Poly(Vinylamine) derived N-doped of C-dots with antimicrobial and anti-biofilm activities

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1. Blood compatibility analysis

Blood compatibility of 3:1, 1:1, 1:3 ratio of N-doped C-dots were investigated by hemolysis and blood clotting analysis according to the method proposed by Zamani et al. with some modification [30]. For the analysis, fresh blood was taken from the healthy volunteer by approval from Human Research Ethics Committee of Canakkale Onsekiz Mart University (011-KAEK-27/2020-E.2000045671) and placed into EDTA containing tubes immediately.

In the hemolysis process, 4 mL of fresh blood was diluted with 5 mL of 0.9 % NaCl aqueous solution. Separately, CA:PVAm C-dots suspension at 100 mg/mL concentration was prepared in 0.9 % NaCl aqueous solution and various amounts of this stock N-doped C-dots suspension from 100 µL to 5 µL was added to the 10 mL of 0.9 % NaCl solution containing tubes. Then, 200 µL of diluted blood was slowly added to these tubes to interact of blood with N-doped C-dots. These tubes were incubated at 37 °C for 1h and the C-dots-blood suspension was centrifuged at 100 g for 5 min to precipitated of healthy erythrocytes. The absorbance of supernatant was determined by using UV-Vis spectroscopy (T80+UV/Vis spectrometer, PG Instrument Ltd) at 542 nm wavelength to calculate hemolysis ratio of materials. All experiments were performed as three replicates. DI water and 0.9 % NaCl solution were used as positive and negative control groups and hemolysis ratio % was evaluated by the following Equation 2.

$$\text{Hemolysis ratio}\% = ((A_{\text{sample}} - A_{\text{negative control}})/(A_{\text{positive control}} - A_{\text{negative control}}))*100 \quad (2)$$

In the blood clotting analysis, N-doped C-dots suspension at 100 mg/mL concentration was prepared in 0.9 % NaCl aqueous solution and various amounts of this stock C-dot suspension from 100 µL to 5 µL was added to the flat bottom tubes. Then, 64.8 µL of 0.2 M $CaCl_2$ aqueous solution was added to 810 µL of fresh blood and immediately 270 µL of this was dropped on the C-dots suspension. After 10 min, 10 mL of DI water was added on the tubes slowly and tubes was centrifuged at 100 g for 1 min. Then, supernatant solution was slowly separated from the precipitated part and diluted with 40 mL of DI water in another tube. After 1 h, the absorbance of supernatant was determined by using UV-Vis spectroscopy at 542 nm wavelength. All experiments were performed three replicates. Only 270 µL of fresh blood in 50 mL of DI water was used as control group and blood clotting index was evaluated by the following Equation 3.

$$\text{Blood clotting index} = (A_{\text{sample}}/A_{\text{control}})*100 \quad (3)$$

2. Antimicrobial susceptibility of N-doped C-dots

Antibacterial activity of N-doped C-dots prepared at 3:1, 1:1, 1:3 ratio of CA:PVAm was evaluated by disc diffusion and microtiter broth dilution assays against *E. coli* ATCC 8739 and *P. aeruginosa* ATCC 10145 as gram-negative bacteria and *S. aureus* ATCC 6538 and *B. subtilis* ATCC 6633 as gram-positive bacteria based on the procedure described by Sun et al. with some modification [23]. Bacteria were cultured in NB liquid medium at 35 °C overnight and the concentration of bacteria was adjusted as nearly $0.5X10^8$ CFU/mL by using McFarland 0.5 standard. As an antimicrobial material, N-

doped C-dots suspension was prepared in 0.9 % NaCl aqueous solution at 100 mg/mL concentration and then sterilized by irradiation under photoreactor at 420 nm for 2 min before the analysis.

In the disc diffusion process, 100 µL of this bacteria suspension in the concentration of $0.5X10^8$ CFU/mL was inoculated on agar plate and then three peace of sterile filter discs at 9 mm dimension were placed on the plate. To each N-doped C-dots suspension, 50 µL of 100 mg/mL concentration were dropped on these discs. As a positive control, 20 µL of 1 mg/mL concentration of gentamicin in 0.9 % NaCl solution was used. The plates were incubated at 35 °C for 24 h. The diameter (mm) of the zone of inhibition was measured due to the transparent area around the filter discs.

Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values of N-doped C-dots was determined by microtiter broth dilution assay with a 96-well plate. Briefly, N-doped C-dots suspension was prepared in a concentration of 100 mg/mL in liquid medium of NB. Then, 100 µL aliquot of this N-doped C-dots suspension was two-fold serially diluted in liquid growth medium from 50 to 0.31 mg/mL concentrations with a final volume of 100 µL per well. For each well 5 µL of bacteria suspension in the concentration of $0.5X10^8$ CFU/mL was added to the wells and the plate was incubated at 35 °C for 24 h. Minimum inhibition concentration (MIC) value was defined as the lowest concentration of N-doped C-dots containing well which prevents the visible bacterial growth. Then, all transparent wells were inoculated on nutrient agar and minimum bactericidal concentration (MBC) value was defined as the lowest concentration which kills totally of bacteria. Gentamicin antibiotic was used as a positive control. All experiments were performed three replicates.

3. Biofilm assays

Biofilm biomass analysis by crystal violet (CV) staining was applied to determine of biofilm eradication and inhibition% of N-doped C-dots prepared at 1:3 ratio of CA:PVAm on *B. subtilis* and *E. coli* strains. These processes were performed according to the procedure proposed by Ran et al. with some modification [32].

In the biofilm eradication test, bacteria suspension was adjusted as nearly $0.5X10^8$ CFU/mL by dilution with rich growing medium as NB:RPMI (50:50, v:v) supplemented with 2 wt% glucose. These bacteria suspension, 200 µL for each well was placed into 96-well plate and incubated for 72 h at 35 °C to provide growing of biofilm bacteria. After the incubation, planktonic suspension cells were carefully removed from the well and each well was washed with 0.9 % NaCl solution at three times. Then, 100 µL aliquots of different concentration of N-doped C-dots from 50 mg/mL to 1.56 in liquid growth medium and 100 µL liquid growth medium was also added into the wells to interacted with attached maturated biofilm bacteria. The plate was incubated 35 °C for 24 h again and liquid parts was carefully taken from the wells. In this step, attached maturated cells was fixed on the plate by 200 µL of methanol treatment for 10 min and after that methanol was removed from the well and 200 µL of 0.01 wt% CV aqueous solution was added to stain the biofilm biomass. Then, CV solution removed from the wells and each well gently washed with 0.9 % NaCl solution again at three times. The plate was dried for 2 h at 35 °C and the biofilm was imaged by digital camera. To measure % biomass amount of biofilm, 200 µL of 33.3 vol% acetic acid

aqueous solution was added to the each well to dissolve of CV adsorbed from biofilm. The plate was measured by a microplate reader (Thermo, Multiskan Go) at 590 nm to calculate biofilm %. Bacteria was cultured for 72 h in the same condition as a control group. All experiments were performed three replicates.

In the biofilm inhibition assay, $0.5X10^8$ CFU/mL concentration of 100 µL of bacteria culture was inoculated for each well and 100 µL of CA:PVAm C-dots suspension in liquid growth medium at 50-1.56 mg/mL concentrations was added to the well plate. The plate was incubated 35 °C for 72 h and the planktonic suspension cells was carefully removed from the well and each well was washed with 0.9 % NaCl solution at three times. The attached maturated cells were fixed on the plate by 200 µL of methanol treatment for 10 min and after that methanol was removed from the well and 200 µL of 0.01 wt % CV aqueous solution was added to stain the biofilm biomass. Then, CV solution removed from the wells and each well gently washed with 0.9 % NaCl solution again at three times. The plate was dried for 2 h at 35 °C and the biofilm was imaged by digital camera. To measure % biomass amount of biofilm, 200 µL of 33.3 vol% acetic acid aqueous solution was added to the each well to dissolve of CV adsorbed from biofilm. The plate was measured by a microplate reader (Thermo, Multiskan Go) at 590 nm to calculate biofilm % after the inhibition. Bacteria was cultured for 72 h in the same condition as a control group. All experiments were performed three replicates.

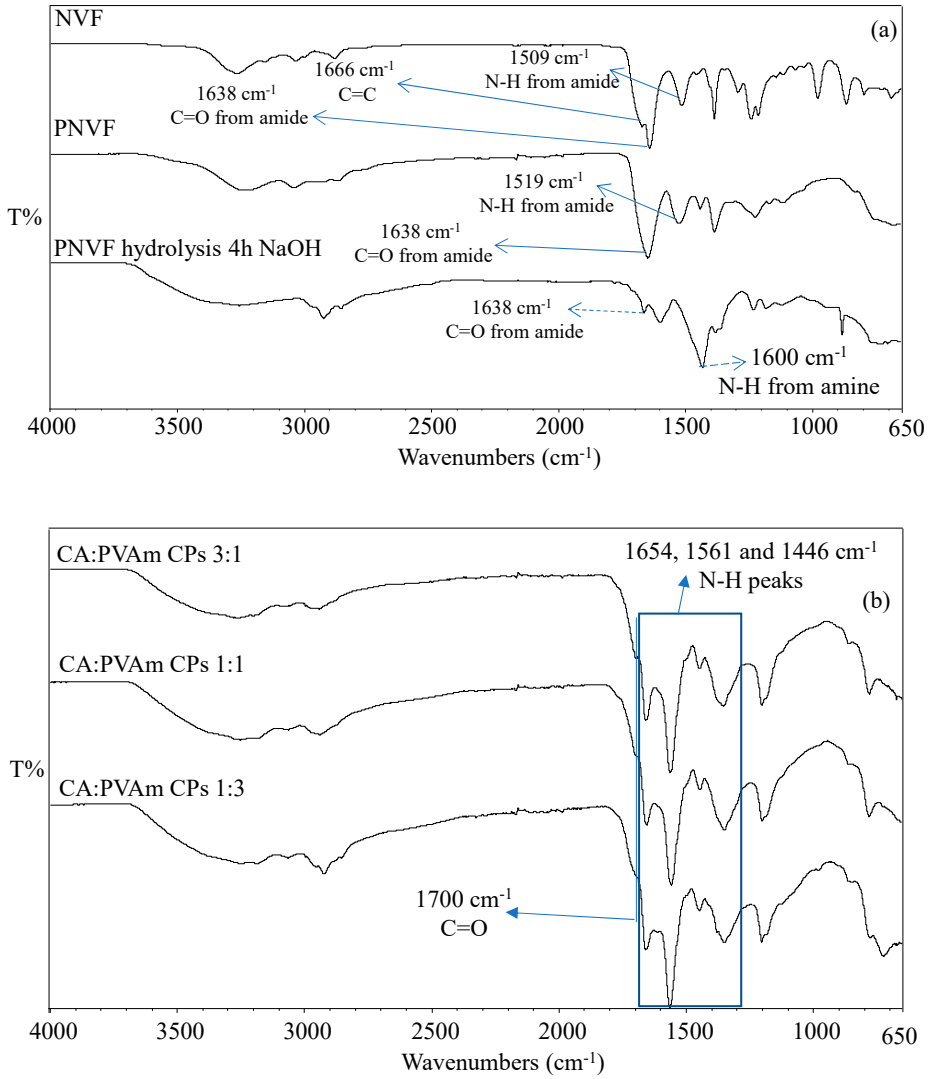

Figure S1. The FT-IR spectra of (a) NVF, PNVF, and PVAm molecules, and (b) CA:PVAm CPs in various ratios of CA and PVAm.

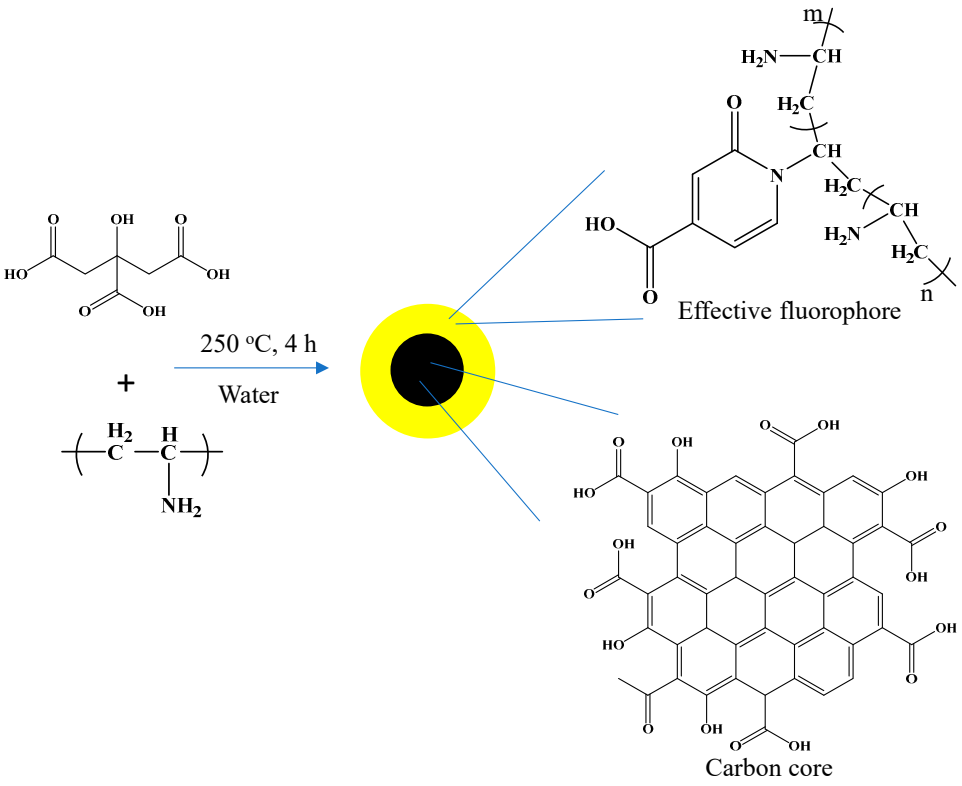

Figure S2. The possible mechanism of synthesis of CA:PVAm Cdots.

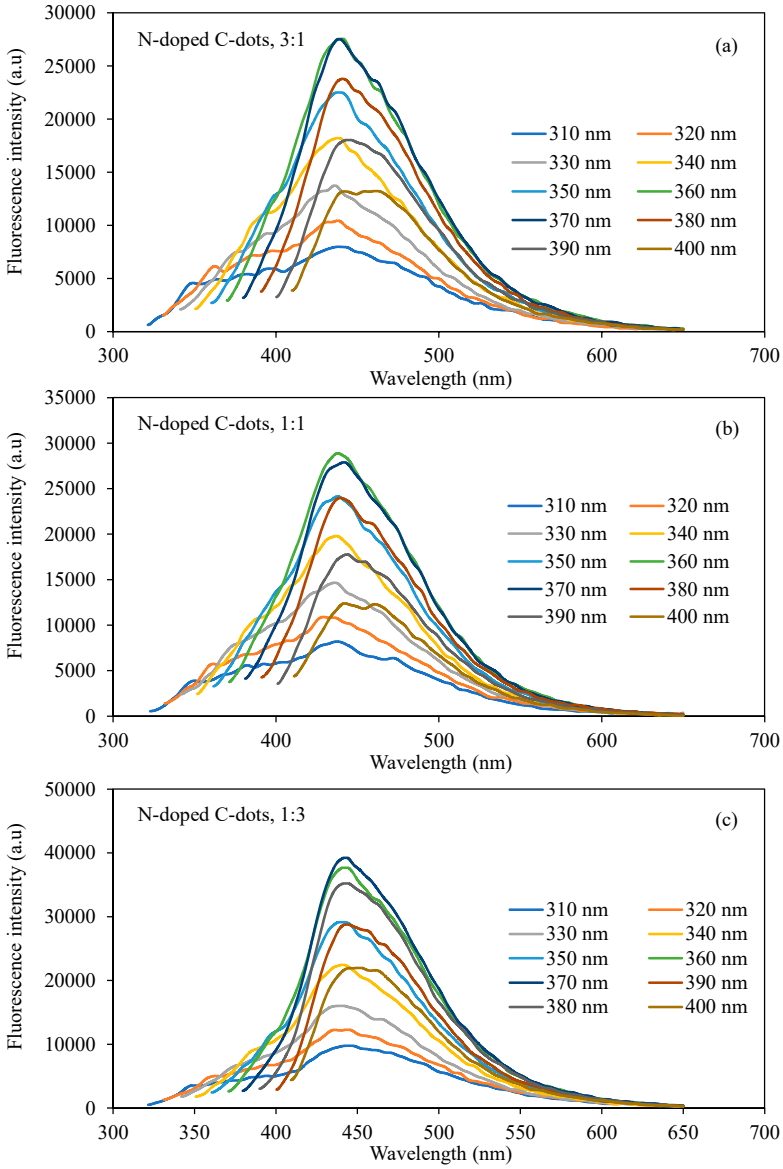

Figure S3. The fluorescence emission spectra of N-doped C-dots in prepared (a) 3:1, (b) 1:1, and (c) 1:3 w/w ratio of CA:PVAm at various excitation wavelengths.

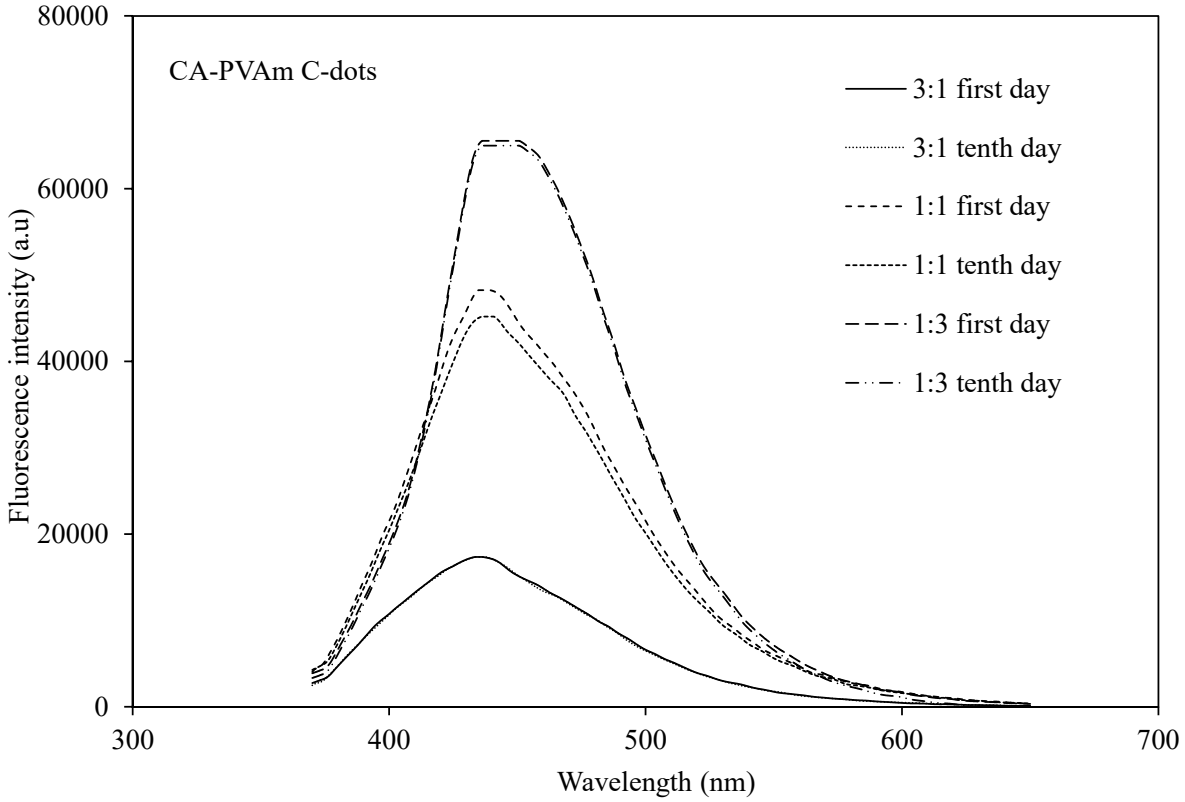

Figure S4. The fluorescence emission spectra of CA-PVAm Cdots at various ratio of CA-PVAm in pH 7.4 PBS solution at 360 nm excitation wavelengths.