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ELECTRONIC SUPPLEMENTERY INFORMATION

PERFLUOROCARBON FUNCTIONALIZED HYALURONIC ACID DERIVATIVES AS OXYGENATING SYSTEMS FOR CELL CULTURE

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Chemicals

All reagents were of analytical grade, unless otherwise stated. High molecular weight hyaluronic acid (HA_{HMW}, M_W 1500 kDa) was a generous gift of Novagenit (Milano, Italy), Bis(4-nitrophenyl) carbonate (4-NPBC), divinyl sulfone (DV), triethylamine (TEA), Trinitrobenzensulfonic acid (TNBS), Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), trypsin–EDTA solution, amphotericin B solution, penicillin-streptomycin, fetal bovine serum (FBS) were obtained from Sigma-Aldrich (Italy). Inulin from Dahlia Tubers Mw 5000 Da, Ethylenediamine (EDA), anhydrous dimethylsulfoxide (DMSO), anhydrous DMF, Dowex sulphonic acid exchange resin were all from Fluka (Milano, Italy).

CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was purchased by Promega.

2-(2,3,5,6-Tetrafluoro-4-(3-Perfluoroheptyl)-1,2,4-Oxadiazol-5-yl)Phenylamino)Acetic Acid oxadiazole 1 (OXA) was prepared as previously reported [1].

Apparatus

UV-VIS absorption spectra were recorded by using a Jasco 7800 UV/VIS Spectrophotometer.

Proton nuclear magnetic resonance (^{1}H NMR) was performed using a Brucker AC-300 instrument operating at 300 MHz, using D_2O as a solvent.

Microwave-assisted synthesis was performed by microwaves generated by a Lab-Mate apparatus (CEM, Matthews, NC, USA).

Size exclusion chromatography (SEC) analysis for the determination of weight average molecular weight (M_w) of HA, HA based polymers and inulin was performed using a multidetector SEC system equipped with a Water 600 pump, a Water 410 refractive index detector and a Universal column from Waters (particle size 5 μm). The calibration curve was determined by using Pullulan standards. The elution medium was 200 mM phosphate buffer pH 6.5/MeOH 90:10 (v/v), with a flow rate of 0.6 ml/min at 35 °C.

Oxygen solubility measurements were performed by using a Delta OHM model HD2109.1 oxymeter. The oxygen solubility probe was a Schott Gerade 120 mm probe having a membrane with an exterior Teflon layer.

Scanning electron microscopy (SEM) images were recorded by using a scanning electron microscope (ESEM QUANTA FEI 200F) with an accelerating voltage of 15 kV. Samples were dehydrated by hexamethyldisilazane (Sigma-Aldrich) and dried under vacuum prior to be gold sputtered and analyzed.

A Thermo Labsystems Multiskan Ex 96-well microplate photometer was used to evaluate cell viability after MTS test.

Synthesis of reference compound oxadiazole 2

To a mixture of oxadiazole **1** (OXA) (1 mmol) in dry DMSO (2 mL), butylamine (1.1 mmol) was slowly added. After stirring for 3 h at room temperature, the mixture was diluted with water (50 mL) and the formed precipitate filtered and crystallized from EtOH giving 2-(3-(butylamino)-2,5,6-

trifluoro-4-(3-perfluoroheptyl-1,2,4-oxadiazol-5-yl)phenylamino)acetic acid **2**: Yield: 73%. Mp 93–95°C (EtOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.77 (t, 3H, J = 7.2 Hz), 1.26-1.33 (m, 2H), 1.39-1.45 (m, 2H), 1.39-1.45 (m, 2H), 3.42-3.49 (m, 2H), 4.00 (s, 2H), 6.81 (bs, 1H, exch. with D_2O), 6.95 (bs, 1H, exch. with D_2O), 12.85 (bs, 1H, exch. with D_2O); UV (DMSO/H_2O, 1:1) λ_{max}= 328 nm (ε = 1450 $M^{-1}cm^{-1}$); MS (m/z): 712 (M^+). Anal. Calcd for $C_{21}H_{14}F_{18}N_4O_3$: C, 35.41; H, 1.98; N, 7.87. Found: C, 35.50; H, 1.95; N, 7.85.

Synthesis of vinyl sulfone derivative of inulin (INUDV)

Vinyl sulfone moieties have been tethered to inulin backbone according to a previously published procedure [2]. Briefly, inulin has been solved in anhydrous DMF at 5% w/v. Then, divinyl sulfone (DV) and triethylamine (TEA) have been added both with a molar ratio equal to 5 respect to inulin repeating units and the reaction has been carried out at 60 °C for 24 h under argon. The pure product has been recovered after precipitation and several washings in diethyl ether and finally dried under vacuum. ^{1}H-NMR spectrum in D_2O showed peaks at δ 3.50 - 4.0 (5H, m: -$\underline{CH_2}$-OH; -$\underline{CH}$-CH_2-OH; -CH_2-$\underline{CH_2}$-O-), 4.14 (1H, t: $\underline{CH}$-OH), 4.25 (1H, d: $\underline{CH}$-OH), 6.43 and 6.90 (3H, 2q: $\underline{CH_2}$=$\underline{CH}$-). The degree of derivatization (DD%) in DV was determined by comparing the peak integrals at δ 6.43 and 6.90 (3H, 2q: $\underline{CH_2}$=CH–) relative to DV double-bond protons, with the peaks between δ 3.5-4.25 relative to inulin fructose unit protons (7H). The value of DD% in DV was 30±1 mol-%.

Synthesis ethylendiamino derivatives of hyaluronic acid (HA-EDA, HA-TBA-EDA)

HA low molecular weight (Mw 250 kDa, polydispersity 2.1) and ethylendiamino derivatives HA-EDA and HA-TBA-EDA have been obtained according to already published synthetic protocols [3]. Briefly, 1% w/v HA-TBA in anhydrous DMSO has been reacted for 4 h at 40 °C with 4-NPBC, at 0.5 molar ratio respect to HA repeating units, in order to activate primary hydroxyl groups of N-acetyl glucosamine residues. Then, ethylenediamine has been added to the activated polymer

solution and left to react for 3 h at 40 °C. The pure product has been recovered after precipitation, washings in acetone and freeze-drying. To obtain HA-EDA, TBA has been removed by adding aqueous NaCl saturated solution after reaction completion. The product has been recovered after washings with a mixture of acetone/water 8:2, with acetone alone and final freeze-drying. ^{1}H-NMR spectrum in D_2O showed principal peaks at δ 2.0 (-NH-CO-$\underline{CH_3}$), δ 3.1 (-CO-NH-CH_2-$\underline{CH_2}$-NH_2-), δ 3.3-3.8 (pyranosyl CH of HA). The derivatization degree in EDA portions linked to HA was calculated by ^{1}H-NMR comparing the peak at δ 3.1 attributable to the methylene groups of EDA portion with the peak at δ 2.0 attributable to acetamido group of HA and by TNBS colorimetric assay and resulted to be 50±2 mol%.

Synthesis of fluorinated derivatives of hyaluronic acid (HA-EDA-OXA)

Derivatization of HA-TBA-EDA with OXA to obtain HA-EDA-OXA derivatives was carried out in anhydrous DMSO with two different reaction temperature. A solution of OXA in anhydrous DMSO and TEA has been added to previously dispersed HA-TBA-EDA in DMSO, the molar ratios between OXA and EDA and between TEA and OXA was set equal to 2.3 and 1 respectively. The reaction was carried out for 24 h at 40 or 60 °C under continuous stirring. After 24 h, the reaction was stopped adding aqueous NaCl saturated solution, then precipitated with an excess of acetone (to remove TBA). Samples were washed with acetone/water 8:2 and then acetone alone. Obtained products were dried under vacuum, dissolved in water and finally freeze-dried.

Enhanced Microwave Synthesis was used as a tool to increase OXA derivatization degree. In this case, the reaction was carried out in a microwave reactor at 40 W for 1h, keeping the reaction mixture temperature at 60 °C via external cooling.

The amount of oxadiazole linked was determined by means of UV spectroscopy by comparing the absorbance at 328 nm of each polymer in DMSO/H_2O (1:1) solution with a calibration curve of the reference compound **2**. The molar amount of unreacted amino groups was finally confirmed by ^{1}H-NMR analysis and TNBS colorimetric assay.

Oxygen solubility measurements

Oxygen solubility measurements were performed on oxygen saturated aqueous dispersions containing HA-EDA and HA-EDA-OXA derivatives at concentration of 1 and 30 mg/ml, at atmospheric pressure, accordingly to a method reported elsewhere [4]. In particular, each solution was stirred with a magnetic stir bar while pure oxygen was continuously bubbled. The temperature of each solution was adjusted either at 25 or 37 ± 0.1 °C by using a thermostated oil bath. Once the dispersions reached a stable maximum oxygen concentration (saturated solution), bubbling was stopped and the release of dissolved oxygen was determined by evaluating the change in the oxygen solubility (desaturation) as a function of time.

Hydrogel formation and gelation time

Stock dispersions of HA-EDA, HA-EDA-OXA and INUDV each at the concentration of 3 % w/v in Dulbecco's Phosphate Buffer Solution (DPBS) at pH 7.4 were obtained, then the opportune volumes of these dispersions were mixed to obtain HA-EDA/INUDV and HA-EDA-OXA/INUDV gel forming aliquots having weight ratios 70:30 or 80:20 respectively. The time needed to obtain a stable crosslinked hydrogel was qualitatively monitored by the inversion tube test [5].

Mechanical characterization of hydrogels

Compression tests were performed using an Instron apparatus (Model 3345, Instron, Norwood, MA) on crosslinked hydrogel. Compression moduli were calculated as the slope of the stress-strain plot in the elastic region, i.e. within the first 20% of strain. [6] In particular, stress and strain (ε) values between ε=0.1-0.2 were used to calculate initial compression modulus [7]. All hydrogel samples were prepared according to the above mentioned procedure in order to obtain cylindrical specimens with 13 mm diameter and 3 mm height. After crosslinking, hydrogels were left to equilibrate in DPBS pH 7,4 at 37 °C for 24 h prior to testing; compression measurements were performed in the same conditions of aqueous medium and temperature. The stress, sc, was

calculated by sc ¼ Load/pr2, where r is the original radius of the specimen. The strain (ε) under compression is defined as the change in the thickness (h) relative to the original thickness (h0) of the freestanding specimen, ε ¼ (h0_h)/h0. Stress and strain between ε ¼ 0.2 e 0.3 were used to calculate initial elastic modulus (E).

Rat dermal fibroblasts

Rat fibroblasts were isolated as described [8]: dermis was accurately shaved, cut into 1 cm^2 pieces and immersed in sterile DPBS with 1% v/v of penicillin/streptomycin solution. Specimens were treated with an aqueous cold ethanol solution (70% v/v) for 2 minutes, washed several times with sterile DPBS and reduced in small pieces with a scalpel. So obtained pieces were incubated with a Dispase II solution (2,5 U ml^{-1}) for 1 hour then the epidermis was separated from the dermis with the use of forceps.

Dermis pieces was kept in the bottom of T-75 culture flask for 1 h prior to add DMEM supplemented with 10% (v/v) of FBS, 1% (v/v) of penicillin–streptomycin solution, 1% (v/v) of glutamine solution and 0,1 % v/v amphotericin B solution. Specimens were cultured for 2 weeks by changing the culture medium every 2-3 days until fibroblast migrate from the dermis to the culture flask. The so obtained cells were cultured from passage 1 to 7 in standard fibroblast medium with the above mentioned composition. Cells were used within passage 7.

Cell encapsulation and cytotoxicity

Rat fibroblasts (passage 6) were suspended in 200 µl of 3% w/v gel forming mixture of either HA-EDA/INUDV or HA-EDA-OXA/INUDV at a concentration of 1 x 106 cells ml-1. After 1 h cell medium was added to crosslinked hydrogels and all constructs were cultured in 1 ml of complete DMEM at 37 °C. In particular, two different culture environments were chosen, normoxic condition (5% CO2 in humidified incubators) or reduced oxygen culture using the BD GasPak EZ Gas Generating System sachet. The latter system allow to generate a hypoxic condition inside a

resealable pouch (atmosphere with approximately 1% oxygen after 24 h of incubation). In the experiment performed incubating in normoxic condition 2 groups of constructs were set: the first one received only cell medium exchange every day without further supply; the cell/hydrogel constructs belonging to the second group, in addition to daily fresh medium exchange, were bubbled everyday with pure O_2 for 4 minutes in order to saturate the systems. After 7 days the viability of encapsulated cells was measured by MTS assay on HA-EDA-OXA/INUDV and HA-EDA/INUDV weight ratios 80/20 and 70/30 respectively. In the experiment performed in the anaerobic pouch the medium was changed every 2 days, while the Gas Generating System was replaced after 3 days. In this latter experiment viability of cells inside the hydrogel constructs was assayed via MTS after 24, 72 and 168 hours on HA-EDA-OXA/INU-DV and HA-EDA/INUDV weight ratio 70/30.

Statistical analysis

All results are reported as mean ± standard deviation and, when applicable, statistical analysis for significance was performed by means of Student's t-test, using Microsoft Excel statistical function for t-tests, assuming unequal variance and two tailed distribution; values of $p < 0.05$ were considered statistically significant

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