Supplementary Materials

Formation of carotenoid supramolecular aggregates in nanocarriers monitored via aggregation-sensitive chiroptical output of enantiopure (3S,3'S)-astaxanthin

Aleksandra Orlef, [a] Ewa Stanek, [b] Krzysztof Czamara, [b] Aleksandra Wajda, [a] and Agnieszka Kaczor [*a, b]

[a.] *Faculty of Chemistry, Jagiellonian University, Gronostajowa 2, 30-387 Krakow, Poland.*
[b.] *Jagiellonian Centre for Experimental Therapeutics, Jagiellonian University, Bobrzynskiego 14, 30-348 Krakow, Poland.*

Content

1. Experimental

1.1. Preparation of micelles

1.1.1 Preparation of micelles

1.1.2 Preparation of samples with the carotenoid

1.2. Preparation of adipocytes

1.3. Spectral and microscopic measurements

1.3.1 Electronic Circular Dichroism

1.3.2. Raman Optical Activity

1.3.3. Raman Imaging

1.3.4. Transmission Electron Microscopy

2. Figures

Fig. S1. UV-vis spectra of AXT in DMSO:water solution (**A**, 1:49) and AXT in micelles (**B**: c_{AXT} = 100 μM, c_m = 1 mM) freshly prepared and after stabilization in time.

Fig. S2. ECD and UV-vis spectra of AXT in DMSO:water solution (**A,** 1:200) and AXT in micelles (**B**: c_{AXT} = 10 μM, c_m = 1 mM) freshly prepared and after stabilization in time.

Fig. S3. Evolution of the ECD (**A**) and UV-vis (**B**) signal of AXT in micelles (**B**: c_{AXT} = 100 μM, c_m = 1 mM) during 24 hours after system preparation.

Fig. S4. UV-vis spectra of AXT in micelles during 'feeding' of nanoparticles with the carotenoid i.e. increasing daily the concentration of AXT up to 100 μM, c_m = 1 mM.

Fig. S5. Anisotropy spectra of AXT in micelles during 'feeding' of nanoparticles with the carotenoid i.e. increasing daily the concentration of AXT up to 100 μM, c_m = 1 mM.

Fig. S6. Stability of *J*-aggregates of AXT in micelles at room temperature (**A**) and spectral changes due to thermal treatment of the *J*-aggregates of AXT in micelles (**B**) (c_{AXT} = 100 μM, c_m= 1 mM) – UV-VIS spectra.

Fig. S7. Comparison of resonance Raman and ROA spectra of J_m and J'_m aggregates.

1. Experimental

1.1. Preparation of micelles

1.1.1. Preparation of micelles:

Pluronic F-127 forms spontaneously spherical micelles in an aqueous solution at the CMC (critical micelle concentration), which is 0.7 w/v % at 25°C [A. M. Bodratti, P. Alexandridis, Formulation of Poloxamers for Drug Delivery, *J. Funct. Biomater.*, 2018, 9, 11]. Pluronic F-127 was purchased from Sigma-Aldrich and used as received. To prepare micelle solutions, the required amount of copolymer was dissolved in ultrapure water and vigorously stirred using a magnetic stirrer for 30 minutes at room temperature. The final concentration of Pluronic F-127 solutions were 1 mM (1.3 w/v %).

1.1.2. Preparation of samples with the carotenoid:

(3*S*,3'*S*)-astaxanthin (AXT) was obtained from BASF, Germany. To prepare the AXT stock solution, AXT was dissolved in DMSO to obtain a final concentration of 5 mM. Then, 80 µL of the AXT stock solution was added to 3.920 mL of micelles solutions. In each sample, the AXT concentration was 100 µM and the micelles concentration was 1 mM. In addition, 80 µL of the AXT stock solution was dissolved in 3.920 ml of ultrapure water to obtain the reference sample. Additionally, samples with a lower concentration of AXT (10 µM) were prepared in the same way. All samples were stored at room temperatures and protected from light.

1.2. Preparation of adipocytes:

Epididymal white adipose tissue (eWAT) was isolated from 26 weeks-old C57Bl/6J mice (*n* = 4, Medical University of Warsaw, Warsaw, Poland), minced and then kept for 1h in the enzymatic solution consisting of 3.5 mg/mL collagenase D (Roche Holding AG, Basel, Switzerland), 2% bovine serum albumin (BSA, Sigma), and 150 µM $CaCl_2$ dissolved in PBS. Digestion was conducted at 37°C with gently shaking every 10 minutes then tissue residues were filtrated through a 100 µm nylon cell strainer. After that, floating primary adipocytes were resuspended in a medium (DMEM:F12 containing 20% FBS, 8 µg/mL biotin, and 50 µg/mL gentamycin) containing 10 µM of AXT in DMSO or 1 mM micelles with 100 µM of AXT diluted to final concentration of 10 µM of AXT. Overall 24 h stimulation process was maintained at 37 °C/5% CO_2 in a cell culture incubator. To perform Raman imaging cells were put between CaF_2 microscope slide and the coverslip and measured in chosen time points. Within each group at least 4 representative cells were measured.

1.3. Spectral and microscopic measurements

1.3.1. Electronic Circular Dichroism

UV-vis and ECD spectra were measured in quartz cells with a path length of 1 cm, in the 300-600 nm spectral range using the Jasco J-815 spectrometer. The spectra were recorded using 100 nm/min scanning speed, 2 nm bandwidth, and 0,5 nm data pitch, by averaging 3 scans. All spectral measurements were made (or started) 1 hour after sample preparation and on the following days. A JASCO Peltier cell holder PFD-425S with thermostatic bath Julabo F25-HE was used for heating/cooling the sample during measurements to investigate the effect of thermal treatment.

To analyze UV-vis and ECD spectra, JASCO version 1.52.00 [Build 4] Copyright JASCO Corporation 1995-2000, OPUS 7.2 [Build: 7, 2, 139, 1294] Copyright Bruker Optik GmbH 2012, and OriginPro 2022 9.9.0.220 (Academic) Copyright OriginLab Corporation 1991-2021 softwares were used. The spectra were background-corrected using spectra of micelles solution or water recorded under the same conditions. Additionally, the spectra were smoothed using the Savitzky-Golay (number of points: 25, polynomial order: 3).

1.3.2. Raman Optical Activity

Resonance Raman and Raman Optical Activity (RROA) spectra were measured in ROA optical cells with anti-reflective coating with dimensions of 3x4 mm, in the 250-2550 cm^{-1} spectral range using ChiramRAMAN-2X^{TM} spectrometer with a 532 nm excitation wavelength and 7 cm^{-1} spectral resolution. Laser power of 24 mW, integration time of 3.0215 s, and 2 h acquisition time were used for ROA/Raman measurements. The resonance Raman and ROA spectra were collected and saved in 5 min blocks. The final spectra were averaged over all blocks. ROA/Raman measurements were acquired 1 hour and 48 hours after the preparation of the samples.

To analyse resonance Raman and ROA spectra OriginPro 2022 9.9.0.220 (Academic) Copyright OriginLab Corporation 1991-2021 was used.

1.3.3. Raman Imaging

Raman imaging was carried out with the use of the confocal Raman microscope (WITec alpha300, Ulm, Germany) equipped with a 532 nm laser, a UHTS 300 spectrograph (600 grooves·mm−1 grating), and a CCD detector (DU401A-BV-352, Andor, UK). Raman spectra were acquired with a 0.2 s exposure time per spectrum using low power (*ca.* 3 mW) at each sample to avoid bleaching the AXT signal. Primary adipocytes were measured on CaF_2 glass slides under the coverslip using a 20× air objective (NA = 0.45, Nikon CFI S Plan Fluor ELWD, Japan).

Preprocessing of obtained spectra was conducted *via* the WITec Project Plus software, with baseline correction using autopolynomial of degree 3. Average spectra gathered from whole adipocytes were normalized using vector normalization 1800–400 cm^{-1} spectral range

with the OPUS 7.2 program. Raman distribution images of the signal of the cells (excluding their surroundings) was obtained by integration of the 3030-2830 and 1505-1535 cm^{-1} range for lipids and AXT, respectively.

For analysis of the kinetics of delivery, the band at 1518 cm^{-1} was integrated in the 1496.5-1546.4 cm^{-1} range with the OPUS 7.2 program after background removal (option B).

1.3.4. Transmission Electron Microscopy

Before microscopic analysis, the samples were diluted tenfold. Then, they were dropped on a carbon film supported on a copper grid (Lacey Carbon Film 300 Mesh Cu (Qty 25) from Agar Scientific). The grids with the samples were then counterstained with UranyLess staining solution (UranyLess EM Stain, 30 ml Airless bottle). TEM observations were performed using an analytical transmission electron microscope (FEI TecnaiOsiris). The camera was length in the range of 50−100 mm.

To analyze TEM micrographs, we used Gatan's DigitalMicrograph application with the Gatan Microscopy Suite (GMS) software suite.

TOC was created with BioRender.com.

2. Figures

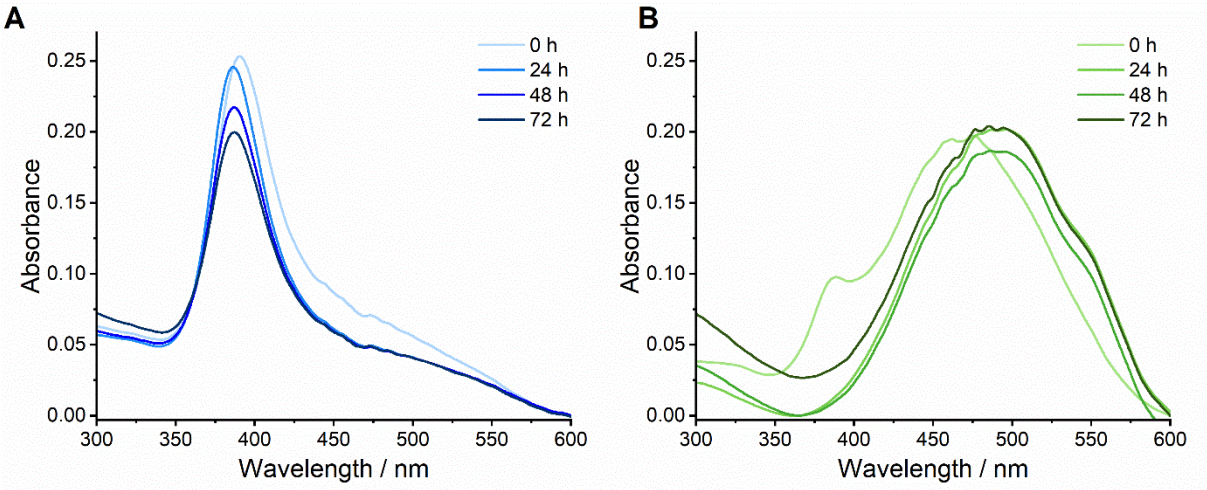

Fig. S1. UV-vis spectra of AXT in DMSO:water solution (**A,** 1:49) and AXT in micelles (**B**: c_{AXT} = 100 μM, c_m = 1 mM) freshly prepared and after stabilization in time.

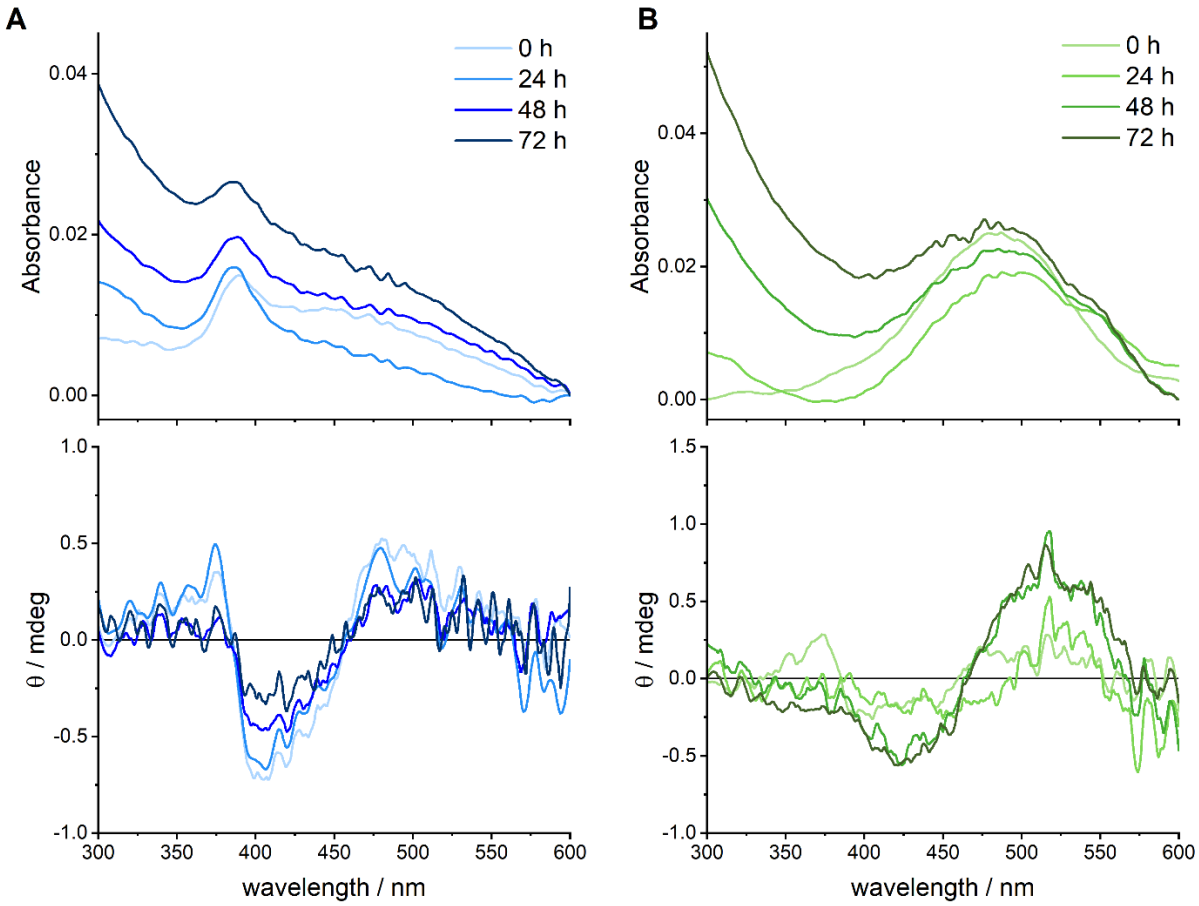

Fig. S2. ECD and UV-vis spectra of AXT in DMSO:water solution (**A,** 1:200) and AXT in micelles (**B**: c_{AXT} = 10 μM, c_m = 1 mM) freshly prepared and after stabilization in time.

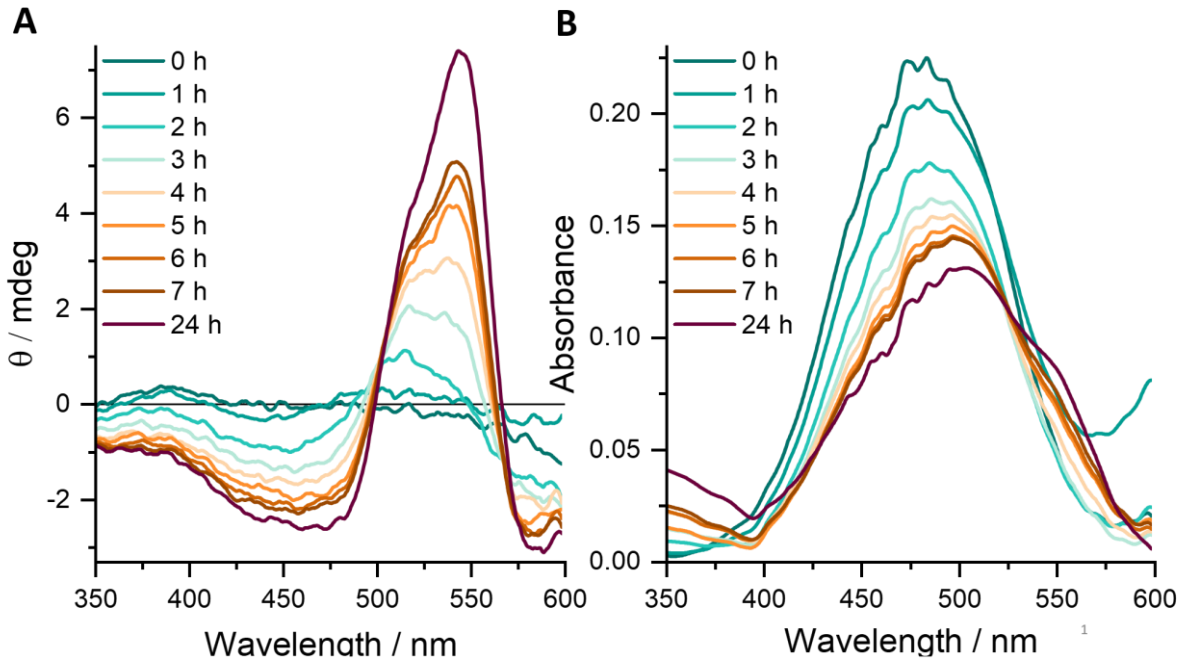

Fig. S3. Evolution of the ECD (**A**) and UV-vis (**B**) signal of AXT in micelles (**B**: c_{AXT} = 100 μM, c_m = 1 mM) during 24 hours after system preparation.

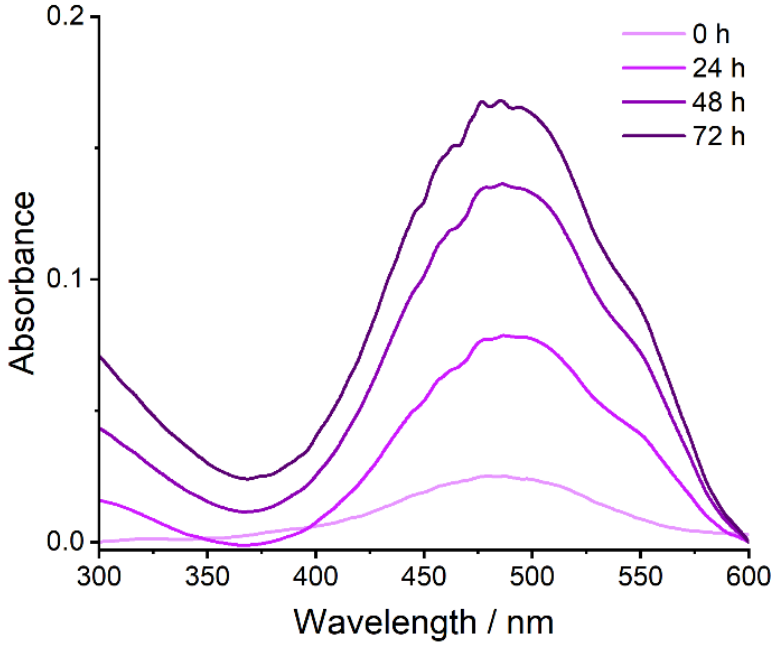

Fig. S4. UV-vis spectra of AXT in micelles during 'feeding' of nanoparticles with the carotenoid i.e. increasing daily the concentration of AXT up to 100 µM, c_m = 1 mM.

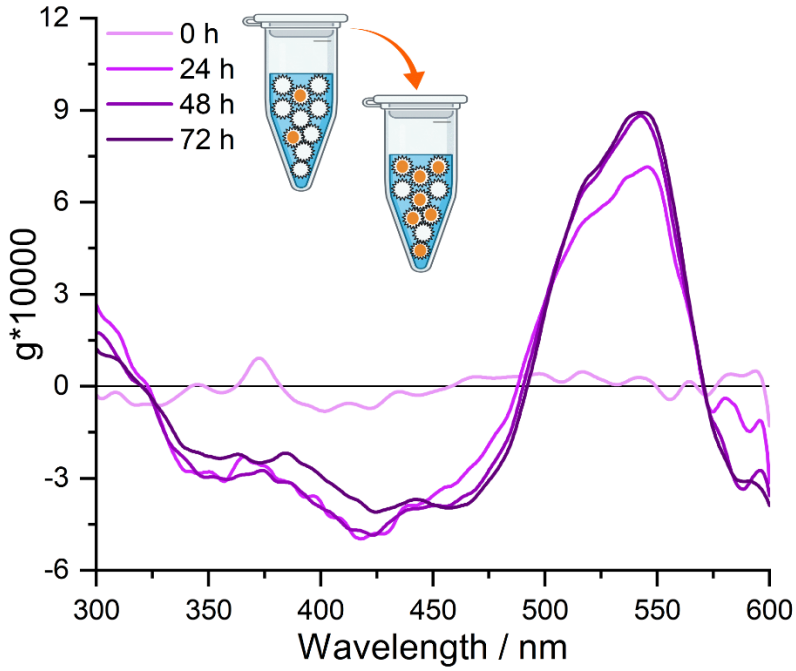

Fig. S5. Anisotropy spectra of AXT in micelles during 'feeding' of nanoparticles with the carotenoid i.e. increasing daily the concentration of AXT up to 100 µM, c_m = 1 mM.

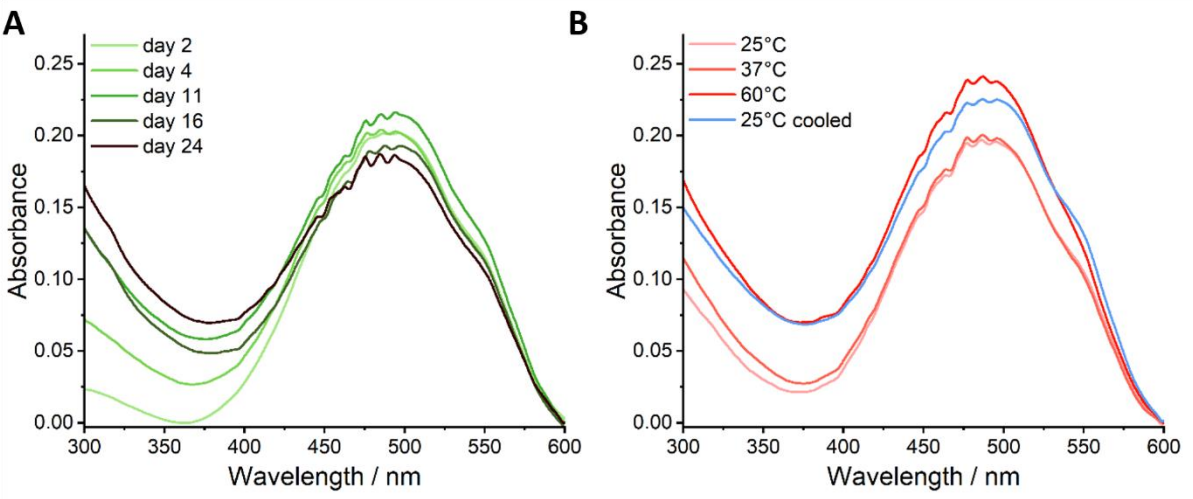

Fig. S6. Stability of *J*-aggregates of AXT in micelles at room temperature (**A**) and spectral changes due to thermal treatment of the system (**B**) (c_{AXT} = 100 μM, c_m= 1 mM) – UV-VIS spectra.

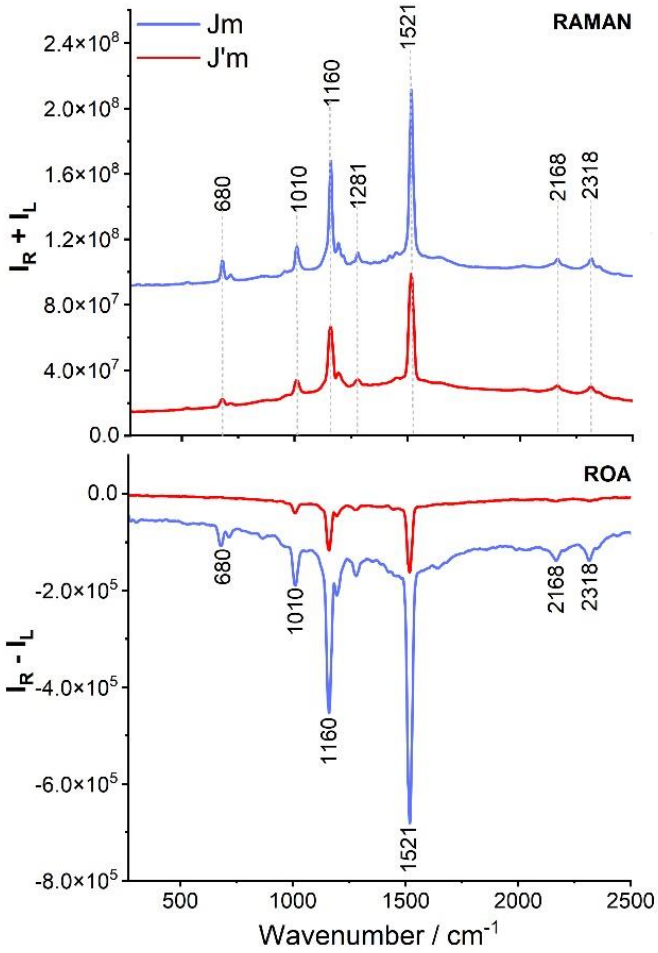

Fig. S7. Comparison of resonance Raman and ROA spectra of J_m and J'_m aggregates.