Electronic Supplementary Material (ESI) for Nanoscale.

Supplementary Information

Polyoxometalate-Polypeptide Nanoassemblies as Peroxidase Surrogates with Antibiofilm Properties

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Materials and instruments

3,3′,5,5′-Tetramethylbenzidine (TMB) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich, 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) was bought from Fluorochem and hydrogen peroxide (H_2O_2, 30 %) purchased from Panreac. Reagents were purchased from Sigma-Aldrich or Fluorochem and used as received. CM-H_2DCFDA was acquired from Invitrogen (C6827), and 5 mM solutions were prepared by dissolving the powder in DMSO just before each assay. Crystal violet and resazurin sodium salt were purchased from Merck and Sigma Aldrich respectively and solutions were freshly prepared at an adequate concentration in sterile ddH_2O.

All data were plotted and analysed using Origin 2019. Polymers were prepared as reported previously[1].

Sample	Mw (g mol^{-1})
$POMK_{10}$	5920
K_{10}	5196
POM	1223

Nanoparticle preparation

$POMK_{10}$ and K_{10} assemblies were prepared injecting 5 µL of a HFIP solution (20 mg mL^{-1}) into 95 µL of the corresponding milieu and homogenised until a homogeneous suspension was obtained. K_{10}/POM was prepared similarly, that is injecting 5 µL of K_{10} HFIP solution (20 mg mL^{-1}) into a medium containing 168 µM of POM to reach the same POM concentration as in $POMK_{10}$.

Kinetic assays

Kinetic assays. TMB as substrate

Kinetic assays were typically performed in a buffered solution of acetate (pH 4.6, 10 mM). Particles were prepared as before (1 mg mL^{-1}) and diluted with 100 µL of reaction buffer, yielding a final concentration of 0.5 mg mL^{-1}. Then, TMB absorbance was registered in a plate reader at the absorbance wavelength of the oxidation product at 652 nm every 2 seconds for 3.3 minutes.

Initial velocity was calculated using the linear part of the curve through a linear fitting and transformed into concentration using the extinction molar coefficient (39000 M^{-1} cm^{-1}). Absorbances were transformed to 1 cm path length by dividing the reaction volume (200 µL) by the total well volume (310 µL).

The reaction buffer consists of TMB at different concentrations ranging from 0.026 to 0.8 mM, H_2O_2 (9.8 mM) and buffered solution of acetate (pH 4.6, 10 mM Reaction buffers were freshly prepared the day of the experiment.

$POMK_{10}$-concentration dependent kinetic experiments of TMB oxidation.

A stock of assemblies' solution at 2 mg mL^{-1} was prepared injecting 10 µL of $POMK_{10}$ (20 mg mL^{-1} in HFIP) into 90 µL of acetate buffer (pH 4.6, 10 mM). Then successively diluted 1:2 in the same buffer. Then 100 µL of a solution containing 0.106 mM of TMB and 9.8 mM of H_2O_2 were added and the absorbance of the oxidation product at 652 nm was registered every 2 seconds for 3.3 minutes.

Kinetic assays containing POM were performed at a concentration of 0.1 mg mL^{-1} (84 µM). 20 µL of a stock solution in HFIP (1.0 mg mL^{-1}) were diluted with 80 µL of acetate buffer (pH 4.6, 10 mM) and 100 µL of reaction buffer was then added.

Studies on the effect of nanoassemblies presence on the oxidation of TMB

$POMK_{10}$ disassembly was performed as follows. Particles were prepared by injecting 10 µL (20.0 mg mL^{-1}) in 190 µL of acetate buffer and centrifuged (6 min,10000 rpm). Then the pellet was redissolved in HFIP (200 µL), evaporated under a N_2 stream, and dried under vacuum overnight to remove any folding. The next day it was redissolved in acetate buffer (200 µL) under sonication and submitted to kinetic analysis as stated above.

The reaction buffer consists of TMB at different concentrations ranging from 0.026 to 0.8 mM, H_2O_2 (9.8 mM) and buffered solution of acetate (pH 4.6, 10 mM). The final concentration is divided by 2. Reaction buffers were freshly prepared the day of the experiment.

Kinetic assays. ABTS as substrate

Kinetic assays with ABTS were performed as with TMB but modifying the concentration range from 0.16 mM to 1.52 mM. It is worth noticing that at high ABTS concentration the system tended to precipitate due to ionic crosslinking. In the case of ABTS concentrations of oxidised substrate were calculated by measuring the absorbance at 420 nm with extinction molar coefficient (36000 M^{-1} cm^{-1}). Absorbances were transformed to 1 cm path length by dividing the reaction volume (200 µL) by the total well volume (310 µL).

CD

Circular dichroism spectra were measured in a *JASCO 810* equipment in a 0.1 cm quartz cuvette. Spectra are the average of two successive measurements. Signal was expressed in mean ellipticity (θ). The samples were prepared in the corresponding medium at 0.1 mg mL^{-1}, which is the highest concentration that was not turbid. All experiments were carried out at room temperature.

DLS and ζ potential

Dynamic light scattering was measured in a Brookhaven 90plus DLS instrument by means of the Photo Correlation Spectroscopy (PCS). All measurements were performed at 1 mg mL^{-1} in the desired medium using 50 µL in a quartz cuvette with a 0.3 cm path length. ζ potentials were measured diluting the stock particle solution to 0.01 mg mL^{-1} in KCl (1 mM).

All measurements were the result of at least two independent replicates and expressed as mean ± sd. All measurements were carried out at room temperature.

Critical aggregation concentration (CAC)

Briefly, 20 µL of Nile red (100 µM) in chloroform were dried in a 96-well polypropylene plate. Then 100 µL of aggregates, at different concentrations, were added to the evaporated dye and incubated overnight at room temperature. The next day, samples containing the dye were transferred into Greiner 96 Black Flat Bottom Fluotrac plate to measure the fluorescence emission (Excitation 570 nm and emission 643 nm). The maximum of emission was plotted against the concentration of sample. CAC was calculated as the concentration corresponding to the intersection point of the lower horizontal and the slope tangent. To measure CAC of samples containing TMB we prepared $POMK_{10}$ dilutions as before and incubated with the reaction buffers at different TMB concentrations. Next, we incubated with the evaporated Nile red and registered the fluorescence emission. All measurements were the result of at least two independent replicates and expressed as mean ± sd.

Fluorescence spectra were registered in a Synergy H1 microplate reader from BioTek

SEM sample preparation

Scanning electron microscopy samples were prepared at 0.25 mg mL^{-1} and casted onto a 0.25 cm^2 silicon wafers and air dried. Then covered with thin film of Pd or C and imaged in a SEM Inspect F50 (FEI Company, Eindhoven, The Netherlands).

Bacterial Proliferation Assays

The bacteria strain used, *Staphylococcus epidermidis* NCIMB 8853 CECT 231, was acquired from the Colección Española de Cultivos Tipo (CECT) as lyophilized bacteria. The bacteria cell bank suspensions were thawed and inoculated on a Nutrient Broth (NB) agar plate and on liquid Nutrient Broth for 24 h at 37 ºC with mild agitation. A first subculture was performed to assure viability of the strain. A dilution from these culture solutions (second subculture and so on) was used for the following tests, corresponding to an inoculum of 1 × 10^7 CFU mL^{-1}. Stock solutions of all the tested compounds were prepared in HFIP - except for POM which was prepared in MQ-H_2O - at a concentration of 20 mg mL^{-1}, aliquoted, and stored at −20 ºC to avoid evaporation of the solvent.

(A) Bacterial growth inhibition assay (MIC): Conditions here described are for testing 7 different concentrations of the compounds, with triplicates of each condition. Therefore, 4 compounds were tested per plate. An adapted version of the microdilution method was used. Firstly, the materials were dissolved in 100 µL of NB at 2× the highest tested concentration in the wells of the first row (A) of a 96-well plate. In addition, 50 µL of liquid medium had been previously added to rows B to F. Subsequent dilutions at 1:2 are prepared in rows B to G, by withdrawal of 50 µL from the previous row (more concentrated) to the next row (half diluted), mixing well. By now, there are 50 µL in each well. Then, 50 µL of bacterial suspension at 10^7 CFU mL^{-1} were added to each well. The 96-well plates were incubated for 24 h at 37 ºC under mild agitation. Bacterial growth was controlled by visual observation

of the turbidity in each well at time 0 h and 24 h. Results (MIC) are recorded as the lowest concentration of antimicrobial agent that inhibits visible growth of the bacteria and were compared with the variation of a control culture containing *S. epidermidis* (+ control) and of solution of the tested compounds without bacteria as well as only NB (- control).

(B) Bacterial cell viability assay (MBC): Cell viability was analysed using a Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) assay in a 96-well plate. Once the bacterial cultures of the growth inhibition assay had been grown for a total of 24 h, 25 µL of a 0.1 mg mL^{-1} resazurin (prepared in LB or NB medium) were added to each well and incubated in the dark at 37 ºC for 1 h under stirring. Resazurin has a blue colour at the testing pH and turns pink when reduced by the viable bacteria to resorufin. Therefore, pink wells indicate metabolizing bacteria, while blue wells are indicative of bacteria that have lost their ability to convert resazurin to resorufin. Different controls were made in order to corroborate the MBC value obtained by the resazurin assay. The change of colour was confirmed at 1, 4, and 24 h after its addition. The viability of bacteria was verified (either confirmed or rejected) by the colony plate-counting method, by seeding 10 µL from the cell culture onto NB agar plates and observing the presence or absence of bacterial growth after 24 h at 37 ºC.

Intracellular ROS assay

The intracellular ROS assay was performed following a modified version of a previously published protocol.[2] Mid-log phase *S. epidermidis* were treated with CM-H_2DCFDA (20 µM) and incubated for 60 min at 37 °C in the dark. After the incubation time, the bacteria suspension was washed x3 with PBS to remove any remaining CM-H_2DCFDA outside the cells, maintaining the same final volume. 100 µL of this suspension were transferred into each well of a 96-wells plate. Black microplates were used to avoid autofluorescence and well-to-well crosstalk issues. Then, the corresponding volume from the hybrids stock solutions (in HFIP) and parent POM (in ultrapure H_2O) was added for the final conditions. Control wells, with (1.25 µL) and without HFIP were included as well. Immediately after addition of all the different components, the plate was covered with a sealing film to avoid evaporation and contamination and the kinetic of the fluorescence intensity of DCF was measured on a Synergy H1 microplate reader from BioTek, with an excitation wavelength of 488 nm and emission wavelength of 530 nm. The experiment was conducted in triplicates and repeated at least twice for each condition.

ROS quenching assay

Ascorbic acid (2.5 mM) was used to study the impact of the addition of an antioxidant or ROS scavenger in the MBC values. Briefly, the same protocol described for the bacterial proliferation assays was followed, including the addition of ascorbic acid at a final concentration in the well of 2.5 mM, just after the addition of all the conditions. Previously, the viability of *S. epidermidis* in presence of a range of concentrations of three common antioxidants (*N*-acetylcysteine, ascorbic acid and glutathione) was tested. For all of them, important bacterial reduction was observed at concentration ≥ 5 mM (see Fig. S24). Therefore, a concentration of 2.5 mM was considered safe for the bacteria cells and

sufficient to act as ROS scavenger. Once the plate was prepared, bacteria were incubated for 24 hours at 37 °C. After this time, resazurin was added as detailed before and the MBC was further verified by seeding 10 μL of each solution on NB agar plates.

Antibiofilm activity

The pellicle biofilm prevention activity of the different materials was studied using a Crystal Violet assay. Briefly, 100 μL of *S. epidermidis* culture (10^7 CFU mL^{-1}) were incubated for 72 h at room temperature with mild agitation in a 96-well plate with addition of a concentration equal to the 1/2x of each material. Samples without any treatment and containing only NB were used as control. After the incubation period, the planktonic cells were discarded, and the wells were washed twice with distilled water. Then, the plate was dried for 10 minutes and followed by addition of 225 μL of a 0.1% crystal violet solution (in distilled water) were added into each well. The crystal violet was incubated for 20 min and then the excess of dye was removed by washing the plate four times with distilled water. After drying the plate for at least two hours the crystal violet was dissolved by adding 200 μL of ethanol 96% to each well. After 10 min of incubation to fully homogenize each solution, the absorbance was measured at 595 nm. Absorbance intensity was recorded using a SPECTROstar Nano microplate reader (BMG Labtech) at 590 nm, with a baseline subtraction at 650 nm to remove any interference. The values were normalized and expressed as % of biofilm mass produced, taking as a value of 0% that of the negative control with only NB and as 100% that of *S. epidermidis* without any treatment.

Preparation of *S. epidermidis* for Scanning Electron Microscopy

300 μL of a *S. epidermidis* culture (10^7 CFU mL^{-1}) were added per well to a 96-wells plate and were further treated with the materials at their MIC and 1/2x MIC. A positive control of bacteria without any treatment and a negative control of only NB were included, following the same protocol as the rest of conditions. Then, the plate was incubated at room temperature (ca. 25 °C) with mild agitation (oscillating) for 72 hours. After this time, the bacteria suspensions were transfer into 1.5 mL centrifugation tubes. Then, bacteria suspensions were washed twice with PBS and the pellet was resuspended in 600 μL of a 2.5% glutaraldehyde solution in PB 10 mM pH 7.2. The suspensions were incubated in a tube rotator for 2.5 hours, preserved from exposure to light. After this time, the suspensions were washed with PBS, centrifuged again, and postfixed in 2% OsO_4 in H_2O for 1 h in a volume enough to cover the pellet of bacteria. The fixed bacteria were then washed with PBS (x3) and dehydrated with increasing concentrations of EtOH (30%, 50%, 70%, 90%, 2x 96%, 3x 100%) for 10 minutes each. Finally, 10 μL of the suspensions were transferred onto silicon wafer squares, let dry and coated with 14 nm of Pd.

Images

Images in Fig. 1 were prepared taking inspiration from a viral capsid (PDB 6EGV) using Protein Imager.[3]

Additional information

DLS and ζ potential

DLS and ζ potential $POMK_{10}$, K_{10}, POM/K_{10}, POM.

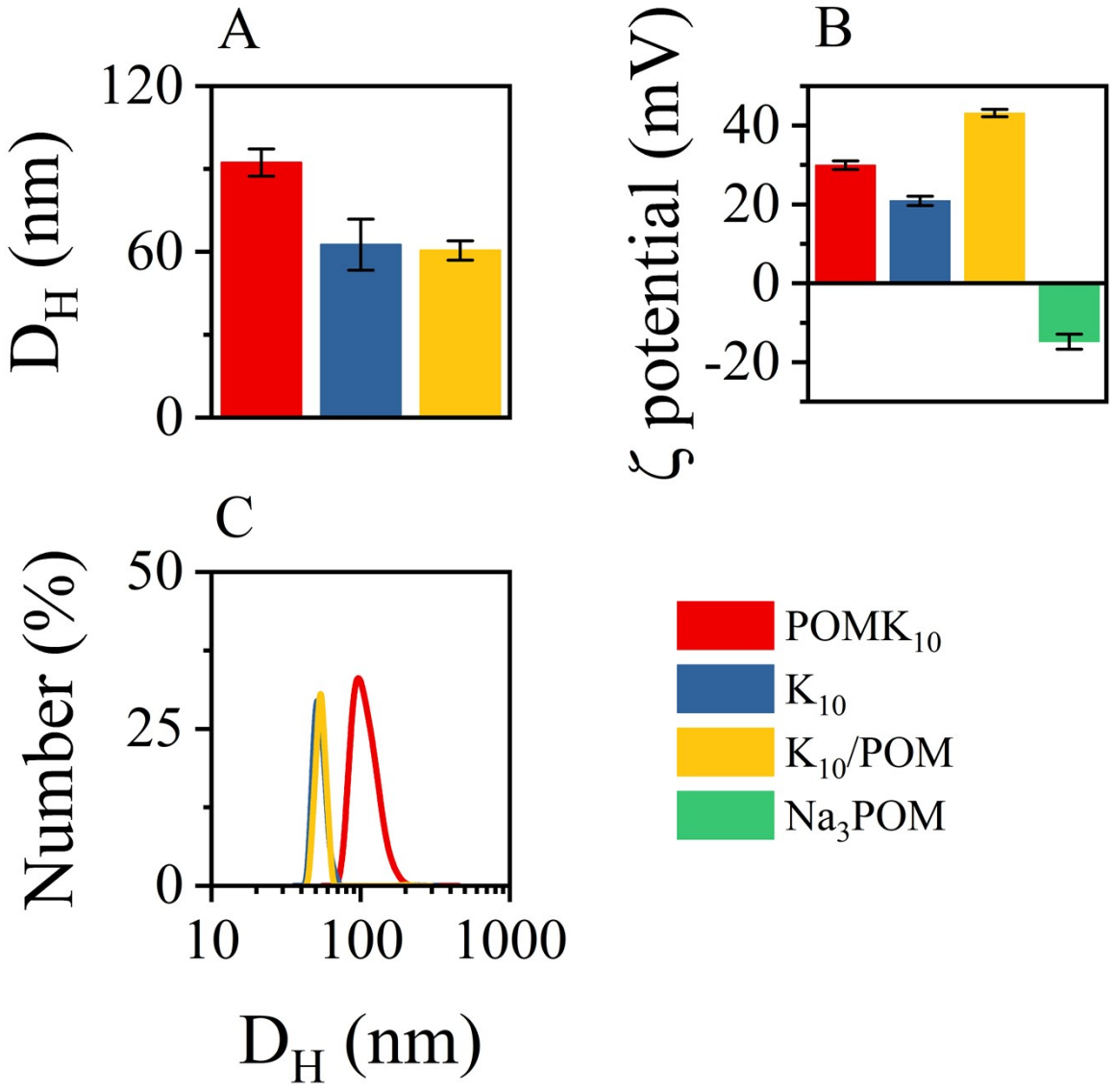


Fig. S1 (A) (C) Hydrodynamic size of $POMK_{10}$, K_{10} and POM/K_{10}. (B) ζ potential of $POMK_{10}$, K_{10}, POM/K_{10}, POM.

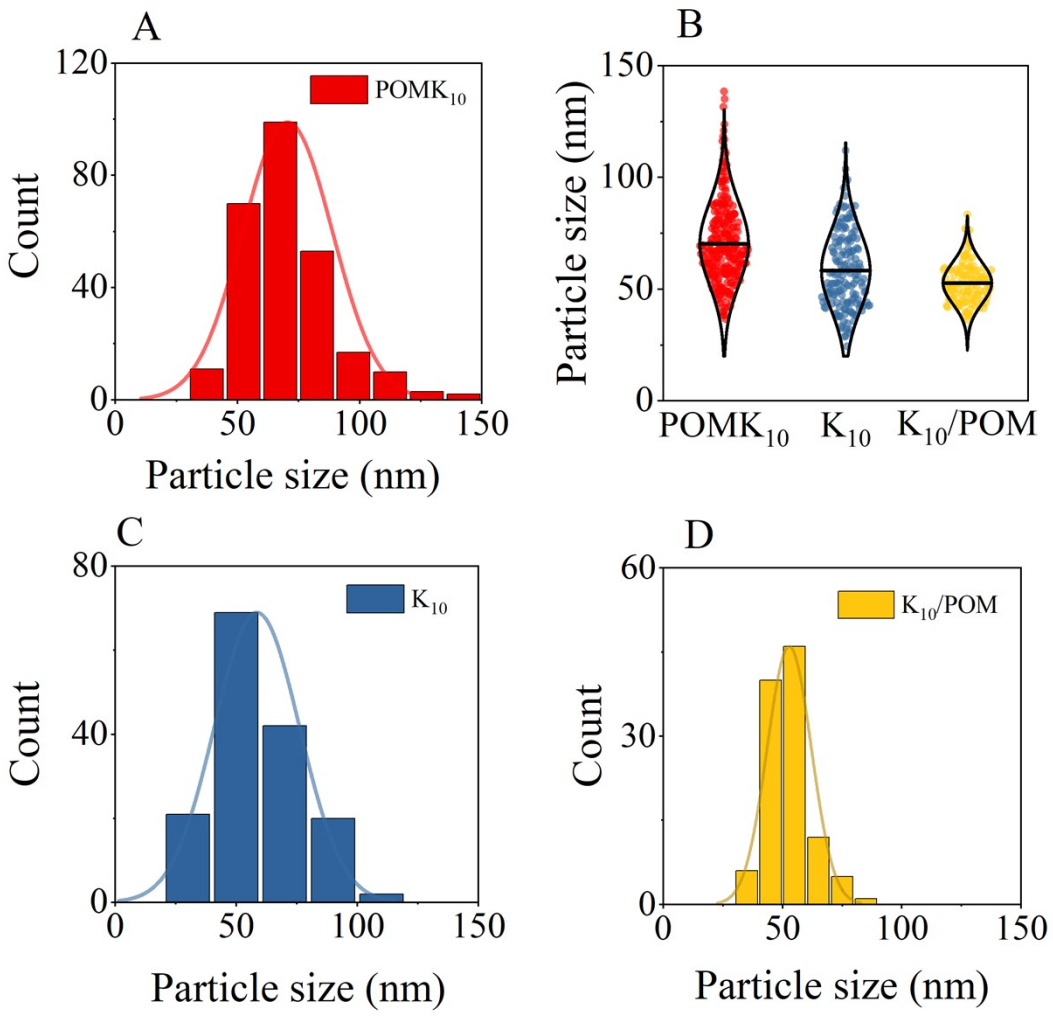


Fig. S 2 Particle size histogram (N > 120 particles). A) D_{POMK10} = 70.3 ± 18.4 nm, C) D_{K10} = 52.8 ± 9.26 nm, D) $D_{K10/POM}$ = 58.4 ± 17.6 nm. B) Violin plots of the particles counted in SEM images fitted to a normal distibution. The black line represent the mean value of the distribution.

DLS and ζ potential varying concentration ($POMK_{10}$)

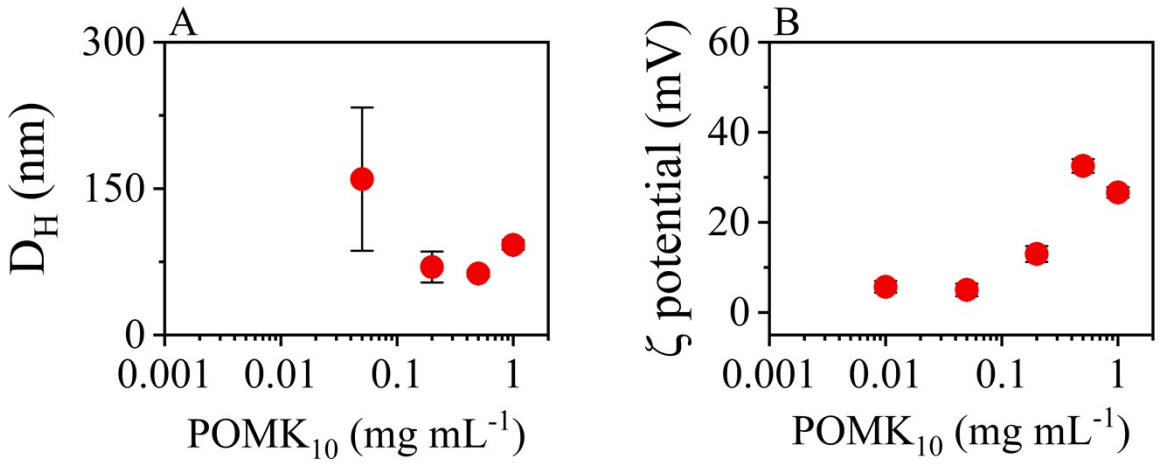


Fig. S 3 (A) Summary of hydrodynamic diameters of $POMK_{10}$ at different concentrations in water. (B) Summary of ζ potential values of $POMK_{10}$ at different concentrations. Each point is the mean value of at least 2 independent samples (repetition).

CAC

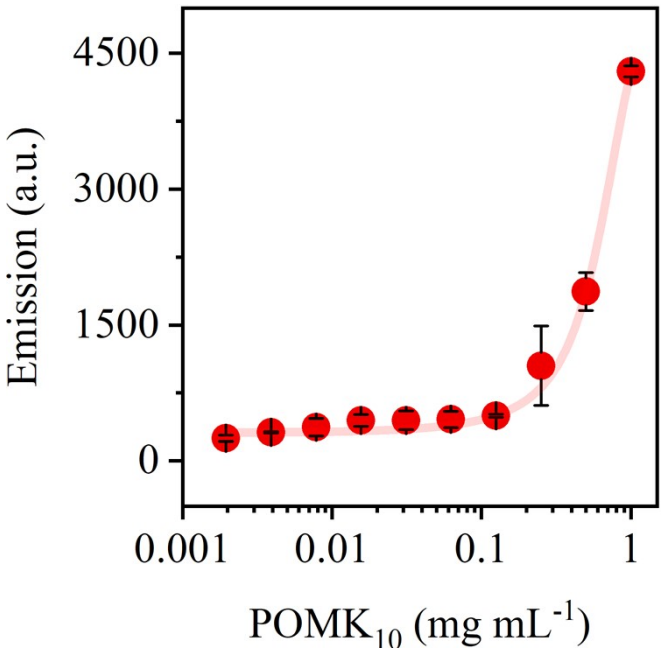


Fig. S 4 Critical aggregation concentration. $POMK_{10}$

DLS and ζ potential varying temperature ($POMK_{10}$)

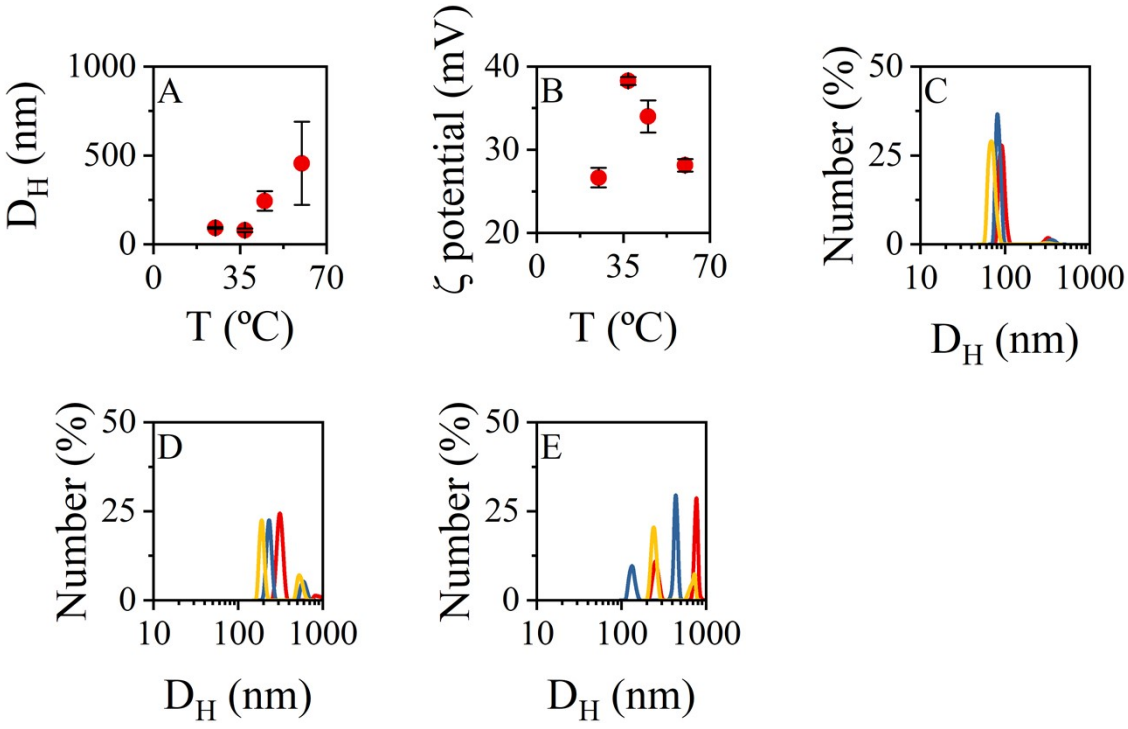


Fig. S 5 (A) Summary of hydrodynamic diameters of $POMK_{10}$ at different temperatures. (B) Summary of ζ potential values of $POMK_{10}$ at different temperatures. (C) 37 ºC (D) 45 ºC (E) 60 ºC. Each curve within (C) to (E) represents an independent sample (repetition).

DLS and ζ potential in NaCl ($POMK_{10}$)

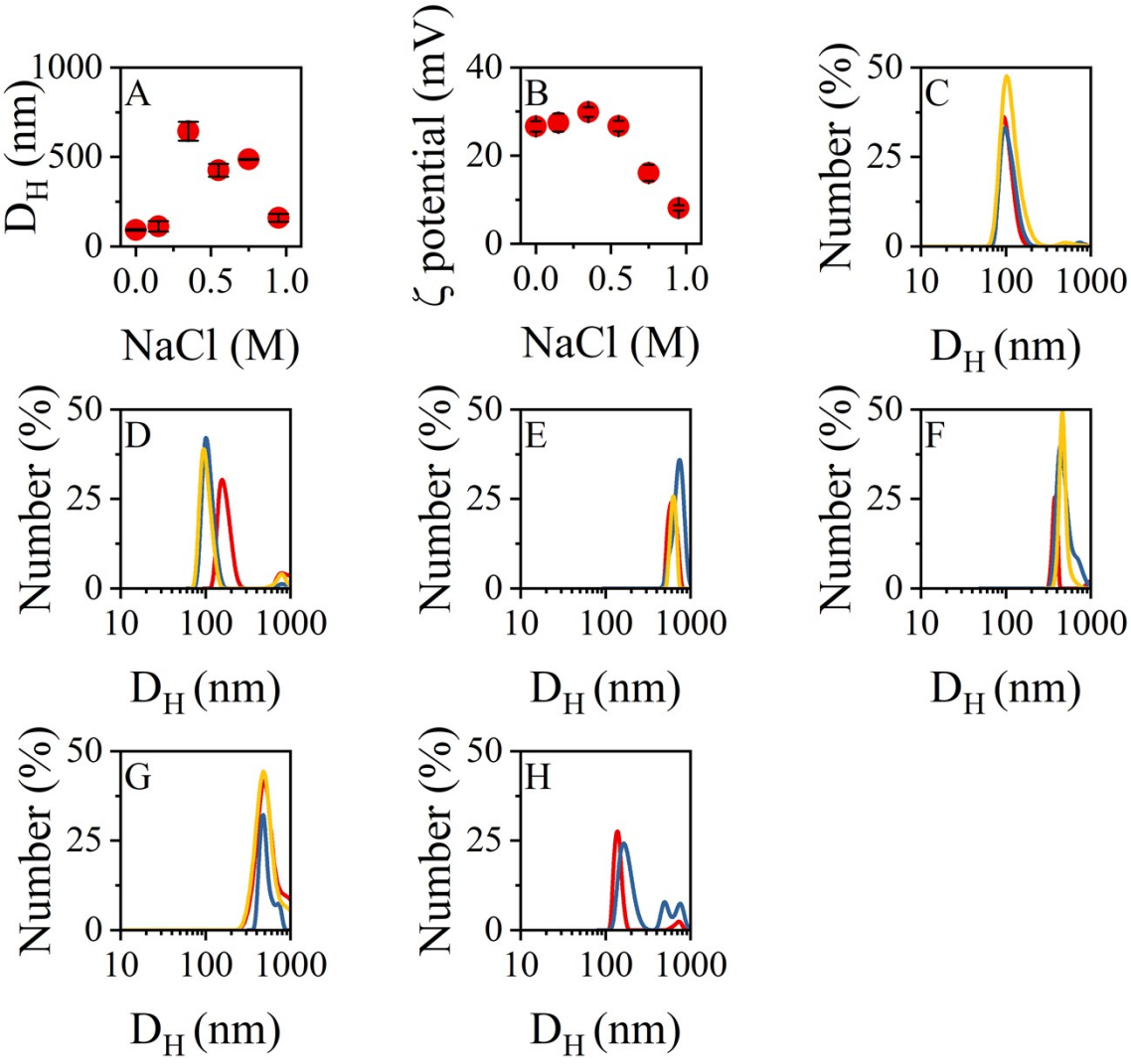


Fig. S 6 (A) Summary of hydrodynamic diameters of $POMK_{10}$ at different NaCl concentrations. (B) Summary of ζ potential values of $POMK_{10}$ at different NaCl concentrations. (C) 0 M (D) 0.15 M (E) 0.35 M (F) 0.55 M (G) 0.75 M (H) 0.95 M. Each curve within (C) to (H) represents an independent sample (repetition).

DLS and ζ potential in Thiourea ($POMK_{10}$)

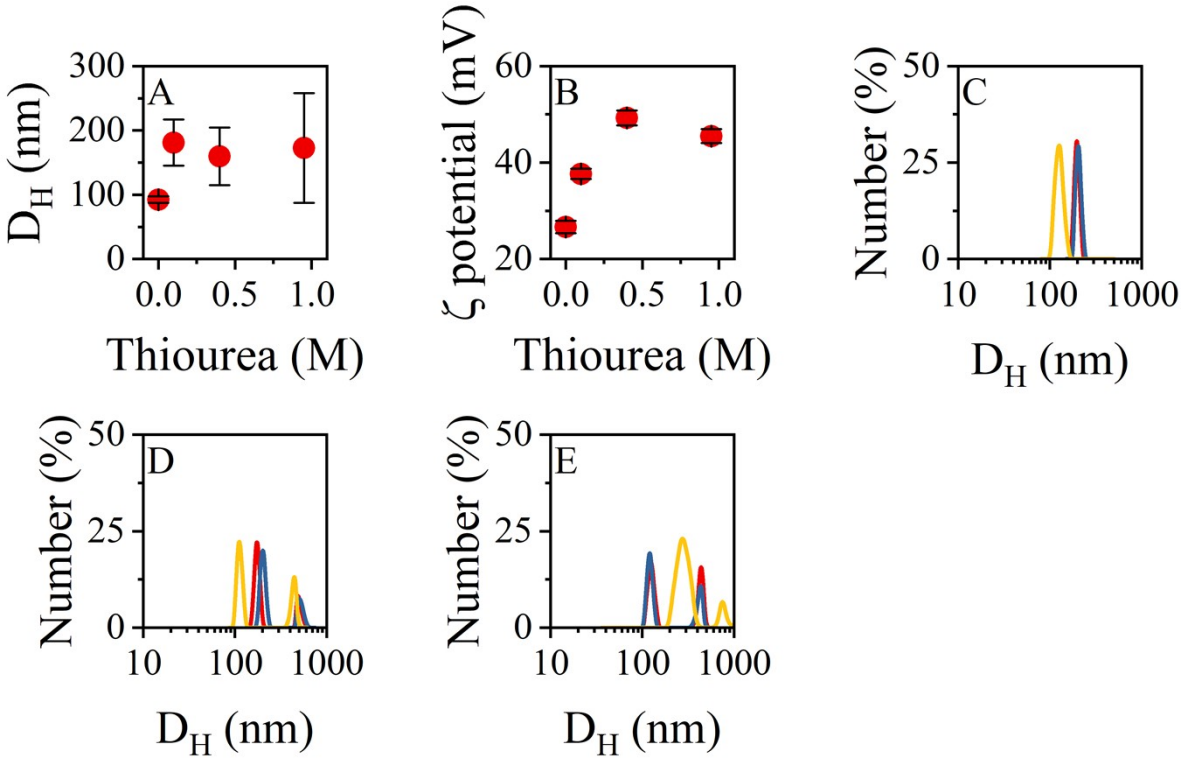


Fig. S 7 (A) Summary of hydrodynamic diameters of $POMK_{10}$ at different thiourea concentrations. (B) Summary of ζ potential values of $POMK_{10}$ at different thiourea concentrations. (C) 0.15 M (D) 0.40 M (E) 0.55 M (F) 0.95 M. Each curve within (C) to (E) represents an independent sample (repetition).

DLS and ζ potential in HFIP ($POMK_{10}$)

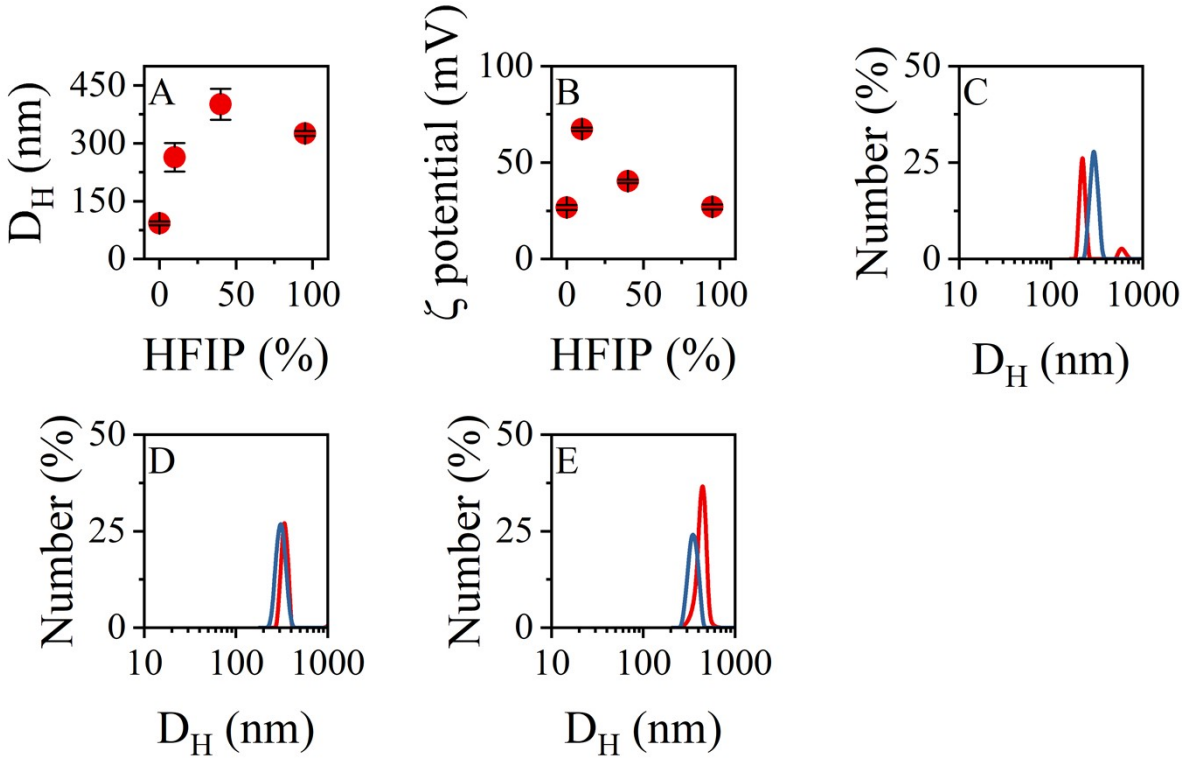


Fig. S 8 (A) Summary of hydrodynamic diameters of $POMK_{10}$ at different HFIP concentrations. (B) Summary of ζ potential values of POMK10 at different HFIP concentrations. (C) 15 % (D) 40 % (E) 95 %. Each curve within (C) to (E) represents an independent sample (repetition).

DLS and ζ potential varying pH ($POMK_{10}$)

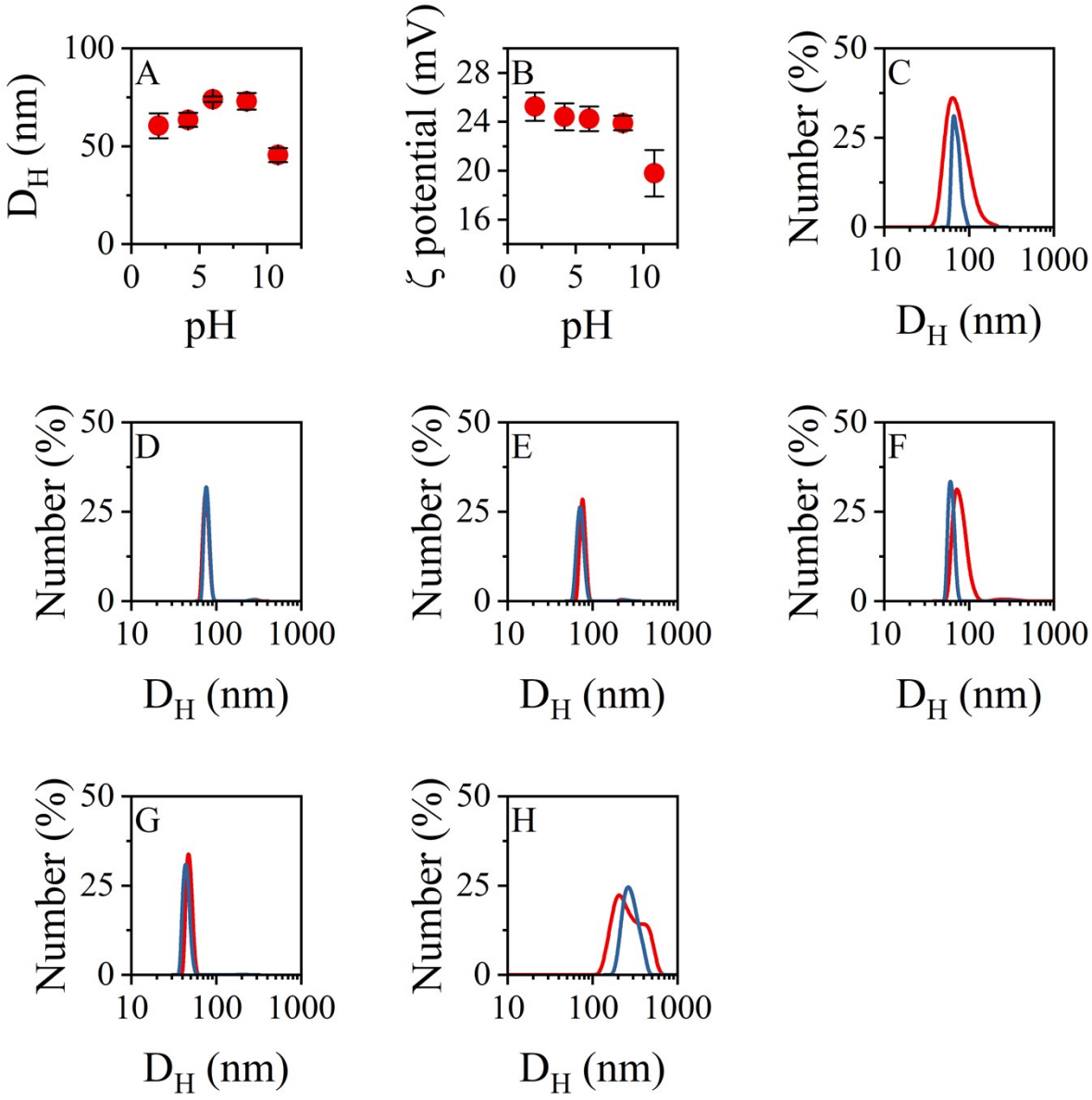


Fig. S 9 (A) Summary of hydrodynamic diameters of $POMK_{10}$ at different pH conditions. (B) Summary of ζ potential values of $POMK_{10}$ at different pH conditions. DLS curves at (C) pH 2 (HCl 100 mM) (D) pH 4.6 (Acetate 20 mM) (E) pH 6 (MES 20 mM), (F) pH 7 (TRIS 20 mM), (G) pH 8.8 (Carbonate 20 mM). (H) pH 12 (NaOH 100 mM). Each curve within (C) to (H) represents an independent sample (repetition).

Circular dichroism

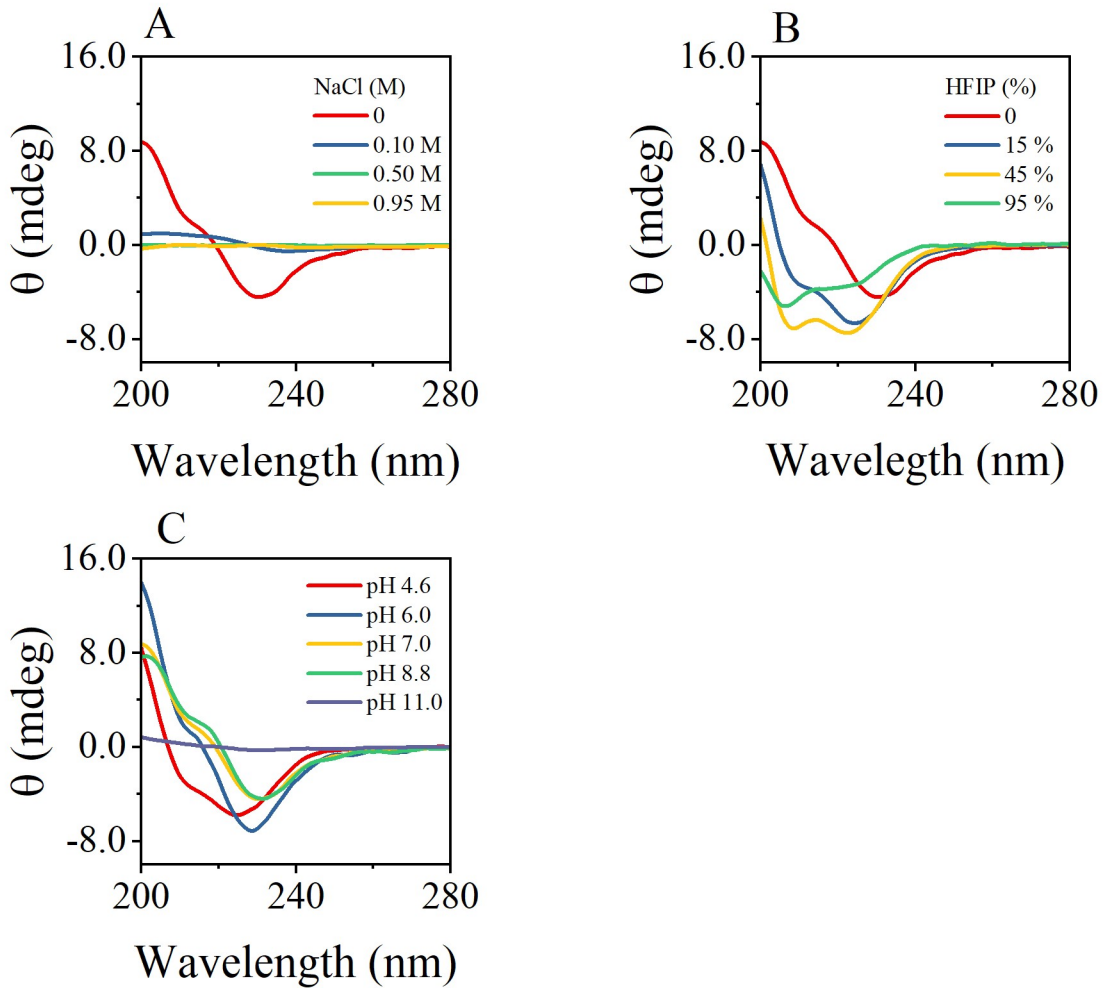


Fig. S 10 (A) CD spectra of $POMK_{10}$ at different NaCl concentrations. (B) CD spectra of $POMK_{10}$ at different HFIP concentrations. (C) CD spectra of $POMK_{10}$ at different pH conditions. .

DLS and ζ potential K_{10}/POM at different ratios

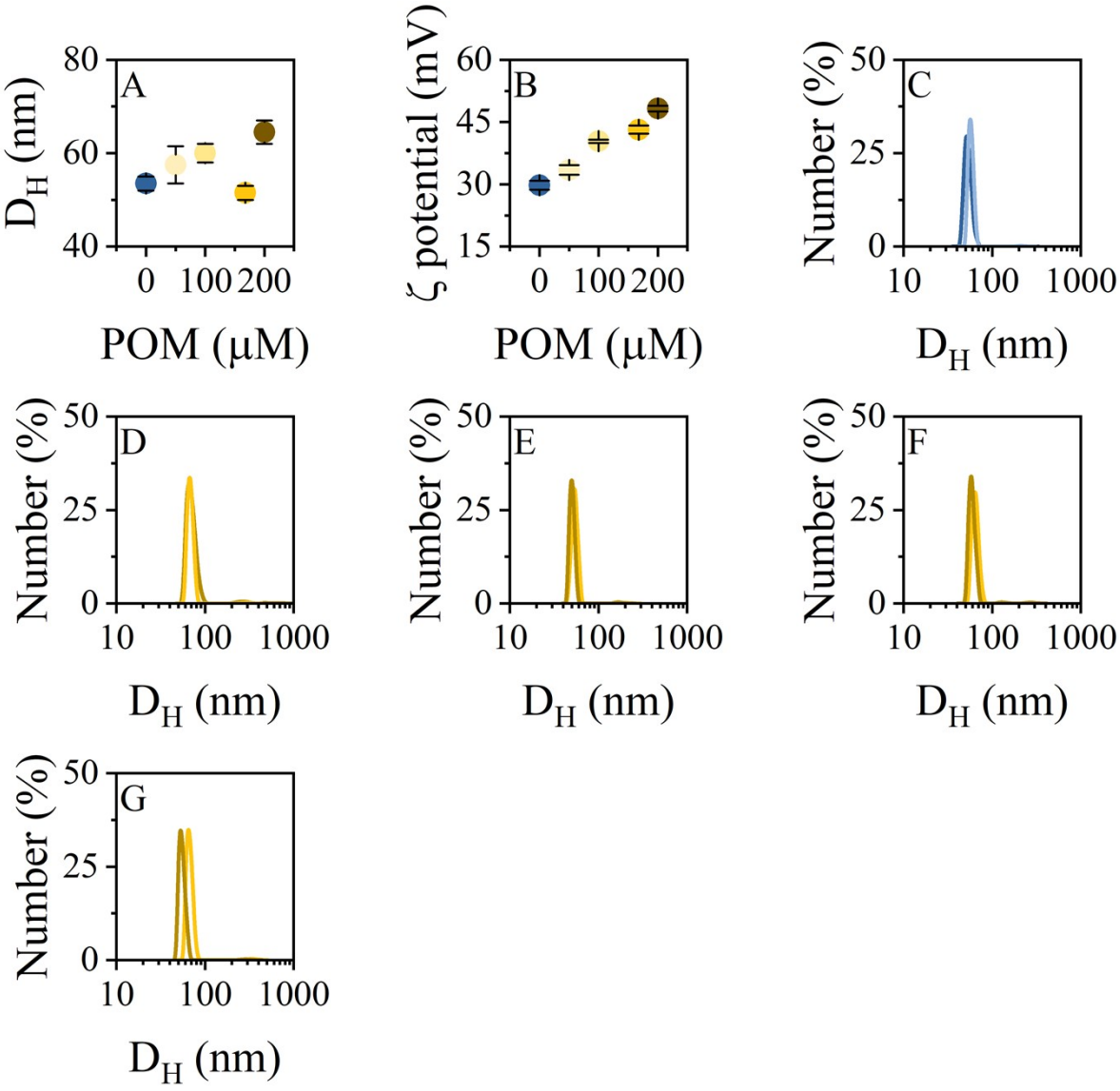


Fig. S 11 (A) Summary of hydrodynamic diameters of K_{10}/POM at different POM concentrations. K10 concentration is fixed to 1 mg mL^{-1} and the mass ratio is 6, 12, 20, 24 %wt. (B) Summary of ζ potential values of K_{10}/POM at different POM concentrations. (C) 0 (D) 50 μM (E) 100 μM, (F) 168 μM, (G) 200 μM. Each curve from (C) to (G) represents an independent sample (repetition).

Kinetic assays

$POMK_{10}(TMB/H_2O_2)$

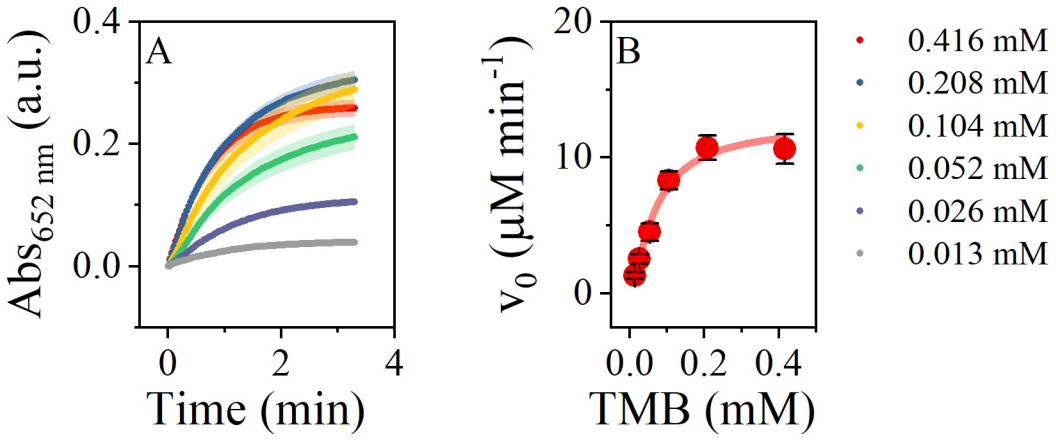


Fig. S 12 (A) Kinetics of TMB oxidation by $POMK_{10}$ at different concentrations. (B) initial rates against TMB concentrations. K_{Half} = 69.0 ± 15.2 µM. R^2 = 0.99056

POM (TMB/H_2O_2)

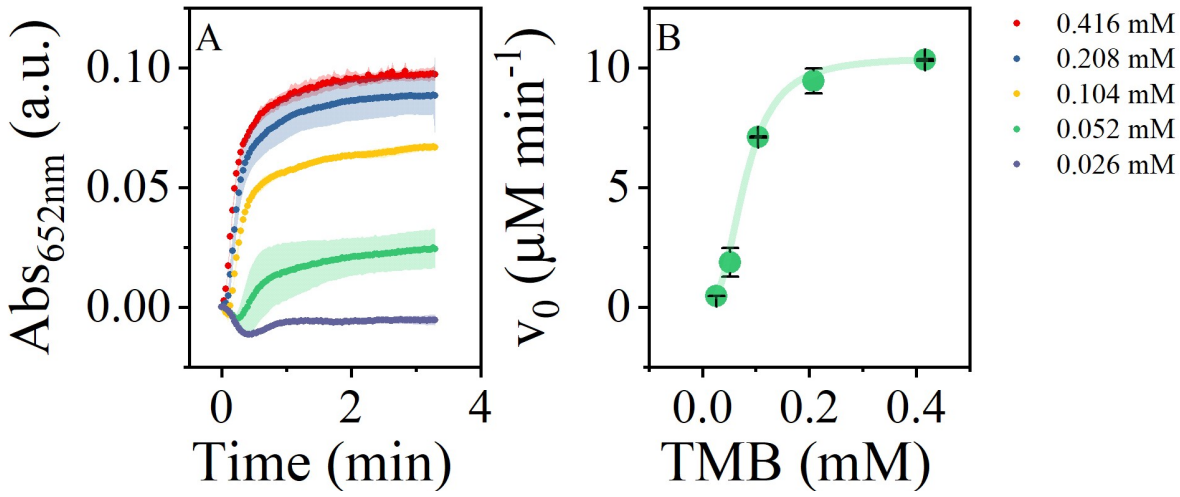


Fig. S 13 (A) Kinetics of TMB oxidation by POM at different concentrations. (B) initial rates against TMB concentrations. K_{Half} = 79.2 ± 0.3 µM, R^2 = 0. 0.99999

K_{10}/POM (TMB/H_2O_2)

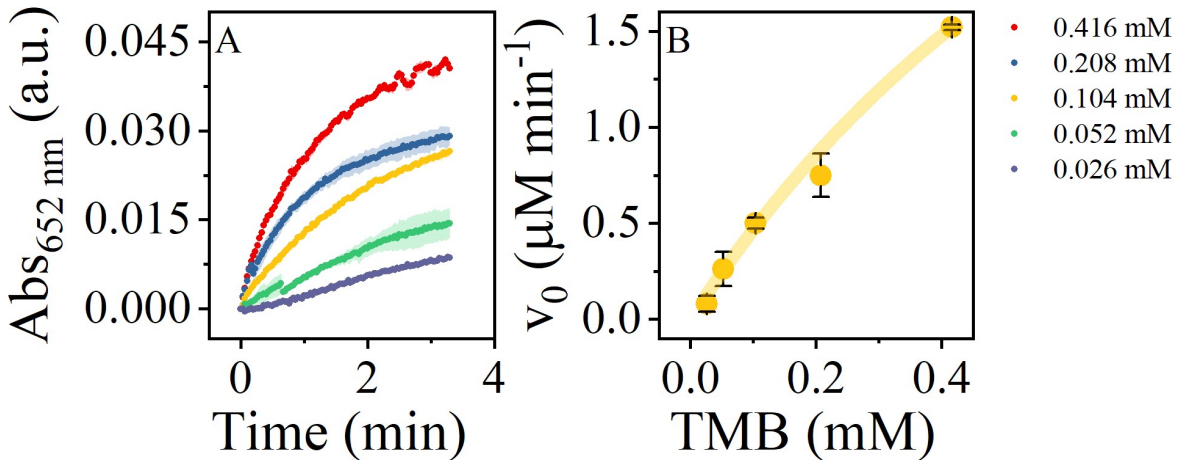


Fig. S 14 (A) Kinetics of TMB oxidation by K_{10}/POM at different concentrations. (B) initial rates against TMB concentrations. POM = 168 μM. K_M = 1040 ± 30 μM, R^2 = 0.99838. The ionic hybrid does fit to a Michaelis Menten kinetic.

DLS after TMB oxidation ($POMK_{10}$)

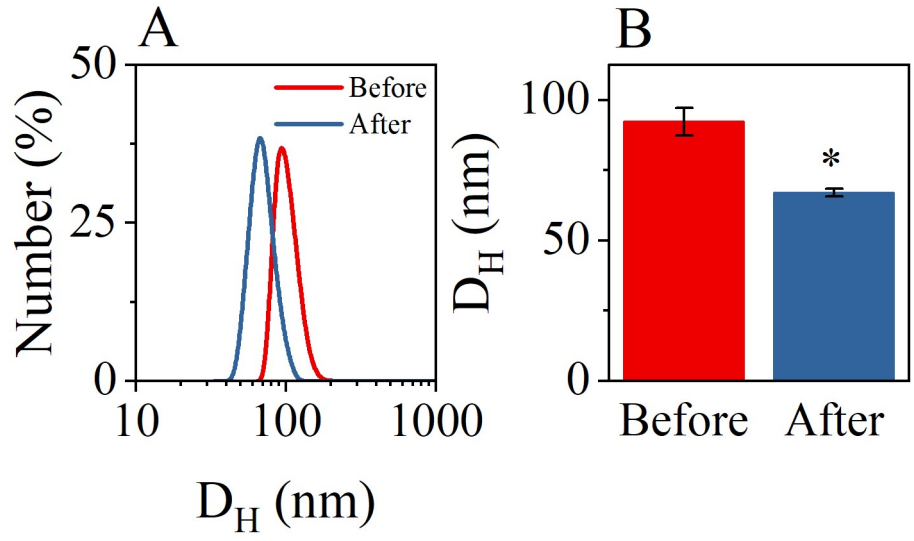


Fig. S 15. A) DLS curves of $POMK_{10}$ after the reaction with TMB. We had to lower TMB concentration since the absorbance at 615 nm interferes with the equipment's laser. [TMB] = 26 μM. B) Samples are significantly different using an unpaired t-test (p = 0.0213).

POMK$_{10}$ reaction (TMB/H_2O_2): dependence on pH

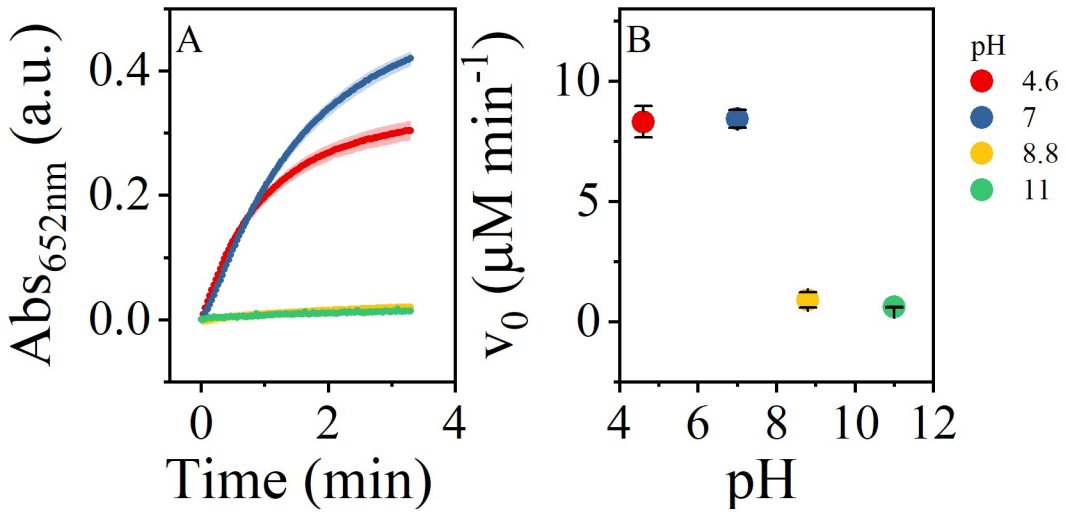


Fig. S 16 (A) POMK$_{10}$ activity at different pH. (B) initial rates against pH

POMK$_{10}$ reaction (ABTS/H_2O_2)

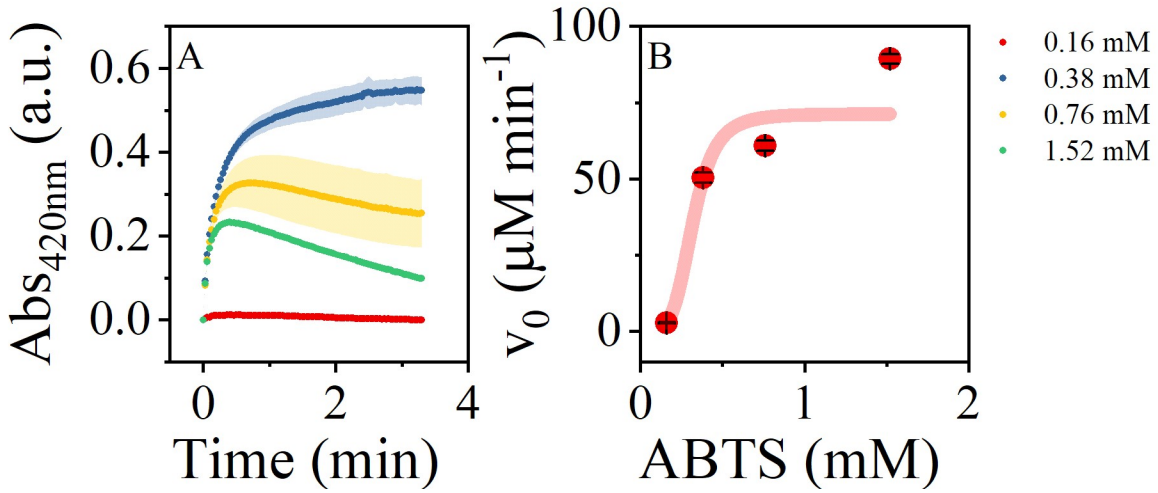


Fig. S 17 (A) Kinetics of ABTS oxidation by POMK$_{10}$ at different concentrations. (B) initial rates against ABTS concentrations. K_{half} = 347.0 ± 88.1 μM, R^2 = 0.97316. Note that the last point is unreliable due to precipitation

K_{10}/POM reaction (ABTS/H_2O_2)

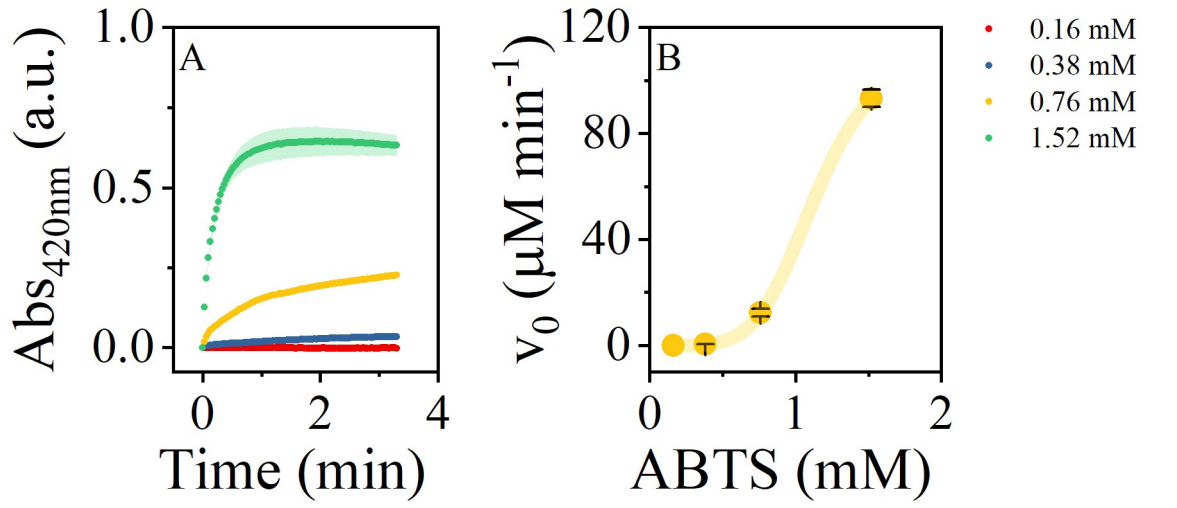


Fig. S 18 (A) Kinetics of ABTS oxidation by K_{10}/POM at different concentrations. (B) initial rates against ABTS concentrations. K_{half} = 347.0 ± 88.1 μM, R^2 = 0.97316. Note that the last point is unreliable due to precipitation

POM reaction (ABTS/H_2O_2)

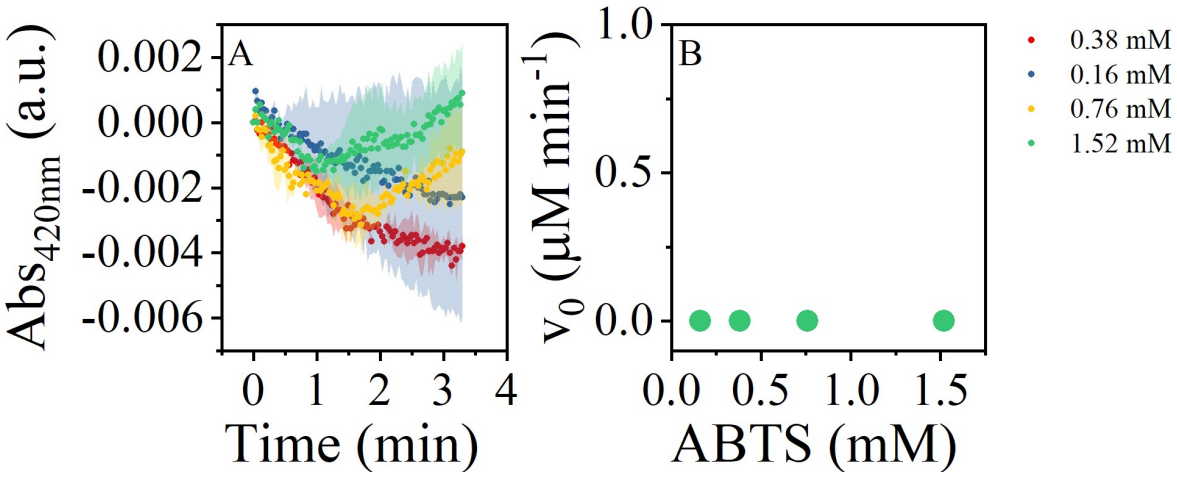


Fig. S 19 (A) Kinetics of ABTS oxidation by POM at different concentrations. (B) initial rates against ABTS concentrations.

POMK$_{10}$ reaction (TMB/H_2O_2) with concentration

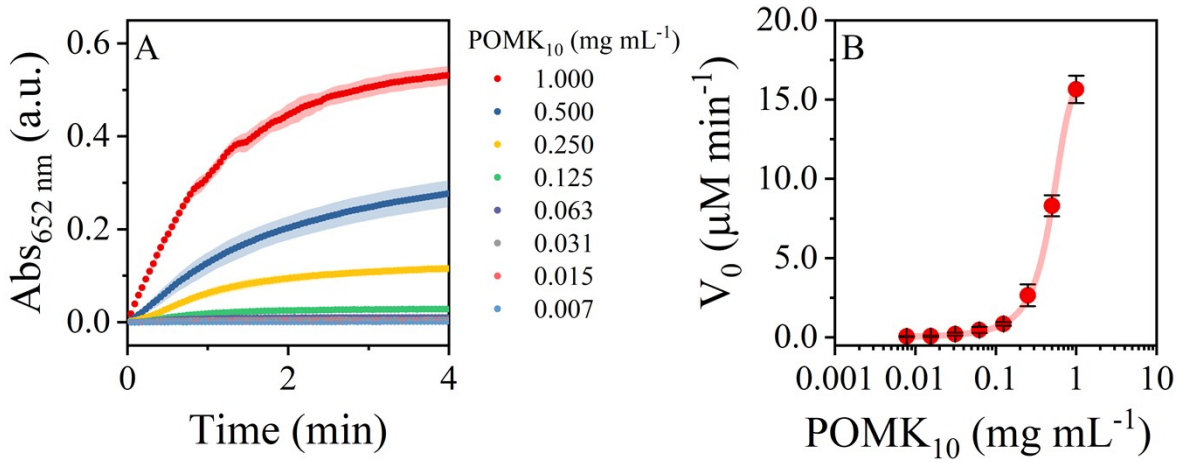


Fig. S 20 (A) Kinetics of TMB oxidation by POMK$_{10}$ at different concentrations. (B) initial rates against POMK$_{10}$ concentrations, X axis is in Log scale. R^2 = 0.99862 for Boltzmann sigmoidal curve.

Critical aggregation concentration

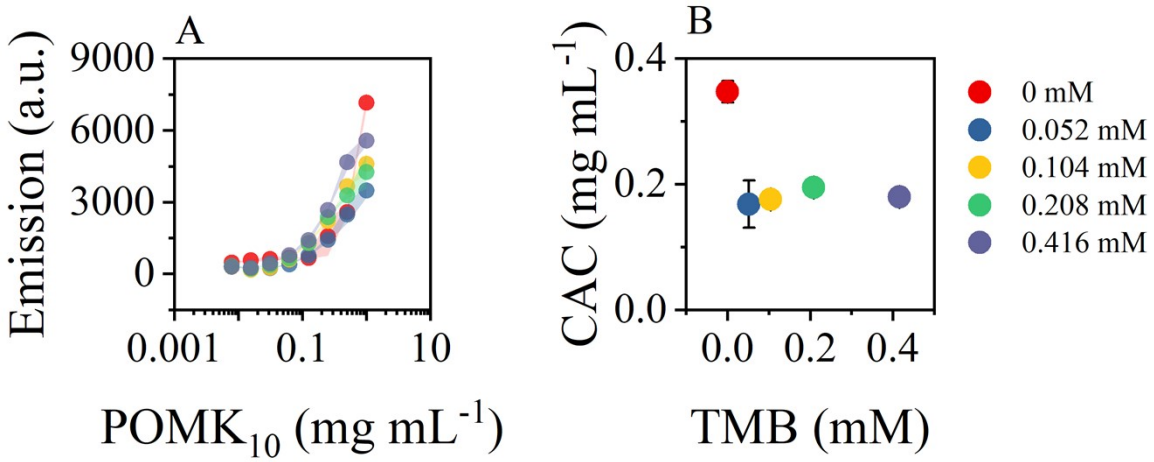


Fig. S 21 (A) Nile red emission at different TMB concentrations. (B) Critical aggregation concentration. In all experiments [H_2O_2] was fixed at 9.8 mM.

$POMK_{10}$ assemblies, $POMK_{10}$ disassembled reaction (TMB/H_2O_2)

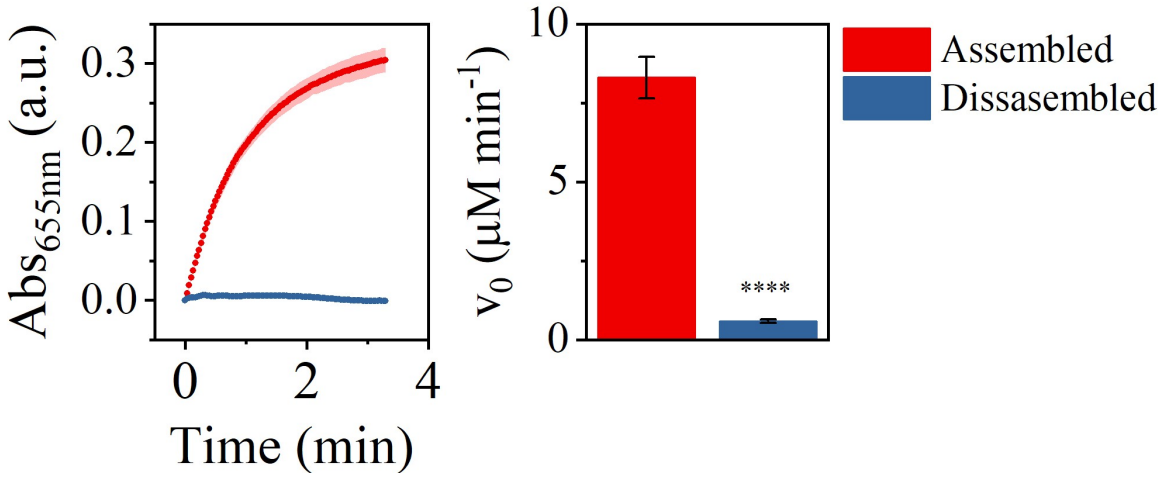
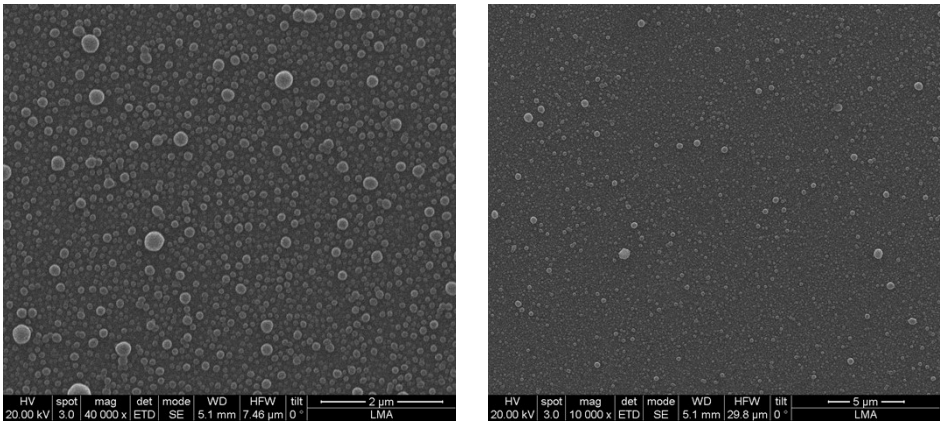


Fig. S 22 Kinetic profile of $POMK_{10}$ when assembled and disassembled.

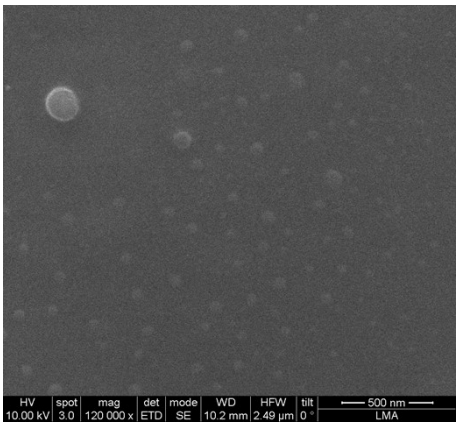
SEM images

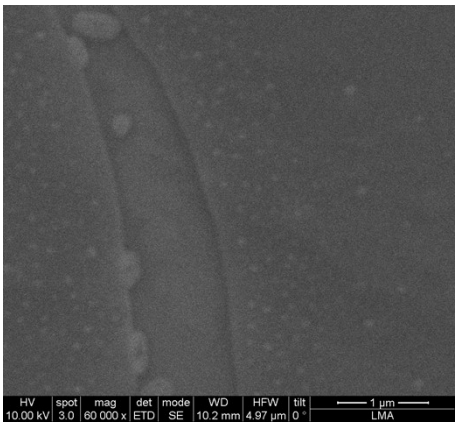
Complementary SEM images of $POMK_{10}$, K_{10}, K_{10}/POM nanoparticles.

$POMK_{10}$

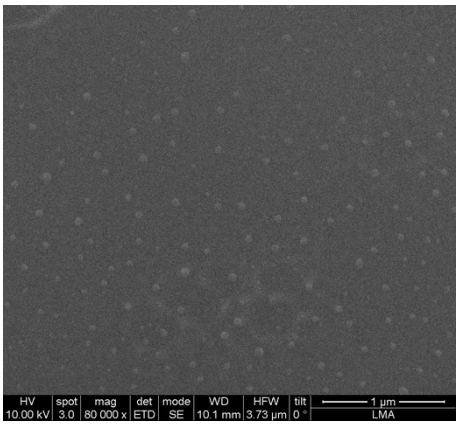


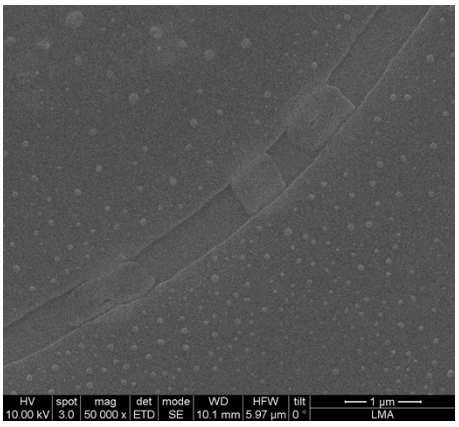
K_{10}



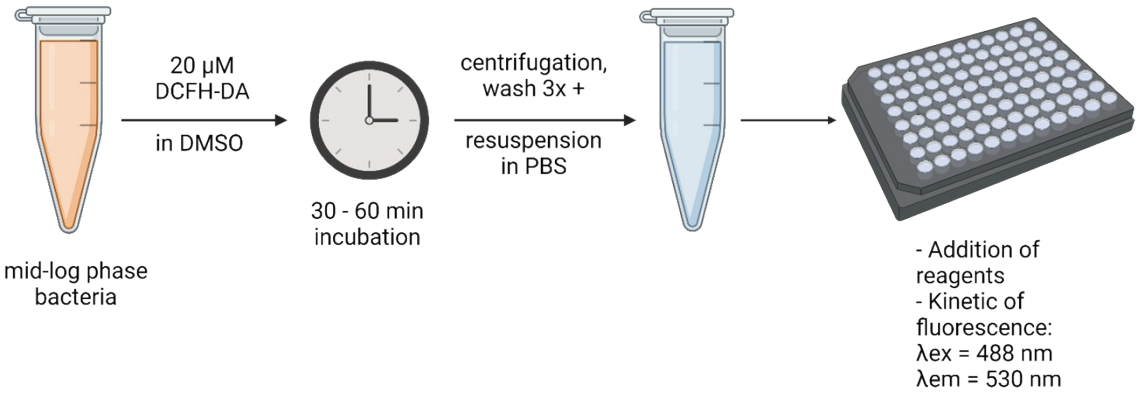


K_{10}/POM





Antimicrobial activity: MIC and MBC



Scheme S 1. Summary of the procedure for the DCFH-DA assay

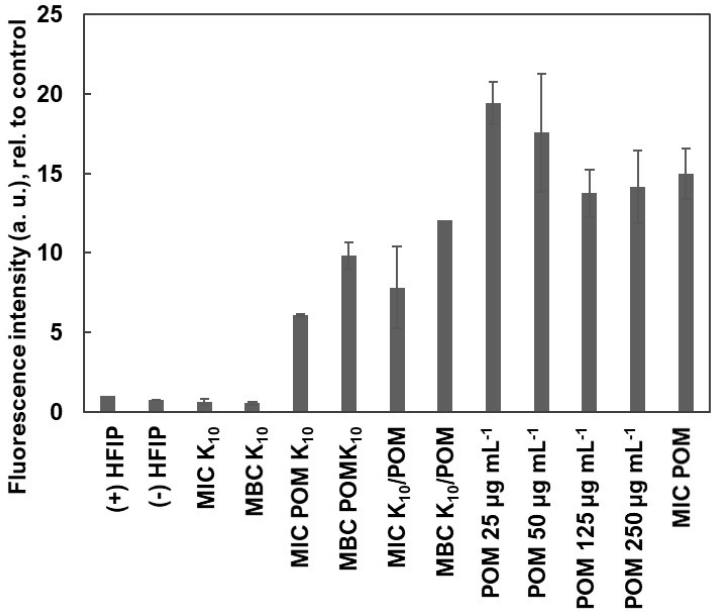


Fig. S 23. Intracellular ROS produced by S. epidermidis under different conditions. λ_{ex} = 488 nm, λ_{em} = 530 nm. The intensity values have been normalized taking as a value of 1 that of the control of S. epidermidis treated with 1.25 µL of HFIP [(+) HFIP].

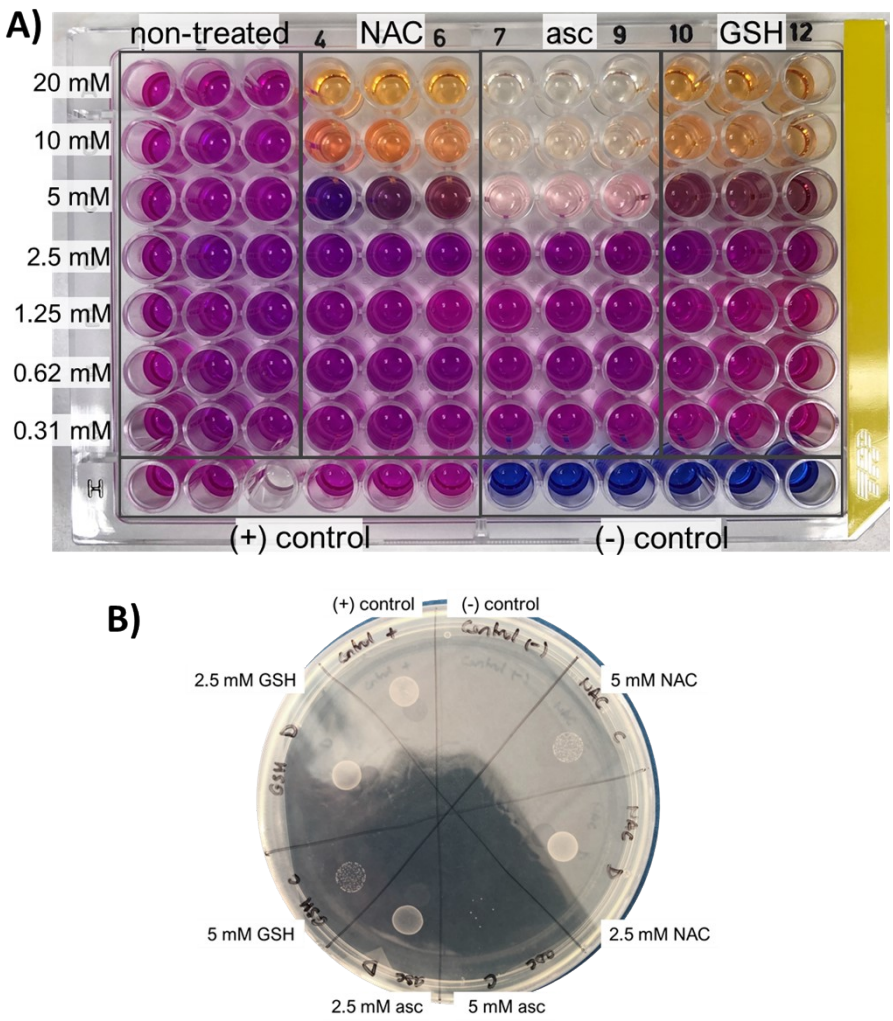


Fig. S 24. MBC assay for different antioxidants against S. epidermidis. (A) resazurin assay, (B) control of bacterial growth on agar plate.

The effect of three different antioxidants to be used as ROS scavengers was tested against *S. epidermidis* to choose the most effective concentration of use with non-toxic for bacteria. The three antioxidants tested were: *N*-acetylcysteine (NAC), ascorbic acid (asc) and glutathione (GSH). The powder products were dissolved in PBS at 400 mM, and the assay was carried out as described previously. Important bacterial growth reduction is observed already at 5 mM.

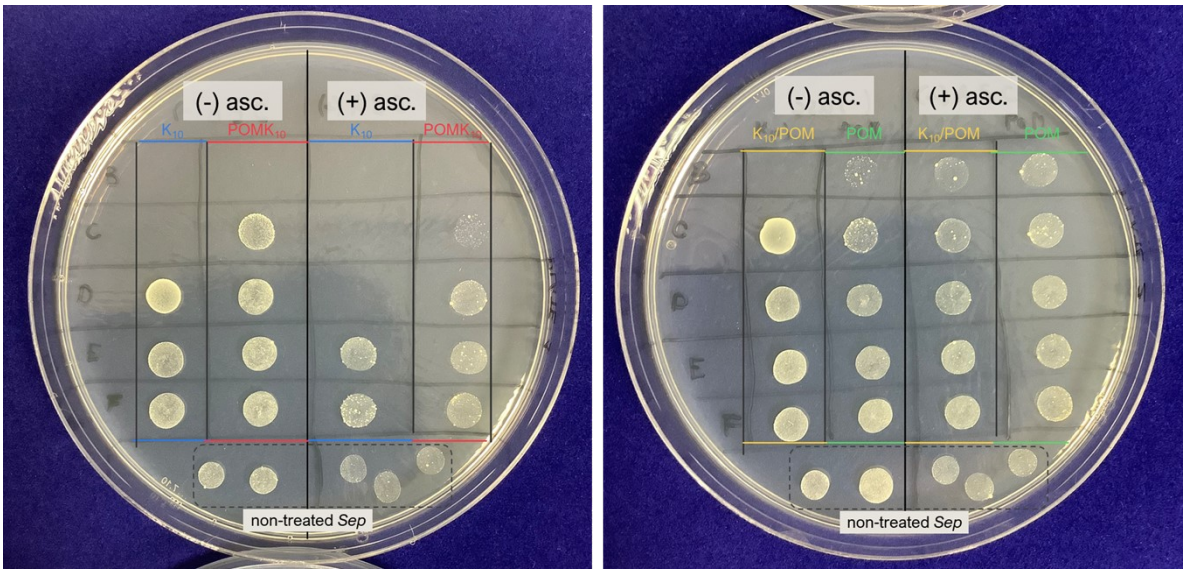


Fig. S 25. Verification of MBCs of K_{10}, $POMK_{10}$, K_{10}/POM and POM on agar plate, by seeding 10 µL of solution of each condition from a 96-wells plate on NB agar plate, with and without addition of ascorbic acid 2.5 mM.

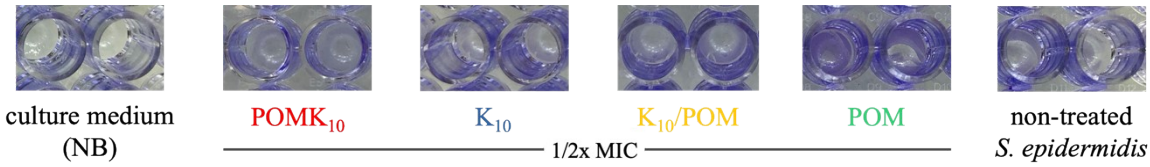
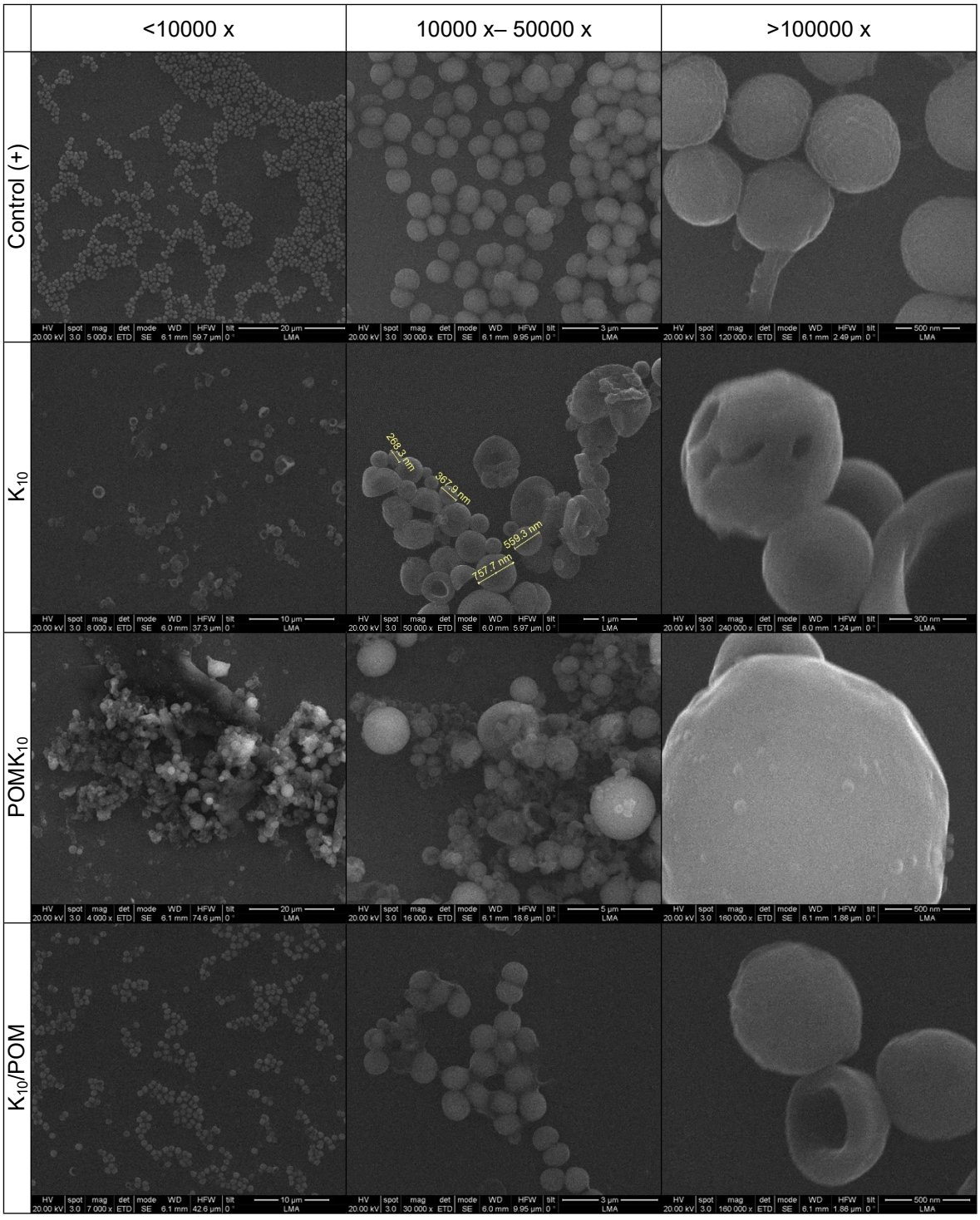
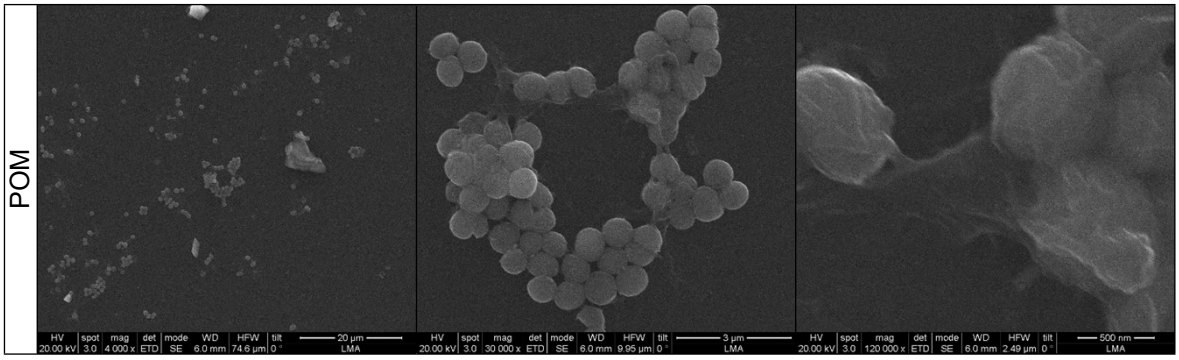


Fig. S 26. Crystal violet staining of S. epidermidis biofilm before solution in EtOH 96%.

Table S 1. Complementary SEM images of S. epidermidis untreated and treated with MIC of K_{10}, $POMK_{10}$, K_{10}/POM and POM.





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