Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications

Fluorescence Turn-on Detection of Live Cell Apoptosis with Hyperbranched Conjugated Polyelectrolyte

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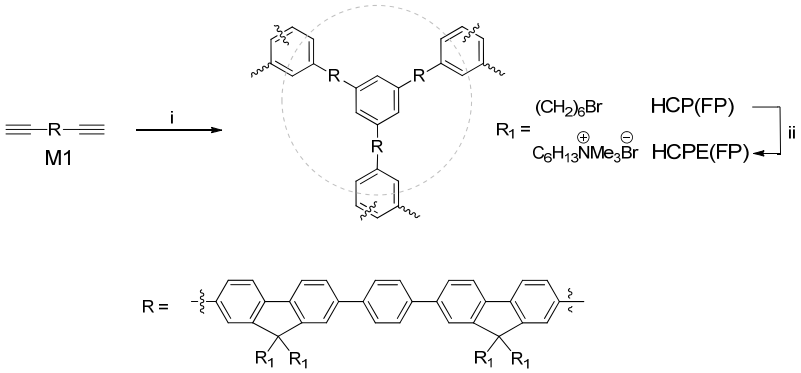
Experimental Section

Materials. Bovine serum albumin (BSA), lysozyme, pepsin, thrombin, trypsin, and cationic 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma. Recombinant human caspase-3 and caspase-7 were purchased from R&D Systems. Inhibitor 5-[(S)-(+)-2-(methoxymethyl)pyrrolidino]sulfonylisatin was purchased from Calbiochem. Fetal bovine serum (FBS) and trypsin-EDTA solution were purchased from Gibco (Lige Technologies, AG, Switzerland). Staurosporine was purchased from Biovision. Milli-Q water was supplied by Milli-Q Plus System (Millipore Corporation, Breford, USA). Dulbecco's Modified Eagle Medium (DMEM) medium was purchased from National University Medical Institutes (NUMI, Singapore). MCF-7 breast cancer cell line was provided by American Type Culture Collection. Dichloromethane (DCM) was distilled over calcium hydride. *N*,*N*-diisopropylethylamine (DIEA), dimethyl sulfoxide (DMSO), trifluoroacetic acid (TFA), triisopropylsilane (TIS), piperazine-*N,N′*-bis(2-ethanesulfonic acid (PIPES), ethylenediaminetetraacetic acid (EDTA), 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS), hex-5-ynoic acid were all purchased from Sigma-Aldrich and used as received without further purification. Rink-amide resin, *O*-benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate (HBTU), *N*-hydroxybenzotriazole (HOBt) and Fmoc-protective amino acids were purchased from GL Biochem Ltd.

Characterization. ^{1}H and ^{13}C NMR spectra were measured on a Bruker ARX 400 NMR spectrometer. Chemical shifts were reported in parts per million (ppm) referenced with respect to residual solvent ($CDCl_3$ = 7.26 ppm, $(CD_3)_2SO$ = 2.50 ppm or tetramethylsilane $Si(CH_3)_4$ = 0 ppm). All analytical HPLC were carried out on Shimadzu LCMS (IT-TOF) system or Shimadzu LCMS-2010EV system equipped with an auto-sampler using reverse-phase Phenomenex Luna 5 μm C18(2) 100 Å 50 × 3.0 mm columns. A 0.1% TFA/H_2O and 0.1% TFA/acetonitrile were used as eluents for all HPLC experiments. UV-vis absorption spectra were taken on a Milton Ray Spectronic 3000 array spectrophotometer. Photoluminescence (PL) spectra were measured on a Perkin-Elmer LS 55 spectrofluorometer. All PL spectra were measured with an excitation wavelength of 350 nm. The cells were imaged by fluorescence microscope (Nikon A1 Confocal microscope).

Synthesis and Characterization to HCPE(FP).



Scheme S1. Synthetic route to HCPE(FP). Reagents and conditions: i) $CpCo(CO)_2$, toluene, 65 °C, UV radiation, 8 h; ii) THF/H_2O, NMe_3, 24 h.

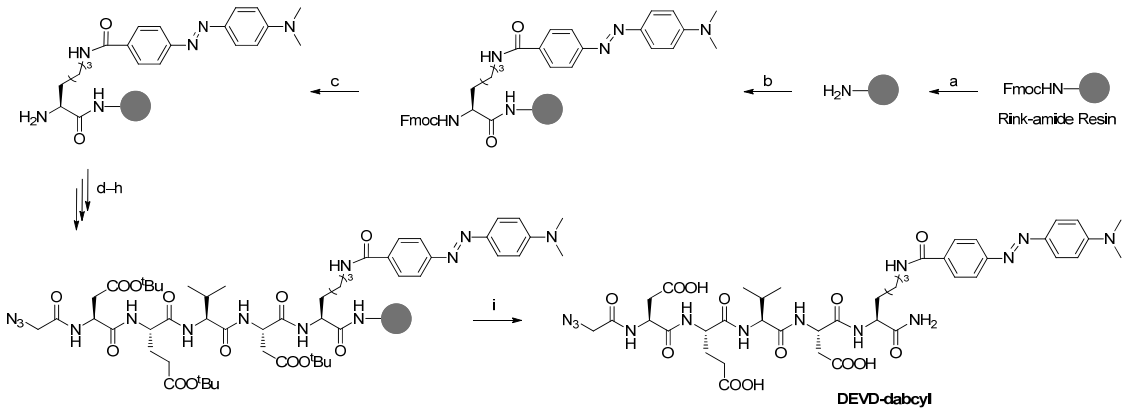
The synthetic route towards HCPE(FP) is depicted in Scheme S1. Cyclotrimerization of M1 using cyclopentadienylcobaltdicarbonyl [$CpCo(CO)_2$] as catalysis under UV radiation afforded neutral hyperbranched polymer HCP(FP) in 60% yield. According to the ^{1}H NMR spectrum of HCP(FP), the ratio of integration for peak at 3.17 ppm (terminal acetylene proton) to that at 2.03 ppm ($C\underline{H}_2$ next to the fluorenyl 9-position) is ~0.06,

indicating that the percentage of triple bond involved in "new" benzene formation is 76%. The number of newly formed benzene ring is estimated to be 7, and the degree of polymerization is 15, which yields the number average molecular weight of HCP as ~16000. The subsequent quaternization of HCP(FP) with NMe_3 afforded cationic HCPE(FP) in 90% yield. The ^{1}H NMR spectrum for HCPE(FP) shows an integration ratio of ~4.5 for peak at 3.03 ppm ($N^+(C\underline{H}_3)_3$, and embedded terminal alkyne protons) to that at 2.20 ppm ($C\underline{H}_2$ next to the fluorenyl 9-position), which indicates almost a 100% quaternization degree.

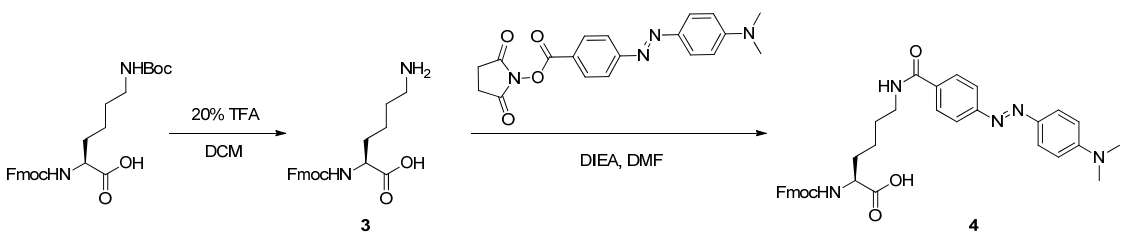
Synthesis of HCP(FP): A Schlenk tube charged with cyclopentadienylcobaltdicarbonyl ($CpCo(CO)_2$) was degassed with three vacuum-nitrogen cycles. A solution of compound M1 (100 mg, 0.1 mmol) in anhydrous toluene (1.5 mL, 0.01 M) was then added to the tube, and the system was further degassed three times. The mixture was vigorously stirred at 65 °C under irradiation with a 200 W Hg lamp (operating at 100 V) placed close to the tube for 8 h. After the mixture was cooled to room temperature, it was dropped in methanol (100 mL) through a cotton filter. The precipitate was collected and redissolved in tetrahydrofuran. The resultant solution was filtered through a 0.22 μm filter, and poured in hexane to precipitate the product. After being dried in vacuum at 40 °C, HCP (FP) was obtained as a brown powder (60 mg, yield = 60%). ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm): 7.79 (m, 1H, ArH), 3.28 (m, 8H, $C\underline{H}_2Br$), 3.17 (s, 0.49 H, acetylene proton), 2.03 (m, 8H, $C\underline{H}_2$), 1.67 (m, 8H, $C\underline{H}_2$), 1.22 (m, 16H, $C\underline{H}_2$), 0.68 (m, 8H, $C\underline{H}_2$).

Synthesis of HCPE(FP): Trimethylamine (2 mL) was added dropwise to a solution of HCP (50 mg) in THF (20 mL) at −78 °C. The mixture was stirred for 12 h, and then

allowed to warm to room temperature. The precipitate was redissolved by the addition of MeOH (10 mL). After the mixture was cooled to −78 °C, additional trimethylamine (2 mL) was added, and the mixture was stirred at room temperature for 24 h. After solvent removal, acetone was added to precipitate HCPE as yellow solid (55 mg, yield = 90%). ^{1}H NMR (CD_3OD, 500 MHz, δ ppm): 7.87 (m, 18H, ArH), 3.25 (m, 8H, C$\underline{H}_2$), 3.03 (br, 36H, $N^+(C\underline{H}_3)$), 2.20 (m, 8H, C$\underline{H}_2$), 1.57 (m, 8 H, C$\underline{H}_2$), 1.18 (m, 16H, C$\underline{H}_2$), 0.66 (m, 8H, C$\underline{H}_2$).



Scheme S2. Solid-phase synthesis of DEVD-dabcyl. Reagents and Conditions: (a) 20% piperidine in DMF; (b) Fmoc-Lys(dabcyl)-COOH, HBTU, HOBt, DIEA, DMF; (c) 20% piperidine in DMF; (d) i. Fmoc-Asp(O^{t}Bu)-OH, HBTU, HOBt, DIEA, DMF; ii. 20% piperidine in DMF; (e) i. Fmoc-Val-OH, HBTU, HOBt, DIEA, DMF; ii. 20% piperidine in DMF; (f) i. Fmoc-Glu(O^{t}Bu)-OH, HBTU, HOBt, DIEA, DMF; ii. 20% piperidine in DMF; (g) i. Fmoc-Asp(O^{t}Bu)-OH, HBTU, HOBt, DIEA, DMF; ii. 20% piperidine in DMF; (h). 2-azidoacetic acid, HBTU, HOBt, DIEA, DMF; (i) TFA/TIS/H_2O (v/v/v = 95:2.5:2.5), 3 h; Abbreviation: HBTU (O-benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluorophosphate), HOBt (hydroxybenzotriazole), *N,N*-diisopropylethylamine (DIEA), TIS (triisopropylsilane).



Scheme S3. Synthesis of compound **4**.

Synthesis of Fmoc-Lys(dabcyl)-COOH (4). Fmoc-Lys(Boc)-COOH (0.47 g, 1.0 mmol) was vigorously stirred in 20% TFA/DCM solution for 3 h. The reaction solution was then concentrated and dried *in vacuum* to afford compound **3**. The crude compound **3** was directly used without further purification. To a solution of compound **3** in DMF, dabcyl acid-NHS (0.44 g, 1.2 mmol) and DIEA (0.4 mL, 2.5 mmol) were added. The reaction mixture was stirred at room temperature overnight. The resulting solution was then concentrated *in vacuum* and purified by flash chromatography (EA : Hexane = 1 : 2) to afford **4** as red solid (0.41 g, 67%). ^{1}H NMR (300 MHz, $CDCl_3$), δ (TMS, ppm): 8.61 (s, 1 H), 8.06 (d, J = 3.0 Hz, 2 H), 7.87-7.81 (m, 7H), 7.75 (d, J = 3.0 Hz, 2H), 7.67 (d, J = 6.0 Hz, 1H), 7.40 (t, J = 6.0 Hz, 2H), 7.33 (t, J = 6.0 Hz, 2H), 6.81 (d, J = 3.0 Hz, 2H), 5.74 (s, 1H), 4.33 (d, J = 3.0 Hz, 2H), 4.25 (t, J = 6.0 Hz, 1H), 3.35 (d, J = 3.0 Hz, 2H), 3.07 (s, 6H), 1.82-1.72 (m, 2H), 1.61 (t, J = 3.0 Hz, 2H), 1.47 (d, J = 6.0 Hz, 2H). MS (IT-TOF): *m/z* 620.3010 [$(M+H)^+$, calcd. 620.2795].

Synthesis, Purification, and Characterization of DEVD-dabcyl. DEVD-dabcyl was synthesized using standard Fmoc strategy with rink amide resin as the solid support. Standard HOBT/HBTU/DIEA coupling method was used throughout the whole process. The resin (100 mg, loading ~0.5 mmol/g) was swelled in HPLC-grade DMF for 1 h at room temperature. Subsequently, Fmoc group was deprotected in piperidine/DMF (v/v = 1/4) for 2 h at room temperature. Following piperidine removal, the resin was washed extensively with DMF and DCM and dried thoroughly under high vacuum. Next, dabcyl-containing lysine **4** (SI Scheme S3) was dissolved in dry DMF (1.5 mL) together with HBTU (4 equiv), HOBt (4 equiv), and DIEA (8 equiv). The dry resin was then added and the resulting mixture was shaken at room temperature. After overnight reaction, the resin

was filtered and washed thoroughly with DMF (3×), DCM (3×) and DMF (3×) until the filtrate became colorless. After drying thoroughly under high vacuum, the resin was deprotected again with 20% piperidine in DMF for the next coupling cycle. The above cycle was repeated until the last 2-azidoacetic acid has been coupled. After the whole coupling process, the resin was washed thoroughly with DMF and dried under high vacuum for 2 h at room temperature. The peptide was then cleaved in a mixture of 95% TFA, 2.5% triisopropylsilane (TIS) and 2.5% H_2O for 4 h at room temperature. Following prolonged concentration *in vacuum* until >80% of cleavage cocktail was removed, cold ether (chilled to –20 °C) was added to the liquid residue to precipitate the peptide. The ether layer was then decanted and the precipitates were dried thoroughly *in vacuum*. The resulting peptide was further purified by prep-HPLC and characterized by LC-MS. IT-TOF m/z [$(M+H)^+$], calcd: 938.4042, found 938.3734. The HPLC condition is: 10–100% B for 10 min, then 100% B for 2 min, 10% B for 5 min (Solvent A: 100% H_2O with 0.1% TFA; Solvent B: 100% CH_3CN with 0.1% TFA).

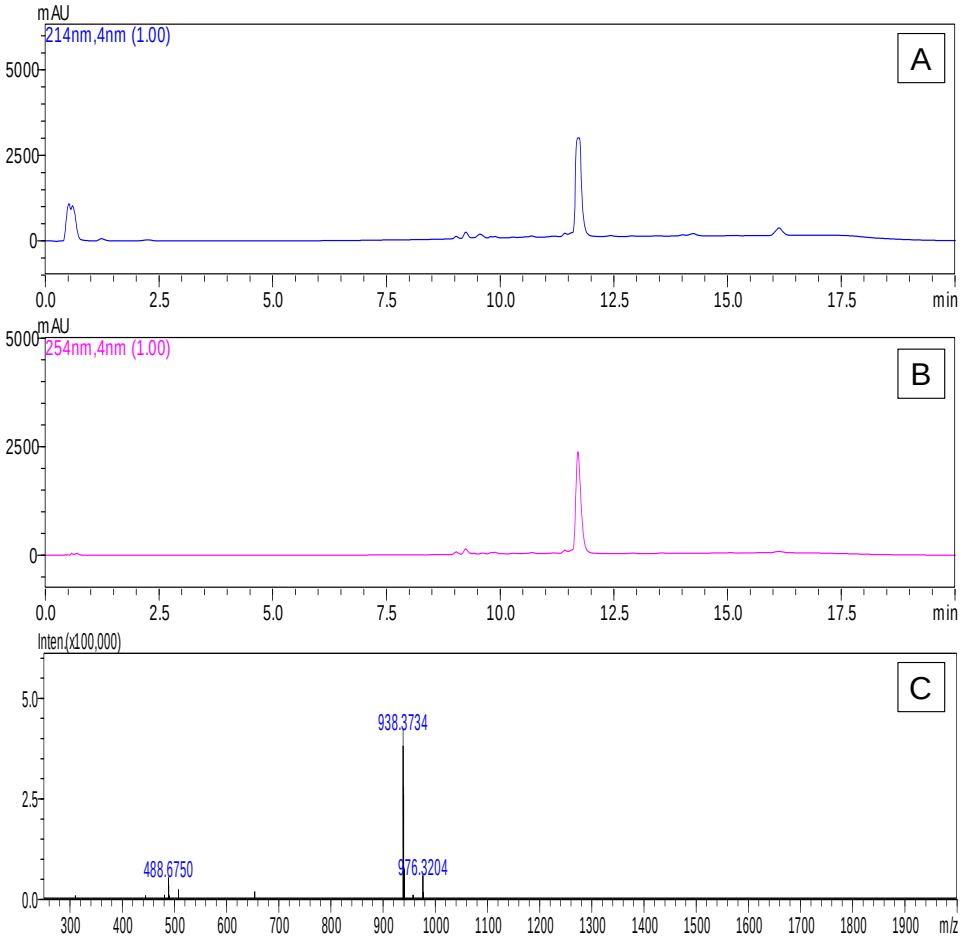


Figure S1. (A and B) HPLC spectra and (C) mass spectrum (IT-TOF) of DEVD-dabcyl.

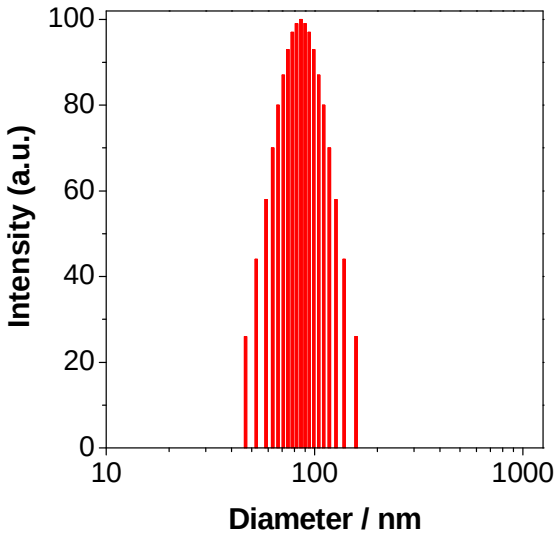


Figure S2. Hydrodynamic diameters of HCPE/DEVD-dabcyl complex particles in DMSO/water (v/v = 1/199) obtained from LLS.

General Procedure for Enzymatic Assay. A mixture of HCPE(FP) (10 nM) and DEVD-dabcyl (12 μM) were diluted with caspase-3/7 assay buffer (50 mM PIPES, 100 mM NaCl, 1 mM EDTA, 0.1% w/v CHAPS, 25% w/v sucrose, pH = 7.2) to make 45 μL working solutions. 5 μL of the recombinant caspase-3 (~0.04 μg/μL stock solution in assay buffer) was added into the above working solution. The reaction mixture was incubated at room temperature for 60 min and was then diluted to a total of 300 μL with deionized water for photoluminescence measurement. The solution was excited at 350 nm, and the emission was collected from 390 to 650 nm.

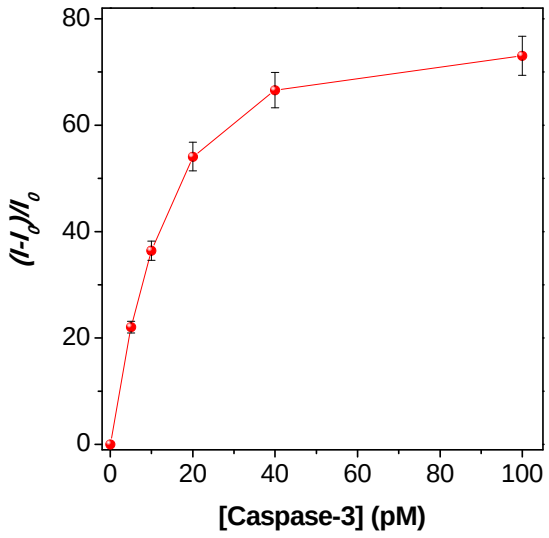


Figure S3. Plot of $(I-I_0)/I_0$ versus concentration of caspase-3 in PIPES buffer. I and I_0 are the PL intensities of HCPE/DEVD-dabcyl in the presence and absence of caspase-3, respectively. λ_{ex} = 350 nm.

Cell Culture. MCF-7 cell lines were provided by American Type Culture Collection. MCF-7 breast cancer cells were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 100 U/mL

penicillin and 100 μg/mL streptomycin (Thermo Scientific) and maintained in a humidified incubator at 37 °C with 5% CO_2. Before experiment, the cells were pre-cultured until confluence was reached.

Microscopy Imaging. MCF-7 cells were cultured in the chambers (LAB-TEK, Chambered Coverglass System) at 37 ^{o}C. After 80% confluence, the adherent cells were washed twice with 1× PBS buffer. The mixture of HCPE(FP) (2 nM) and DEVD-dabcyl solution (2.4 μM) were then added to the chamber. After incubation for 2 h at 37 ^{o}C, the cells were washed once with 1× PBS buffer, and treated with 1 μM staurosporine as apoptosis inducers for 1 h. The cells were then imaged immediately by fluorescence microscope (Nikon A1 confocal microscope).

Cytotoxicity Assay. 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were used to assess the metabolic activity of MCF-7 cancer cells or study the cytotoxicity of mixture of HCPE(FP) and DEVD-dabcyl. MCF-7 cells were seeded in 96-well plates (Costar, IL, USA) at an intensity of 4×10^{4} cells mL^{-1}. After 24 h incubation, the medium was replaced by HCPE/dabcyl (1:1200) complexes at different concentrations of HCPE(FP) (2, 5 and 10 nM). The cells were then incubated for 12, 24 and 48 h. After the designated time intervals, the wells were washed twice with 1×PBS buffer, and 100 μL of freshly prepared MTT (0.5 mg mL^{-1}) solution in culture medium was added into each well. The MTT medium solution was carefully removed after 3 h incubation in the incubator at 37 ^{o}C. DMSO (100 μL) was then added into each well and the plate was gently shaken to dissolve all the precipitates formed. The absorbance of MTT at 570 nm was monitored by the microplate reader (GeniosTecan). Cell viability

was expressed by the ratio of absorbance of the cells incubated with HCPE/dabcyl suspension to that of the cells incubated with culture medium only.

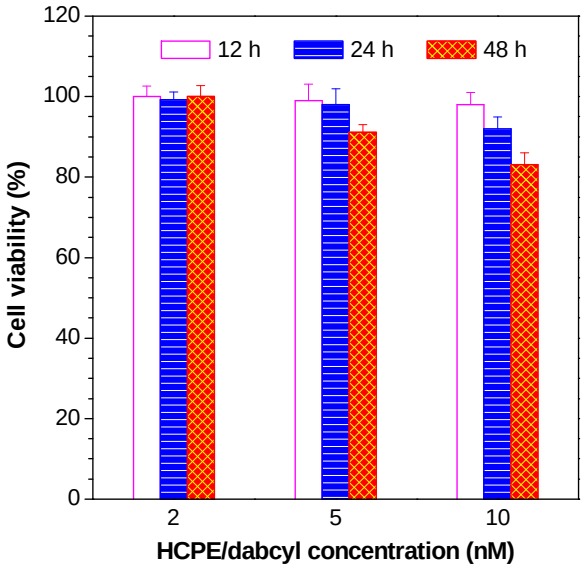


Figure S4. Metabolic viability of MCF-7 cancer cells after incubation with HCPE/dabcyl (mole ratio = 1:1200) complexes at different concentrations of HCPE(2, 5 and 10 nM) for 12, 24 and 48 h.