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Dendrimer Hydrazides as Multivalent Transient Inter-Cellular Linkers

D Zhao

S-M Ong

Zhilian Yue
University of Wollongong, zyue@uow.edu.au

Z Jiang

Y-C Toh

See next page for additional authors

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Abstract

Three-dimensional (3D) multi-cellular aggregates (MCAs), as a model scaffold-free tissue construct, are useful for engineering cell-dense and matrix-poor tissues for repair and regeneration applications. To facilitate rapid MCA formation with high degrees of linker consistency and performance, we synthesized a class of dendrimer hydrazides with 8, 16 and 32 arms that can react with the aldehyde on the modified cell surfaces to form MCAs. DAB-AM-16 hydrazide with 32 arms demonstrated the best cell aggregation ability as compared to the dendrimer hydrazides with fewer arms, facilitating MCA formation at lower linker concentrations, minimizing cytotoxicity. Characterization of the MCAs formed with 2 μm of DAB-AM-16 hydrazide indicated that the cells proliferated well, maintained 3D cell-cell interaction and 3D cell morphology even as the inter-cellular linker gradually disappeared from the cell surfaces. Cells cultured as MCAs also demonstrated improved cell functions than the cells cultured in 2D monolayer. The dendrimer hydrazides would be a class of consistent, economical, and high performance multivalent transient inter-cellular linkers useful in forming scaffold-free 3D tissue constructs for soft-tissue engineering and regenerative medicine.

Keywords

linkers, cellular, inter, dendrimer, transient, hydrazides, multivalent

Disciplines

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Authors

D Zhao, S-M Ong, Zhilian Yue, Z Jiang, Y-C Toh, M A. Khan, J Shi, C H Tan, J Chen, and H Yu

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Deqiang Zhao[1,2], Siew-Min Ong[1,3] , Zhilian Yue[1], Zhiyong Jiang[4], Yi-Chin Toh[1], Khan Majad[1], Jiahua Shi[4], Choon-Hong Tan[4], J. Paul Chen[2,5†], and Hanry Yu[1,3, 6,7,8,9, 10†]

1 Institute of Bioengineering and Nanotechnology, A*STAR, The Nanos, #04-01, 31 Biopolis Way, Singapore 138669, Singapore;

2 Department of Chemical and Bio-molecular Engineering, Faculty of Engineering, National University of Singapore, 9 Engineering Drive 1, Singapore 117576;

3 NUS Graduate School for Integrative Sciences and Engineering, Centre for Life Sciences (CeLS) #05-01, 28 Medical Drive, Singapore 117456;

4 Department of Chemistry, Faculty of Science, National University of Singapore, 3 Science Drive 3, Singapore 117543;

5 Division of Environmental Science Engineering, Faculty of Engineering, National University of Singapore, 9 Engineering Drive 1, Singapore 117576;

6 Graduate Program in Bioengineering, Centre for Life Sciences (CeLS) #05-01, 28 Medical Drive, Singapore 117456;

7 Department of Physiology, Yong Loo Lin School of Medicine, #03-03, 2 Medical Drive, Singapore 117597, Singapore;

8 Singapore-MIT Alliance, E4-04-10, 4 Engineering Drive 3, Singapore 117576, Singapore;

9 NUS Tissue-Engineering Programme, DSO Labs, National University of Singapore, Singapore 117597, Singapore;

10 Department of Haematology-Oncology, National University Hospital, Singapore 119074, Singapore;

† Corresponding authors: Tel: +65-65163466; Fax: +65-68727150

Emails: phsyuh@nus.edu.sg; esecjp@nus.edu.sg

Abstract

Three-dimensional (3D) multi-cellular aggregates (MCAs), as a model scaffold-free tissue construct, are useful for engineering cell-dense and matrix-poor tissues for repair and regeneration applications. To facilitate rapid MCA formation with high degrees of linker consistency and performance, we synthesized a class of dendrimer hydrazides with 8, 16 and 32 arms that can react with the aldehyde on the modified cell surfaces to form MCAs. DAB-AM-16 hydrazide with 32 arms demonstrated the best cell aggregation ability as compared to the dendrimer hydrazides with fewer arms, facilitating MCA formation at lower linker concentrations, minimizing cytotoxicity. Characterization of the MCAs formed with 2 μM of DAB-AM-16 hydrazide indicated that the cells proliferated well; maintained 3D cell-cell interaction and 3D cell morphology even as the inter-cellular linker gradually disappeared from the cell surfaces. Cells cultured as MCAs also demonstrated improved cell functions than the cells cultured in 2D monolayer. The dendrimer hydrazides would be a class of consistent, economical, and high performance multivalent transient inter-cellular linkers useful in forming scaffold-free 3D tissue constructs for soft-tissue engineering and regenerative medicine.

Keywords: tissue construct, cell glue, dendrimer, multivalent linker

1. Introduction

Classical tissue engineering uses scaffolds to culture cells and eventually forms tissue-like constructs [1, 2] for transplantation and other extra-corporeal applications [3]. While some tissues and organs such as cartilage have extensive extra-cellular matrices (ECM), internal organs such as liver, heart, and kidney are mostly cell-dense and ECM-poor; therefore engineering tissue constructs with minimal biomaterials and even as scaffold-free cellular constructs would be important to recapitulate the complex structure-functional relationship in these internal organs. Multi-cellular aggregates (MCAs) has been a model scaffold-free micro-tissue construct [4] that exhibits some tissue-like properties, such as 3D cell morphology, maintenance of 3D cell-cell and cell-ECM interactions, and improved cell viabilities and functions [5, 6]. MCAs have found applications in bio-artificial liver-assisted devices [7], organ printing [8], cartilage regeneration [9], wound healing [10], tumour modelling [11] and drug discovery [12]. MCAs can be formed in either stationary culture on special substrata, such as the non-adherent or positively-charged surfaces [13, 14], ligand-coated surfaces [15], and thermo-responsive polymeric surfaces [16]; or in suspension culture using orbital shaker [9], spinner flasks [17], rotating wall vessels [18], and hanging drops [19]. Both stationary and suspension cultures depend on cells' natural aggregation ability, thus requiring days to form MCAs. Polymeric scaffolds with cell binding moieties such as Arg–Gly–Asp (RGD) or Tyr-Ile-Gly-Ser-Arg (YIGSR) peptides [20, 21], ligand [22] or cell adhesion receptors [23] can be used to accelerate the MCA formation in suspension. Another approach to

facilitate MCA formation is to use synthetic linker molecules as “cell glues” [24-26]. Mammalian cells are surface engineered to present non-native functional groups, such as aldehyde [27], which can then react with the inter-cellular linkers, such as avidin-biotin-hydrazide [24, 25] or polyetheleneimine hydrazide (PEI-hydrazide) [26], to rapidly glue the cells into MCAs within hours [24, 25] or minutes [26]. However, these linkers either require multiple steps to form MCAs [24, 25], or exhibit heterogeneity and inconsistency in linker synthesis with a narrow window of linker concentration [26] for the formation of MCAs without compromising cell viability significantly.

It is therefore critical to develop inter-cellular linkers with high degrees of consistency, economy and performance that facilitate rapid MCA formation for large-scale applications [7-12]. The effective linker concentration for MCA formation must be low enough to minimize cytotoxicity; the synthesis process must be simple and consistent for cost and quality controls. Since many biological systems involve polyvalent molecular interactions [28], we hypothesize that a highly efficient inter-cellular linker should be multivalent, i.e. with multiple reactive functional groups (e.g. hydrazide) per linker molecule. Dendrimer is a multivalent molecule that allows functionalization of its multiple arms to confer many reactive functional groups per dendrimer molecule, while exhibiting low cytotoxicity [29]. We therefore functionalized a class of dendrimers with hydrazide functional groups (from 4 to 32 arms) and explored their suitability to form MCAs. DAB-AM-16 hydrazide with 32

arms demonstrated best efficiency to form MCAs (i.e., cell aggregation ability) than other dendrimer hydrazides with fewer arms. The high cell aggregation ability of the 32-arm dendrimer hydrazide allows MCA formation at less than 10 μM with negligible cytotoxicity. Cells in MCAs proliferated well; maintained 3D cell-cell interactions and 3D cell morphology with improved cellular functions, even as the bulk of inter-cellular linkers disappeared within 3 days. This dendrimer hydrazide inter-cellular linker would be very useful for forming scaffold-free 3D tissue constructs for soft-tissue engineering and regenerative medicine.

2. Materials and methods

All reagents were purchased from Sigma-Aldrich unless otherwise stated.

2.1 Cell culture

HepG2 cells (ATCC, USA) were cultured in 75 cm^2 tissue culture flasks (Nunc, Denmark) in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Invitrogen, USA) supplemented with fetal calf serum (10 %) (Hyclone, USA), 1.5 g/L glucose, penicillin (100 units/mL), 100 (μg/mL) streptomycin, sodium bicarbonate (1.3 g/L) and Hepes (1.2 g/L) (GIBCO, Invitrogen, USA) at 37 ºC in a humidified environment with CO_2 (5 %).

2.2 Synthesis of dendrimer hydrazides

^{1}H and ^{13}C NMR (nuclear magnetic resonance) spectra were recorded on a 400 MHz Avance III NMR spectrometer (Bruker, Germany). High or low resolution mass spectra were obtained on a LCQ spectrometer (Finnigan MAT, USA) in ESI mode, a 95XL-T mass spectrometer (Finnigan MAT, USA) in FAB mode or an Autoflex II TOT/TOF 50 MALDI-TOF system (Bruker, Germany). Infrared spectra were recorded on a FTS 165 FTIR (Fourier Transform Infrared) spectrometer (BIO-RAD, USA).

General procedure of synthesis

A solution of primary amines (50 mg) (Fig. 1**a-d**) in methanol (3 ml) was chilled in ice bath, followed by the addition of methyl acrylate (primary amine group: methyl acrylate = 1: 5) in three portions over 1 h [30]. The solution was stirred at room temperature under nitrogen for 4 days; and then the solvent and excess methyl acrylate was removed *in vacuo* to yield the intermediate methyl esters as clear oils (Fig. 1**e-h**). The solution of intermediate methyl ester in absolute ethanol (3 ml) were refluxed with hydrazine monohydrate (methyl ester group: hydrazine hydrate = 1: 10) for 12 or 24 h for ethylenediamine ester and DAB esters respectively [31]. Ethylenediamine hydrazide was precipitated with adding hexane, and further dried with pump to get white solid (Fig. 1**i**). DAB hydrazides (Fig. 1**j-l**) were purified with dialysis (Spectropro 7, MWCO 500) to remove the residual reactant, before lyophilisation to get semi-solid product.

2.2.1 Ethylenediamine hydrazide

Ethylenediamine ester (Fig. 1**e**) (yield = 95 %, clear yellow oil) : ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.61 (s, 12H), 2.71 (t, *J* = 7.2 *Hz*, 8H), 2.45 (s, 4H), 2.39 (t, *J* = 7.2 Hz, 8H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.8, 52.1, 51.4, 49.6, 32.4 ppm; IR (KBr): 2953, 2832, 1736, 1437, 1197, 1040, 1039, 844 cm^{-1}; HRMS (ESI) m/z 405.2243 ($M+H^+$), calc. for $C_{18}H_{32}N_2O_4$ 404.2231; Ethylenediamine hydrazide (Fig. 1**i**) : (yield = 93 %). ^{1}H NMR (400 MHz, d_6-DMSO) δ 9.03 (s, 4H), 3.99 (s, 8H), 2.60 (t, *J* = 7.0 *Hz*, 8H), 2.37 (s, 4H), 2.13 (t, *J* = 7.0 *Hz*, 8H) ppm; ^{13}C NMR (100 MHz, d_6-DMSO)

δ 171.1, 50.9, 49.6, 31.5 ppm; IR (KBr): 3398, 1645, 1216, 1025, 755 cm^{-1} ; HRMS (ESI) m/z 405.2673 ($M + H^+$), calc. for $C_{14}H_{32}N_{10}O_4$ 404.2681.

2.2.2 DAB-AM-4 hydrazide

DAB-AM-4 ester (Fig. 1**f**) (yield = 95 %, clear yellow oil): ^1H NMR (400 MHz, $CDCl_3$) δ 3.60 (s, 24H), 2.70 (t, *J* = 7.2 *Hz*, 16H), 2.40-2.32 (m, 36H), 1.50 (dd, J_1 = 7.5 *Hz* , J_2 =7.6 *Hz*, 8H), 1.32 (s, 4H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.9, 53.9, 51.8, 51.8, 51.4, 49.0, 32.3, 24.8, 24.4 ppm; IR (KBr): 2954, 2811, 1738, 1437, 1046, 846 cm^{-1}; HRMS (ESI) m/z 1005.6331 ($M+H^+$), calc. for $C_{48}H_{88}N_6O_{16}$ 1004.6330; DAB-AM-4 hydrazide (Fig. 1**j**) (yield = 66 %): ^1H NMR (400 MHz, d_6-DMSO) δ 8.97 (s, 8H), 4.16 (s, 16H), 2.59 (t, *J* = 7.0 *Hz*, 16H), 2.33 (s, 20H), 2.12 (t, *J* = 6.8 *Hz*, 16H), 1.44 (d, *J* = 6.0 *Hz*, 8H), 1.33 (s, 4H) ppm; ^{13}C NMR (100 MHz, d_6-DMSO) δ 171.1, 51.6, 51.1, 49.3, 31.4, 24.0 ppm; IR (KBr): 3437, 2952, 2830, 1659, 1014 cm^{-1}; HRMS (ESI) m/z 1005.7227 ($M + H^+$), calc. for $C_{40}H_{88}N_{22}O_8$ 1004.7228.

2.2.3 DAB-AM-8 hydrazide

DAB-AM-8 ester (Fig. 1**g**) (yield = 95 %, clear yellow oil) : ^1H NMR (400MHz, $CDCl_3$) δ 3.60 (s, 48H), 2.70 (t, *J* = 7.20 *Hz*, 32H), 2.40 - 2.32 (m, 84H), 1.49 (s, 24H), 1.32(s, 4H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.8, 54.1, 52.2, 51.8, 51.7, 51.4, 49.0, 32.2, 24.3 ppm; IR (KBr): 2950, 2808, 1734, 1435, 1197, 1166, 1043, 843 cm^{-1}, HRMS (ESI) m/z 2150.3928 ($M+H^+$), calc. for $C_{104}H_{192}N_{14}O_{32}$ 2149.3900;

DAB-AM-8 hydrazide (Fig. 1**k**) (yield = 73 %) : ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.98 (s, 16H), 4.21 (s, 32H), 2.59 (t, *J* = 6.8 *Hz*, 32H), 2.33 (s, 52H), 2.13 (t, *J* = 6.8 *Hz*, 32H), 1.44 (s, 24H), 1.32 (s, 4H) ppm; ^{13}C NMR (100 MHz, d_6-DMSO) δ 172.8, 54.1, 52.2, 51.8, 51.7, 49.0, 32.2, 24.3 ppm; IR (KBr) : 3434, 2953, 2837, 1652, 1017 cm^{-1}; LRMS (FAB) m/z 2150.1, calc. for $C_{88}H_{192}N_{46}O_{16}$ 2149.56.

2.2.4 DAB-AM-16 hydrazide

DAB-AM-16 ester (Fig. 1**h**) (yield = 95 %, clear yellow oil) : ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.62 (s, 96H), 2.72 (t, *J* = 7.2 *Hz*, 64H), 2..40 (t, *J* = 7.0 *Hz*, 192H), 1.53 (s, 56H), 1.35 (s, 4H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.9, 52.3, 52.2, 51.7, 32.3, 24.4, 24.1 ppm; IR (KBr): 2951, 2812, 1743, 1435, 1197, 1158, 1043, 843 cm^{-1}; MALDI-TOF MS, 4446.48, calc. for $C_{216}H_{400}N_{30}O_{64}$ 4438.9; DAB-AM-16 hydrazide (Fig. 1**l**) (yield = 78 %) : ^{1}H NMR (400 MHz, d_6-DMSO): δ 9.00 (s, 32H), 4.18 (s, 64H), 2.59 (s, 64H), 2.33 (s, 116H), 2.13 (t, *J* = 6.0 *Hz*, 64H), 1.45 (s, 56H), 1.33 (s, 4H) ppm; ^{13}C NMR (100 MHz, d_6-DMSO) δ 171.2, 51.6, 51.2, 49.3, 31.4, 23.9 ppm; IR (KBr): 3434, 2956, 2832, 1650, 1373, 1019 cm^{-1}; MALDI-TOF MS [32], found 4446.20, calc. for $C_{216}H_{400}N_{30}O_{64}$ 4438.9.

2.3 MCA formation and size distribution

HepG2 cells were trypsinized and resuspended in cold phosphate-buffered saline (PBS) in 1.5 ml micro-centrifuge tubes (Greiner Bio-one, Netherlands). Cells were treated with 0.5mM sodium periodate (Boekel Scientific, USA) for 15 min at 4 °C in

the dark on an orbital shaker. After washing twice with cold PBS, linkers of specific concentration were added and the suspension was placed on an orbital shaker for 30 min at 4°C. The size of MCAs was quantified by counting the number of observable cells per MCA under phase-contrast microscope (Olympus, Japan). For every linker at a certain concentration, the size distribution of MCAs was plotted as histogram by counting ≥ 50 MCAs. Only MCAs with > 5 cells were considered. Data plotted represent the mean ± s.e.m of 3 independent experiments.

2.4 Assessment of cytotoxicity of inter-cellular linkers

HepG2 cells were cultured in 96-well plates (Nunc, Denmark) at a density of 1.0×10^4 cells/well. After 24 h,, medium was removed and the linkers in serum-free medium were added to each plate at different concentrations (0 mM, 0.001 mM, 0.01 mM, 1.0 mM, 5.0 mM, and 10.0 mM), with a final volume of 200 µl. After 24 h of incubation, the medium was removed and the cells were rinsed with 150 µl sterile PBS twice. 200µl of 3- (3,4-dimethylthiazol-2yl)- 2,5-diphenyltetrazolium bromide (MTT) in PBS solution (1 mg/ml) was then added to each well. After incubation for 4 h, the formazon was dissolved with DMSO and the absorbance was measured at 570 nm using microplate reader (Tecan, UK). Data plotted represent the mean ± s.e.m of 3 independent experiments.

2.5 Live/dead assay on MCAs

MCAs were co-stained with CellTracker™ Green (CTG, 20 μM)) (Molecular Probes, USA) and Propidium Iodide (PI, 25μg/ml) (Molecular Probes, USA) for live and dead cells respectively in 8-well Lab-Tek® chambers (Nunc, Denmark) for 30 min at 37 ºC. After staining, MCAs were rinsed with PBS twice to remove excess dyes, fixed with paraformaldehyde (PFA) (3.7 %) for 15 min at 37 ºC and rinsed with PBS twice to remove the excess PFA. CTG and PI images are acquired by confocal laser scanning microscopy (CLSM) (Zeiss LSM510, Germany) at 488 nm and 543 nm excitation wavelengths respectively. Cell viability was quantified with Image-Pro Plus (IPP, Media Cybernetics Inc, USA) by expressing the ratio of the number of green pixels to the sum of green and red pixels. Data plotted represent the mean ± s.e.m of 3 independent experiments.

2.6 MTS cell proliferation assay

CellTiter96® aqueous one solution reagent (Promega, USA), diluted 5 times with PBS, was added to the cells and incubated at 37 ºC in the dark [32]. After 3 hours, the solution was collected and the absorbance of the supernatant was measured at 490 nm using a Safire^{II} microplate reader (Tecan, Switzerland). Data plotted represent the mean ± s.e.m of 3 independent experiments.

2.7 PicoGreen DNA quantification

MCAs were lysed in 1 ml of 0.5 % Triton-X DNA-free DI-water. DNA concentration was determined using PicoGreen dsDNA quantification kit (Molecular Probe, USA)

according to the manufacturer's protocol. Data plotted represent the mean ± s.e.m of 3 independent experiments.

2.8 Scanning Electron Microscopy

MCAs were fixed with 3.7 % glutaraldehyde in PBS (pH 7.0) at room temperature for 30 min. After treating with osmium tetroxide (1 %) (OsO_4) for 1 h, MCAs were dehydrated sequentially in ethanol (25 %, 50 %, and 75 % for 15 min respectively; and 95 % and 100 % for 30 min respectively). MCAs were then fixed onto the double-sided tape and dried in vacuum for 1 h. After sputter coated with gold/palladium, the samples were viewed with scanning electron microscope (JEOL JSM-5600, Japan).

2.9 F-actin staining

MCAs were fixed in PFA (3.7 %) for 30 min and permeabilized with Triton-X (0.5 %) for 15 min before staining with TRITC-Phalloidin (0.2 μg/ml) for 20 min at room temperature. MCAs were then imaged by CLSM (Zeiss, CLSM410, Germany).

2.10 DAB-AM-16 hydrazide labeled with Oregon Green 488 labeling

DAB-AM-16 hydrazide (10 mg/ml in 0.1 M sodium bicarbonate) was reacted with Oregon Green 488 carboxylic acid, succinimidyl ester (Molecular Probes, USA) (10 mg/ml, 50 μl in DMSO) for 1 h in the dark, followed by an additional incubation of 1 h [33]. Un-reacted Oregon Green was removed by dialysis with MWCO 1000

(Spectrum Laboratories, USA) for 24 h at room temperature. The labeled DAB-AM-16 hydrazide was then freeze-dried and stored at - 30 ºC.

2.11 Albumin secretion and cytochrome P-450 1A1/2 activity of MCAs

The albumin secretion and cytochrome P-450 1A1/2 activity of MCAs were measured on days 1, 3, 5 and 7. 2D monolayer cells were used as control. The albumin secretions over 24-h culture were determined using the Human Albumin ELISA Quantitation Kit (Bethyl Laboratories Inc., USA). Cytochrome P-450 1A1/2 activity was measured by 7-ethoxyresorufin-O-deethylation (EROD) assay. MCAs were incubated with 30 μM of ethoxyresorufin in serum-free DMEM for 4 h in 12-well plate on orbital shaker and the amount of fluorescent product resorufin produced was measured using a Safire^{II} microplate reader (Tecan, Switzerland) at 480 nm excitation and 520 nm emission. Both albumin secretion and cytochrome P-450 1A1/2 activity were normalized to the total cell number, quantified using the PicoGreen DNA quantification kit (Invitrogen, USA). Data plotted represent the mean ± s.e.m of 3 independent experiments.

3. Results

3.1 Synthesis of dendrimer hydrazides

The multivalent inter-cellular linkers were hydrazide derivatives of ethylenediamine (Fig. 1**a**), DAB-AM-4 (Fig. 1**b**), DAB-AM-8 (Fig. 1**c**), and DAB-AM-16 (Fig. 1**d**), synthesized in a 2-step reaction. Firstly, the primary amines were reacted with methyl acrylate to yield the intermediate esters of ethylenediamine ester, DAB-AM-4 ester, DAB-AM-8 ester, and DAB-AM-16 ester (Fig. 1**e-h** respectively). They were verified by the appearance of a methyl ester peak at δ = ~ 3.62 ppm in the ^{1}H NMR spectra [30] and wave number = ~ 1743 cm^{-1} in the FT-IR (fourier transform infrared) spectra [34]. ^{1}H NMR spectra demonstrated the successful synthesis of 4, 8, 16 and 32 arms intermediate esters (Fig. 1**i-l** respectively), further confirmed by mass spectrometry. In the second step, all the methyl esters were treated with hydrazine monohydrate to yield the final hydrazide derivatives of ethylenediamine hydrazides, DAB-AM-4 hydrazides, DAB-AM-8 hydrazides, and DAB-AM-16 hydrazides (Fig. 1**i-l** respectively). The hydrazide derivatives were verified by a hydrazide hydrogen peak at δ = ~ 9.00 ppm in the ^{1}H NMR spectra [35] and wave number = ~ 1650 cm^{-1} in the FT-IR spectra [36]. ^{1}H NMR spectra demonstrated the successful synthesis of 4, 8, 16 and 32 arms hydrazide (Fig. 1**i-l** respectively), further confirmed by mass spectrometry. Therefore, we have successfully synthesized the hydrazide derivatives of ethylenediamine, DAB-AM-4, DAB-AM-8, and DAB-AM-16 with 4, 8, 16 and 32 arms hydrazide respectively as multivalent inter-cellular linkers.

3.2 Assessment of cytotoxicity of inter-cellular linkers

Cytotoxicity of the inter-cellular linkers was assessed with MTT assay (Fig. 2) [37]. At linker concentration ([linker]) ≤ 0.01 mM, cell viabilities were maintained at > 90 % in all the linkers (Fig. 2). At 0.01 mM < [linker] ≤ 0.05 mM, cell viability in DAB-AM-16 hydrazide deteriorated to 60.4 ± 8.2 %, while cell viabilities in the other 3 linkers were maintained at > 80 %. At 0.05 mM < [linker] ≤ 0.5 mM, cell viability in DAB-AM-16 hydrazide deteriorated further to 3.3 ± 1.2 %, while cell viabilities in other linkers were maintained at > 50 %. At 0.5 mM < [linker] ≤ 1 mM, cell viability of DAB-AM-8 further deteriorated to 8.2 ± 3.1 %, while cell viabilities of ethylenediamine hydrazide and DAB-AM-4 hydrazide were still maintained at > 50 %. In general, dendrimer hydrazides with more functional arms demonstrated higher cytotoxicity at a particular linker concentration than the dendrimer hydrazides with fewer arms. We have used [linker] ≤ 0.01 mM in subsequent studies to ensure high cell viability in all linkers.

3.3 Assessment of multivalency on MCA formation

The inter-cellular linker-facilitated rapid MCA formation is a two-step process: single HepG2 cells were modified with 0.5 mM sodium periodate [26] to introduce aldehyde groups onto the cell surfaces, which were then reacted with an inter-cellular linker to form MCAs (Fig. 3A). To investigate linkers' efficiency to form MCAs, surface-modified cells were incubated in 0.01 mM of linkers for 30 min at 4 ºC, and the MCA size distribution were measured by manually quantifying the cell number

per MCA under a phase contrast microscope. The MCA size distribution was indicated as histogram (Fig. 3B). DAB-AM-16 hydrazide can form larger MCAs (47.63 ± 33.50 cells/MCA) than DAB-AM-4 hydrazide and DAB-AM-8 hydrazide (9.25 ± 1.25 cells/MCA, 28.34 ± 16.70 cells/MCA respectively), while ethylenediamine hydrazide could not form MCAs even at high linker concentrations of up to 0.1 mM. With 2 μM DAB-AM-16 hydrazide, 88 ± 4 % of the cells were effectively aggregated and further characterized.

3.4 Cell viability and proliferation

To assess the cell viability of MCAs over a 7-day culture period, confocal laser scanning microscopy (CLSM) was used to image live and dead cells stained with CTG and PI respectively, and the cell viability was quantified with image processing. MCAs demonstrated a high cell viability of 91 ± 3 % on day 0, immediately after MCA formation (Fig. 4a). The cell viability improved to ~100 % from day 3 onwards (Fig 4d-h). The size of MCAs also doubled from ~ 100 μm on day 0 to ~ 200 μm after the 7-day culture period. To confirm if the increase in the size of MCAs is due to cell proliferation, both MTS cell proliferation and PicoGreen DNA quantification assay [32] were conducted over a 7-day culture period. The MTS assay is indicative of the total cell mitochondrial activity, which increased 17.6 fold from an absorbance reading of 0.18 ± 0.05 on day 0 to 3.17 ± 0.28 on day 7 (Fig. 5A). Total cell number was also measured as the DNA content using the PicoGreen assay. The DNA content increased from 8.21 ± 1.80 μg on day 0, to 138.37 ± 27.28 μg on day 7(Fig. 5B). Both

the MTS assay and DNA quantification assay indicated that total cell number increased approximately 17 folds during the 7-day culture with a cell doubling time of ~ 2 days.

3.5 Cell morphology in MCAs

To observe the cell morphology of the MCAs more closely, SEM images were taken over the 7-day culture. The SEM images indicated that cell-cell contacts were relatively loose immediately after MCA formation on day 0 (Fig. 6a). Compact cell-cell contacts were achieved from day 1 onwards, resulting in compact cell spheroids (Fig. 6b-d). Cell-cell boundaries gradually disappeared over days (Fig. 6b-d) as cells reassembled into cell spheroids. To further observe the cell morphology, F-actin in the MCAs was visualized by TRITC-phalloidin staining. Cells in MCAs exhibited a cortical F-actin distribution, indicative of 3D cell morphology. From the F-actin staining (Fig. 6e-h), we also observed a similar trend on the establishment of cell-cell contacts within the MCAs as observed in the SEM images. Cells in the MCAs were loosely aggregated on day 0 (Fig. 6e); and further formed compact cell-cell contacts from day 1 onwards (Fig. 6f-h), reminiscent of that seen in naturally formed spheroids [38]. Throughout the 7-day culture, cells exhibited the typical cortical F-actin distribution of the 3D cells (Fig. 6e-h).

3.6 Tracking of linker's existence on the cell surfaces

To investigate the fate of the inter-cellular linker, DAB-AM-16 hydrazide was labeled

with a fluorescent tag, Oregon Green 488 [33], via a stable amide bond. The fluorescence signal was monitored with confocal microscopy over a 7-day culture (Fig. 7A a-h). Quantification of the fluorescence remaining on the cell surfaces showed a reduction from 100 % on day 0 to 36 ± 11 % on day 1, 2 ± 0.2 % on day 4, and almost 0 % on day 5 (Fig. 7B). The control MCAs which were fixed on day 0 still displayed strong fluorescence signal (Fig. 7A h insert) on day 7, indicating that the decrease in fluorescence observed in viable MCAs over time is not due to the photo-bleaching or photo-damage effects during the culture or imaging procedures. This transient residence property of the hydrazide dendrimer on cell surfaces is desirable for tissue engineering and other cell- or tissue-based applications.

3.7 Albumin secretion and cytochrome P-450 1A1/2 activity

To investigate the functional maintenance of the engineered MCAs, we monitored albumin secretion and cytochrome P-450 activity of HepG2 cells cultured as MCAs and 2D confluent monolayer (as a control) over the 7-day culture. Both functions were normalized against the cell number. The albumin secretion of HepG2 MCAs were 2.68 ± 0.16, 0.62 ± 0.13, 1.67 ± 0.18, and 2.67 ± 0.60 $\mu g/10^6$ cells/day on days 1, 3, 5 and 7 respectively, as compared to 1.02 ± 0.04 $\mu g/10^6$ cells/day secreted by cells cultured in 2D confluent monolayer (Fig. 8A). Even though there was a transitional decrease in albumin secretion by the MCAs from day 1 to day 3, the albumin secretion after prolonged culture on day 7 was approximately 2.5 times that of 2D monolayer cells. Cytochrome P-450 1A1/2 activity of HepG2 MCAs and 2D

monolayer were measured by 7-ethoxyresorufin-O-deethylation (EROD) assay. The amount of resorufin produced by MCAs culture were 0.272 ± 0.036, 0.323 ± 0.005, 0.314 ± 0.067, and 0.266 ± 0.022 nmole/10^6 cells on days 1, 3, 5, and 7 respectively, while that of the 2D monolayer cell culture was 0.096 ± 0.013 nmole/10^6 cells (Fig. 8B). The cytochrome P-450 1A1/2 activities of the HepG2 cells cultured as MCAs over the 7-day culture were consistently higher (i.e. > 2.5 times) than that of the 2D monolayer.

4. Discussion

We have developed a class of multivalent inter-cellular linkers based on dendrimers that can aggregate cells effectively at low concentrations with low cytotoxicity. The cells in MCAs maintained high cell viability; proliferated well; exhibited 3D cell morphology and 3D cell-cell contacts; and showed improved detoxification function (EROD) than the 2D monolayer-cultured cells. The bulk of the inter-cellular linkers also disappeared within three days of culture. Although the cell viability and proliferation activities, and the detoxification functions (as indicated by EROD data, Fig. 8B) of HepG2 MCAs, remained stable throughout the culture period, albumin level declined on day 3, indicating that certain cellular functions can potentially be affected by the transient inter-cellular linkers. Further investigations to unravel the mechanisms of the effects would eventually allow development of newer linkers that completely alleviate any adverse effects of the linkers to the cellular functions. This class of novel transient multivalent inter-cellular linkers are sufficiently non-toxic and effective in forming scaffold-free multi-cellular aggregates or structures for applications such as regenerative medicines and drug screening [39, 40].

Effective MCA formation is critically dependent on the successful establishment of cell-linker-cell contacts, as demonstrated by avidin-biotin-hydrazide [24, 25] and PEI-hydrazide [26] . Since the cell-surface sialic acids is limited [41], the cell-surface aldehyde groups generated after cell surface modification is also limited; thus the reactivity between the inter-cellular linkers and cell-surface aldehydes is critical in

determining the effectiveness of establishing cell-linker-cell contacts, which results in MCA formation. Here, we increase the efficiency of inter-cellular linker by increasing the number of functional arms per linker molecule, so as to increase the chances of achieving simultaneous multivalent interactions between the linker molecule and two adjacent cells to form MCAs rapidly. We chose the dendrimer as our polymeric backbone, as the dendrimer's specific "open" molecular structure allows all the functional groups like hydrazides to be exposed on the outside of molecule surface for reacting with cell-surface aldehydes to form MCAs rapidly (Fig. 3A). While for linear polymers, such as PEI-hydrazide, the hydrazide functional arms can be entangled within certain higher order structures and rendered inaccessible for gluing cells [42].

Another conceivable way to improve MCA formation is by increasing the linker concentration. This might not always work. In a simple scenario of cell-linker-cell contacts, very high initial linker concentration will saturate the cell-surfaces with the linkers, which may diminish the availability of the cell-surface aldehyde for reacting with linker for effective MCA formation. We have indeed observed that very high linker concentrations (> 10 mM) rendered cell aggregation ineffective (data not shown). In comparison, increasing the number of functional arms on the inter-cellular linker is a better method to improve the effectiveness of MCA formation.

While linkers with more hydrazide arms exhibit more effective cell aggregation capability than the ones with fewer arms, the linkers' multivalency also correspond to

cytotoxicity at a given linker concentration (Fig. 2) [43] . Therefore, dendrimer hydrazide with more arms is ideal for rapid and large MCA formation involving cells that are more tolerant to exposure to the linkers (e.g. cancer cell lines forming multi-cellular tumor spheroid (MCTS) as drug testing model); while the dendrimer hydrazide linkers with fewer arms that exhibit lower cell aggregation ability and low cytotoxicity should be useful for highly sensitive cell types (e.g. primary cells) in applications where only small cellular aggregates or structures would be sufficient.

The dendrimer hydrazide inter-cellular linkers are synthesized via a simple 2-step reaction using more economical chemicals, such as DAB dendrimers, methyl acrylate, and hydrazine monohydrate, as compared to the expensive avidin, biotin [24, 25]and emaleimidocaproic acid hydrazide (EMCH) [26]. Compared to linear polymer (with a broad distribution of molecular mass) like PEI-hydrazide, dendrimers such as DAB-AM-16 hydrazide has well-defined unimolecular structure, readily verified via NMR, FTIR, and Mass Spectroscopy. The ease of chemical characterization ensures high quality controls of materials, and if necessary, relevant purification schemes can be readily established to ensure very low variance from batch to batch. Such high quality synthesis, purification, characterization and quality control of this biomaterial are critical for further regulatory approval and use in respective applications.

5. Conclusion

We have developed a class of multivalent dendrimer hydrazides as inter-cellular linkers for rapid formation of MCAs. DAB-AM-16 hydrazide is the most effective amongst the dendrimer hydrazides tested, indicating a positive correlation between available multivalent arms per dendrimer hydrazide and the linker's cell aggregation ability. The MCAs maintained high cell viability, proliferation, tissue-like phenotypes, such as tight cell-cell contacts and 3D cell morphology, and improved cell functions over a 7-day culture period, making the dendrimer hydrazides potentially useful in cell- or tissue-based biomedical applications. Further development of dendrimer hydrazide as multivalent transient inter-cellular linkers will produce sophisticated scaffold-free micro-tissue constructs with multiple cell types as biomimicry drug testing platforms or for controlling complex tissue regeneration in regenerative medicine.

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