Fucoidan/bis-MPA-based dendrimer nanoparticles with intrinsic anti-angiogenic activity for oncology applications

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Materials and Methods

Origin, extraction, characterization and hydrolysis of fucoidan

High purity fucoidan from two species of brown seaweeds, *Fucus vesiculosus* (bladderwrack, wild seaweed harvested at Nova Scotia in Canada) and *Undaria pinnatifida* (wakame, wild seaweed harvested at Australian coast), was used in the studies (Marinova Pty Ltd, Australia). A mild, organic-solvent free, aqueous process was used in fucoidan extraction, followed by filtration, concentration and dehydration steps, according to a proprietary procedure. The resulting powders were analysed for total carbohydrate via the phenol-sulfuric method,[1] uronic acid via a colorimetric assay,[2] polyphenol via the Folin-Ciocalteu assay,[3] sulfate via the barium sulfate turbidimetric assay[4] and cations content via inductively coupled plasma mass spectrometry (ICP-MS), as well as concerning their carbohydrate profile via gas chromatography with flame ionization detection (GC-FID) of fucoidan monosaccharides derivatised to alditol acetates.[5] Native molecular weight fucoidan from *F. vesiculosus* (FVF, peak molecular weight 51.1 kDa, batch number DPFVF2017505) and native molecular weight fucoidan from *U. pinnatifida* (UPF, peak molecular weight 84.3 kDa, batch number DPGFS2018515) were compared to fractionated fucoidan from *F. vesiculosus* (FVF-F with a peak molecular weight of 20.7 kDa, batch number SK1601013A) and *U. pinnatifida* (UPF-F with peak molecular weight 8.2 kDa, batch number SK1101084B). The peak molecular weight of the fucoidans was assessed by HPLC-SEC-RI using 0.1 M LiCl on a Shodex SB-806M HQ column. Molecular weight was calculated based on elution time of the peak molecular weight compared to a dextran calibration curve. NOTES: Since native fucoidans are polydisperse, all experiments were consistently performed using the same batches; moreover, native and fractionated fucoidans from the same species originated from different extraction batches.

Dendrimers

Biodegradable dendrimers based on the 2,2-bis(hydroxymethyl)propionic acid monomer (bis-MPA based dendrimers) were acquired to Polymer Factory (Sweden). The dendrimers were synthetized using a trimethylol propane core and presented ammonium functional end-groups. Generation 2 dendrimers were used which presented 12 ammonium surface groups (associated to 12 trifluoroacetate counterions) and 2044.3 Da as molecular weight (>95% based on NMR analysis, >97% based on MALDI-TOF analysis).

Preparation and characterization of fucoidan/dendrimer nanoparticles

Following an optimization study, fucoidan/dendrimer nanoparticles were prepared at 1:2 and 2:1 fucoidan/dendrimer (F/D) mass ratios. For the 1:2 ratio, 1 mL of a 1 mg/mL fucoidan solution was mixed with 1 mL of a 2 mg/mL dendrimer solution (corresponding to a F/D molar ratio of 0.033 and 0.020 when using fucoidan from *F. vesiculosus* and *U. pinnatifida*, respectively). For the 2:1 ratio, 1 mL of a 2 mg/mL fucoidan solution was mixed with 1 mL of a 1 mg/mL dendrimer solution (corresponding to a F/D molar ratio of 0.133 and 0.081 when using fucoidan from *F. vesiculosus* and *U. pinnatifida*, respectively). After mixing, the solutions were ultrasonicated for 1 min at constant magnetic stirring. All solutions were prepared in distilled water.

The hydrodynamic diameter of the nanoparticles was measured, at 25ºC, by dynamic light scattering (DLS), using a 633 nm laser beam and a detection angle of 173° in a Zetasizer Nano ZS equipment (Malvern Instruments). For the size distribution analysis, the cumulants method was used to calculate the mean particle size (Z-average) and the polydispersity index (PDI). Zeta potential measurements were performed by electrophoretic light scattering (ELS) using the same instrument and a detection angle of 17°. The *Smoluchowsky* model for aqueous suspensions was applied to convert the electrophoretic mobility into zeta potential values. The data presented are means of three independent sample measurements.

Nanoparticle´s morphology and size were assessed by transmission electron microscopy (TEM). Nanoparticles were prepared as previously described and the obtained solution was dropped onto a 200-mesh carbon-coated copper grid. After solvent evaporation at room temperature, the grid was treated with uranyl acetate staining solution to provide better contrast in the image. The samples were then imaged under high vacuum conditions at an accelerating voltage of 80-120 kV in a Jeol JEM 1400 electron microscopy equipment.

Lyophilized nanoparticles were also analysed by Fourier Transform Infrared spectroscopy with Attenuated Reflectance Module (FTIR-ATR) using a Spectrum Two (Perkin Elmer) equipment connected with an ATR apparatus with a diamond crystal (UATR Two, Perkin Elmer). For freeze-drying, the solutions containing the nanoparticles were frozen at -80 °C and lyophilized for three days using a Labconco® FreeZone 4.5L freeze-dryer.

Cytotoxicity studies of fucoidan and fucoidan/dendrimer nanoparticles using HUVEC cells

Human Umbilical Vein Endothelial Cells (HUVEC, Gibco) were cultured in Medium 200 (Gibco) supplemented with 1x Large Vessel Endothelial Supplement (LVES, Gibco, containing contains foetal bovine serum, hydrocortisone, human epidermal growth factor, basic fibroblast growth factor, heparin, and ascorbic acid) and 1% Antibiotic-Antimycotic (Gibco). This supplemented culture medium will be simply referred to as culture medium throughout this work. HUVEC cells were always incubated in a humified incubator at 37ºC and 5% CO_2 during all the experiments.

For cytotoxicity evaluation of the four types of fucoidan, cells were previously seeded in 96 well-plates (VWR) at a density of 5000 cells/well and incubated for 24h. Then, media were substituted by fucoidan solutions prepared in fresh cell culture medium at the final concentration in the range of 10 to 1000 μg/mL (8 wells for each concentration). The cells were then incubated for more 24h, after which metabolic activity was assessed by the rezasurin reduction assay. The cytotoxicity experiments of the fucoidan/dendrimer nanoparticles were performed in a similar manner. In this case, experiments were based on the final fucoidan concentration in the cell culture media and a lower range of fucoidan concentrations (10 to 50 μg/mL) was tested when compared with the experiments with fucoidan alone. Positive (0.1% (v/v) Triton-X-100 solution prepared in cell culture medium, Sigma-Aldrich) and negative (only cell culture medium) controls were used in both types of experiments. Control experiments were also performed with the bis-MPA based dendrimers alone at the concentrations present in the tested solutions containing the nanoparticles.

For the rezasurin reduction assay, the medium in each well was replaced by 200 μL of fresh medium containing resazurin (Sigma-Aldrich) at a final concentration of 0.01mg/mL. The plates were then incubated for 4h, after which 100 μL of medium from each well was transferred to a white opaque plate (VWR). Fluorescence measurement (λ_{ex}=530nm/λ_{em}=590nm) was done using a microplate reader (Victor^3 1420, PerkinElmer). Results are presented as a percentage of those obtained for the negative control (cells cultured only in cell culture medium).

Anti-angiogenic activity evaluation of fucoidan and fucoidan/dendrimer nanoparticles

The tube formation assay was used to assess the anti-angiogenic activity of normal molecular weight and hydrolysed fucoidan molecules, as well as of fucoidan/dendrimer nanoparticles. Fucoidans' anti-angiogenic activity was evaluated at a final concentration in the wells of 100, 500, and 1000 μg/mL, whereas experiments with nanoparticles used 10 and 50 μg/mL (values based on fucoidan

concentration). Briefly, 50 µL of Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Gibco) were added per well in a 96 well-plate (pre-chilled overnight) and then incubated for 30 min at 37ºC. After Geltrex™ gelling, HUVEC cells were seeded at the density of 10,000 cells/well with cell culture medium containing the solutions to be tested. The cells were then incubated for 6h in a humified incubator at 37ºC and 5% CO_2. After this period, cells were labelled with a solution of calcein-AM (Sigma-Aldrich) prepared in *Dulbecco*'s phosphate buffered saline solution (DPBS, Sigma-Aldrich, without calcium and magnesium) and incubated for another 30 min. The final concentration of calcein-AM in solution was 2 µg/mL. Then, cells were washed with DPBS, and samples were visualized in an epifluorescence inverted microscope (Nikon Eclipse TE2000-E). The control consisted of cells in Geltrex™ incubated only with cell culture medium. The raw data were first treated with ImageJ for contrast adjustment and background subtraction.[6,7] Then, the treated images were quantitatively analysed using the AngioTool software[8] to obtain data regarding vessel area, number of junctions and lacunarity (results were normalized to the corresponding control value). In case of the fucoidans, the medium supernatants were collected, and angiopoietin-2 (Ang-2) was also quantified using an ELISA kit (Human Angiopoietin 2 ELISA Kit, Invitrogen) according to the manufacturer's instructions.

Evaluation of the hemocompatibility of the fucoidan/dendrimer nanoparticles

Haemolysis and coagulation assays used fresh human blood and a pool of citrated human plasma, respectively. These samples were collected from healthy donors at the Funchal Central Hospital with authorization from the Local Ethics Commission (anonymous samples with individuals' consent). The human plasma was frozen until use.

A modification of the cyanmethemoglobin assay was used for haemolysis assessment.[9] First, C reagent (cyanmethemoglobin reagent) was prepared with 50 mg potassium ferricyanide, 12.5 mg potassium cyanide, and 35 mg potassium dihydrogen phosphate (all from Sigma-Aldrich) in 250 ml of distilled water. Reagent C was prepared in an amber bottle with 250 µl Triton-X (Sigma-Aldrich), and its pH was adjusted to 7.4. For evaluation of the extent of haemolysis caused by the fucoidan/dendrimer nanoparticles, 10 µL of diluted human blood (10% (v/v) in Mg^{2+}/Ca^{2+} free DPBS), was distributed by several microcentrifuge tubes. After, 70 µL of each test solution (previously prepared in 0.9 % (w/v) NaCl at concentrations of 10, 25 and 50 µg/mL, being these values based on fucoidan concentration) and the controls (DPBS as negative control and water as positive control) were added to the corresponding tube (the assay was done in triplicate for each situation). After incubation

at 37°C for 3 h, the tubes were centrifuged at 3800 rpm for 5 min. The supernatants (40 µL) were transferred to clear microplates, 160 µL of reagent C was added to each well and the absorbance was measured at 550 nm. Results are expressed as the percentage of haemolysis relative to the water-treated blood (control), which represents 100% haemolysis.

For the coagulation assay,[10] 50 µL of each nanoparticle solution previously prepared in 0.9 % (w/v) NaCl were added to the wells of a clear 96-well plate (in triplicate) at the concentration of 50 µg/mL (based on fucoidan concentration). Then, 100 µL of thawed pooled human plasma was aliquoted to each well. To initiate the process of coagulation, 50 µL of calcium chloride (final concentration of 20 mM) was added to each well, and the absorbance was monitored each minute in a microplate reader at 405 nm (Victor3 1420, PerkinElmer) until a plateau was reached. The time necessary to reach the plateau was considered the clotting time. Controls were performed without the presence of calcium chloride and using only a 0.9 % (w/v) NaCl solution (the reference control).

Statistical analysis

Statistical analyses were performed using RStudio software. Data are presented as mean ± standard deviation (SD). Independent Student's t-test, one-way analysis of variance (ANOVA), or two-way ANOVA were used for statistical analysis, followed by Bonferroni or Tukey post-hoc tests to evaluate the significance of the experimental data. The significance level was set at 0.05, and p-values are indicated accordingly.

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FTIR SPECTRA

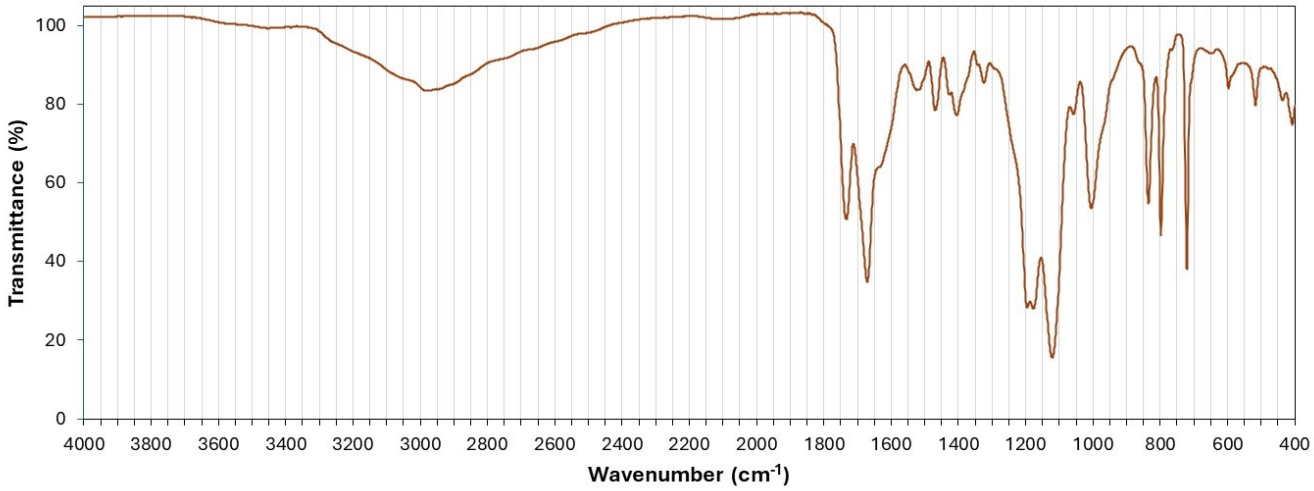

Figure S1. FTIR spectrum of the bis-MPA dendrimer.

Table S1. Interpretation of the FTIR spectrum from the bis-MPA dendrimer.

Wavenumber (cm^{-1})	**Assignment**	**Comment**
3200 - 3400	N-H stretching	Broad band from NH_3^+ groups
2850 - 2950	C-H stretching	Aliphatic C-H stretching from methyl and methylene groups
1790 - 1810	C=O stretching	Strong and sharp peaks from carboxylate groups in TFA counter ion
1730 – 1750	C=O stretching	Ester groups from dendrimer
1550 - 1650	N-H bending	Strong to medium peaks from NH_3^+ groups
1370 - 1450	C-H bending	Methyl and Methylene groups
1100 - 1350	C-F stretching	Strong peaks from CF_3 groups in TFA counter ion
1010 - 1200	C-O-C stretching	Strong or medium peaks from ester linkages

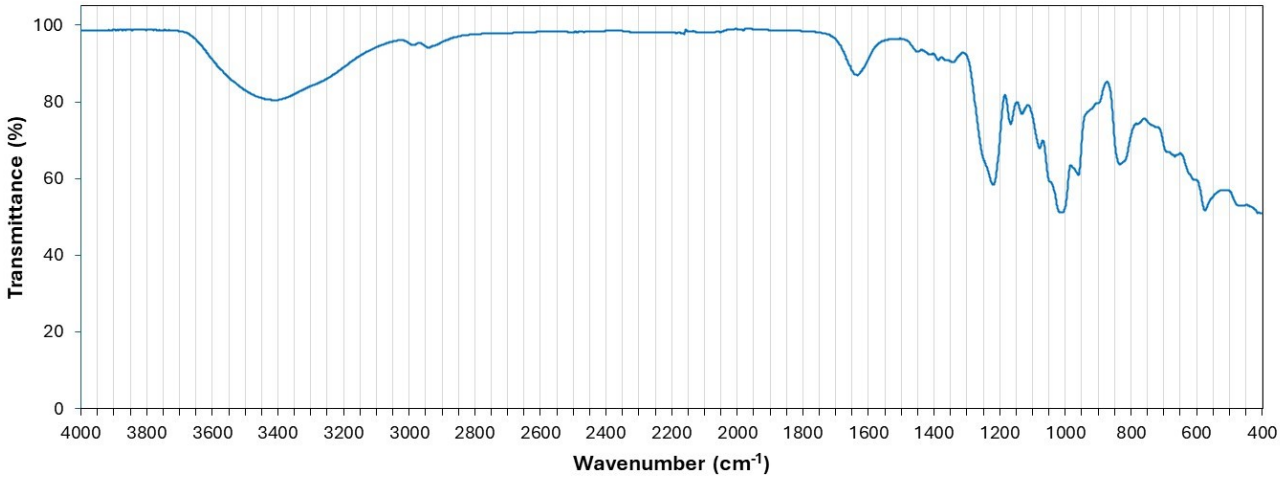

Figure S2. FTIR spectrum of the fucoidan from *F. vesiculosus.*

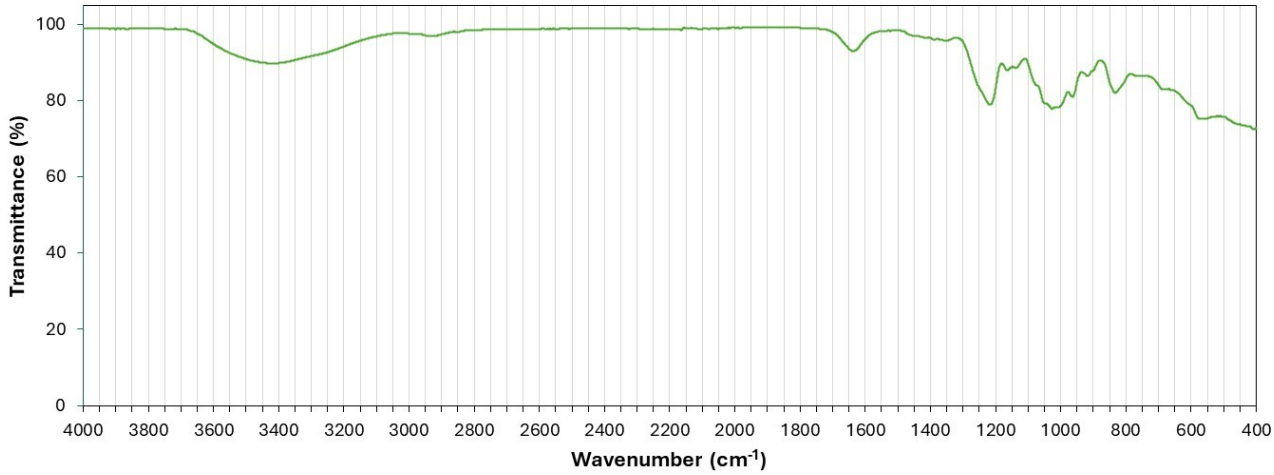

Figure S3. FTIR spectrum of the fucoidan from *U. pinnatifida.*

Table S2. Interpretation of the FTIR spectra of fucoidan from *F. vesiculosus* and U. *pinnatifida*.

Wavenumber (cm^{-1})	**Assignment**	**Comment**
3200 - 3400	O-H stretching	Broad band from hydroxyl groups in fucoidan/water
2900	C-H stretching	Polysaccharide backbone
1650	O-H bending	Water molecules
1400	C-H bending	Polysaccharide structure
1220 - 1260	S=O asymmetric stretching	Strong band from sulfates
1030 - 1080	C-O-S symmetric stretching	Sulfate ester groups
1000 - 1150	C-O-C and C-OH stretching	Carbohydrate rings, “fingerprint” for polysaccharides
800 - 850	C-O-S bending	Sulfate ester groups

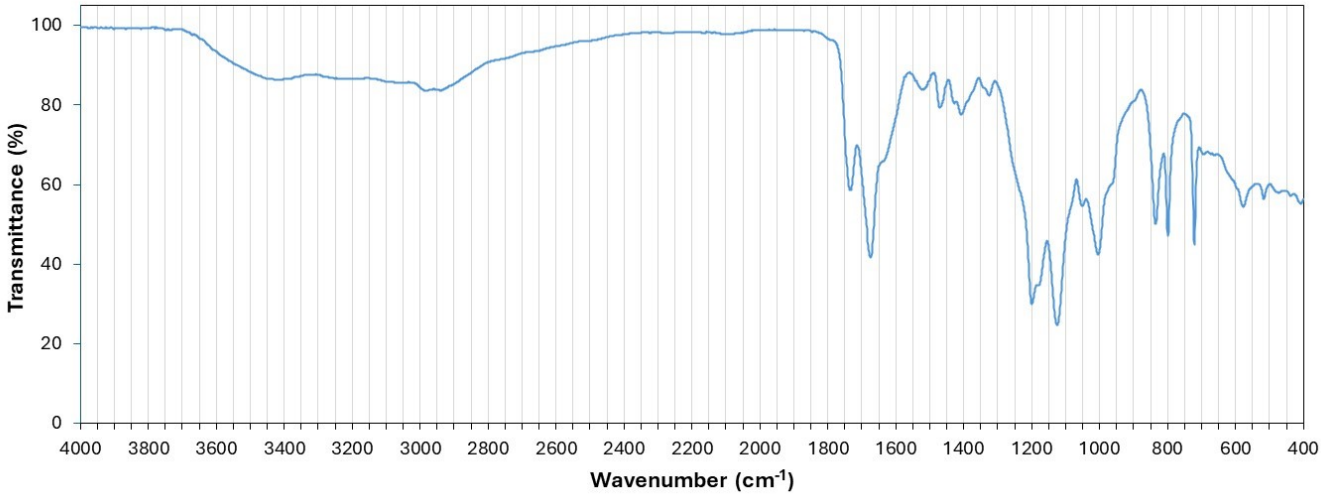

Figure S4. FTIR spectrum of the FVF 1:2 nanoparticles.

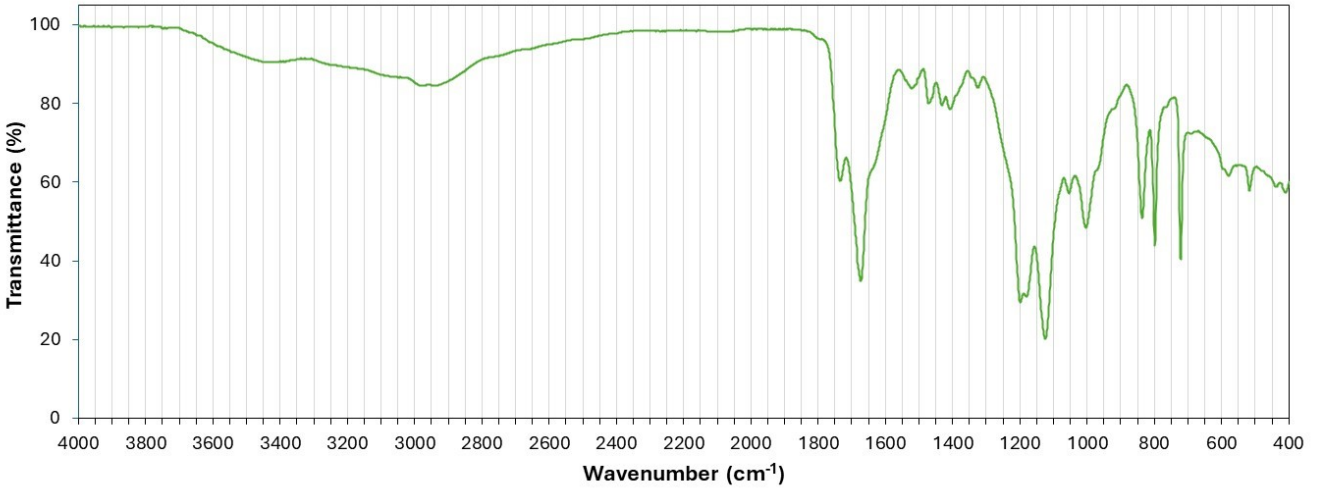

Figure S5. FTIR spectrum of the UPF 1:2 nanoparticles.

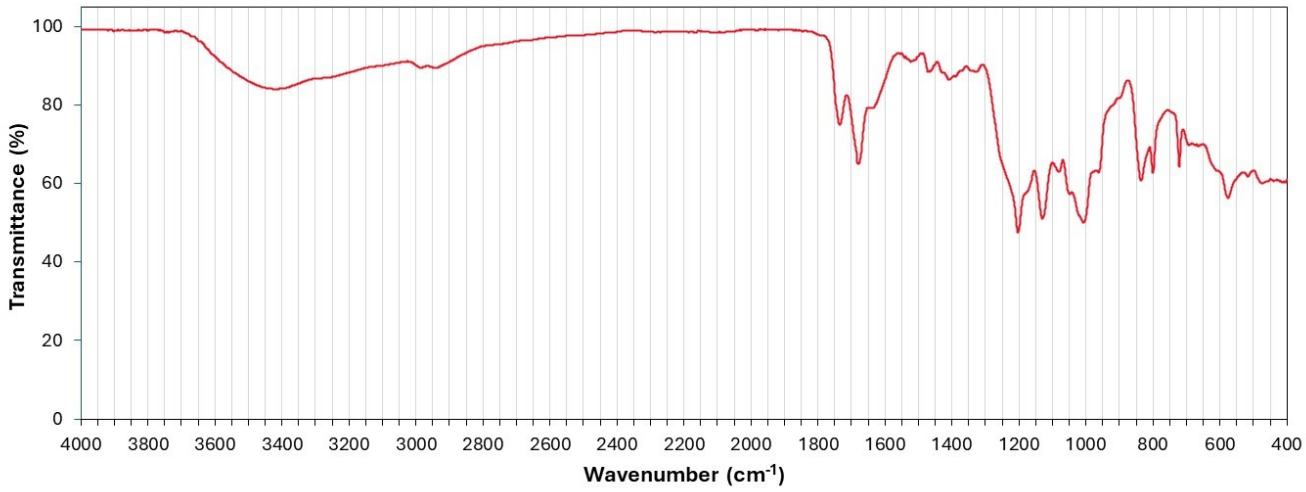

Figure S6. FTIR spectrum of the FVF 2:1 nanoparticles.

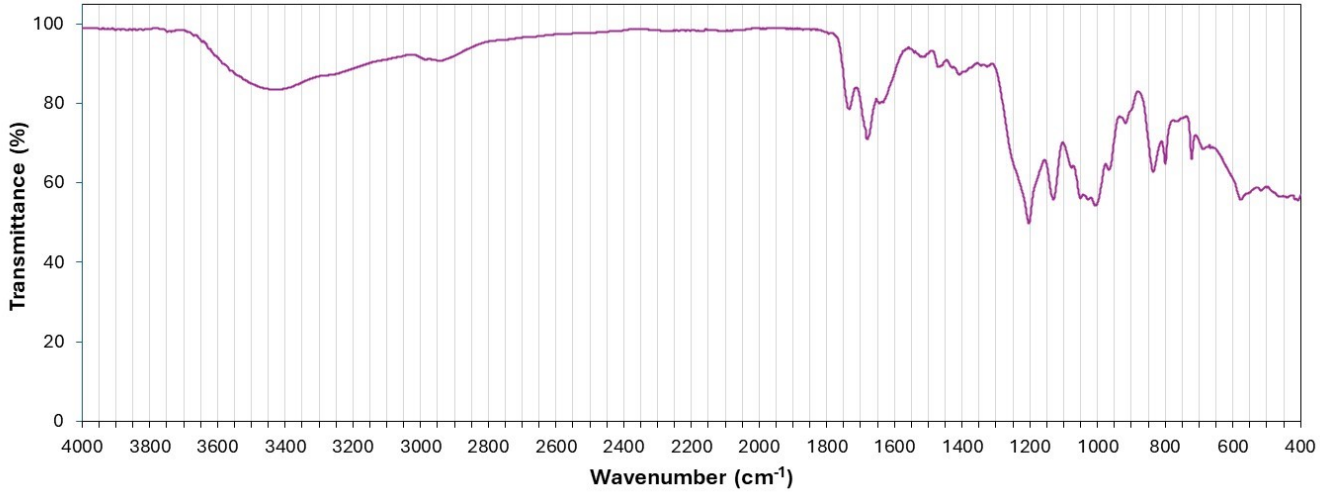

Figure S7. FTIR spectrum of the UPF 2:1 nanoparticles.

Table S3. Interpretation of the FTIR spectra of the FVF and UPF nanoparticles..

Wavenumber (cm^{-1})	**Assignment**	**Nanoparticles**
3200–3400	O–H stretching (fucoidan/water)	Confirms fucoidan/water
1730–1750	C=O stretching (dendrimer)	Confirms dendrimer
1220–1260	S=O asymmetric stretch (sulfates)	Confirms fucoidan

1000–1100	C–O–C and C–O–S (polysaccharides)	Confirms fucoidan

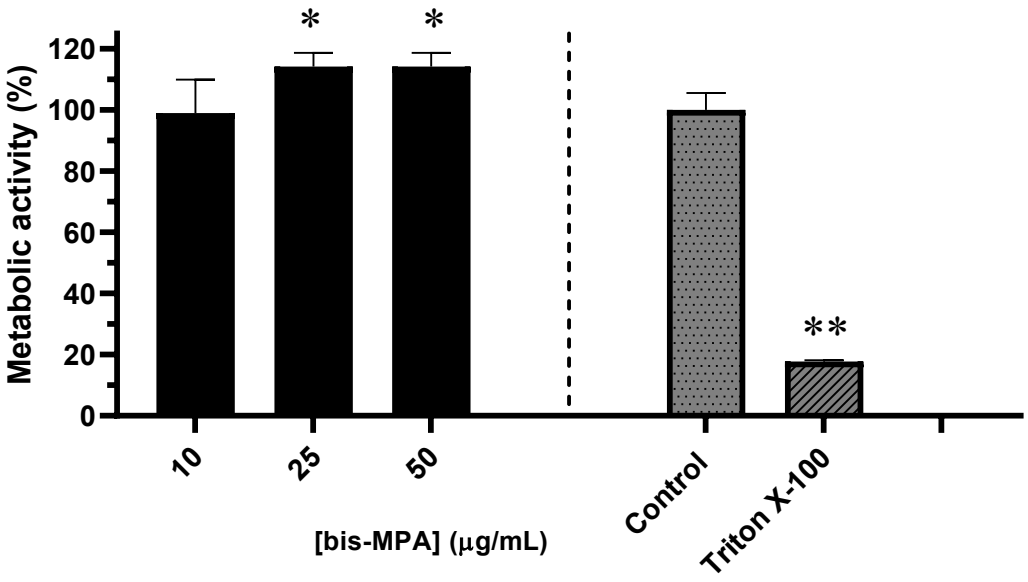

Figure S8. Effect of the concentration of bis-MPA dendrimer on the metabolic activity of HUVEC cells after 24h. Results are expressed as a percentage of the negative control (only cell culture medium); medium containing Triton-X was used as positive control. Data represents the mean ± SD (n=3, *p<0.05, **p<0.001 as compared to the control).