

Sono-assembly of nanostructures by tyrosine-tyrosine coupling reaction at the interface of acoustic cavitation Bubbles

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Experimental Section

Materials

N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) ($\geq 98\%$), Lysozyme from hen egg white ($\geq 90\%$, ≈ 14.3 kDa), Bovine serum albumin (BSA) (99% , ≈ 66 kDa), DL-Dithiothreitol (DTT) ($\geq 98\%$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Methanol HPLC grade, Hydrogen Peroxide (30%), Iron(II) Chloride (98%), Trifluoro Acetic Acid ($\geq 99.0\%$) were purchased from Sigma-Aldrich.

Ultrasonic oxidation of BTTE and proteins

BTTE, tyrosine, lysozyme and BSA were sonicated using ELAC Nautik USW 51-052 transducer (transducer surface area 23.3 cm^2) connected to an amplifier LVG 60 A (ELAC) operated at 355/1056 kHz acoustic frequencies and 1.8 W cm^{-2} acoustic intensities (calorimetrically measured). All solutions were prepared using Milli-Q water (conductivity $<10^{-6}\text{ S cm}^{-1}$). The solutions (10 mL) were placed in the ultrasonic reaction vessel equipped with a cooling jacket to allow circulation of thermostated water ($37 \pm 2\text{ }^\circ\text{C}$). The pH of the solutions was kept at 4.5. Hydrogen peroxide concentration was determined using a standard

spectroscopic method, as previously reported.^[1] Under the experimental conditions used in this study, acoustic cavitation (growth of pre-existing gas bubbles followed by their violent collapse) is generated in the sonication medium. The collapse of the cavitation bubbles is near adiabatic and generates temperatures of thousands of degrees within the bubbles for a short period of time. Under this extreme temperature condition, highly reactive radicals are generated. If water saturated with air is the medium, the primary reaction step is the thermal decomposition of water vapour within imploding cavitation bubbles to generate $\cdot\text{OH}$ and $\cdot\text{H}$ primary radicals which on further reaction leads to the formation of hydrogen peroxide and superoxide. If water saturated with nitrogen is the medium only $\cdot\text{OH}$ and $\cdot\text{H}$ primary radicals and hydrogen peroxide are generated.

In order to study the effect of ultrasound on BTTE, tyrosine, lysozyme and BSA, 1 mM aqueous solutions were prepared. In the sonicated protein solutions, the actual concentration of tyrosine moieties was kept at 1 mM taking into account the number of tyrosine units per protein chain (3 for lysozyme and 19 for bovine serum albumin). BTTE oxidation via Fenton reaction was carried out as follows: 30 ml solution of 1 mM BTEE and 50 mg/mL FeCl_2 were prepared. Next, 0.1 mL of H_2O_2 (6 mM) solution was added at intervals of 10 minutes, to produce a radical concentration at the rate of 120 μM per hour.

The antioxidant properties of BTTE and BTTE nanoparticles were determined by using the colorimetric radical scavenging tests based on 2,2-diphenyl-1-picrylhydrazyl (DPPH).^{2,3} Briefly, a 250 μM DPPH solution was prepared by dissolving DPPH in methanol. BTTE or BTTE nanoparticles were dissolved in a mixture 50: 50 (v/v) ethanol:water, at a final concentration of 125 μM . 2 ml of DPPH solution was added to 4 mL of BTTE or BTTE nanoparticles solutions. The solution was kept under stirring for half an hour before measuring the absorbance data at 521 nm. Four samples were analysed: a blank, BTEE, BTEE NPs and ascorbic acid. All experiments were performed in triplicate. We calculate the antioxidant activity, AA, from:

$$\% \text{ AA} = [A_{30} - A_0]_{\text{antioxidant}} / [A_{30} - A_0]_{\text{ascorbic acid}}$$

where A_{30} is the absorbance at $t = 30$ minutes and A_0 is the absorbance at $t = 0$.

Characterisation of samples treated by high frequency ultrasound

Samples sonicated up to 3 hours were analysed by mass spectroscopy (MS), HPLC and fluorescence spectroscopy. The MS analyses were performed using an Agilent 6500 Q-TOF LC/MS system in positive ion mode. HPLC analyses were performed using a Shimadzu SCL-10AVP high-performance liquid chromatography (HPLC) equipped with a Phenomenex column model “Jupiter 5u C18 300A” and with an ultraviolet detector (UV) set at 205 nm. All chromatograms were generated by LabSolution software (Shimadzu). The injection volume was 20 μ L and, the flow rate was 0.8 mL/min with MeOH:H₂O:TFA (49.5:49.5:1) as the eluent. Absorption measurements were carried out on a Cary 100 SCAN (Varian, Palo Alto, CA) spectrophotometer. Fluorescence spectra were acquired using a Shimadzu RF-5301PC fluorescence spectrophotometer (Shimadzu) equipped with a xenon lamp and 1.0 cm optical length quartz cell. All the measurements were carried out after dilution in PBS (Phosphate Buffered Saline) solution to a final concentration of 16 μ M in terms of tyrosine moieties. Samples were excited at different wavelengths ranging from 320 to 380 nm, and the fluorescence spectra were recorded from 340 to 600 nm using a bandwidth of 5 nm for excitation slit and 10 nm for the emission slit. Excitation spectra of the samples were acquired at λ_{em} =400-480 nm and collected from 230 to 310 nm using a bandwidth of 10 nm for both excitation and emission slits. SEM images were acquired using a high-resolution field emission environmental Scanning Electron Microscope (Quanta 200 FEI) with gold sputter coating pre-treatment.

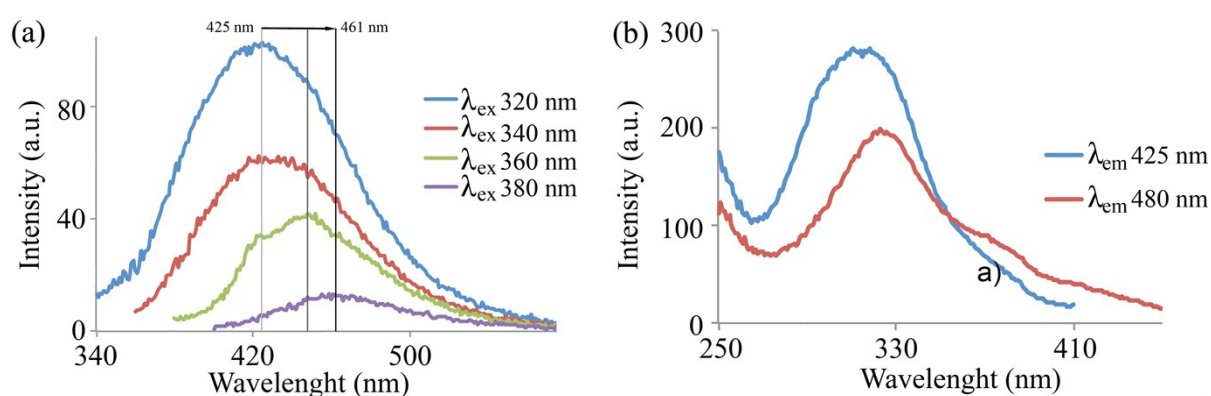


Figure S1: a) The dependence of emission spectrum on excitation wavelengths; b) Excitation spectra of BTEE dimerization products.

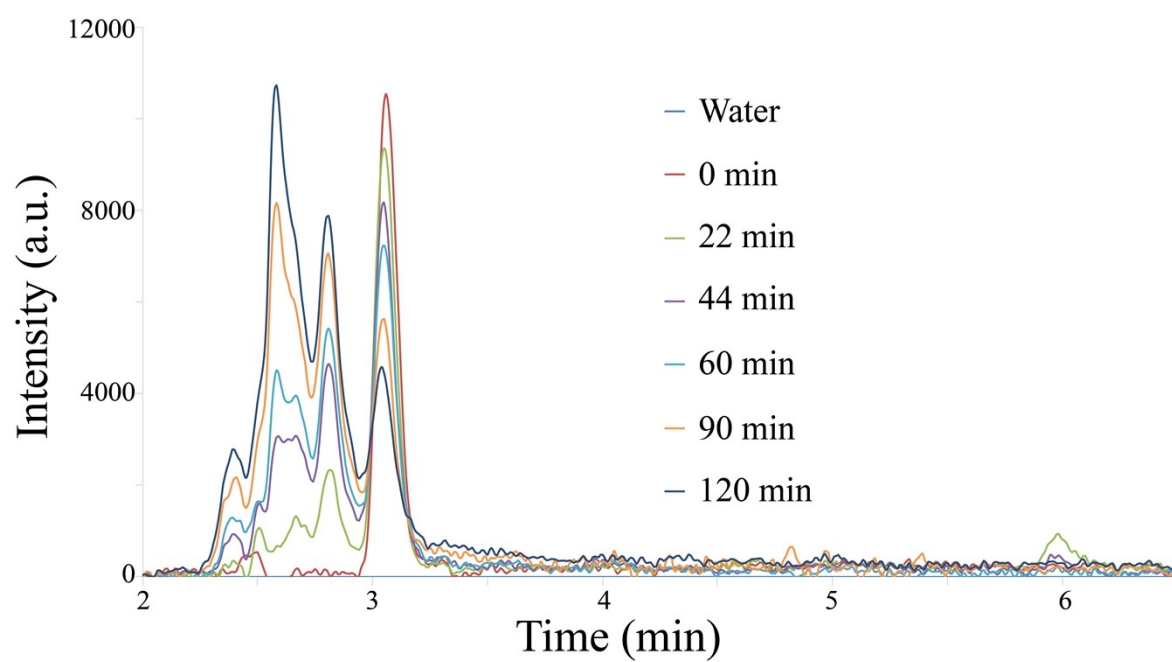


Figure S2: HPLC analysis of tyrosine sonicated at 355 kHz up to 2 hours

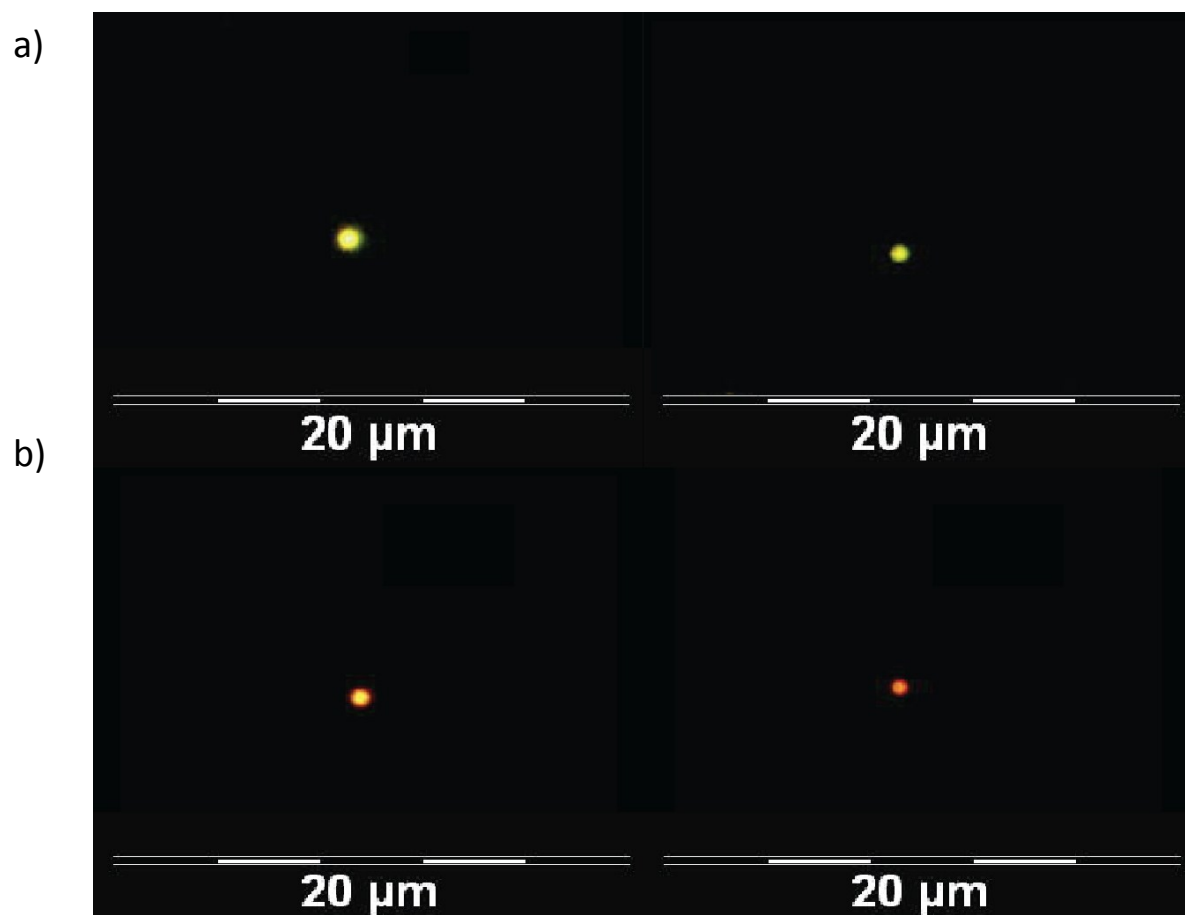


Figure S3: Photostability of a single nanoparticles after continuous irradiation for 10 min under microscope light using a) FITC filter and b) TRITC filter.

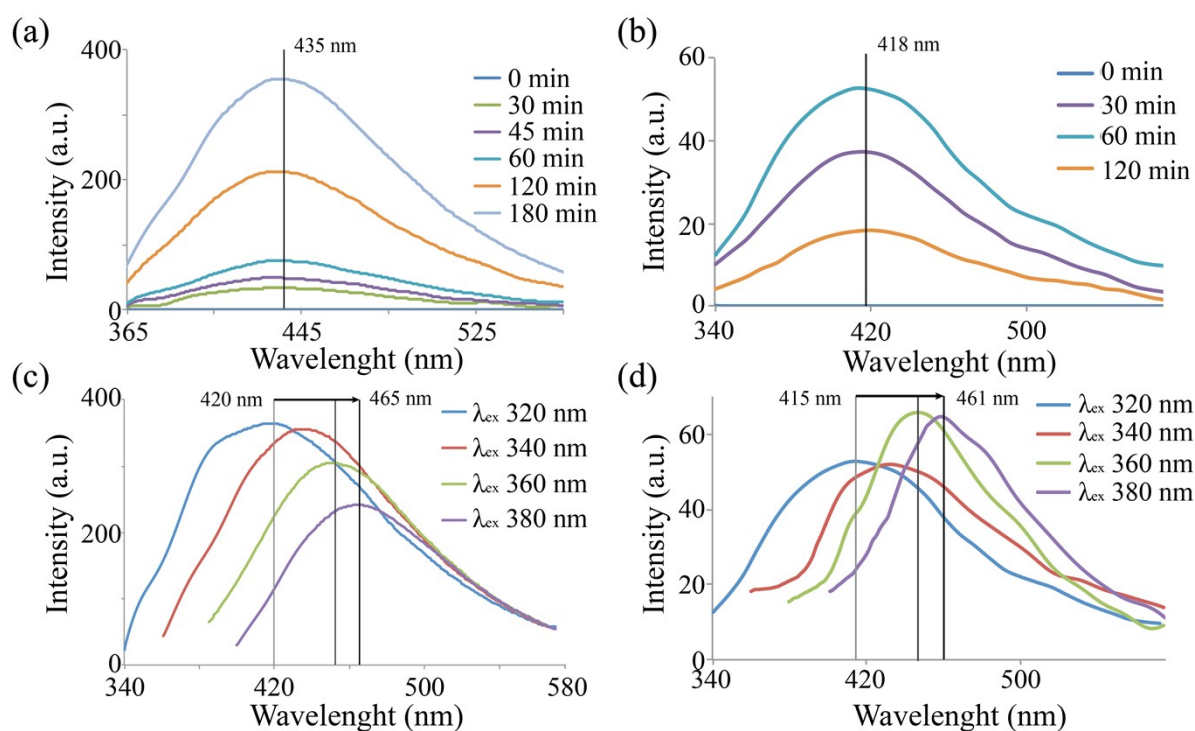


Figure S4: The fluorescence spectra of sonicated lysozyme (a,b) and BSA (c,d), measured in PBS pH 7.4 as a function of sonication time, show peaks centred at 435 nm and 418 nm, respectively. In both cases the peaks show an increase in intensity as a function of sonication time indicating the formation of dimeric species. Unlike BTTE, the peak is not red-shifted as a function of sonication time. However, a red shift in the emission peak is observed while changing the excitation wavelength (b,d). This suggests at least two fluorescent species are formed within the protein with a similar emission spectra namely DOPA-DOPA and Tyr-

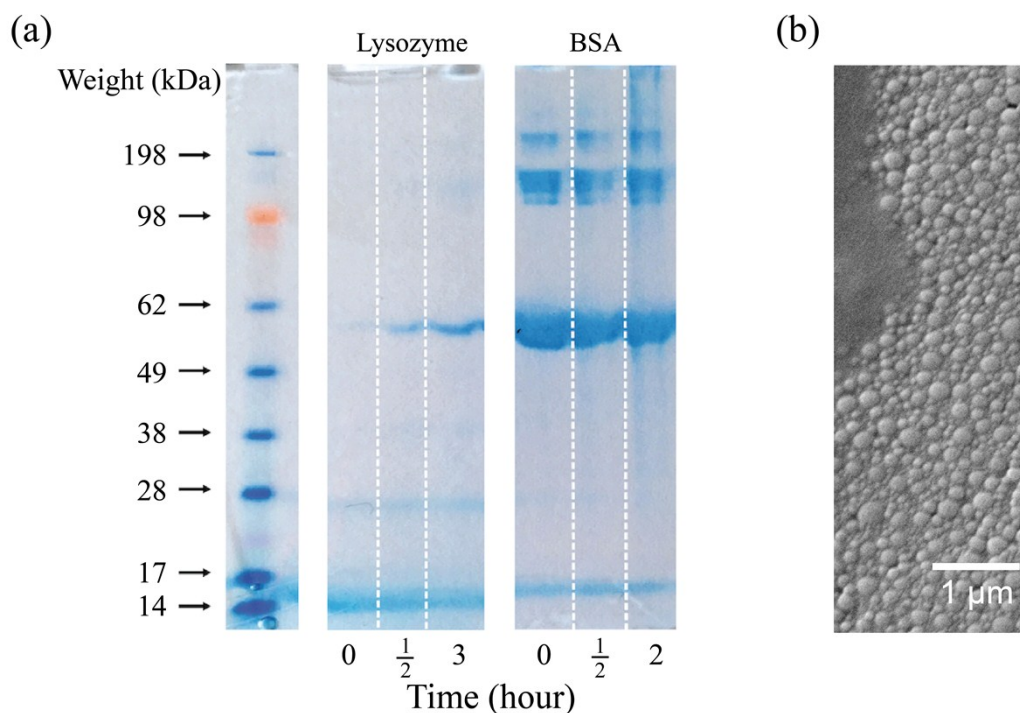


Figure S5: a) SDS PAGE gel of sonicated lysozyme and bovine serum albumin, b) SEM image of BSA nanoparticles crosslinked via Tyr-Tyr coupling.

References

- [1] L. Dharmarathne, M. Ashokkumar, F. Grieser, *J Phys Chem A* **2012**, *116*, 7775-7782.
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- [3] Saito, S., Kawabata, J., *Tetrahedron*, **2005**, *61*, 8101–8108.