Macromolecular Research Article

DOI 10.1007/s13233-017-5160-3 www.springer.com/13233 pISSN 1598-5032 eISSN 2092-7673

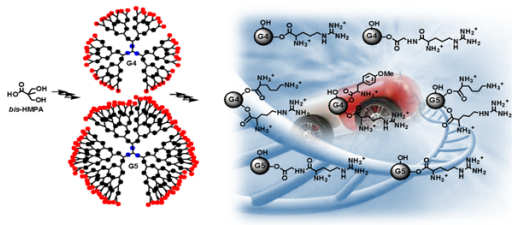
Synthesis and Characterization of Polyester-Based Dendrimers Containing Peripheral Arginine or Mixed Amino Acids as Potential Vectors for Gene and Drug Delivery

Silvana Alfei*,†
Sara Castellaro†

Dipartimento di Farmacia, Sezione di Chimica e Tecnologie Farmaceutiche e Alimentari, Università di Genova, Viale Cembrano 4, I-16148 Genova, Italy

Received May 16, 2017 / Revised July 29, 2017 / Accepted July 31, 2017

Abstract: Macromolecular systems with well-defined sizes, shapes and high controlled architecture like dendrimers are of eminent interest in nanomedical applications such as drug delivery, gene transfection, and imaging. In this paper versatile protocols for the synthesis of polyester-based, hydrolysable, polycationic dendrimers have been setup. Fourth and fifth generation dendrimers equipped with several peripheral hydroxyl groups were prepared from 2, 2-*bis*(hydroxymethyl)propanoic acid. They were successfully esterified with arginine alone or mixed with lysine or *O*-methyltyrosine and with dipeptide arginine-glycine and seven polycationic dendrimers were finally obtained as hydrochlorides. Their structures and composition were confirmed by NMR analysis and by experimental molecular weight computed by volumetric titration and their buffer capacity was higher than fourth generation polyamidoamine (G4-PAMAM) derivatives taken as reference. The synthesized dendrimers harmonize a polycationic character and a buffer capacity which presuppose a good transfection efficiency with a degradable scaffold thus appearing as a promising team of new non-toxic vectors for biomedical applications.



Keywords: polyester-based dendrimers, *L*-arginine, amino acids, buffer capacity.

1. Introduction

Dendrimers[1,2] are a class of synthetic polymers characterized by tree-like branched structure, globular shape, low polydispersity, interior cavities and a large number of functions at the periphery. The typical high controlled architecture of dendrimers makes them different from linear polymers in various respects: their viscosity does not increase linearly with the molar mass but reaches a maximum in correspondence of a precise generation; their globular shape avoids the entanglement of chains as it occurs in linear polymers; their high density of functional groups at the periphery favours the interaction with solvents and reagents thus facilitating their functionalization for example with drugs or target hydrophobic molecules; their cavities can accommodate small natural or synthetic molecules with pharmacological activity protecting them from premature degradation, increasing their solubility in biological fluids, decreasing their toxicity and favouring their bioavailability. These features make dendrimers suitable materials for various biomedical applications such as drug delivery nanocarriers (prodrugs),[3] biosensors,[4,5] bio-imaging agents,[6] and theranostics.[7] Furthermore dendrimers containing nitrogen atoms which can be protonated at physiological pH are deeply investigated as non-viral polymeric vectors[8-17] for delivering nucleic acids such as plasmid DNA, antisense oligonucleotides and RNA into specific defective cell of patients (gene therapy) for treating several severe diseases, including cancer. The interaction between the dendrimers and the nucleic acids is electrostatic so the cationic dendrimer condenses the anionic nucleic acids. Because cell membranes are negatively charged, the net positive charge of the dendrimer nucleic acid complex contributes to the transfection efficiency, although highly cationic systems are also cytotoxic.

Polyamidoamines (PAMAM)[18,19] are among the most investigated dendrimer vectors. PAMAM are considered as good references both in the field of gene delivery[20] and of drug delivery[21-25] thanks to their efficient transfection activity but they are also endowed with high cytotoxicity mainly deriving from the high density of protonated amino groups in the polymeric framework. To improve biodegradability and to decrease toxicity of PAMAM, research efforts focused on their chemical modification such as acetylation,[26,27] PEGylation,[28] introduction of pyrrolidone,[29] carbohydrate,[30,31] amino acid,[32-34] and peptide[35] residues. PAMAM modified in periphery by acetylation,[21] PEGylation,[22] introduction of saccharide residues such as glycidol,[23] D-glucoheptono-1,4-lactone[24] or by conjugation with targeted moieties such as interleukin 6 antibody (IL-6) or RGD peptide[25] have been successfully used to bind covalently or encapsulate therapeutics such as histone deacetylase inhibitors (HDACi), methotrexate (MTX), doxorubicin (DOX) or ursolic acid obtaining promising cancer-specific prodrugs. A different approach to reduce cyto-

Acknowledgments: This work has been supported by University of Genoa (Progetti di Ateneo). The authors are very thankful to Dr Gaby Brice Taptue for language help and to Mr Gagliardo Osvaldo for Elemental analysis.

***Corresponding Author:** Silvana Alfei (alfei@difar.unige.it)
†These authors equally contributed to this work.

toxicity in polycationic vectors was to introduce hydrolysable linkages, as the ester groups, as applied both to PAMAM[36] itself and to non dendrimer vectors such as poly(β-amino esters).[37-39] Moreover, dendrimers containing amino acid residues[32,40-42] have aroused great interest since the diversity of amino acid residues can meet the recognized requirement for modulating the buffer capacity of the vector and facilitate its escape from endosomal acidic compartments through membrane disruption of endosomes.[10] About this *L*-histidine incorporation in *N*-(2-hydroxypropyl)methacrylamide (HPMA)-*co*-oligolysine brush polymers led to an increased buffer capacity and gene transfection efficiency in different endosomal pH ranges in *in vitro* test.[43]

Also *L*-arginine appears particularly appealing to functionalize synthetic vectors. *L*-arginine rich peptides are widely used as carriers for genetic materials[44] and *short interfering* RNA (*si*RNA)[45] and there are many examples of dendrimers with *L*-arginine moieties that improved cellular uptake of *si*RNA[46,47] and transfection activity.[48,49] *L*-arginine-rich hyperbranched copolymers (P-lys-arg0.20) resulted in significantly improved in their transfection efficiency, which depends on the content of arginine in the polymers itself, under all the condition studied and showed lower toxicity in COS-7 cells.[50]

On this ground, with the aim of preparing hydrolysable dendrimer structures which could harmonize the presence of protonated amino groups with low cytotoxicity levels and incorporate drugs or bind DNA we report in this work versatile chemical protocols for the synthesis of 7 dendrimers of fourth and fifth generation derived from 2,2-*bis*(hydroxymethyl)propanoic acid (*bis*-HMPA), a monomer of the AB2 type used to prepare a variety of dendritic architectures.[51]

The here reported prepared dendrimers have an internal polyester structure known for its biocompatibility[52] and a set of peripheral amino acids which include arginine, lysine and *O*-methyltyrosine, also mixed together, or the arginine-glycine dipeptide which produce basic centres protonable at different pKa values. Molecular weight determinations, mean size and surface charge measurements and buffer capacity of the new materials are also reported. The structures of prepared dendrimers have been confirmed also by IR, NMR, and elemental analysis.

It's obvious to think that, thanks to the polycationic but biodegradable structure and to the optimal buffer capacity, which presupposes efficient transfection power, the amino acids-modified dendrimers prepared represent a new appealing and promising team of potential non cytotoxic vectors for gene and drug delivery.

2. Experimental

2.1. Chemical materials

4-Dimethylaminopiridinium *p*-toluenesulfonate (DPTS),[53] isopropylidene-2,2-*bis*(methoxy)propanoic acid D1(A) (**1**),[54] (mp 124-126 °C), benzyl 2,2-*bis*(hydroxymethyl)propanoate D1(Bn) (**2**),[54] (mp 88-90 °C), dendrons D2(BnA) (**3**),[54] D2(A) (**4**),[54] D2(Bn) (**5**),[54] (mp 74-76 °C), D4(BnA) (**6**),[54] D4(A) (**7**),[54] $^{\alpha}$*N*-Boc-*L*-lysine (**17b**), $^{\alpha}$*N*-Boc-*L*-*O*-methyltyrosine (**17c**) and dipeptide $^{\alpha}$*N*-Boc-$^{\omega}$*N*-nitro-*L*-arginine-glycine (**18**)[55] were prepared according to known procedures. Experimental and analytical data for compounds **17b**, **17c**, and **18** are available in Supporting information (Sections 1 and 2, Figure S1, and Table S1).

All the reagents and solvents were purchased from Aldrich or Merck and were used without further purifications. The solvents were dried and distilled according to standard procedures. Petroleum ether refers to the fraction with boiling point 40-60 °C.

2.2. Chemical methods

Melting points, determined on Leica Galen III hot stage apparatus or Mettler Toledo MP50 Melting Point System, are uncorrected. FTIR spectra were recorded as films or KBr pellets on a Perkin Elmer System 2000 spectrophotometer. ^{1}H and ^{13}C NMR spectra were acquired on a Bruker Avance DPX 300 Spectrometer at 300 and 75.5 MHz respectively and assigned through DEPT-135 and decoupling experiments. Coupling constant values were given in Hertz. Fully decoupled ^{13}C NMR spectra were reported. Chemical shifts were reported in δ (parts per million) units relative to the internal standard tetramethylsilane (δ= 0.00 ppm) and the splitting patterns were described as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad signal). Freeze-drying was performed on an EDWARDS Super Modulyo Freeze Dryer, Ice capacity 8 kg, 8 kg/24 h, refrigeration down to -55 °C with 24-place Drum Manifold. Centrifugations were performed on an ALC 4236-V1D Centrifuge at 3,400-3,500 rpm. Dynamic light scattering (DLS) and zeta potential were performed on a Malvern Zetasizer Nano ZS instrument (Southborough, MA). Thin layer chromatography (TLC) system for routine monitoring the course of reactions and confirming the purity of analytical samples employed aluminium-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F254) and detection of spots was made by UV light and/or by ninhydrin solution 0.2% in ethanol and heating in stove at 100 °C. Flash chromatography (FC) was performed on Merck Silica gel (0.040-0.063 mm).

Elemental analyses were performed with an EA1110 Elemental Analyser (Fison-Instruments).

Organic solutions were dried over anhydrous sodium sulphate and were evaporated using a rotatory evaporator operating at reduced pressure of about 10-20 Torr.

2.3. Determination of molecular weights of dendrimers

Molecular weights of dendrimers in the form of hydrochlorides were obtained by volumetric titrations with $HClO_4$ in acetic acid (AcOH).[56] A sample of the dendrimer (10-30 mg) was dissolved in AcOH (5 mL), treated with 2-4 mL of a solution of mercury acetate (1.5 g) in AcOH (25 mL), added with a few drops of a solution of quinaldine red (100 mg) in AcOH (25 mL) and titrated with a standardized 0.17-0.18 N solution of $HClO_4$ in AcOH. The very sharp end points were detected by observing the disappearance of the red colour or its change to yellow and in some cases the appearance of a fine white precipitate.

2.4. Potentiometric titrations of dendrimers

Potentiometric titrations to determine the buffer capacity [β=

$dc_{(HCl)}/d(pH)$][57] and then the average buffer capacity [=$dV_{(HCl)}/dpH(1)$][58] of dendrimers in the form of hydrochlorides were performed at room temperature with a Hanna Microprocessor Bench pH Meter. The dendrimer (20-30 mg) was dissolved in Milli-Q water (30 mL) then was treated with standard 0.1 N NaOH (1-1.5 mL, pH=10-12). The solution was potentiometrically titrated by adding 0.2 mL samples of standard 0.1 N HCl up to total 3.0 mL and measuring the corresponding pH values.[59]

2.5. Dynamic light scattering (DLS) and zeta potential

The hydrodynamic size (diameter) of the amino acids-modified dendrimers was measured in batch mode at 25 °C in a low volume quartz cuvette (pathlength 10 mm) using a Malvern Zetasizer Nano ZS instrument with back scattering detector (173°, 633 nm laser wavelength). Dendrimer samples were prepared at a concentration of 1 mg/mL in PBS and filtered through a 0.02 μm filter. A minimum of twelve measurements per sample were made. Z average diameter, derived from a cumulants analysis of the measured correlation curve, is reported as the intensity-weighted average (Int-Peak) hydrodynamic radius. The zeta potential was measured at 25 °C in deionized water. An applied voltage of 100 V was used. Samples were loaded into pre-rinsed folded capillary cells and a minimum of twelve measurements were made per sample.

2.6. Synthesis of dendrimer G4(OH) (10)

A mixture of D4(A) (**7**)[54] (3.52 g, 1.69 mmol), **8** (0.062 g, 0.52 mmol), DPTS (0.347 g, 1.18 mmol) in CH_2Cl_2 (26 mL) was treated with dicyclohexylcarbodiimide (DCC) (0.38 g, 1.84 mmol) at room temperature under N_2 and magnetic stirring for 24 h. The precipitated dicyclohexylurea (DCU) was removed by filtration and washed with CH_2Cl_2. Filtrate and washings were combined, concentrated at reduced pressure and taken with ethyl acetate (EtOAc) (25 mL) to make precipitate DPTS which was filtered and washed with EtOAc. The solvent was removed at reduced pressure to give dendrimer G4(A) (**9**) which was submitted to a careful column chromatography to eliminate traces of unreacted DCC and *N*-acylureic adduct of **7** performed as follows. Dendrimer **9** was dissolved in the minimum quantity of a mixture petroleum ether/EtOAc=1:1 and passed through a short silica gel column (*h*=20 cm, ø=2 cm) using the same mixture of solvents (100 mL) collecting 1 mL fractions up to disappearance of IR bands at 2118 cm^{-1} (DCC) and at 1648, 1527 cm^{-1} (*N*-acylureic adduct of **7**). The chromatography was completed with petroleum ether/EtOAc=2:3 (100 mL) and EtOAc 100% (200 mL) collected as a single fraction. The removal of the solvent at reduced pressure afforded **9** which was brought to constant weight under vacuum.

2.6.1. Compound 9

Glassy white solid (2.67 g, 82% isolated yield). FTIR (KBr, cm^{-1}): 1739 (C=O). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.89 (s, 3H, CH_3 of *core*), 1.14 (s, 72H, CH_3 of fourth generation), 1.28 (s, 36H, CH_3 of third generation), 1.32 (s, 18H, CH_3 of second generation), 1.35 (s, 72H, CH_3 acetonide), 1.41 (s, 72H, CH_3 acetonide), 1.45 (s, 9H, CH_3 of first generation), 3.62 (d, 48H *J*=11.3 Hz, CH_2O acetonide), 4.15 (d, 48H, *J*=11.8 Hz, CH_2O acetonide), 4.13-4.36 (m, 90H, CH_2O dendrimer + *core*). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 16.88 (CH_3 of *core*); 17.52, 17.55, 17.68, 18.49 (CH_3 dendrimer generations), 21.90-22.10 (CH_3 acetonide), 25.18-25.32 (CH_3 acetonide), 42.06 and 42.09 (quaternary C), 46.06 (quaternary C of *core*), 46.71 (quaternary C), 46.78 (quaternary C), 46.87 (quaternary C), 64.93, 65.72, 65.92, and 65.96 (CH_2O of dendrimer generations), CH_2O *core* not detected, 98.10, 98.12, and 98.19 (quaternary C acetonide), 171.49, 171.71, 171.80, and 173.48-173.60 (C=O). Found: C, 57.38; H, 7.35. $C_{302}H_{468}O_{138}$ requires C, 57.51; H, 7.48%.

A solution of **9** (1.37 g, 0.22 mmol) in MeOH (34 mL) was treated with four spatula tips of acid resin Dowex 50 WX2-200 at room temperature with magnetic stirring for 24 h. The resins were removed by filtration and washed with fresh MeOH. Filtrate and washings were combined, concentrated at reduced pressure to give a pink glassy solid which was left under magnetic stirring overnight in excess of dry Et_2O, filtered to give **10** as a pink hygroscopic solid (1.20 g) which was further purified by dissolution in H_2O (80 mL), centrifuged to remove insoluble residues and freeze-dried to obtain **10** which was stored in a dryer on P_2O_5.

2.6.2. Compound 10

Fluffy white solid (1.14 g, 98% isolated yield). Mp 77 °C. FTIR (KBr, cm^{-1}): 3424 (OH), 1739 (C=O). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 0.79 (s, 3H, CH_3 of *core*), 1.01 (s 72H, CH_3 of fourth generation), 1.16 (s, 36H, CH_3 of third generation), 1.18 (s, 18H, CH_3 of second generation), 1.22 (s, 9H, CH_3 of first generation), 3.28-3.48 (m, 96H, CH_2OH); 4.11-4.19 (m, 90H, CH_2O of dendrimer), 4.44 (br, 48H, OH). ^{13}C NMR (DMSO-d_6, 75.5 MHz): δ 16.37, 16.67, 16.87 (two signals overlapped) and 17.11 (CH_3), 46.17, 46.23 (two signals overlapped) and 50.21 (quaternary C), 63.64 (CH_2OH), 64.35, 64.86 and 65.29 (two signals overlapped) (CH_2O), 171.42 (two signals overlapped), 171.78 and 174.00 (C=O). Found: C, 51.86; H 7.18. $C_{230}H_{372}O_{138}$ requires C, 51.68; H, 7.01%.

2.7. Synthesis of G4 dendrimers 19-22 containing protected arginine residues

2.7.1. General procedure

A solution of **10** in dry DMF (25 mg/mL) was added with the reagent containing *N*-BocArg(NO_2) residues (**17a**, or **18** or a 1/1 feed molar ratio mixture **17a/17b,** or a 1/1 feed molar ratio mixture **17a/17c**) (1.05 equiv./OH of **10**) and DPTS (0.2 equiv./OH of **10**), then treated with a solution of DCC in CH_2Cl_2 (54 mg/mL, 1.21 equiv./OH of **10**). The solution was kept under magnetic stirring at room temperature for 24 h (60 h for the mixture **17a/17c**) then the precipitated DCU was removed by filtration and washed with fresh acetone (30 mL). Filtrate and washings were combined, concentrated at reduced pressure, taken with the minimum quantity of acetone and filtered through a short silica gel column (*h*=8 cm, ø=2 cm) with the help of acetone (25 mL) to remove last traces of DCU. The solvent was removed by evaporation at reduced pressure to give a solid which was crushed with Et_2O, filtered and washed again with

fresh Et_2O (**21**) or simply separated from the solvent by decantation (**19**, **20**, and **22**). The solid was brought to constant weight under vacuum to give the dendrimer. To avoid loss of material dendrimer **20** was not subjected to filtration through silica gel.

2.7.2. Dendrimer G4[17a(36)OH(12)] (19)

Off white glassy solid, 90% isolated yield. FTIR (KBr, cm^{-1}): 3391 (NH), 1741 (C=O ester), 1714 (C=O urethane), 1631 (NH), 1533 (NO_2), 1368 (NO_2).^{1}H NMR (acetone-d_6, 300 MHz): δ0.80-1.53 (m, 138H, CH_3 of dendrimer), 1.42 (s, 324H, CH_3 of Boc), 1.58-2.05 (m, 144H, CH_2CH_2 Arg), 3.43 (br, 72H, CH_2NH Arg), 3.72 (br, 24H, CH_2OH), 4.00-4.60 (m, 210H, CH_2O of dendrimer+ CHNH Arg+12 OH), 6.39 (m, 36H, NHBoc Arg); 7.38-8.90 (m, 108H, $^{\delta}$NH Arg+$^{\omega}NH_2$ Arg). Found: C, 46.11; H, 6.49; N, 15.43. $C_{626}H_{1056}N_{180}O_{318}$ requires C, 46.43; H, 6.57; N, 15.57%.

2.7.3. Dendrimer G4[18(29)OH(19)] (20)

Off white glassy solid, 86% isolated yield. FTIR (KBr, cm^{-1}): 3397 (NH), 1743 (C=O ester), 1629 (NH), 1534 (NO_2), 1368 (NO_2). ^{1}H NMR (DMSO-d_6, 300 MHz): δ0.67-2.04 (m, 254H, CH_3 of dendrimer + CH_2CH_2 Arg), 1.38 (s, 261H, CH_3 of Boc), 3.11 (m, 58H, CH_2NH Arg), 3.42 (m, 38H, CH_2OH), 3.69-4.40 (m, 235H, CH_2O of dendrimer+CHNH Arg+CH_2NH Gly), 4.60 and 4.95 (two br, 19H, OH), 6.87 (br, 29H, NHBoc Arg), 7.58-8.70 (m, 116H, NH Gly+$^{\delta}$NH Arg+$^{\omega}NH_2$ Arg). Found: C, 46.33; H, 6.66; N, 15.78. $C_{607}H_{1010}N_{174}O_{312}$ requires C, 46.32; H, 6.47; N, 15.49%.

2.7.4. Dendrimer G4[17a(25)17b(23)] (21)

Off white solid, 97% isolated yield. FTIR (KBr, cm^{-1}): 3389 (NH), 1745 (C=O ester), 1712 (C=O urethane), 1628 (NH), 1528 (NO_2), 1368 (NO_2). ^{1}H NMR ($CDCl_3$, 300 MHz): δ0.91-2.07 (m, 376H, CH_3 of dendrimer + CH_2CH_2 Arg + $CH_2CH_2CH_2$ Lys), 1.42 (s, 639H, CH_3 of Boc), 3.09 (m, 46H, CH_2NH Lys), 3.26-3.56 (m, 50H, CH_2NH Arg), 3.85-4.82 (m, 234H, CH_2O of dendrimer+CHNH Lys+CHNH Arg), 4.91, 5.46 and 5.65 (m, 71H, NHBoc Lys+NHBoc Arg), 7.34-9.00 (m, 75H, $^{\delta}$NH Arg+$^{\omega}NH_2$ Arg). Found: C, 51.34; H, 7.48; N, 11.49. $C_{873}H_{1491}N_{171}O_{378}$ requires C, 51.32; H, 7.36; N, 11.73%.

2.7.5. Dendrimer G4[17a(17)17c(23)OH(8)] (22)

Off white glassy solid, 98% isolated yield. FTIR (KBr, cm^{-1}): 3390 (NH+OH), 1744 (C=O ester), 1711 (C=O urethane), 1628 (NH), 1515 (NO_2), 1368 (NO_2).^{1}H NMR (DMSO-d_6, 300 MHz,): δ 0.70-1.24 (m, 138H, CH_3 of dendrimer), 1.30 (s, 207H, CH_3 of Boc *O*-Me-Tyr), 1.36 (s, 153H, CH_3 of Boc Arg), 1.38-1.90 (m, 68H, CH_2CH_2 Arg), 2.60-3.00 (m, 46H, CH_2 *O*-Me-Tyr), 3.11 (br, 34H, CH_2NH Arg), 3.49 (br, 16H, CH_2OH), 3.69 (s, 69H, CH_3O), 3.80-4.50 (m, 210H, CH_2O of dendrimer + CHNH Arg + CHNH *O*-Me-Tyr), 4.90 (br, 8H, OH), 6.70-7.30 (m, 132H, CH phenyl+ NHBoc Arg+NHBoc *O*-Me-Tyr), 7.50-8.20 (m, 34H, $^{\omega}NH_2$ Arg), 8.45 (br, 17H, $^{\delta}$NH Arg). Found: C, 55.12; H, 6.83; N, 10.08. $C_{762}H_{1132}N_{108}O_{315}$ requires C, 54.33; H, 6.77; N, 8.98%.

2.8. Removal of NO_2 and Boc groups from dendrimers 19-22

2.8.1. General procedure

A solution of dendrimer containing BocArg(NO_2) residues (**19**, **20**, **21**, or **22**) in 8.8% HCOOH in methanol (35 mg/mL) was added to a slurry of 10% Pd/C (weight of catalyst/weight of substrate=1/1) in 8.8% HCOOH in methanol (20 mg/mL) and stirred at room temperature overnight. The slurry was then filtered through a silica plug (h=8 cm, ø=2 cm) to remove the catalyst which was washed with fresh methanol (30 mL). Filtrate and washings were combined and evaporated at reduced pressure to remove the solvent. The crude product was dissolved in dry methanol (6 mL) and treated under stirring at room temperature for 24 h with excess of acetyl chloride (350 μL). After removal of the solvent at reduced pressure the solid residue was washed in excess acetone under stirring for 3 h, filtered (**23**) or simply separated from the solvent by decantation (**24**, **25**, and **26**) and brought to constant weight at reduced pressure. The unprotected dendrimers were further purified by dissolution in water (5-10 mL), centrifugation (3,400 rpm, 15 min) to remove impurities, freeze-dried and stored in a dryer on P_2O_5.

2.8.2. Dendrimer G4[15a·2HCl(36)OH(12)] (23)

Very hygroscopic fluffy solid, 68% isolated yield. FTIR (KBr, cm^{-1}): 3364 (NH_3^+), 1741 (C=O ester), 1653 (NH).^{1}H NMR (DMSO-d_6, 300 MHz): δ0.79-1.40 (m, 138H, CH_3 of dendrimer), 1.40-2.02 (m, 144H, CH_2CH_2 Arg), 3.21 (m, 72H, CH_2NH Arg), 3.34-3.62 (m, 24H, CH_2OH), 3.62-4.71 (m, 210H, CH_2O of dendrimer+ $CHNH_3^+$ Arg+12OH), 8.07 and 8.75 (br, 288H, $^{\delta}$NH+$^{\omega'}NH_2^+$+$^{\omega}NH_2$+$^{\alpha}NH_3^+$ Arg).

2.8.3. Dendrimer G4[16·2HCl(29)OH(19)] (24)

Very hygroscopic fluffy solid, 74% isolated yield. FTIR (KBr, cm^{-1}): 3412 (NH_3^+), 1739 (C=O ester), 1638 (NH). ^{1}H NMR (DMSO-d_6, 300 MHz): δ0.74-1.38 (m, 138H, CH_3 of dendrimer), 1.42-1.98 (m, 116H, CH_2CH_2 Arg), 3.21 (m, 58H, CH_2NH Arg), 3.30-3.59 (m, 38H, CH_2OH), 3.77-4.45 (m, 235H, CH_2O of dendrimer+ $CHNH_3^+$ Arg+CH_2NH Gly), 4.70 and 5.06 (br, 19H, OH), 7.56-9.51 (m, 261H, NH Gly+$^{\delta}$NH+$^{\omega'}NH_2^+$+$^{\omega}NH_2$+$^{\alpha}NH_3^+$ Arg).

2.8.4. Dendrimer G4[15a·2HCl(25)15b·2HCl(23)] (25)

Very hygroscopic fluffy solid, 69% isolated yield. FTIR (KBr, cm^{-1}): 3431 (NH_3^+), 1744 (C=O ester), 1628 (NH).^{1}H NMR (DMSO-d_6, 300 MHz): δ 0.90-2.07 (m, 376H, CH_3 of dendrimer+CH_2CH_2 Arg +$CH_2CH_2CH_2$ Lys), 2.76 (m, 46H, $CH_2NH_3^+$ Lys), 3.21 (m, 50H, CH_2NH Arg), 3.80-4.80 (m, 234H, CH_2O of dendrimer+$CHNH_3^+$ Lys+$CHNH_3^+$ Arg), 7.60-9.20 (m, 338H, $^{\delta}$NH+$^{\omega'}NH_2^+$+$^{\omega}NH_2$+$^{\alpha}NH_3^+$ Arg and $^{\alpha}NH_3^+$+$^{\varepsilon}NH_3^+$Lys).

2.8.5. Dendrimer G4[15a·2HCl(17)15c·HCl(23)OH(8)] (26)

Very hygroscopic fluffy solid, 61% isolated yield. FTIR (KBr, cm^{-1}): 3431 (NH + OH), 1743 (C=O ester), 1648 (NH), 1250 (C-O), 1129 (C-O).^{1}H NMR (DMSO-d_6, 300 MHz): δ 0.80-1.38 (m, 138H, CH_3 of dendrimer), 1.40-2.10 (m, 68H, CH_2CH_2 Arg), 1.90-3.30 (m, 80H, CH_2 *O*-Me-Tyr+CH_2NH Arg), 3.35-3.60 (m, 16H, CH_2OH), 3.73 (s, 69H, CH_3O), 3.86-4.60 (m, 210H, CH_2O of dendrimer + $CHNH_3^+$ Arg+$CHNH_3^+$ Tyr), 6.86-7.17 (m, 92H, CH phenyl), 7.00-7.90, 8.03 and 8.84 (br, 205H, $^{\omega}NH_2$ Arg+$^{\delta}$NH Arg+ $^{\omega'}NH_2^+$ Arg+$^{\alpha}NH_3^+$ Arg+$^{\alpha}NH_3^+$ *O*-Me-Tyr).

2.9. Synthesis of dendrimer G5(OH) (28)

A solution of G4(OH) (**10**) (495.7 mg, 0.093 mmol), D1(A) (**1**)

(814.2 mg, 4.67 mmol) and DPTS (262.0 mg, 0.89 mmol) in a mixture of CH_2Cl_2/DMF 1/1, v/v (11 mL) was treated under N_2 and magnetic stirring with a solution of DCC (1.11 g, 5.38 mmol) in the same solvent mixture (5 mL). A solid precipitate immediately formed and the suspension was stirred for 24 h at room temperature. The white precipitate of DCU was filtered off and washed with fresh CH_2Cl_2 (15 mL) and EtOAc (2×15 mL). Organic phase and washings were combined and hydrolysed with water (30 mL). Additional EtOAc (15 mL) was added to obtain a better separation of phases. After separation, the aqueous phase was extracted with additional EtOAc (3×30 mL) and all the extracts combined and dried (Na_2SO_4). The removal of the solvents at reduced pressure afforded a white solid which was treated with a mixture petroleum ether 40-60 °C/EtOAc=1:4 to precipitate traces of residual insoluble solids which were removed by filtration, then the solution was passed through a short silica gel column (h=15 cm, ø=2 cm) using the above solvent mixture (200 mL) collecting 1 mL fractions up to disappearance of IR bands at 2118 cm^{-1} (DCC) and at 1648, 1527 cm^{-1} (*N*-acylureic adduct). The chromatography was completed with EtOAc 100% (100 mL) and acetone 100% (100 mL) all collected in a single fraction containing the dendrimer. The removal of the solvents at reduced pressure afforded G5(A) (**27**) which was brought to constant weight under vacuum.

2.9.1. Compound 27

Spongy white solid (798.6 mg, 67% isolated yield). FTIR (KBr, cm^{-1}): 1739 (C=O). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.71-1.28 (m, 282H, CH_3 of *core* and dendrimer generations), 1.35 and 1.36 (s, 144H, CH_3 acetonide), 1.41 and 1.43 (s, 144H, CH_3 acetonide), 3.63 (overlapped d, 96H, *J*=11.7 Hz, CH_2O acetonide), 4.15 (overlapped d, 96H, *J*=11.7 Hz, CH_2O acetonide), 4.26-4.36 (m, 186H, CH_2O of dendrimer). ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 17.39-18.50 (CH_3 of dendrimer), 21.52-21.61 (CH_3 acetonide), 24.94-25.62 (CH_3 acetonide), 42.06-42.16, 46.63-49.20 (quaternary C of dendrimer), 64.86-66.15 (CH_2O of dendrimer), 98.14-98.18 (quaternary C of acetonide), 173.54-174.26 (C=O). Found: C, 57.44; H, 7.30. $C_{614}H_{948}O_{282}$ requires C, 57.43; H, 7.44%.

A solution of **27** (753.3 mg, 0.059 mmol) in MeOH (17 mL) was treated with two spatula tips of acid resin Dowex 50 WX2-200 at room temperature with magnetic stirring for 24 h. The resins were removed by filtration and washed with fresh MeOH. Filtrate and washings were combined, concentrated at reduced pressure to give a glassy solid (674.3 mg) which was dissolved in water (40 mL), filtered to remove insoluble material (8.9 mg) and freeze-dried to give **28** which was stored in a dryer on P_2O_5.

2.9.2. Compound 28

White fluffy solid (636.3 mg, 99% isolated yield). FTIR (KBr, cm^{-1}): 3421 (OH), 1736 (C=O). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 0.88 (s, 3H, CH_3 of *core*), 1.00 (s), 1.01 (s), 1.06 (s), 1.16 (s), 1.23 (s) (279H, CH_3 of dendrimer generations), 3.41-3.44 (m, 192 H, CH_2OH), 4.00-4.20 (m, 186H, CH_2O of dendrimer), 4.60-5.00 (br, 96H, OH). ^{13}C NMR (DMSO-d_6, 75.5 MHz): δ 16.67-16.93 (CH_3 of dendrimer), 46.05-50.10 (quaternary C of dendrimer), 63.55 (CH_2O of dendrimer), 171.52-176.55 (C=O). Found: C, 51.66; H, 7.09. $C_{470}H_{756}O_{282}$ requires C, 51.70; H, 6.98%.

2.10. Synthesis of G5 BocArg(NO_2) dendrimers 29-31

Dendrimer G5(OH) **(28)** was treated with **17a**, or **18** or with a 1/1 feed molar ratio mixture **17a/17b** as described for the synthesis of **19-22**. To avoid loss of material due to strong adsorption, dendrimer **30** was not subjected to filtration through silica gel.

2.10.1. Dendrimer G5[17a(66)OH(30)] (29)

Off white glassy solid, 98% isolated yield. FTIR (KBr, cm^{-1}): 3403 (NH), 1741 (C=O ester), 1699 (C=O urethane), 1630 (NH), 1536 (NO_2), 1368 (NO_2). ^{1}H NMR (acetone-d_6, 300 MHz): δ 1.03-1.52 (m, 282H, CH_3 of dendrimer), 1.42 (s, 594H, CH_3 of Boc), 1.56-2.02 (m, 264H, CH_2CH_2 Arg), 3.44 (m, 132H, CH_2NH Arg), 3.72 (m, 60H, CH_2OH), 3.95-4.75 (m, 414H, CH_2O of dendrimer +CHNH Arg+30 OH), 6.40 (m, 66H, NHBoc Arg), 7.41 and 9.00 (m, 198H, $^{\delta}$NH+$^{\omega}$$NH_2$ Arg), Found: C, 46.82; H, 6.39; N, 15.36. $C_{1196}H_{1680}N_{264}O_{612}$ requires C, 46.63; H, 6.58; N, 15.01%.

2.10.2. Dendrimer G5[18(52)OH(44)] (30)

Off white solid, 98% isolated yield. FTIR (KBr, cm^{-1}): 3397 (NH), 1743 (C=O ester), 1629 (NH), 1533 (NO_2), 1368 (NO_2). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 0.85-2.06 (m, 490H, CH_3 of dendrimer + CH_2CH_2 Arg), 1.37 (s, 468H, CH_3 of Boc), 3.14 (m, 104H, CH_2NH Arg), 3.49 (m, 88H, CH_2OH), 3.70-4.40 (m, 446H, CH_2O of dendrimer+CHNH Arg+CH_2NH Gly), 4.58 and 4.92 (br, 44H, OH), 6.85 (m, 52H, NHBoc Arg), 7.54-8.76 (m, 208H, NH Gly+$^{\delta}$NH Arg+$^{\omega}$$NH_2$ Arg). Found: C, 46.76; H, 6.30; N, 14.55. $C_{1146}H_{1900}N_{312}O_{594}$ requires C, 46.57; H, 6.48; N, 14.79%.

2.10.3. Dendrimer G5[17a(38)17b(30)OH(28)] (31)

Off white solid, 96% isolated yield. FTIR (KBr, cm^{-1}): 3392 (NH), 1743 (C=O ester), 1705 (C=O urethane), 1635 (NH), 1526 (NO_2), 1369 (NO_2). ^{1}H NMR (acetone-d_6, 300 MHz): δ 0.95-1.90 (m, 614H, CH_3 of dendrimer + CH_2CH_2 Arg + $CH_2CH_2CH_2$ Lys), 1.39 (s, 882H, CH_3 of Boc), 2.91 (m, 60H, CH_2NH Lys), 3.15 (m, 76H, CH_2NH Arg), 3.49 (m, 56H, CH_2OH), 3.74-5.05 (m, 418H, CH_2O of dendrimer+CHNH Lys+CHNH Arg+28 OH), 6.45-8.79 (m, 212H, NHBoc Lys+NHBoc Arg+$^{\delta}$NH Arg+$^{\omega}$$NH_2$ Arg). Found: C, 50.96; H, 7.21; N, 11.11. $C_{1368}H_{2318}N_{250}O_{622}$ requires C, 50.99; H, 7.25; N, 10.87%.

2.11. Removal of NO_2 and Boc groups from G5 dendrimers 29-31.

NO_2 and Boc groups were removed from dendrimers **29-31** as described for **19-22**.

2.11.1. Dendrimer G5[15a·2HCl(66)OH(30)] (32)

Very hygroscopic fluffy solid, 46% isolated yield. FTIR (KBr, cm^{-1}): 3402 (NH_3^+), 1750 (C=O ester), 1652 (NH). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 0.79-1.39 (m, 282H, CH_3 of dendrimer), 1.47-2.00 (m, 264H, CH_2CH_2 Arg), 3.21 (m, 132H, CH_2NH Arg), 3.47 (br, 60H, CH_2OH), 3.80-4.71 (m, 414H, CH_2O of dendrimer+$CHNH_3^+$ Arg+30 OH), 7.81 and 9.55 (m, 528H, $^{\delta}$NH+$^{\omega'}$$NH_2^+$+$^{\omega}$$NH_2$+$^{\alpha}$$NH_3^+$ Arg).

2.11.2. Dendrimer G5[16·2HCl(52)OH(44)] (33)

Very hygroscopic off white fluffy solid, 71% isolated yield. FTIR

(KBr, cm^{-1}): 3412 (NH_3^+), 1740 (C=O ester), 1638 (NH). ^{1}H NMR (DMSO-d_6, 300 MHz): δ0.76-1.40 (m, 282H, CH_3 of dendrimer), 1.40-1.98 (m, 208H, CH_2CH_2 Arg), 3.20 (m, 104H, CH_2NH Arg), 3.33-3.61 (m, 88H, CH_2OH), 3.70-4.49 (m, 490H, CH_2O of dendrimer+$CHNH_3^+$ Arg+CH_2NH Gly+29 OH), 4.91 (br, 15H, OH), 7.22-9.93 (m, 468H, NH Gly+$^{\alpha}NH_3^+$+$^{\delta}NH$+$^{\omega'}NH_2^+$+$^{\omega}NH_2$ Arg).

2.11.3. Dendrimer G5[15a·2HCl(38)15b·2HCl(30)OH(28)] (34)
Very hygroscopic pale yellow fluffy solid, 51% isolated yield. FTIR (KBr, cm^{-1}): 3411 (NH_3^+), 1743 (C=O ester), 1631 (NH). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 0.90-2.05 (m, 614H, CH_3 of dendrimer+CH_2CH_2 Arg + $CH_2CH_2CH_2$ Lys), 2.75 (m, 60H, $CH_2NH_3^+$ Lys), 3.18 (m, 76H, CH_2NH Arg), 3.39-3.55 (m, 56H, CH_2OH), 3.80-4.70 (m, 418 H, CH_2O of dendrimer+$CHNH_3^+$ Lys+$CHNH_3^+$ Arg + 28 OH), 7.60-9.20 (m, 484H, $^{\delta}NH$+$^{\omega'}NH_2^+$+$^{\omega}NH_2$+$^{\alpha}NH_3^+$ Arg and $^{\alpha}NH_3^+$+$^{\varepsilon}NH_3^+$ Lys).

3. Results and discussion

The target of our work was the setting up of synthetic protocols for the obtainment of polycationic dendrimer vectors able to incorporate and deliver drugs or to bind and deliver genetic material. The desired vectors had to be characterized by hydrolysable inner matrix of the ester type and had to be equipped with an appropriate buffer capacity (β) in order to be able to enter in target cells by an endocytotic process, to escape the endosome thanks to the so-called *proton-sponge* effect when inside and to release the transported material into the cytosol (transfection activity). The ester matrix ensures low cytotoxicity levels of the prepared materials while the amino acids selected for decorating the periphery, having differently protonable basic residues, contribute to electrostatically bind DNA or to encapsulate drugs and to the achievement of the proper β value. Between them, arginine has the commitment of promote cellular up-take. As hydrolysable ester matrix we focused our attention on dendrimers derived from 2,2-*bis*(hydroxymethyl)propanoic acid (*bis*-HMPA), a known building block of the AB2 type used to prepare an ample variety of dendrimer architectures as reviewed in depth recently.[51]

The adoption of the synthetic strategy known as double-stage convergent approach[56] required the synthesis of a fourth generation acetonide-protected dendron starting from *bis*-HMPA, its reaction with a *core* molecule functionalized with three hydroxyl groups and final removal of the acetonide protection to obtain the fourth generation polyhydroxyl dendrimer.

The procedure allowed a good monitoring of the dendron growth through FTIR and NMR spectroscopy and minimized the risk of structural defects of the final dendrimer. Careful column chromatography was a necessary purification step for the obtainment of pure products when in the protected form. Alternative simpler routes based on one-pot reactions exploiting *bis*-HMPA to afford polydispersed hyperbranched dendrimers were not considered in order to avoid a defective macromolecular structures and challenging NMR interpretation of spectra. Chart 1 shows the fourth generation dendron **7**, its precursors, and *core* molecule **8** used in this work. Besides the identification number, for an easier structural identification in the text, to all dendrons (**D**) and dendrimers (**G**) a code made by a letter (**D** or **G**) followed by a number to specify the generation including within parenthesis the type of functionalization at the periphery was assigned. **Bn** was used to indicate benzyl, **A** to indicate acetonide and **OH** to indicate hydroxyl groups.

3.1. Synthesis of dendrimer G4(OH) (10)

Dendron **7**[54] was used to synthesize the new acetonide den-

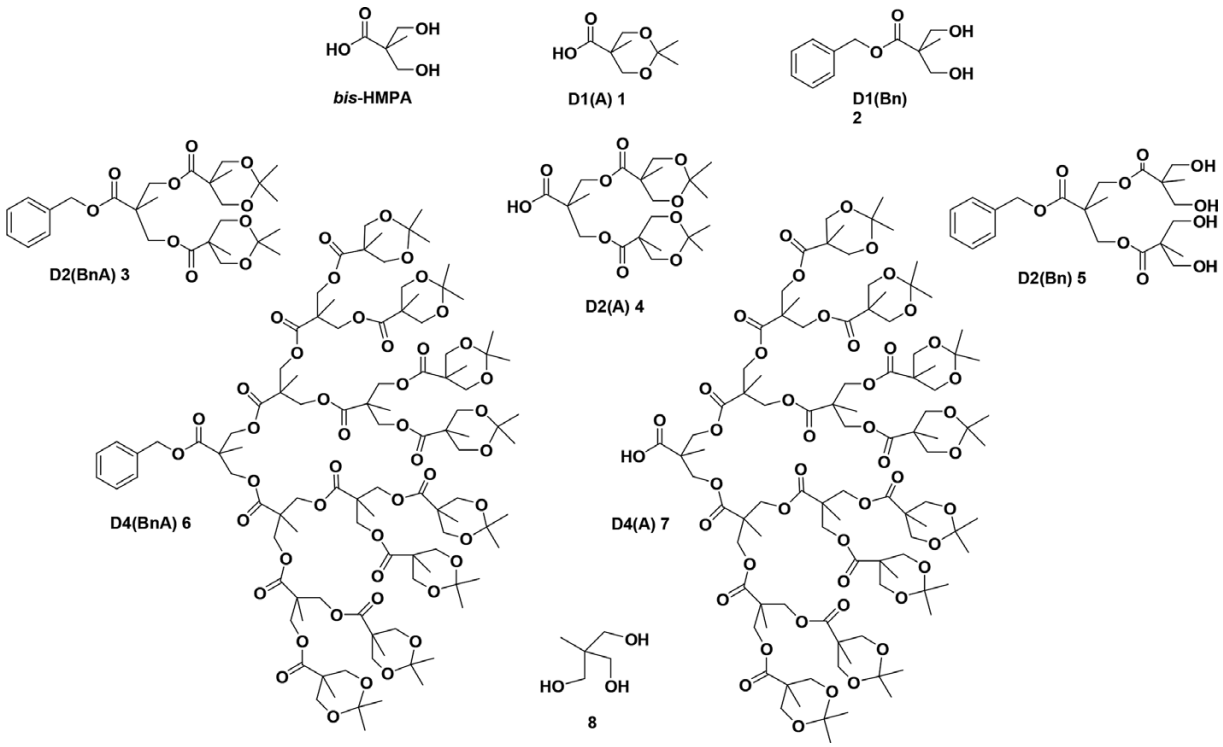
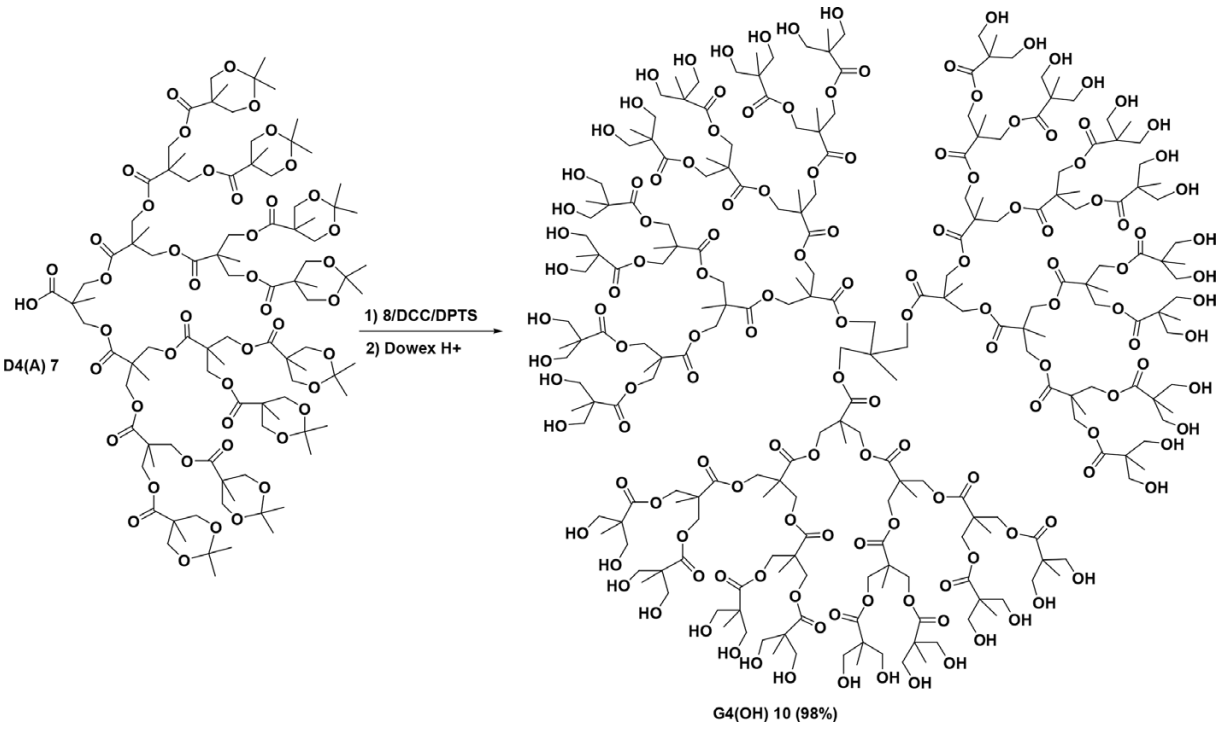


Chart 1. Structures of dendrons **1**-**7** and *core* molecule **8** used to prepare dendrimer G4(OH) (**10**).



Scheme 1. Synthesis of dendrimer G4(OH) (**10**).

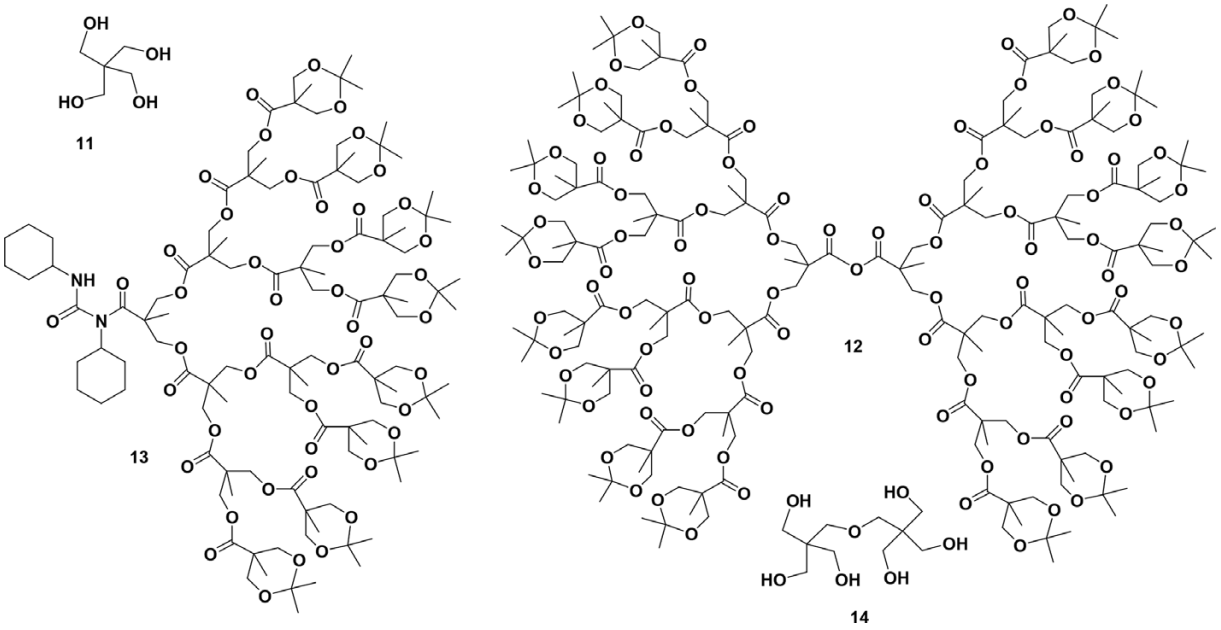


Chart 2. Structures of *core* molecules **11** and **14**, anhydride **12** and adduct **13**.

drimer G4(A) (**9**) by reaction with 2,2-*bis*(hydroxymethyl)propan-3-ol (**8**) as the *core* molecule. The successive removal of the acetal protection with acid resin Dowex 50 WX2-200 afforded G4(OH) (**10**) having 48 hydroxyl groups at the periphery susceptible of functionalization (Scheme 1).

Attempts to bind **7** to other *core* molecules such as 2,2-*bis*(hydroxymethyl)-1,3-propanediol (pentaerythrytol, **11**) and obtain a four-dendron dendrimer failed for steric buttressing, presumably. The reaction of the carboxylic function of **7** with DCC brought only to the isolation of significant quantities of anhydride **12** (60%) and *N*-acylureic adduct **13** (20%) both derived from DCC-activated **7**. The reaction of **7** with 2-{[3-hydroxy-2,2-*bis*(hydroxymethyl)propoxy]methyl}-2-(hydroxymethyl)propane-1,3-diol (dipentaerythrytol, **14**) having less buttressed OH groups was successful but the low yields recorded (16%) and the lack of reactivity of the hydroxyl-free dendrimer towards esterification in preliminary test reaction made us not to consider the molecule further. Chart 2 shows *core* molecules **11** and **14**, anhydride **12** and adduct **13**.

Dendrimer **10** was soluble in DMF, DMSO, H_2O and insoluble in other organic solvents. The ^{1}H NMR spectrum of **10** was very diagnostic of the dendrimer structure (Figure 1) and was char-

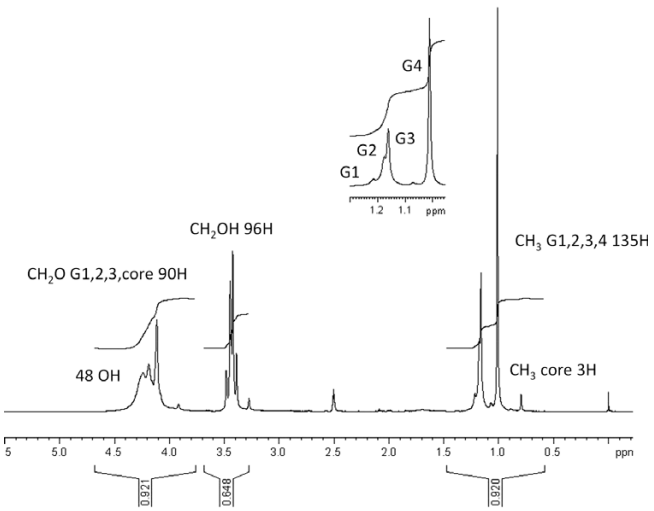


Figure 1. ^{1}H NMR spectrum of G4(OH) (**10**) in DMSO-d_6 (G=generation).

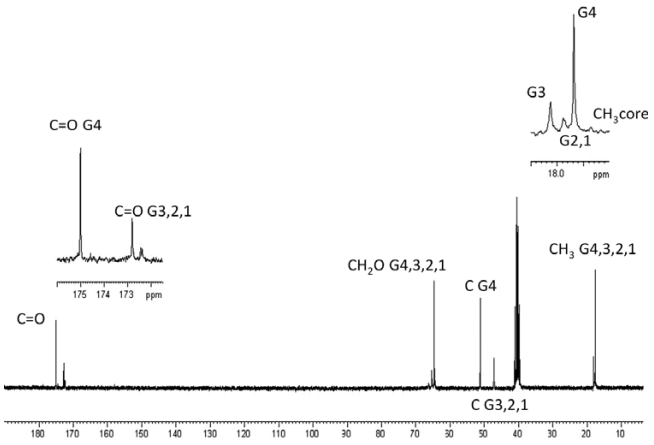


Figure 2. ^{13}C NMR spectrum of G4(OH) (**10**) in DMSO-d_6 (G=generation).

acterized by the multiplets of CH_2 of first, second, third generation and of the *core* in the interval 3.92-4.18 ppm, by the multiplet corresponding to 96 protons of methylene groups in CH_2OH at 3.43 ppm and by the broad singlet at 4.4 ppm of the OH groups, whose number was deduced with precision from the reliable integral value of the proton peak of the CH_2OH groups by dividing by two. The signals of decreasing intensities corresponding to the CH_3 of *bis*-HMPA in the fourth generations and to the CH_3 of the *core* were also visible at 1.01, 1.16, 1.18, 1.21 and 0.80 ppm.

The structure of **10** was further confirmed by ^{13}C NMR spectrum (Figure 2) where the signals relative to C=O of esters of the fourth, third, second and first generation were observed at 175.02, 172.80, 172.44, and 172.38 ppm respectively. The signals of inner CH_2O residues of the three generations were found between 65.39 and 66.30 ppm while those of peripheral CH_2OH at 64.64 ppm. The signals for the quaternary carbons were visible at 51.22 ppm (fourth generation) and between 47.10 and 47.30 ppm (first, second and third generation). Methyl groups of all the generations and of the *core* were observed at 17.68, 18.12, 17.88, 17.85, and 17.37 ppm.

3.2. Selection and protection of amino acids

To functionalize the surface of **10** with residues endowed with different basic properties and structures we chose amino acids **15a-c** and dipeptide **16**. Their structural features are: presence or absence of a second protonable function atom in side chain. The simultaneous presence on the dendrimer of two amino acids was designed to have hetero dendrimers with more variety of protonable groups at different pKa, in order to optimize the buffer capacity values. Furthermore the *O*-methyltyrosine (**15c**) was included in view of preparing a hetero functional dendrimer containing besides amino acid residues hydrophobic residues which could improve cellular uptake.[34] In addition, the presence of an easy detectable signal of the methoxy group could have helped NMR investigations on the dendrimer composition. In dipeptide **16** the glycine residue was introduced as a polar spacer for arginine hopefully to facilitate the functionalization reaction. Chart 3 shows the structures of the free amino acids **15a-c**, dipeptide **16**, protected amino acids **17a-c** and dipeptide **18**.

The arginine moiety appeared particularly appealing since it is known that arginine-rich peptides are extensively used as cell-penetration peptides for nucleic acids[44] and *si*RNA[45] delivery, and dendrimers or copolymers containing arginine are known to improve *si*RNA cellular uptake[46,47] and efficiency of transfection[48-50] and showed lower toxicity[50] and for this are widely used as vectors of genetic materials.[60] These features have been attributed to the ability of protonated guanidine residues of favouring interaction with the negatively charged residues such as phosphate, sulfate and carboxylate groups present in cell mem-

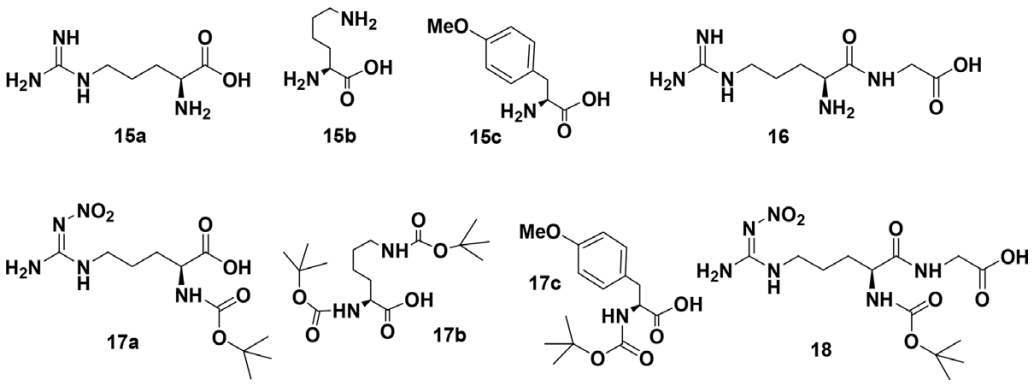


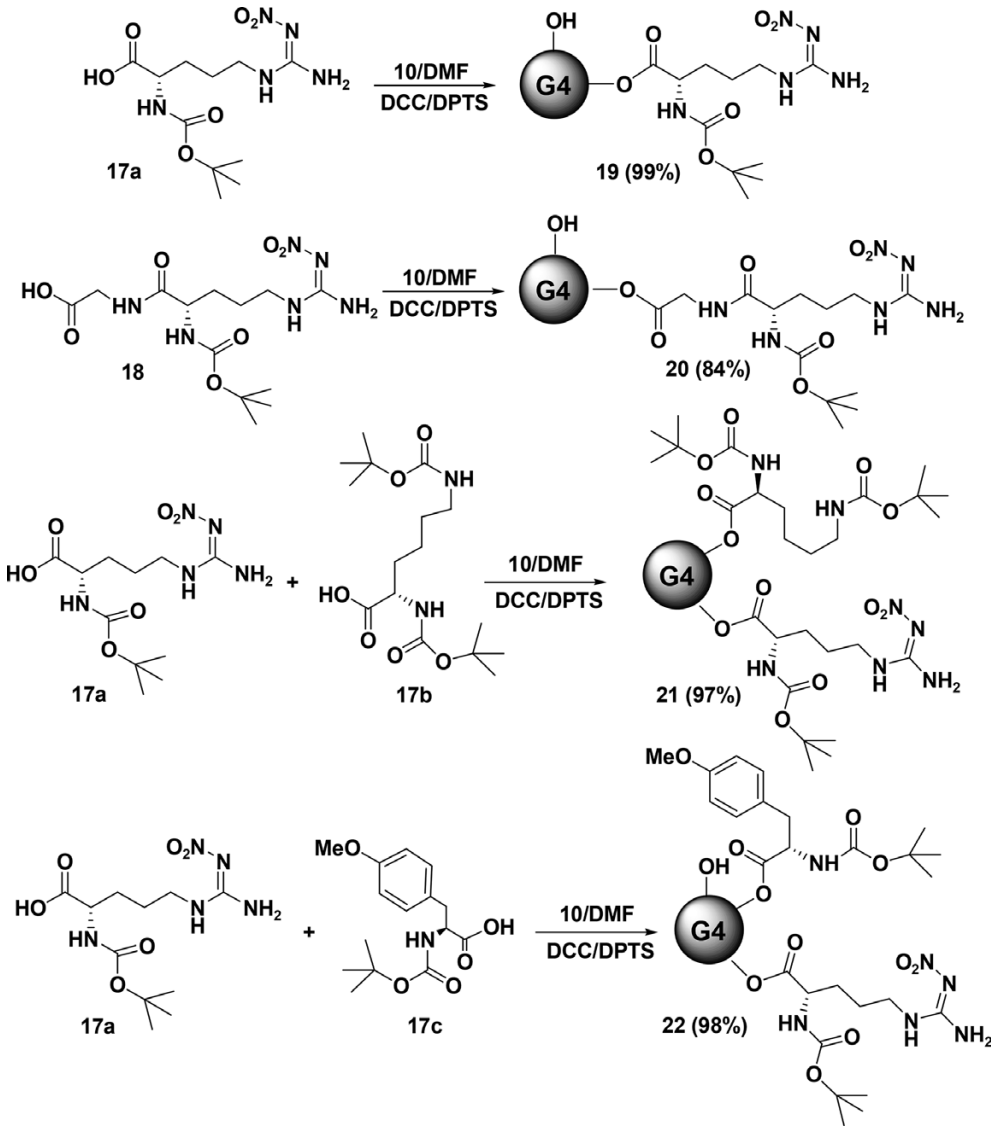
Chart 3. Free and protected amino acids and dipeptide used to functionalize dendrimers.

branes. A primitive strategy thought to obtain dendrimers from amino acids was based on the following three steps: Boc-protection of amino acids, esterification of the dendrimer **10** with Boc-protected amino acids through carbodiimide activation and final removal of the Boc-protection to afford dendrimer in the form of hydrochlorides. So **15b** and **15c** were converted into their *N*-Boc-derivatives **17b** and **17c** (Chart 3) following standard procedures and their structure was confirmed by ^{1}H and ^{13}C NMR spectroscopy (Supporting Information, Section 1.2 and Section 1.3). It is interesting to note the occurrence of rotamers due to restricted rotation of the urethane bond[61] in the case of **17b**. The synthesis of arginine completely protected with three Boc groups to use in the above protocol proved very troublesome and pushed us to consider commercial **17a** and dipeptide **18** as possible arginine-containing reagents to functionalize dendrimer **10** applying proper changes in the deprotection steps. The synthesis of dipeptide **18** was performed by reacting **17a** with methyl ester of Boc-protected glycine[55] (Supporting information, Section 2).

3.3. Synthesis of G4 dendrimers containing arginine residues

For the preparation of fourth generation dendrimers containing arginine residues the grafting of arginine **17a** and dipeptide **18** to **10** was successful with DCC and DPTS as activator and catalyst respectively instead of EDC and DMAP. Dendrimer **10** was also treated in the same reaction conditions with a 1:1 feed molar mixture of **17a** and lysine **17b** to explore the possibility of obtaining hetero-dendrimers containing residues having wider basic properties and with a 1:1 feed molar mixture of **17a** and **17c** to investigate the effect of introducing hydrophobic moieties at the periphery (Scheme 2).

The analysis of ^{1}H NMR spectra of **19**, **20**, and **22** evidenced that not all the OH groups at the periphery were reactive towards the protected arginine reagents. The integration of the proton signals at 3.43 ppm (arginine $CH_2{}^{\delta}N$) and at 3.72 ppm (CH_2OH) of protected **19** allowed to deduce that 12 OH groups were still present at the periphery. In the case of **20** the integration of the signals in the range 3.69-4.40 ppm (CH_2O of inner



Scheme 2. Synthesis of G4 dendrimers **19-22** containing protected arginine residues.

matrix, arginine $CH_2^{\delta}N$ and glycine CH_2N) allowed to deduce that unreacted OH groups were 19. Finally, for **22** the integration of signals at 3.11 ppm (arginine $CH_2^{\delta}N$), 3.69 (CH_3O of *O*-methyltyrosine) and 4.90 (free OH) allowed evidencing the presence of 23 arginine, 17 tyrosine and 8 free hydroxyl residues. The uncomplete functionalization was attributed to a rigidity and bulkiness of the BocArg (NO_2).

The phenomenon was not observed with the mixed dendrimer **21** where the arginine reagent was diluted with lysine in a 1:1 feed molar ratio. In the ^{1}H NMR spectrum of **21** the comparison of integrals of the broad signal of $CH_2^{\varepsilon}N$ group of lysine at 3.09 ppm and of $CH_2^{\delta}N$ arginine at 3.26-3.56 ppm allowed to establish that the lysine/arginine ratio was 1/1.09 corresponding to 23 lysine and 25 arginine residues at the periphery of dendrimer respectively. This indicated that no substantial difference in reactivity of the two amino acids occurred, contrarily to what observed with tyrosine dendrimer **22**.

In Supporting Information (Figure S27(A)) is reported a significant portion of ^{1}H NMR spectrum of **21** showing the signals used to calculate the ratio Arg/Lys with the corresponding integrals.

The successive deprotections of the four dendrimers were performed in two consecutive steps by removing first the NO_2 groups with formic acid in the presence of Pd/C followed by treatment with CH_3COCl and methanol to remove Boc groups (Scheme 3).

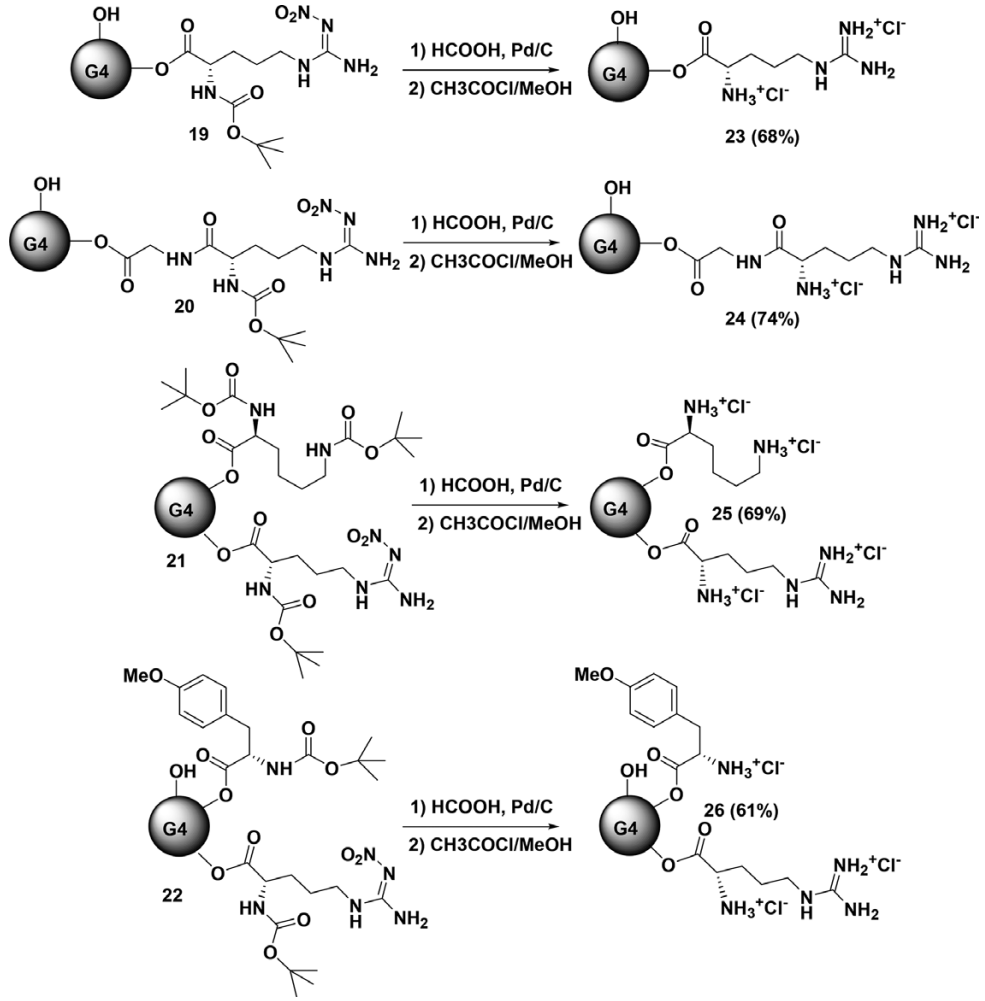
The composition data, confirmed by similar calculations applied to selected ^{1}H NMR signals of deprotected **23-26** were employed to obtain corrected molecular weights of dendrimers and yields. In Supporting Information (Figure S27(B)) is reported a significant portion of ^{1}H NMR spectrum of **25** showing the signals used to calculate the ratio Arg/Lys with the corresponding integrals.

The ^{1}H NMR spectra were not only useful for estimating the dendrimers composition both Boc protected and in the form of hydrochlorides but also to control and confirm the structures obtained in the various steps. As samples, the spectral modifications that occurred both in the phase of esterification and in the phase of protecting groups removal along the preparation of dendrimers **25** and **26** are reported in Figures S24 and S25, respectively (Supporting information).

The polycationic dendrimers **23-26** were obtained as hygroscopic glassy solids after freeze-drying and were soluble in MeOH, DMSO, DMF, H_2O and insoluble in toluene, Et_2O, THF, dioxane, CH_2Cl_2, $CHCl_3$, EtOAc, acetone, acetonitrile, EtOH.

3.4. Synthesis of G5 dendrimers containing arginine residues

Since it is quite difficult to establish a priori which are the opti-



Scheme 3. Synthesis of G4 dendrimers **23-26** containing arginine residues.

mal dimensions of a vector to efficaciously bind plasmid or fragments of DNA and bring it to the nucleus, we included in our research the achievement of higher generations dendrimers. To this end the first synthetic route designed to obtain **27** consisted in removing the acetal protection of D4(BnA) (**6**) to achieve D4(Bn)OH having 16 hydroxyl groups at the periphery, in growing it up of one generation by reaction with D1-type dendron **1** to obtain the fifth generation dendron D5(BnA) and in removing the benzyl protecting group by catalytic hydrogenation to achieve D5(A). Finally the reaction of D5(A) with the *core* **8** it should have provided **27**. The experimental procedure to prepare dendrons D4(Bn)OH, D5(BnA) and D5(A) and their structures with relative yields are available in Supporting information (Section 3 and Figure S2).

The yields reported makes it clear that no obstacle was encountered along this synthesis until obtaining the fifth generation dendron D5(A) but unfortunately the last step didn't provide the desired **27** and only unreacted D5(A) was obtained at the end of the reaction. So we decided to consider the fourth generation dendrimer **10** as a "hypercore"[2] to which graft dendrons of the D1-type. According to this procedure, dendrimer **10** was grown of one generation through reaction with **1** which yielded G5(OH) (**28**) after deprotection (Scheme 4).

The different polarity of polyhydroxyl **10** and acetonide-protected **27** allowed the separation of the two dendrimers by column chromatography.

Dendrimer **28** was functionalized with protected arginine reagents **17a**, **18** and with a 1:1 feed molar ratio mixture of **17a** and **17b** in the same conditions as **10** affording dendrimers **29-31** (Scheme 5). The successive removal of protecting groups gave dendrimers **32-34** (Scheme 6).

Dendrimers **32-34** isolated by freeze-drying appeared as amorphous hygroscopic solids soluble in MeOH, DMSO, DMF, water and insoluble in toluene, Et_2O, THF, dioxane, CH_2Cl_2, $CHCl_3$, EtOAc, acetone, acetonitrile, and EtOH.

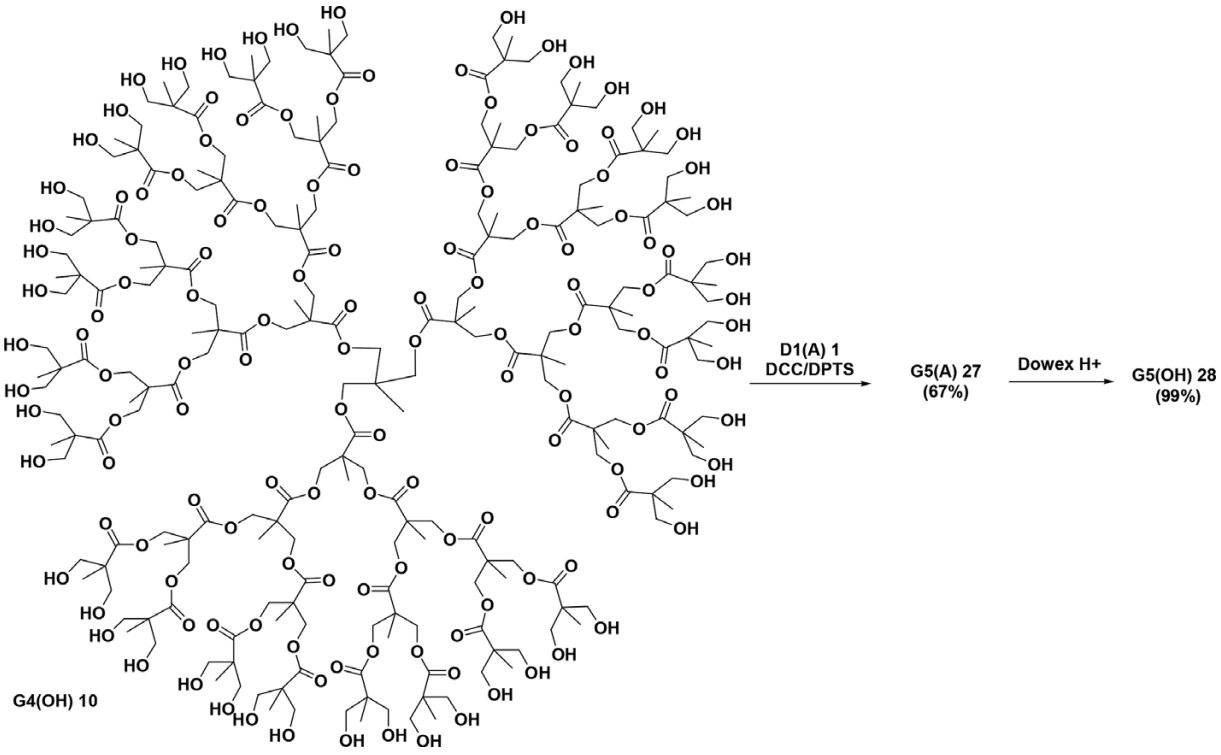
Also in the case of G5-dendrimers ^{1}H NMR spectra were used to check the phases of functionalization and Boc groups removal. As sample, spectral modifications that occurred in the preparation of dendrimers **34** are reported in Figure S26 (Supporting information).

Then in Figure S27 are reported the significant portions of ^{1}H NMR spectra of dendrimers **30** (C) and **33** (D) where the signals and the corresponding integrals used for detect the dendrimer composition (number of free OH groups) are shown. Chart 4 shows simplified structures of all the prepared dendrimers in the form of hydrochlorides.

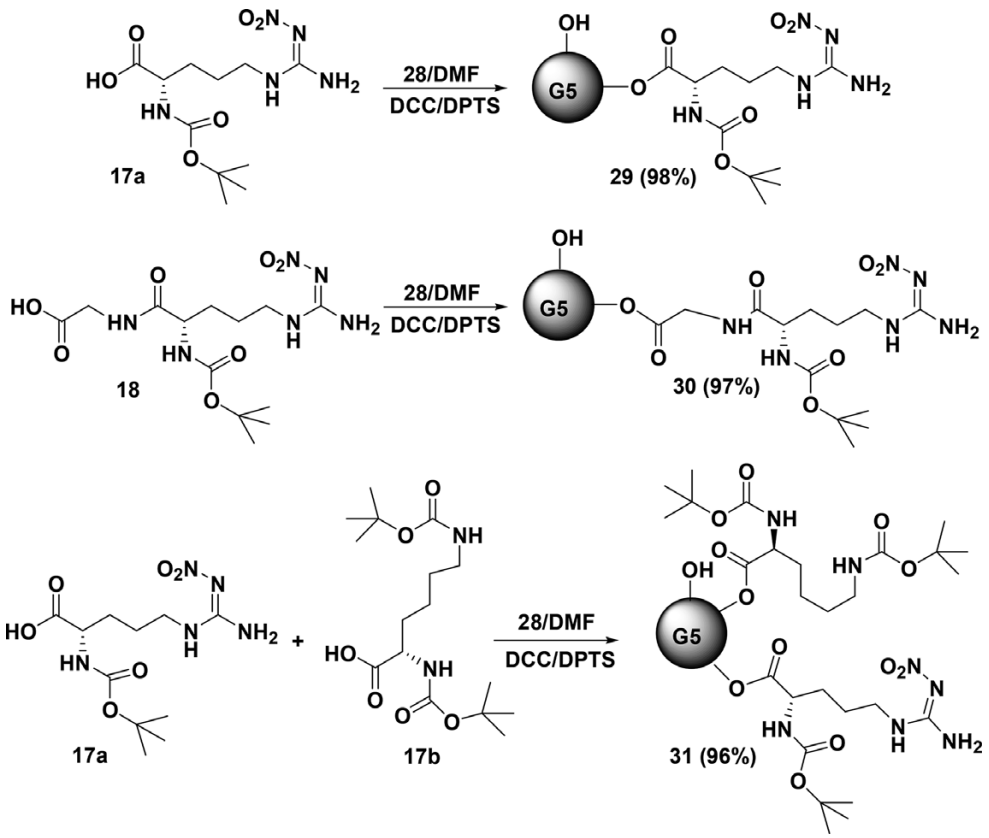
The generally high tendency of these materials to absorb water precluded the obtainment of reliable micro-analytical data.

3.5. Molecular weight determinations of the prepared dendrimers by titrations

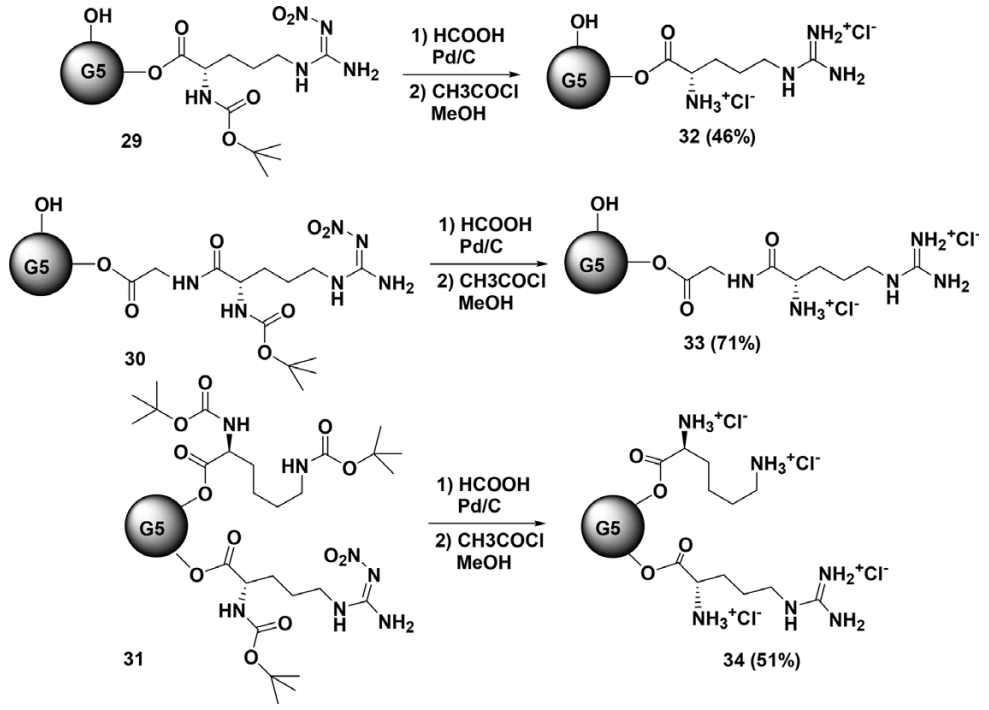
To determine the molecular weight of the 7 dendrimers reported in Chart 4 and have additional evidence of the prepared structures and composition at the periphery without having to resort to routine well known but very expensive techniques like MALDI-TOF, the titration of amine hydrochlorides with $HClO_4$ solutions in AcOH in the presence of mercuric acetate and quinaldine red as indicator[56] proved simple and affordable. There are no references up to day which use this innovative cheap and fast method for dendrimers, but its accuracy has been secured by a sharp endpoint of titration, while its reliability have been demonstrated by the reproducibility of results. Table 1 collects the comparison between calculated and observed



Scheme 4. Synthesis of fifth generation dendrimer G5(OH) (**28**).



Scheme 5. Synthesis of G5 dendrimers containing protected arginine residues.



Scheme 6. Deprotection of G5 dendrimers containing arginine residues.

molecular weights as obtained by titration. The generally good or very good agreement of observed data with the calculated one confirmed the molecular structures of the prepared dendrimers and the goodness of the method. The numbers in the round brackets indicate the dendrimer relative compositions.

3.6. Potentiometric titrations of the prepared dendrimers

It is generally accepted that carriers improve their efficacy of transfection if endowed with a proper buffer capacity [$\beta = dc_{(HCl)}/dpH$][57] and so with an average buffer capacity [$\bar{\beta} = dV_{(HCl)}/dpH(1)$][58] in the pH range 4.5-7.5 suitable to make them escaping from

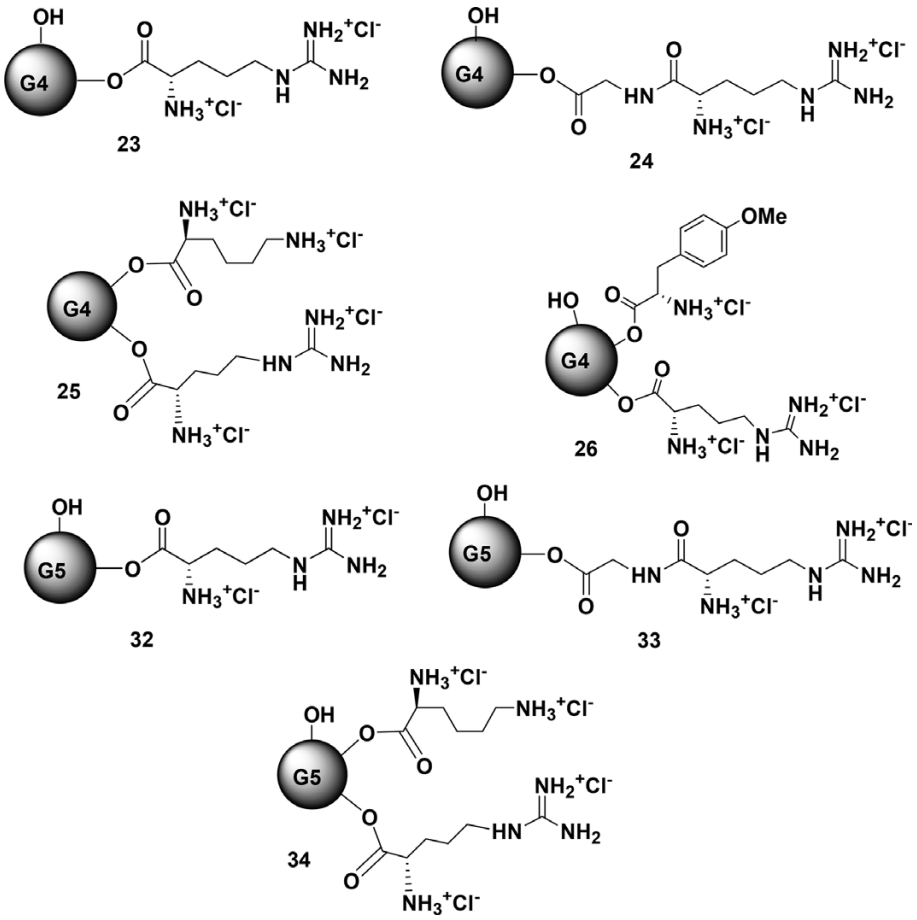


Chart 4. Synopsis of the simplified structures of the prepared dendrimers in the form of hydrochlorides.

Table 1. Molecular weights of dendrimers **23-26** and **32-34** from titration with $HClO_4$[56]

Dendrimer	N[a]	MW (calc.)	MW (obs.)
G4[15a·2HCl(36)OH(12)] (**23**)	72	13593.36	13150
G4[16·2HCl(29)OH(19] (**24**)	58	13644.10	13293
G4[15a·2HCl(25)15b·2HCl(23)] (**25**)	96	15698.37	15155
G4[15a·2HCl(17)15c·HCl(23)OH(8)] (**26**)	57	14152.23	14256
G5[15a·2HCl(66)OH(30)] (**32**)	132	26040.26	26100
G5[16·2HCl(52)OH(44)] (**33**)	104	25799.41	26000
G5[15a·2HCl(38)15b2·HCl(30)OH(28)] (**34**)	136	25661.06	25745

[a]Number of peripheral basic groups as determined by NMR.

endosomes compartments where pH is in the 5-6 range.[10] To have an estimate of the buffer capacity of the prepared dendrimers, potentiometric titrations of **23-26** and **32-34** were performed according to Benns *et al.*[59] Since PAMAM are considered as good reference in the field both of gene delivery[20] and of drug delivery[21-25] literature data of three G4-PAMAM derivatives[62] potentiometrically titrated with the same protocol were used to obtain their titration curve. The β and $\overline{\beta}$ values of each synthetized sample and of G4-PAMAM derivatives were calculated from the titration data. Table 2 collects the β values recorded in the pH range 5-7.

Figures of all the titration and β values curves $\overline{\beta}$ and of the histogram of all the calculate are available in Supporting information (Figures S28-30).

Though no clear, typical patterns came out from data in Table 2

Table 2. Buffer capacity values of dendrimers **23-26**, **32-34** and G4-PAMAM derivatives in the pH range 6-7 from potentiometric titrations

Dendrimer	pH	B
G4[15a·2HCl(36)OH(12)] (**23**)	6.4	0.0833
G4[16·2HCl(29)OH(19] (**24**)	6.0	0.0150
G4[15a·2HCl(25)15b·2HCl(23)] (**25**)	6.5	0.033
G4[15a·2HCl(17)15c·HCl(23)OH(8)] (**26**)	6.5	0.0741
G5[15a·2HCl(66)OH(30)] (**32**)	6.8	0.0426
G5[16·2HCl(52)OH(44)] (**33**)	6.1	0.0227
G5[15a·2HCl(38)15b·2HCl(30)OH(28)] (**34**)	6.3	0.0180
G4-PAMAM[a]	7.0	0.0014
G4-PAMAM-Arg[b]	6.25	0.0024
G4-PAMAM-HisHisArg[c]	6.2	0.0038

[a]Fourth generation PAMAM. [b]G4-PAMAM containing arginine. [c]G4-PAMAM containing the His-His-Arg sequence.

Table 3. Summary of particle characterization: Particle hydrodynamic size (DLS) and zeta-potential at 25°C

Dendrimer	N[a]	MW (calc.)	MW (obs.)	*Z*-potential (mV)[b]	*Z*-AVE size (nm)[b,c]
G4[15a·2HCl(36)OH(12)] (**23**)	72	13593.36	13150	31.2±0.1	4.4±0.1
G4[16·2HCl(29)OH(19] (**24**)	58	13644.10	13293	21.5±0.6	4.7±0.1
G4[15a·2HCl(25)15b·2HCl(23)] (**25**)	96	15698.37	15155	34.8±0.2	4.6±0.1
G4[15a·2HCl(17)15c·HCl(23)OH(8)] (**26**)	57	14152.23	14256	20.8±0.1	4.5±0.1
G5[15a·2HCl(66)OH(30)] (**32**)	132	26040.26	26100	50.0±0.6	5.1±0.1
G5[16·2HCl(52)OH(44)] (**33**)	104	25799.41	26000	43.7±0.7	5.4±0.1
G5[15a·2HCl(38)15b2·HCl(30)OH(28)] (**34**)	136	25661.06	25745	51.8±0.1	5.3±0.1

[a]Number of peripheral basic groups as determined by NMR. [b]Degree of freedom=12. [c]Derived from a cumulants analysis of the measured correlation curve and reported as the intensity-weighted average (Int-Peak) hydrodynamic radius.

correlating buffer capacity (β) with number of generation and nature of amino acid, it is interesting to note the sharp increase of β recorded with dendrimer containing arginine and *O*-methyltyrosine (**26**) with respect to the other dendrimers containing arginine residues indicating the complexity of interaction process of dendrimers with the aqueous environment. However all the dendrimer prepared resulted with a β value much more higher than the G4-PAMAM derivatives taken as reference.

3.7. Dynamic light scattering (DLS) and zeta potential

Particle hydrodynamic size (diameter) and Zeta Potential of all prepared dendrimers were measured and the obtained data together with the deviation standard (N=12) values are summarized in Table 3. Particle size was determined by Dynamic Light Scattering (DLS) and Z average diameter (Z-AVE, nm), derived from a cumulants analysis of the measured correlation curve, was reported as the intensity-weighted average (Int-Peak) hydrodynamic radius. Zeta potential (mV), which provides a measure of the electrostatic potential at the surface of the electrical double layer and the bulk medium and which is related to the nanoparticle surface charge, was performed using zetasizer (N=12).

As expected, the mean diameters of the fourth generation samples were lower than those of the fifth generation ones, with decimal differences depending on the amino acids present on their periphery but in any case they were within range 4.4-5.3 nm and comparable to the values reported for PAMAMs-NH_2 of the same generations.[63,64]

Also the surface charge of the examined samples that was found within range 20-50 mV was in perfect agreement with G4 and G5-PAMAM-NH_2 literature data[64] with higher values for G5 samples than G4. Finally, it can be noticed how within the same generation the Zeta Potential increases with the increase of N.

4. Conclusions

Versatile protocols for the step-wise synthesis of dendrimers containing peripheral amino acid residues have been setup. The fourth generation dendrimers **10** and the fifth generation dendrimer **28** containing 48 and 96 hydroxyl groups at the periphery were prepared from 2,2-*bis*(hydroxymethyl)propanoic acid (*bis*-HMPA). Commercial $^{\alpha}$*N*-Boc-$^{\omega}$*N*-nitro-*L*-arginine (**17a**), a mixture 1/1 of **17a** and Boc-*L*-Lysine and dipeptide $^{\alpha}$*N*-Boc-$^{\omega}$*N*-nitro-*L*-arginine-glycine (**18**) were successfully bound to **10** and to **28** using DCC as the condensation reagent affording fourth and fifth generation dendrimers respectively. Another hetero dendrimer of the fourth generation containing arginine and *O*-methyltyrosine residues was also accomplished to explore the influence of hydrophobic residues in cell up-take.

The removal of nitro and Boc protecting groups was obtained with HCOOH in the presence of Pd/C followed by treatment with acetyl chloride and methanol to achieve the corresponding hydrochloride forms. The structures of the parent dendrimers **9**, **10**, **27**, and **28** and the results of the successive chemical modifications were confirmed by ^{1}H and ^{13}C NMR analysis.

Experimental molecular weight of hydrochlorides **23-26** and **32-34** was obtanined by titration with $HClO_4$ solutions in AcOH in the presence of mercuric acetate and quinaldine red. All the prepared samples were found to have mean size and surface charge in agreement with that of PAMAMs of the same generation and to have a β value much more higher than the three G4-PAMAM derivatives taken as reference and therefore potentially endowed with good transfection efficiency. The seven amino acids-modified polycationic polyester-based dendrimers possess the main features to represent a promising team of new non-toxic carriers for gene and drug delivery.

Supporting information: Information is available regarding: the structures of amino acids, of dipeptide and of corresponding Boc-protected derivatives, general procedure for the preparation of *N*-Boc-amino acids **17b**, **17c** (Section 1), table of experimental data of **17b** and **17c** (Table S1), analytical data of **17b** and **17c** (Sections 1.2 and 1.3), synthesis of $^{\alpha}$*N*-BocArg(NO_2) GlyOH (**18**) (Section 2), structures of dendrons D4(Bn)OH, D5(BnA), and D5(A) with their yields (Figure S2), experimental procedure to prepare dendrons D4(Bn)OH, D5(BnA) and D5(A) (Section 3), NMR spectra of compounds **1-10** (Figures S3-S20) and **27-28** (Figures S21-S23), NMR monitoring of dendrimers **25**, **26**, and **34** synthesis (Figures S24-S26), estimating of composition of dendrimers **21**, **25**, **30** and **33** (Figure S27), acid-base titration curves (Figure S28), buffer capacity (β) curves (Figure S29), histogram of average buffer capacity ($\overline{\beta}$) (Figure S30). The materials are available *via* the Internet at http://www.springer.com/13233.

References

(1) R. Hourani and A. Kakkar, *Macromol. Rapid Commun.*, **31**, 947 (2010).
(2) M. Sowinska and Z. Urbanczyk-Lipkowska, *New J. Chem.*, **38**, 2168 (2014).
(3) P. Kesharwani, K. Jain, and N. Jain, *Progr. Polym. Sci.*, **39**, 268 (2014).
(4) J. Datija, V. V. R. Sai, and S. Mukherji, S. *J. Mater. Chem.*, **21**, 14367 (2011).
(5) A.-M. Caminade, in *Dendrimers: Towards Catalytic, Material and Biomedical Uses*, A-M. Caminade, C-O. Turrin, R. Laurent, A. Ouali, and B. Delavaux-Nicot, Eds., John Wiley & Sons Ltd, Chichester, UK., 2011, Ch. 15, pp 375-392.
(6) J.-H. Kim, K. Park, H. Y. Nam, S. Lee, K. Kim, and I. C. Kwon, *Progr. Polym. Sci.*, **32**, 1031 (2007).
(7) Z. Wang, G. Niu, and X. Chen, *Pharm. Res.*, **31**, 1358 (2014).
(8) C. Dufès, I. F. Uchegbu, and A. G. Schätzlein, *Adv. Drug Deliv. Rev.*, **57**, 2177 (2005).
(9) H. Eliyahu, Y. Barenholz, and A. Domb, *Molecules*, **10**, 34 (2005).
(10) D. W. Pack, A. S. Hoffman, S. Pun, and P. S. Stayton, *Nat. Rev. Drug Disc.*, **4**, 581 (2005).
(11) D. Schaffert and E. Wagner, *Gene Ther.*, **15**, 1131 (2008).
(12) M. A. Mintzer and E. E. Simanek, *Chem. Rev.*, **109**, 259 (2009).
(13) S. O'Rorke, M. Keeney, and A. Pandit, *Progr. Polym. Sci.*, **35**, 441 (2010).
(14) H. M. Marvaniya, P. K: Parikh, V. R. Patel, K. N. Modi, and D. J. Sen, *J. Chem. Pharm. Res.*, **2**, 97 (2010).
(15) X. Guo and L. Huang, *Acc. Chem. Res.*, **45**, 971 (2012).
(16) Y. Yue and C. Wu, *Biomater. Sci.*, **1**, 152 (2013).
(17) S. Biswas and V. P. Torchilin, *Pharmaceuticals*, **6**, 161 (2013).
(18) N. Taghavi Pourianazar, P. Mutulu, and U. Gunduz, *J. Nanopart. Res.*, **16**, 2342/1 (2014).
(19) G. R. Newkome, and C. D. Shreiner, *Polymer*, **49**, 1 (2008).
(20) J. D. Eichman, A. S. U. Bielinska, J. F. Kukowska-Latallo, and J. R. Baker Jr., *Sci. Technol. Today*, **3**, 232 (2000).
(21) H. Zong, D. Shah, K. Selwa, R. E. Tsuchida, R. Rattan, J. Mohan, A. B. Stein, J. B. Otis, and S. N. Goonewardena, *Chem. Open*, **4**, 335 (2015).
(22) L. Han, R. Huang, S. Liu, S. Huang, and C. Jiang, *Mol. Pharm.*, **7**, 2156 (2010).
(23) Y. Gao, Z. Li, X. Xie, C. Wnaga, J. You, F. Moa, B. Jin, J. Chen, J. Shao, H. Chen, and L. Jia, *Eur. J. Pharm. Sci.*, **70**, 55 (2015).
(24) Y. Zhang, T. P. Thomas, K-H. Lee, M. Li, H. Zong, A. M. Desai, A. Kotlyar, B. Huang, H. M. M. Banaszak, and J. R. Baker Jr., *Bioorg. Med. Chem.*, **19**, 2557 (2011).
(25) S. L. Mekuria, T. A. Debele, H-Y. Chou, and H-C. Tsai, *J. Phys. Chem. B*, **120**, 123 (2016).
(26) R. B. Kolhatkar, K. M. Kitchens, P. W. Swaan, and H. Ghandehari, *Bioconj. Chem.*, **18**, 2054 (2007).
(27) C. L. Waite, S. M. Sparks, K. E. Uhrich, and C. M. Roth, *BMC Biotechnol.*, **9**, 9 (2009).
(28) L. Jianfeng, L. Jinjian, C. Liping, T. Lingling, G. Hongjun, Y. Cuihong, W. Dezhi, S. Linqi, K. Deling, and L. Zongjin, *J. Nanosci. Nanotechnol.*, **14**, 3305 (2014).
(29) M. Ciolkowski, J. F. Petersen, M. Ficker, A. Janaszewska, J. B. Christensen, B. Klajnert, and M. Bryszewska, *Nanomed-Nanotechnol.*, **8**, 815 (2012).
(30) A. Ghilardi, D. Pezzoli, M. C. Bellucci, C. Malloggi, A. Negri, A. Sgnappa, G. Tedeschi, G. Candiani, and A. Volonterio, *Bioconjug. Chem.*, **24**, 1928 (2013).
(31) H. Arima, K. Motoyama, and T. Higashi, *Adv. Drug Deliv. Rev.*, **65**, 1204 (2013).
(32) R. S. Navath, A. R. Menjoge, B. Wang, R. Romero, S. Kannan, and R. M. Kannan, *Biomacromolecules*, **11**, 1544 (2010).
(33) J. H. Park, J-S. Park, and J. S. Choi, *Macromol. Res.*, **22**, 500 (2014).
(34) F. Wang, Y. Wang, H. Wang, N. Shao, Y. Chen, and Y. Cheng, *Biomaterials*, **35**, 9187 (2014).
(35) S. J. Lam, A. Sulistio, K. Ladewig, E. H. H. Wong, A. Blencowe, and G. G. Qiao, *Austr. J. Chem.*, **67**, 592 (2014).
(36) H. Y. Nam, K. Nam, H. J. Hahn, B. H. Kim, H. J. Lim, H. J. Kim, J. S. Choi, and J. S. Park, *Biomaterials*, **30**, 665 (2009).
(37) M. Liu, J. Chen, Y-N. Xue, W-M. Liu, R-X. Zhuo, and S-W. Huang, *Bioconjug. Chem.*, **20**, 2317 (2009).
(38) Q. A. A. Eltoukhy, D. J. Siegwart, C. A. Alabi, J. S. Rajan, R. Langer, and D. G. Anderson, *Biomaterials*, **33**, 3594 (2012).
(39) C. J. Bishop, T-M. Ketola, S. Y. Tzeng, J. C. Sunshine, A. Urttio, H. Lemmetyinen, E. Vuorimaa-Laukkanen, M. Yliperttula, and J. J. Green, *J. Am. Chem. Soc.*, **135**, 6951 (2013).
(40) K. L. Chang, Y. Higuchi, S. Kawakami, F. Yamashita, and M. Hashida, *J. Control. Release*, **156**, 195 (2011).
(41) Y. Wen, Z. Guo, Z. Du, R. Fang, H. Wu, X. Zeng, C. Wang, M. Feng, and S. Pan, *Biomaterials*, **33**, 8111 (2012).
(42) F. Wang, Y. Wang, H. Wang, N. Shao, Y. Chen, and Y. Cheng, *Biomaterials*, **35**, 9187 (2014).
(43) J. Shi, J. G. Schellinger, R. N. Johnson, J. L. Choi, B. Chou, E. L. Anghel, and S. H. Pun, *Biomacromolecules*, **14**, 1961 (2013).
(44) I. Nakase, H. Akita, K. Kogure, A. Gräslund, Ü. Langel, H. Harashima, and S. Futaki, *Acc. Chem. Res.*, **45**, 1132 (2012).
(45) I. Nakase, G. Tanaka, and S. Futaki, *Mol. Bio Syst.*, **9**, 855 (2013).
(46) C. Liu, X. Liu, P. Rocchi, F. Qu, J. L. Iovanna, and L. Peng, *Bioconjug. Chem.*, **25**, 521 (2014).
(47) X. Liu, C. Liu, J. Zhou, C. Chen, F. Qu, J. J. Rossi, P. Rocchi, and L. Peng, *Nanoscale*, **7**, 3867 (2015).
(48) J. B. Kim, J. S. Choi, K. Nam, M. Lee, J. S. Park, and J. K. Lee, *J. Control. Release*, **114**, 110 (2006).
(49) T. Kim, C. Z. Bai, K. Nam, and J. Park, *J. Control. Release*, **136**, 132 (2009).
(50) Q. Peng, J. Zhu, Y. Yu, L. Hoffman, and X. Yang, *J. Biomater. Sci. Polym. Ed.*, **26**, 1163 (2015).
(51) A. Carlmark, E. Malström, and M. Malkoch, *Chem. Soc. Rev.*, **42**, 5858 (2013).
(52) N. Feliu, M. V. Walter, M. I. Montañez, A. Kunzmann, A. Hult, A. Nyström, M. Malkoch, and B. Fadeel, *Biomaterials*, **33**, 1970 (2012).
(53) J. S. Moore and S. I. Stupp, *Macromolecules*, **23**, 65 (1990).
(54) H. Ihre, A. Hult, J. M. J Fréchet, and I. Gitsov, *Macromolecules*, **31**, 4061 (1998).
(55) M. Zhao, J. Liu, X. Zhang, L. Peng, C. Li, and S. Peng, *Bioorg. Med. Chem.*, **17**, 3680 (2009).
(56) A. I. Vogel, in *Elementary Practical Organic Chemistry. Part III. Quantitative Organic Analysis*, Longman Ed., 1st ed., London, 1958, Ch. 20, p 702.
(57) F. Von Seel, in *Grundlagen der Analytischen Chemie*, G. Geier, Ed., 5th ed., Verlag Chemie, Weinheim, 1970, Vol. 82, p 962.
(58) L. Aravindana, K. A. Bicknell, G. Brooks, V. V. Khutoryanskiya, and A. C. Williams, *Int. J. Pharm.*, **378**, 201 (2009).
(59) J. M. Benns, J. S. Choi, R. I. Mahato, J. S. Park, and S. W. Kim, *Bioconjug. Chem.*, **11**, 637 (2000).
(60) J. Yang, Q. Zhang, H. Chang, and Y. Cheng, *Chem. Rev.*, **115**, 5274 (2015).
(61) H. Kessler and M. Molter, *J. Am. Chem. Soc.*, **98**, 5969 (1976).
(62) S. Y. Gwang, M. B. Yun, C. Hye, K. Bokyung, S. C. Insung, and S. C. Joon, *Bioconjug. Chem.*, **22**, 1046 (2011).
(63) N. Heigl, S. Bachmann, C. H. Petter, M. Marchetti-Deschmann, G. Allmaier, G. K. Bonn, and C. W. Huck, *Anal. Chem.*, **81**, 5655 (2009).
(64) Y. Zeng, Y. Kurokawa, T. T. Win-Shwe, Q. Zeng, S. Hirano, Z. Zhang, and H. Sone, *J. Toxicol. Sci.*, **41**, 351 (2016).