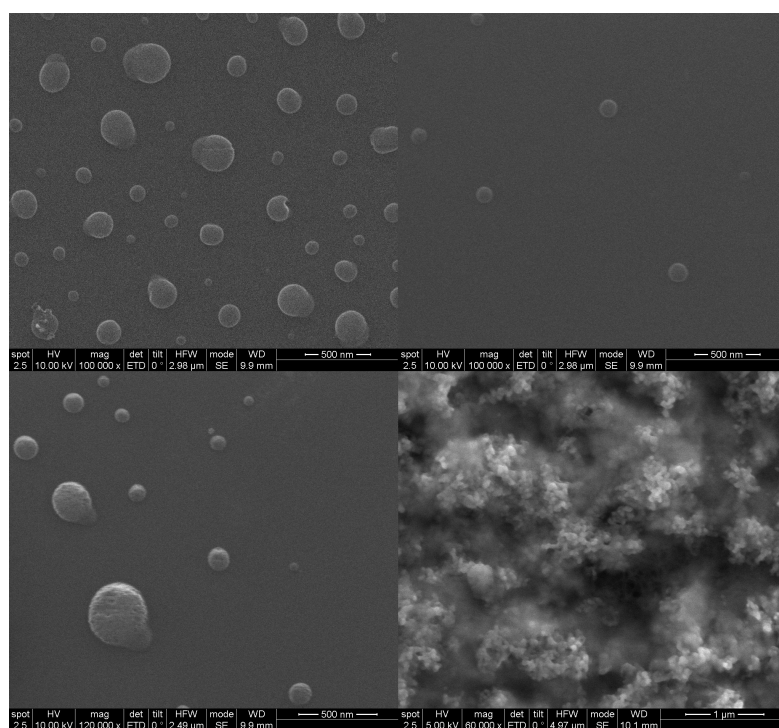


Figure S3. SEM images of PMA capsules deposited on Si-wafer.

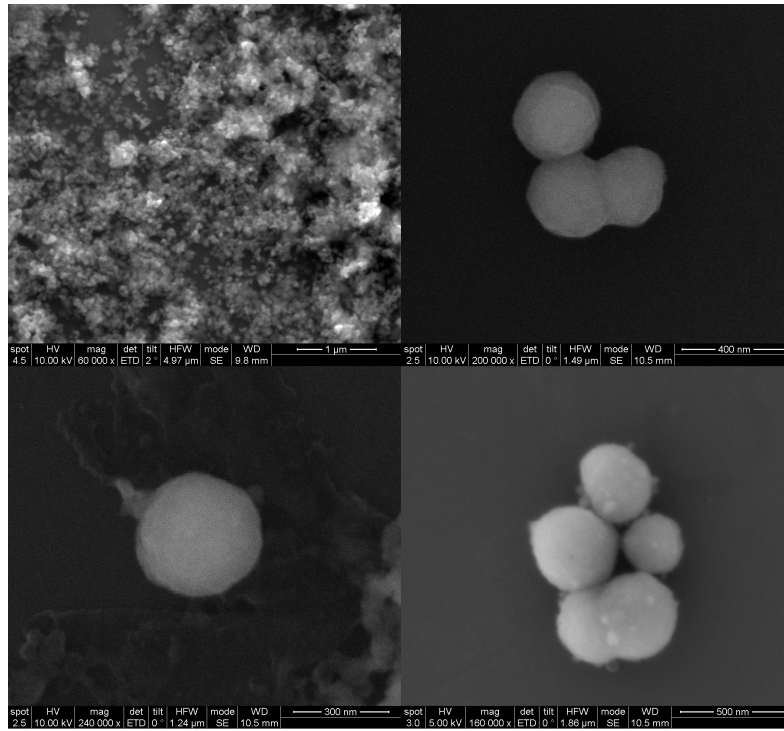


Figure S4. SEM images of PTA capsules deposited on Si-wafer.

Capsule fixation, embedding in gelatine and epoxy resin

Fresh samples were synthesised and they were fixed with glutaraldehyde 0.25 % in phosphate buffer 10 mM at pH 7.2. They were washed three times with PBS and resuspended in 5 % gelatine. Samples were centrifuged to obtain a pellet and were incubated overnight at 4 °C. Gelatine samples were cut in very small pieces before undergoing the subsequent steps. The dehydration of the samples was carried out using the following steps: incubation in ethanol 30 %, ethanol 50 % and incubation overnight in ethanol 70 %; incubation in ethanol 90 % for 1 h and finally incubation (x3) in absolute ethanol. Dehydrated samples in gelatine were used also as prepared for SEM observation. After that samples were incubated overnight in a 1:1 mixture absolute ethanol/epoxy resin (r.t.). The mixture was then removed, changed with absolute epoxy resin and samples were left for impregnation for 8h at room temperature. After another change of medium, the final incubation in epoxy resin was carried out overnight at 60 °C to obtain the polymerisation.

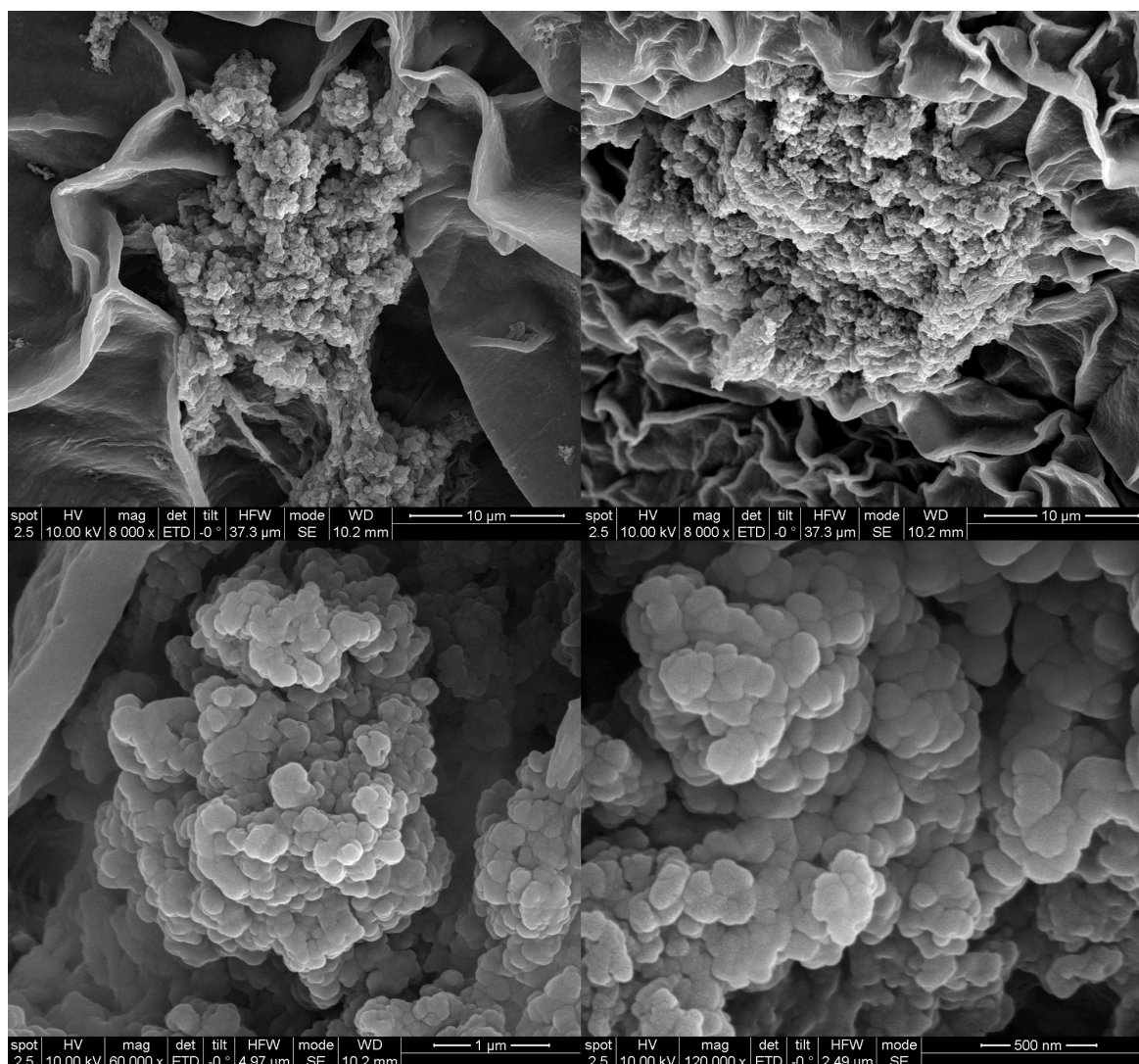


Figure S5. SEM images of Kabanos capsules embedded in gelatine and epoxy resin. The top two images clearly show large clusters of discrete nanocapsules embedded within the folds of the epoxy resin. The lower two higher magnification images show the capsules clustered together but with their shape largely maintained.

Capsule preparation for cryoTEM analysis

Before CryoTEM observation, samples were vitrified in liquid ethane using a FEI vitrobot: a 300 μL drop of an aqueous suspension of the material was placed on a TEM holey-carbon copper grid, the excess of water was blotted away at the vitrobot with filter paper and the grid was freeze-plunged in liquid ethane. Samples were then transferred under liquid nitrogen atmosphere to a Gatan TEM cryo-holder equipped with a liquid nitrogen reservoir so that samples could be handled and observed at 100 K (Figure S6).

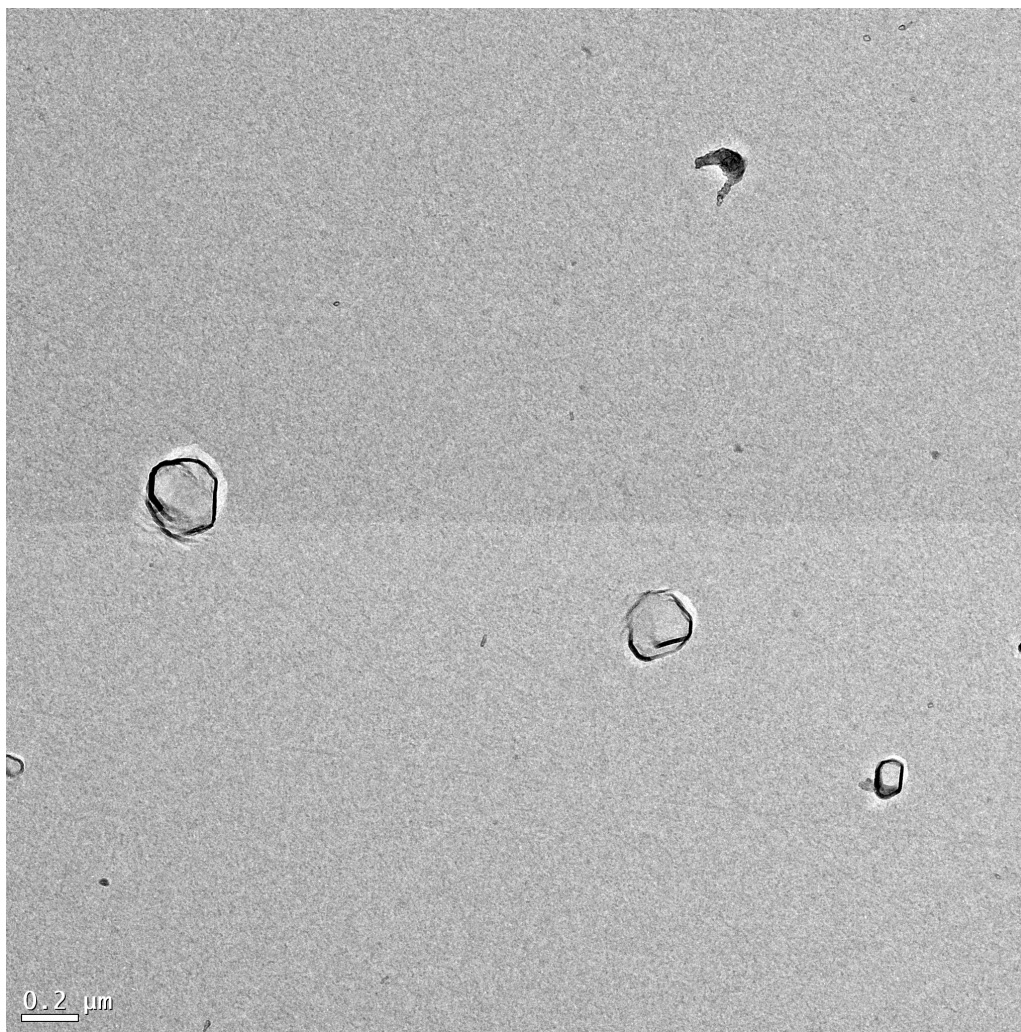


Figure S6. Cryo-TEM images of Kabanos capsules in gelatin and epoxy resin.

4. Size-distribution of capsules

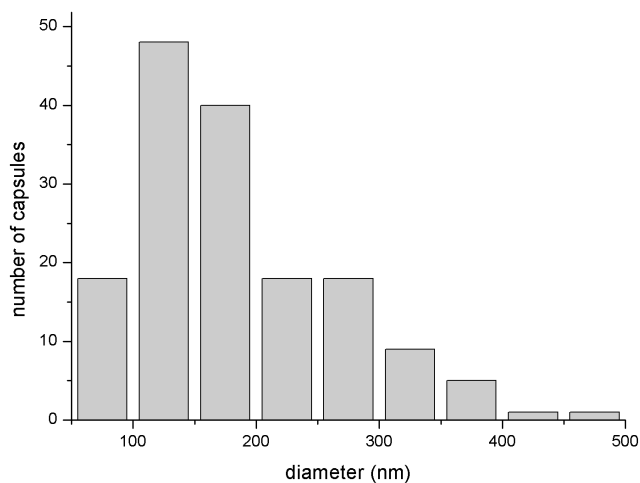


Figure S7. Size distribution of Kabanos-based capsules, which although polydisperse fall largely between 100-200 nm.

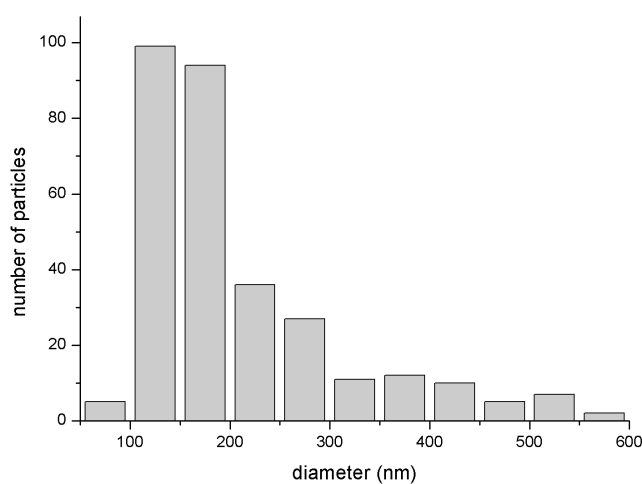


Figure S8. Size distribution of PMA-based capsules which although polydisperse fall largely between 100-200 nm.

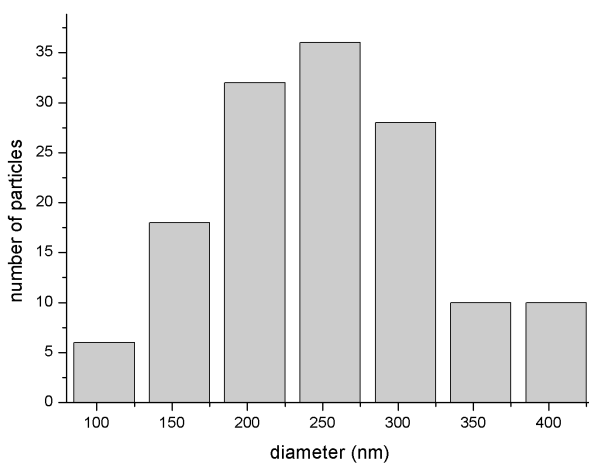


Figure S9. Size distribution of PTA-based capsules, which although polydisperse fall largely between 200-300 nm.

5. Energy dispersive X-ray spectroscopy (EDX) analysis

All results in weight%

Sample	C	O	P	Mo	W	Total
PTA	19.14	24.76	0.80	55.30	100.00
Kabanos	40.72	36.23	4.56	18.48	100.00
PMA	31.79	31.47	1.23	35.51	100.00

All results in atomic%

Sample	C	O	P	Mo	W	Total
PTA	45.95	44.63	0.74	8.67	100.00
Kabanos	56.60	37.81	2.38	3.22	100.00
PMA	52.69	39.15	0.79	7.37	100.00

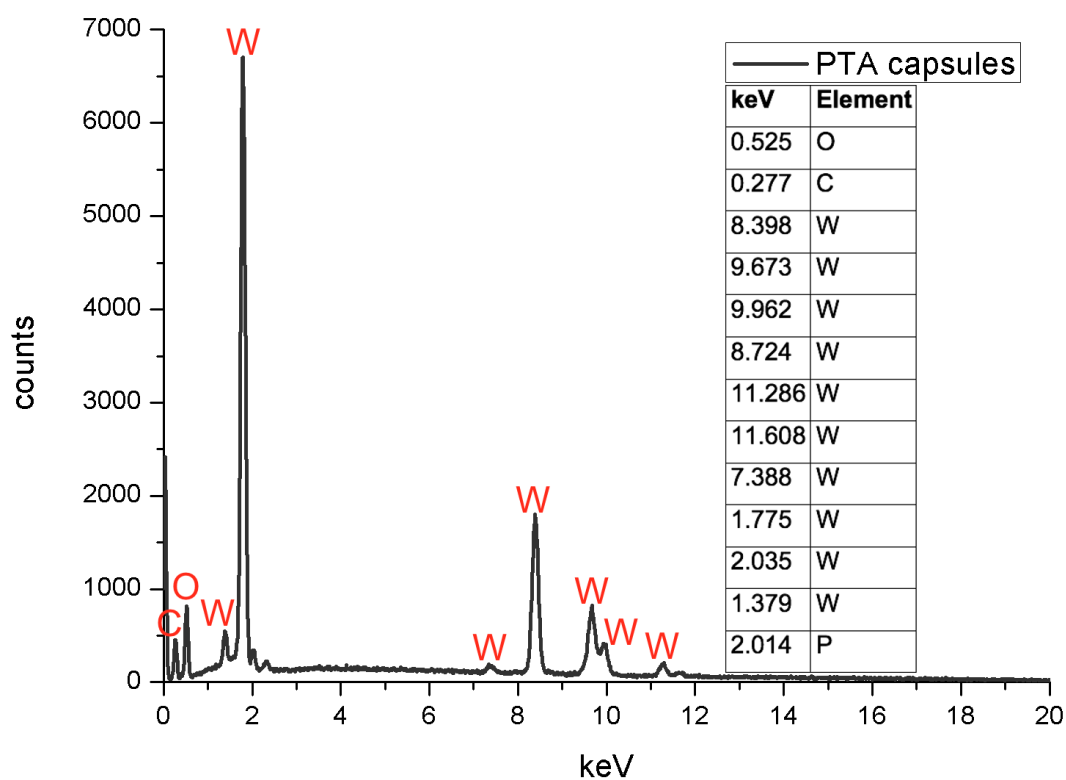


Figure S10. EDX spectrum of PTA-based capsules showing the presence of P, W, C and O.

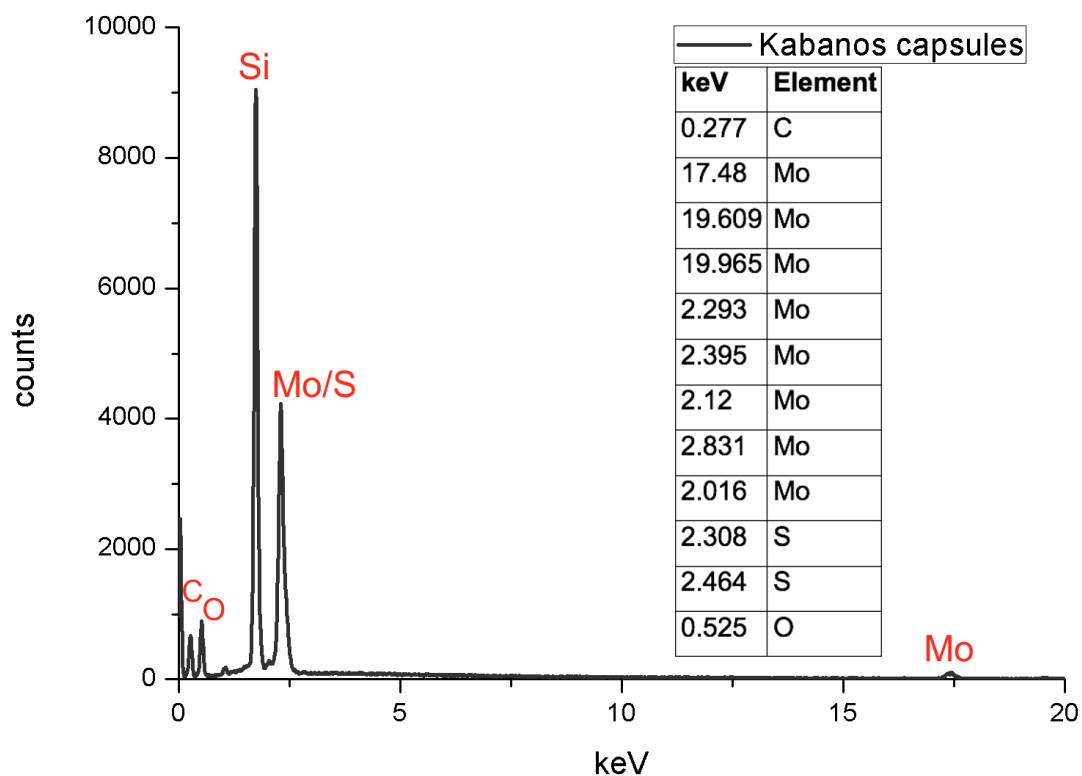


Figure S11. EDX spectrum of Kabanos-based capsules showing the presence of S, Mo, C and O.

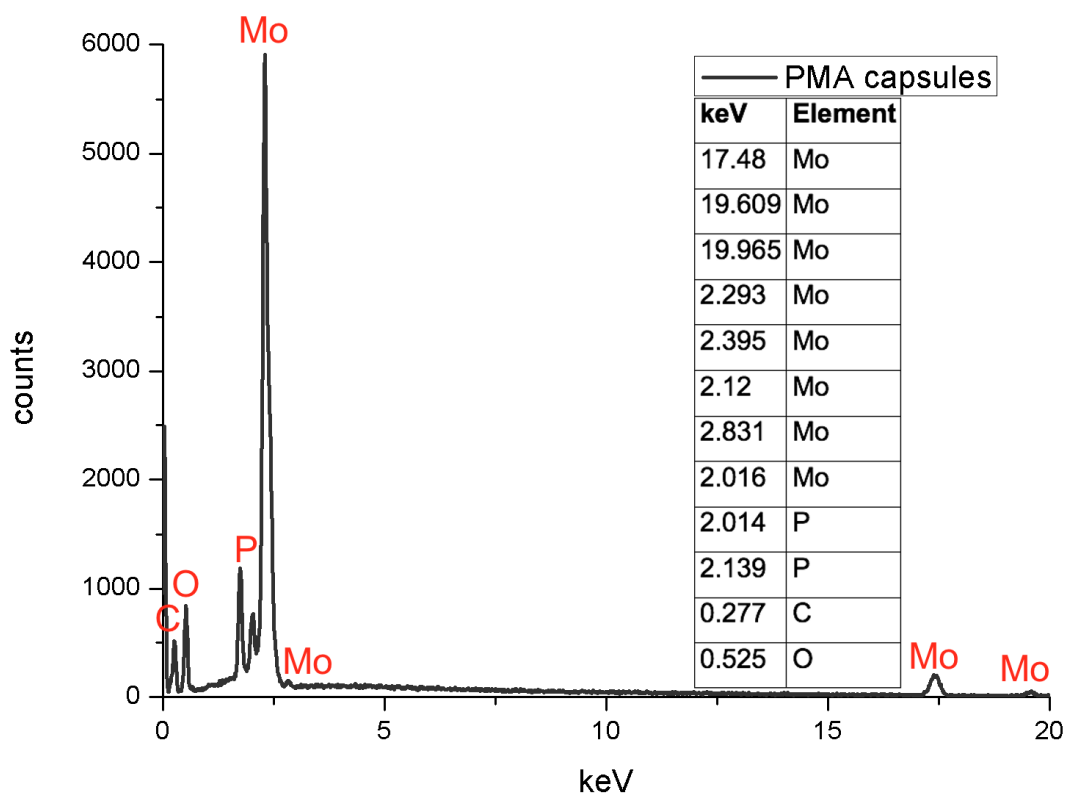


Figure S12. EDX spectrum of PMA-based capsules showing the presence of P, W, C and O.

6. FTIR analysis of capsules

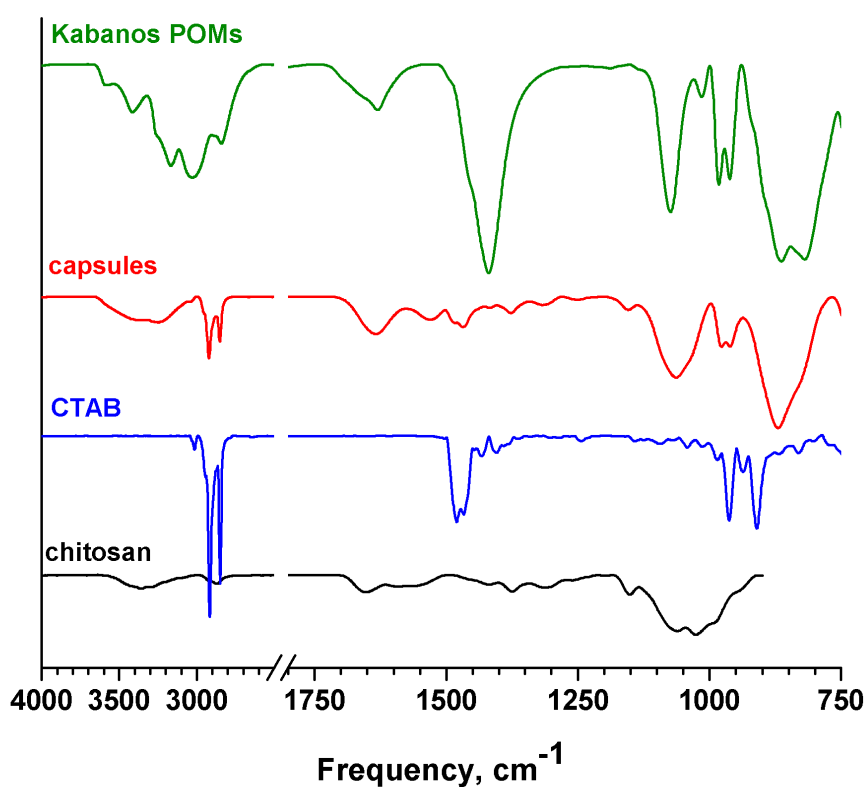


Figure S13. FTIR analysis of Kabanos-based capsules including Kabanos, CTAB and chitosan spectra for comparison.

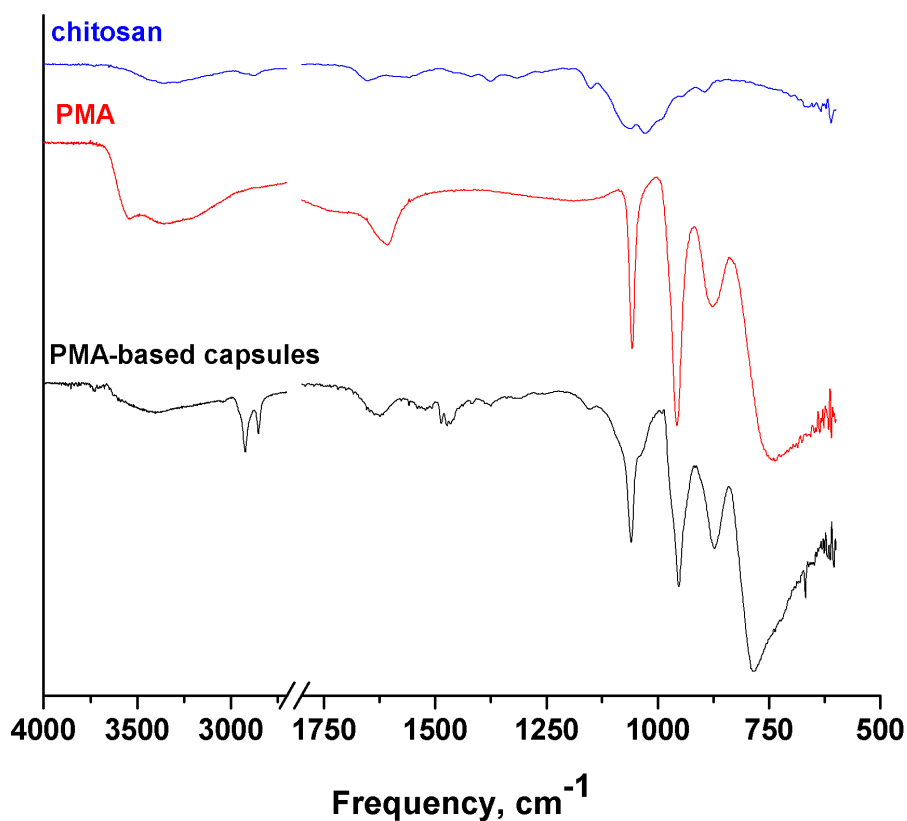


Figure S14. FTIR analysis of PMA-based capsules including PMA and chitosan spectra for comparison.

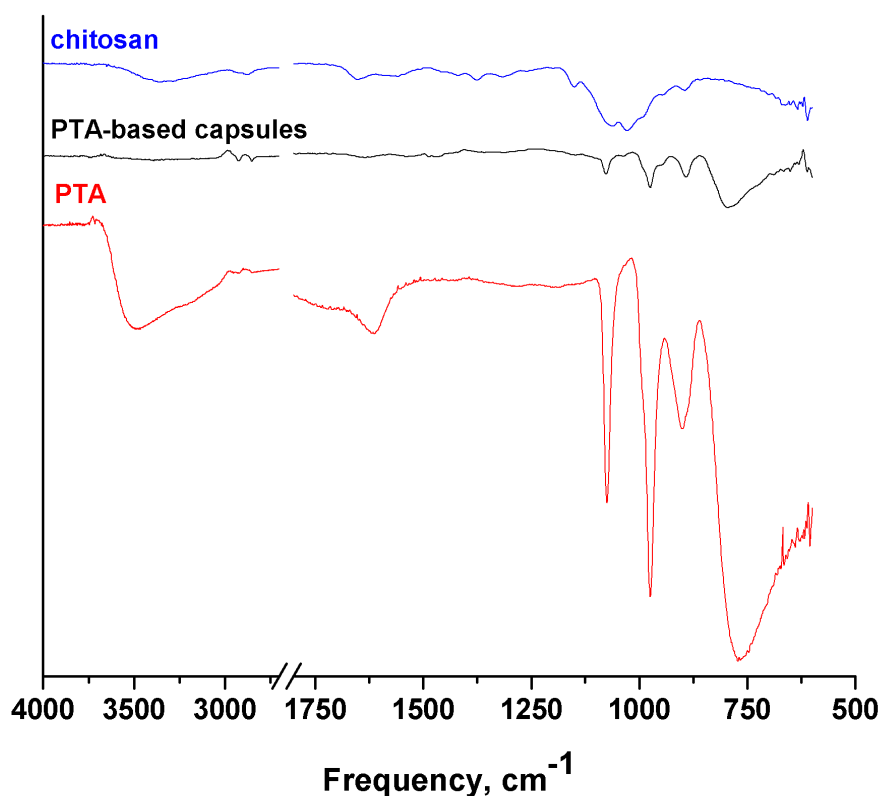


Figure S15. FTIR analysis of PTA-based capsules including PTA and chitosan spectra for comparison.

Compound	Frequencies (cm^{-1})	Additional references
Kabanos POMs	1637 (H_2O), 1408 (NH_4^+), 1088 (SO_3^{2-}), 981 and 963 ($\text{Mo}=\text{O}$), 865 ($\text{Mo}-\text{O}-\text{Mo}$) and 809 (SO_3^{2-})	M. J. Manos, J. D. Woollins, A. M. Z. Slawin, T. A. Kabanos, <i>Angew. Chem. Int. Ed.</i> 2002, 41 , 2801.
PMA	1650 (H_2O), 1066 ($\text{P}-\text{O}$), 968 ($\text{Mo}=\text{O}$), 871 and 789 ($\text{Mo}-\text{O}-\text{Mo}$)	M. Lira-Cantú and P. Gómez-Romero, <i>Chem. Mater.</i> 1998, 10 , 698.
PTA	1650 (H_2O), 1080 ($\text{P}-\text{O}$), 987 ($\text{W}=\text{O}$), 976 ($\text{P}-\text{O}$ and $\text{W}-\text{O}$), 895 ($\text{W}-\text{O}-\text{W}$).	A. J. Bridgeman, <i>Chem. Phys.</i> 2003, 287 , 55.
CTAB	2949 ($\text{N}-\text{CH}_3$) (enhanced in the case of CTAB packing), 2920 and 2850 ($-\text{CH}_2$), 1470 ($\text{RN}(\text{CH}_3)_3^+$), 912 ($\text{C}-\text{N}$), 961, 908, 720, 728 (in-plane swinging $\text{RN}(\text{CH}_3)_3^+\text{Br}$)	R. B. Viana, A.B. F. da Silva, A.S. Pimentel, <i>Advances in Physical Chemistry</i> , 2012, Article ID 903272 (and references therein).
Chitosan	3290 ($\text{O}-\text{H}$ and $\text{N}-\text{H}$ stretch), 2864 ($\text{C}-\text{H}$ stretch), 1645 (primary amide), 1414 ($-\text{CH}_2$ bending), 1375 ($-\text{CH}_3$ symmetrical deformation), 1150 (antisymmetric stretch $\text{C}-\text{O}-\text{C}$ and $\text{C}-\text{N}$ stretch), ($\text{C}-\text{O}$ skeletal vibrations), band at $\sim 1628-1633 \text{ cm}^{-1}$ contains the amide I and asymmetric $-\text{NH}^+$ deformation, while the band at $\sim 1524-1529 \text{ cm}^{-1}$ contains the amide II, $\text{N}-\text{H}$ bending vibration as well as the symmetric $-\text{NH}^+$ deformation, 1100 (skeletal vibration of $\text{C}-\text{O}$ stretching)	G. Lawrie, I. Keen, B. Drew, A. Chandler-Temple, L. Rintoul, P. Fredericks, L. Grøndahl, <i>Biomacromolecules</i> , 2007, 8 , 2533.

Table 1. Summary table of FTIR vibrational frequencies (in cm^{-1}) and assignments.

7. Thermogravimetric analysis (TGA) of capsules

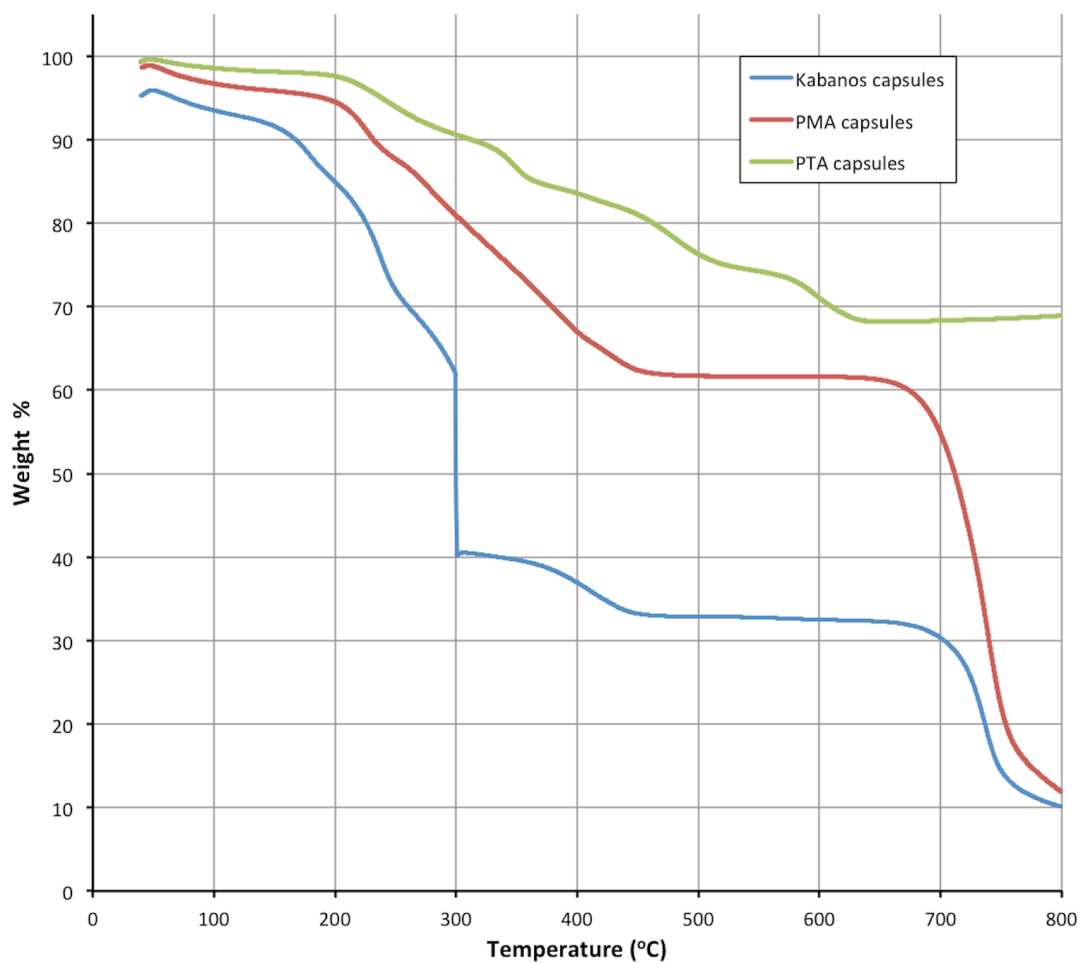


Figure S16. TGA data for Kabanos-, PMA-, and PTA-based capsules from 21-800 °C. All capsules appear to retain stability up to ca. 200 °C, whereupon rapid weight loss occurs. The sharp weight loss seen for the Kabanos-based capsules at 300 °C is not an anomaly or error in the measurement.

8. Antibacterial activity assays

Growth inhibition assay of microbial cells

Escherichia coli DH5-alpha was pre-inoculated in Luria-Bertani (LB) medium and kept under shaking (180 rpm) at 37 °C for 15 hours. A dilution in LB from this culture was used for the following tests, corresponding to an inoculum of 1×10^7 CFU/mL. LB medium was supplemented with proper amounts of capsules (already dispersed in LB medium) to obtain the desired concentration in the sample suspension. The assay was performed in a 96-well plate.

Bacterial growth curves were recorded measuring the optical density (OD) of the samples at 600 nm every one hour (0-8 hours) using a microplate reader (Thermo Scientific MULTISKAN GO). Results were compared with the OD variation of a control culture containing only *E. coli*.

Microbial cell viability assay

Cell viability was analysed by resazurin spectrophotometric assay using a 96-well plate. LB medium was supplemented with proper amounts of capsules as in the growth inhibition assay and a sample without capsules was also included in the assay as control. The microbial cultures have been grown for 6 - 8 h, then 30 mL of 0.1 mg/mL resazurin in LB were added to each well and incubated in the dark at 37 °C for 1 hour. The absorbance of each well was read on a microplate reader (Thermo Scientific MULTISKAN GO) at 605 nm and the contribution of OD just before the incubation with resazurin was subtracted. The spectrophotometer was calibrated to 100 %-absorbance using culture medium with resazurin without cells (control). The relative cell viability (%) related to control wells was calculated by $ABS_{test}/ABS_{control} \times 100$. Results are shown in Figure S17 (below). All controls and antibacterial assays were repeated a total of six times to verify the reproducibility of the results and to calculate the mean values and the standard deviation error bars that are shown on the graphed results of these assays.

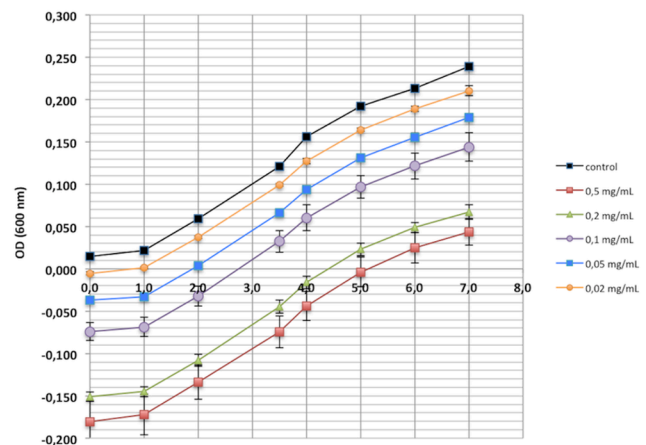
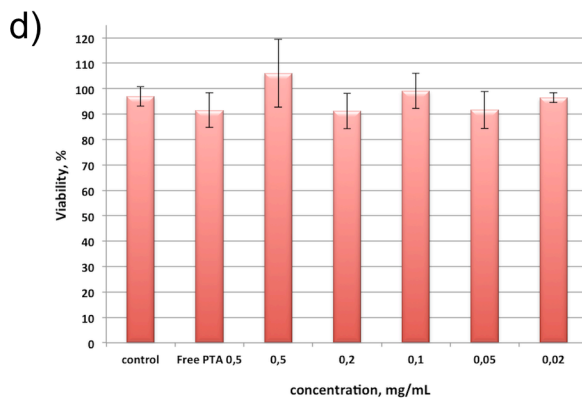
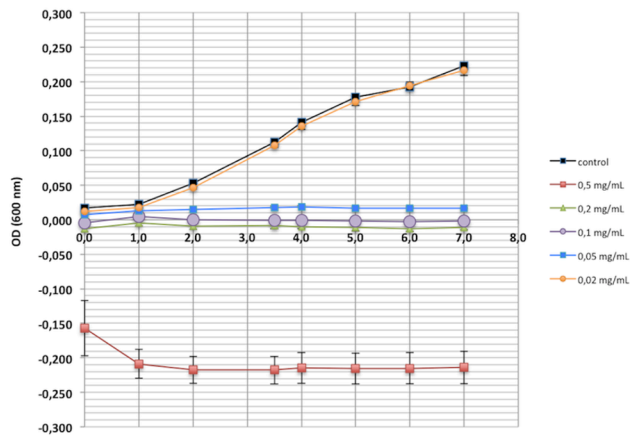
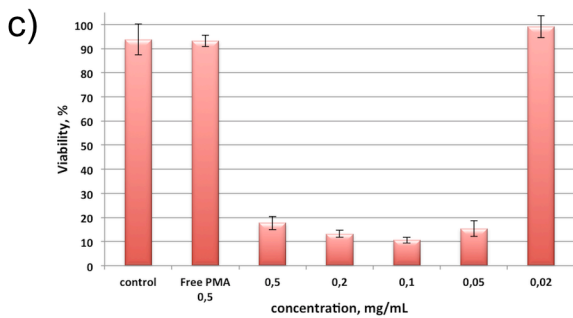
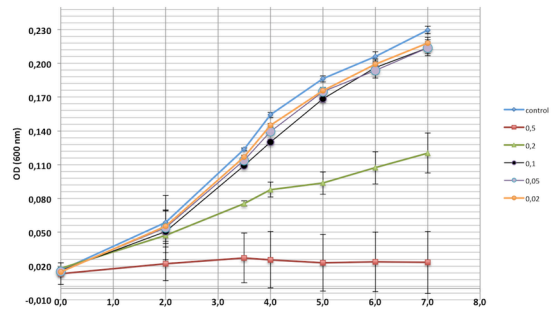
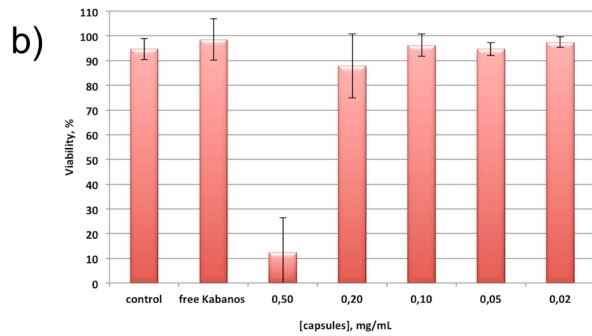
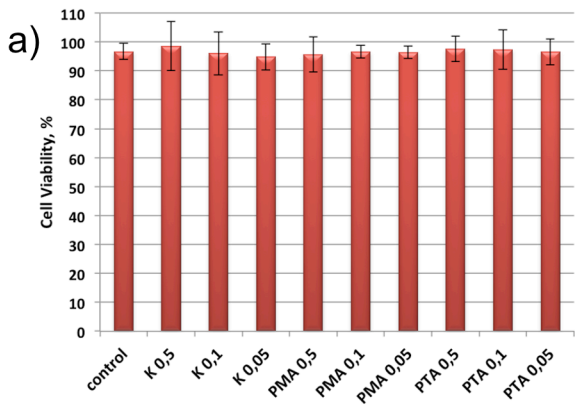


Figure S17. a) Resazurin assay results of antibacterial activity of ‘free’ POMs Kabanos, PMA and PTA against *E.coli*. Resazurin assay results (bar chart) and antibacterial growth inhibition (line graph) of b) Kabanos-based capsules; c) PMA-based capsules; and d) PTA-based capsules.

Bacteria cell fixation for ESEM analysis

In order to further study the structure of the bacterial cells incubated with capsules and inspect their surface composition in more detail, bacterial cells were incubated with 0.5 mg/mL of capsules for 8 h were fixed with 2.5 % glutaraldehyde in phosphate buffer 10 mM at pH 7.2. The fixed bacterial cells were washed three times with PBS and resuspended in water. Control cells (bacteria without capsules) were subject to the same incubation protocol (Figure S18). Figures S19-S21 (below) are representative environmental STEM images of Bacteria incubated with the capsules collected on a FEI Quanta FE6-250 (FEI Company) field emission SEM for high-resolution imaging working at low vacuum mode.

Figure S18 shows that in the absence of capsules, the bacteria are stable under the experimental conditions and that their structure remains intact and undamaged. In Figure S19 the Kabanos capsules are clearly visible and the bacterial cell membranes show signs of stress and damage. The capsules appear somewhat aggregated as a result of the bacteria fixation protocol with glutaraldehyde. Nevertheless, individual capsules can clearly be identified, and can be seen much more clearly in Figure S20, which shows *E.coli* incubated with PMA-based capsules. In the case of PMA-capsules, the signs of membrane stress and damage is much clearer. Figure S21 shows PTA-based capsules and the lack of any obvious signs of stress or damage to the bacterial membranes or internalisation of the capsules.

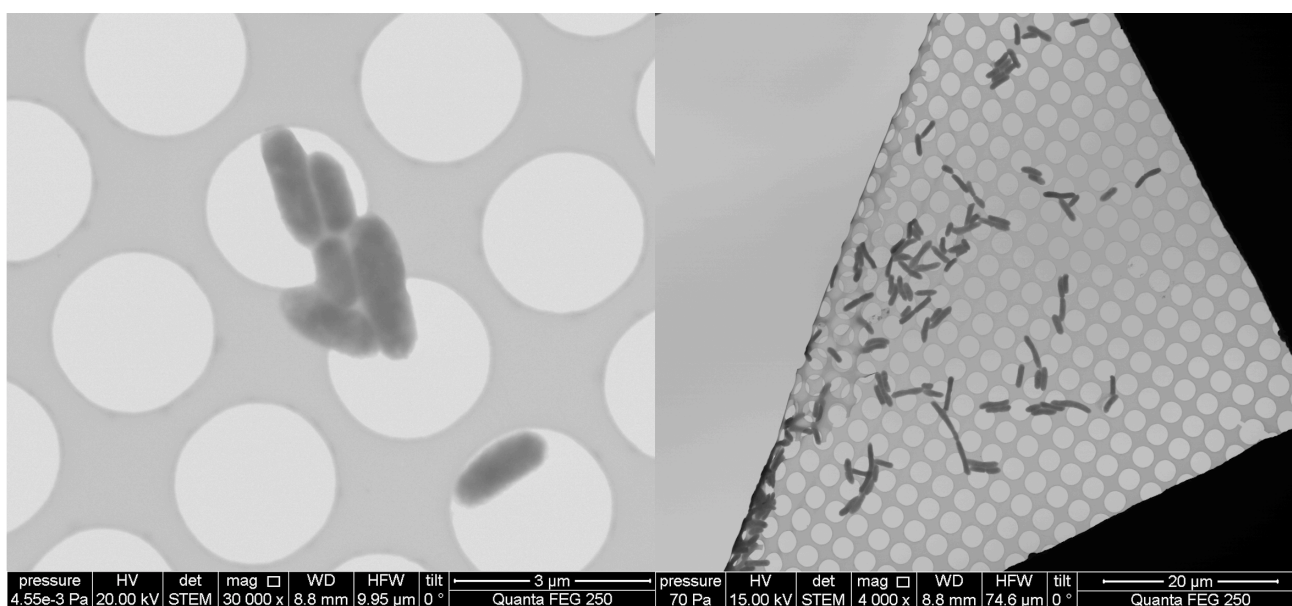


Figure S18. Control STEM image of *E.coli* fixed with glutaraldehyde.

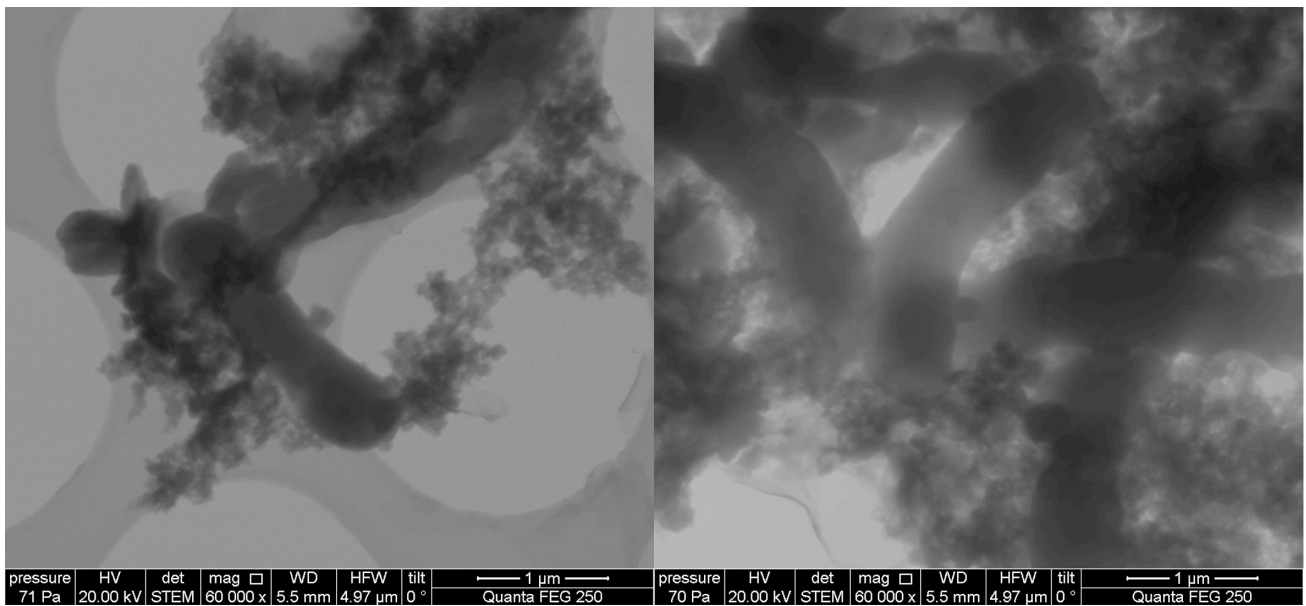


Figure S19. STEM image of *E.coli* incubated with Kabanos-based chitosan capsules and fixed with glutaraldehyde, washed and resuspended in water. The capsules appear somewhat aggregated as a result of the bacterial fixation protocol with glutaraldehyde.

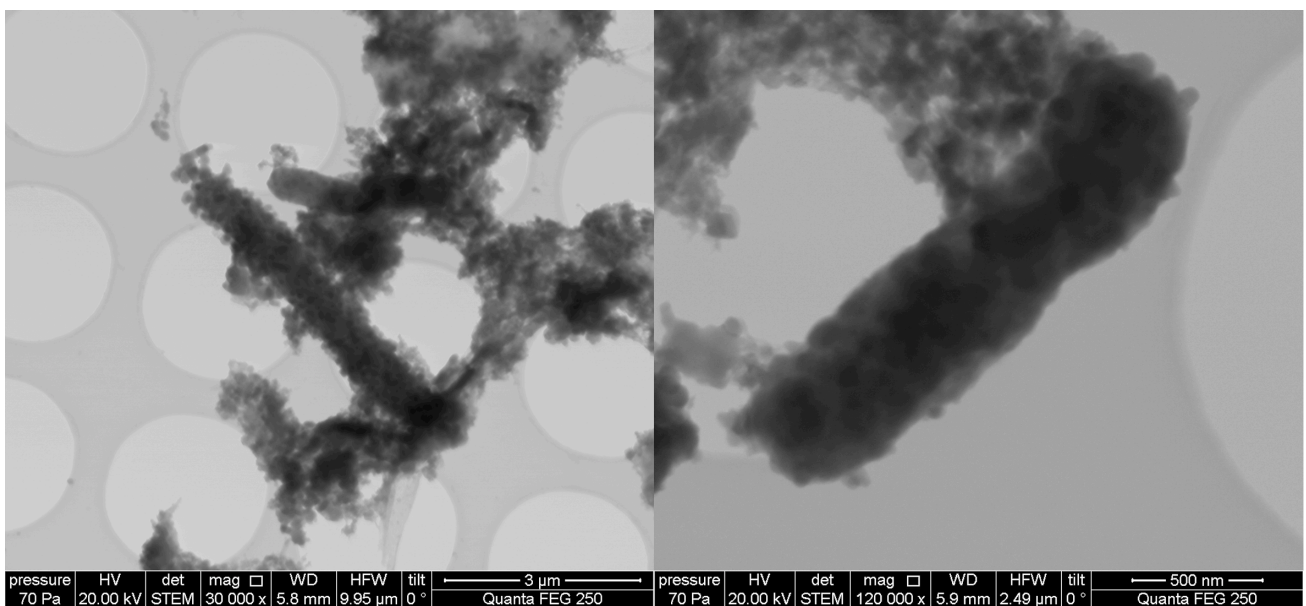


Figure S20. STEM image of *E.coli* incubated with PMA-based chitosan capsules and fixed with glutaraldehyde, washed and resuspended in water. PMA capsules are clearly visible and are intimately associated with the bacterial cells. In the left-hand image the capsules appear somewhat aggregated as a result of the bacterial fixation protocol with glutaraldehyde.

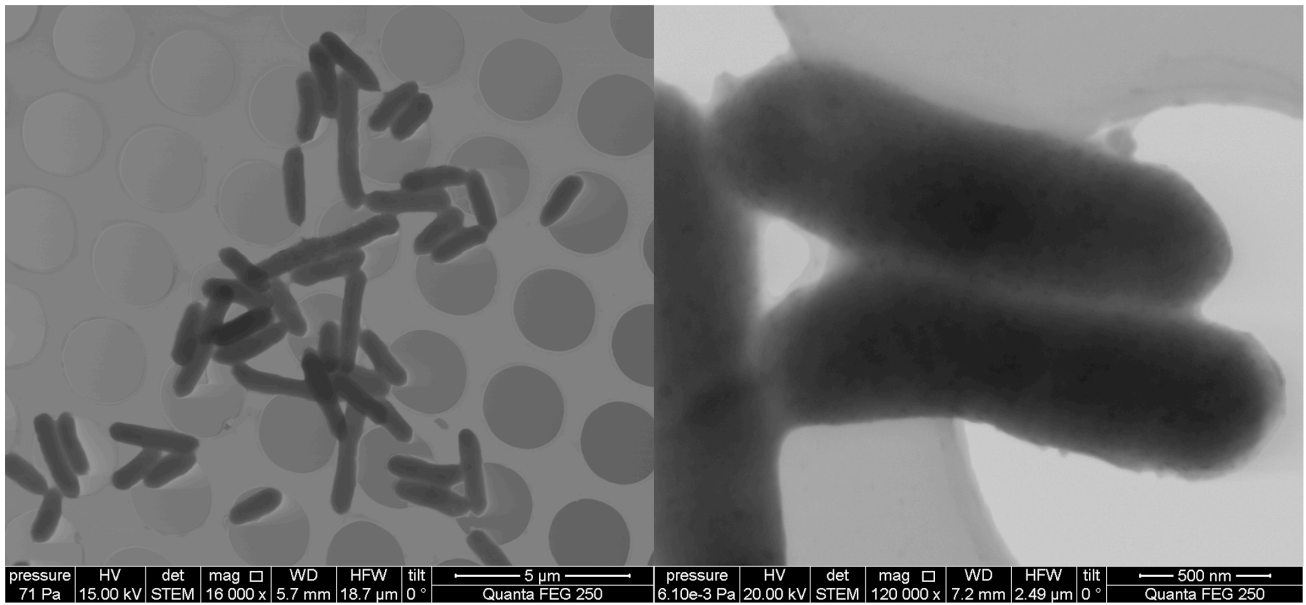


Figure S21. STEM image of *E.coli* incubated with PTA-based chitosan capsules and fixed with glutaraldehyde, washed and resuspended in water. There is an absence of capsules in these ESEM images as the focus of this study is the composition of the bacterial cells.