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Biocompatible, multifunctional, and well-defined OEG-based dendritic platforms for biomedical applications†

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Given the growing importance of drug and gene delivery systems, imaging agents, biosensors, and theranostics, there is a need to develop new multifunctional and biocompatible platforms. Here we synthesized and fully characterized a family of novel multifunctional and completely monodisperse dendritic platforms. Our synthetic methodology, based on compatible protecting groups and the attachment of monodisperse triethylene glycol units, allows the control of the generation and differentiation of terminal groups, thus giving rise to multifunctional and perfectly-defined products. A family of dendrons was synthesized and four distinct dendritic structures were chosen from the family in order to determine the effect of the generation and surface groups on their biocompatibility. The stability in serum, cytotoxicity, and hemocompatibility of these products were studied. Our results indicate that these non-toxic, hemocompatible, non-immunogenic, stable and versatile scaffolds may be very interesting candidates for biomedical applications.

Introduction

While the number of new drugs approved by several agencies each year seems to have reached a plateau, complementary associated technologies, such as *in vitro* diagnostics, contrast agents, and vectors for gene and drug delivery systems, have met with increasing success. Dendrimers show unique behavior that makes them a promising choice for a wide range of biomedical applications. These hyperbranched molecules are characterized by a protein-like globular shape and well-defined structure, multivalency, and capacity to act as nanocontainers, thus allowing their use as drug and gene delivery systems, imaging agents, biosensors, and theranostics.[1–4]

The composition, surface functionality, and size of these macromolecules dictate their toxicity and pharmacokinetic behavior. These factors are crucial to allow their use in diverse biomedical applications. The use of polyethylene glycol (PEG)-based dendrimers or dendrimers functionalized on their surface with PEG units is a very attractive strategy because it combines two important aspects: the theoretically well-defined macromolecular dendrimers and the adoption of well-known polymers in drug delivery systems.[5–13] However, the polydispersity shown by PEG and the difficulty to prepare highly branched dendrimers turn these PEG-decorated dendrimers into polydisperse systems, thereby leading to problems regarding purification, synthesis reproducibility and variations in their *in vivo* behavior.[14] Here we describe the synthesis of oligoethylene glycol (OEG)-based dendrons, prepared and purified as molecules with an exact molecular weight, thereby avoiding the aforementioned issues.

Traditionally, dendrimers are built adopting the growth of a symmetric core, resulting in isotropic structures with equal end-groups.[15] However, the development of materials with higher structural complexity and multiple functionalities that can be precisely modified with diverse molecules (as water inducing-solubilizers, targeting and imaging ligands and different drugs) to confer a range of properties to the final system is very useful and sometimes even necessary for the

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applications explored.[16,17] The chemical strategy that we have optimized allows the controlled differentiation of dendrons, thus giving access to a multifunctional platform. Accordingly, the exact molecular weight and multifunctionality of these dendrons provide an exquisite level of structural control that is expected to eliminate the undesirable effects of size and variation in biomolecule-loading shown by polymer drug delivery systems. Compared with symmetric platforms with all equivalent functional groups on their surface, our current strategy offers considerable advantages for the improved control of the biological applications of these multifunctional nanomaterials. Before these dendrimers are considered for use in diverse therapeutic purposes, we studied the effect of their generation and surface groups on certain factors that are crucial to determine biocompatibility, such as stability in serum, cytotoxicity, and hematocompatibility.

Results and discussion

Synthesis of dendrons

The dendritic platform was synthesized starting from a diethylenetriaminepentaacetic acid (DTPA) derivative (Scheme 1). In this case, one of the five carboxylic acids of the DTPA-based core (**3**) is protected with a benzyl group, while the other four carboxylic acids are protected with a *tert*-butyl group. This protecting scheme allows benzyl group removal preserving the other four carboxylic acids protected with the *tert*-butyl group, or the elimination of the *tert*-butyl groups while the fifth carboxylic acid remains protected. The benzyl-protected carboxylic group is the focal point for dendron synthesis.

Dendron core **3** was obtained following a three-step procedure. Ethanolamine and *tert*-butyl bromoacetate were reacted to obtain *N*,*N*-bis[(*tert*-butyloxycarbonyl)methyl]-2-hydroxyethylamine, and then its bromoderivative (product **2**) was obtained using NBS, attaining 71% of yield after flash chromatography purification. 2-[Bis-(*tert*-butyloxycarbonylmethyl)amino]ethyl bromide (**2**) was mixed with glycine benzyl ester, and the reaction was carried out in a biphasic solvent system composed of CH_3CN and phosphate buffer at pH 8, affording the dendron core (**3**) in a 58% yield (92% of purity). After removal of *tert*-butyl groups with HCl–dioxane, the core dendron with four free carboxylic acids and one carboxylic acid protected with a benzyl group was obtained quantitatively (**4**).

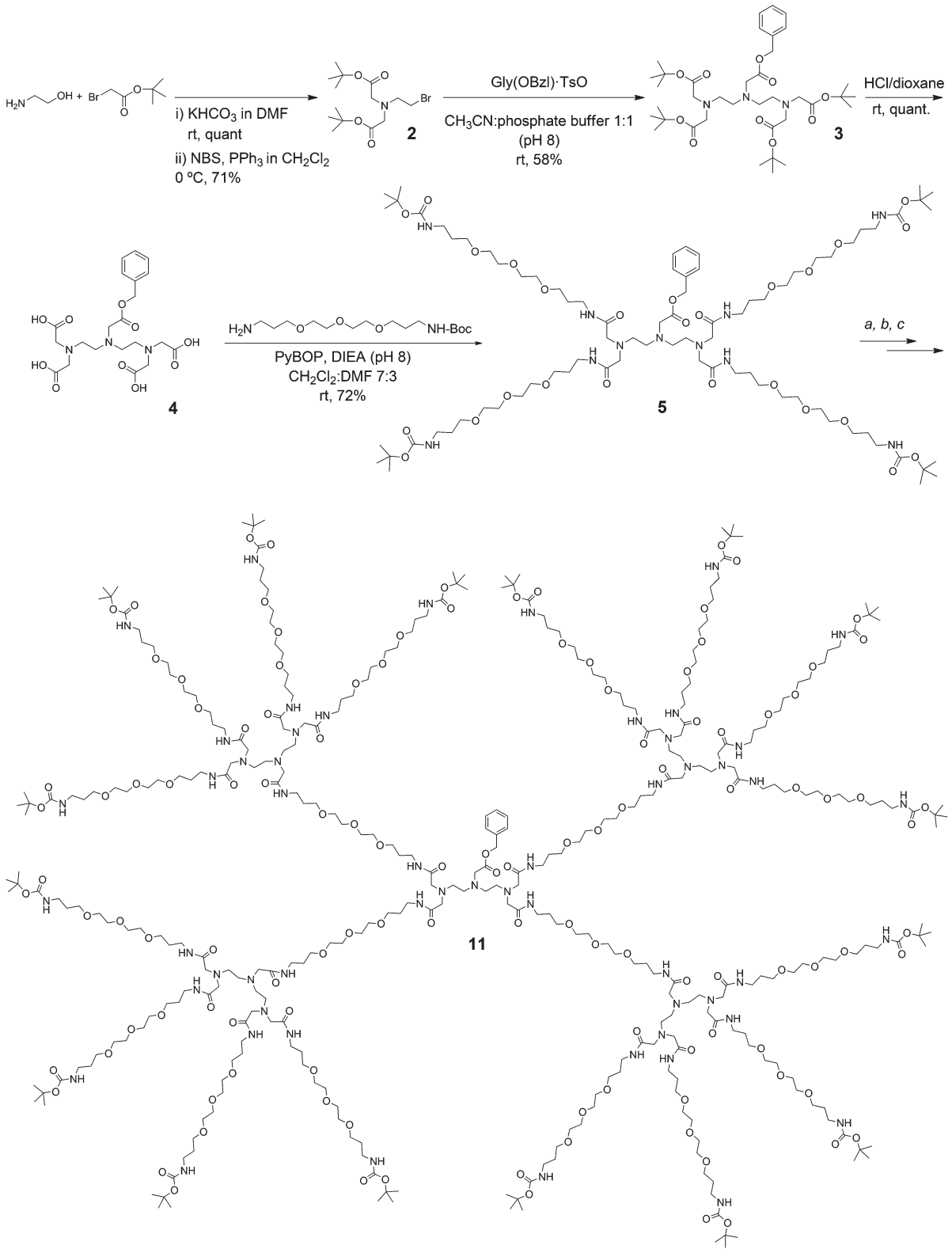
Reaction between **4** and four Boc monoprotected bisaminetriethylene glycol units (1-(*tert*-butyloxycarbonyl-amino)-4,7,10-trioxa-13-tridecanamine) using PyBOP : HOBt as coupling reagents system in the presence of DIEA led to the first generation dendron (**5**, Scheme 1) with an AB_4 architecture. Product **5** was obtained in a multigram scale (3.5 g) with satisfactory yield (72%), excellent purity (99%), and completely monodisperse, as confirmed by HRMS and NMR. The second generation dendron (**11**) was prepared by a "semi-convergent" synthetic strategy (Scheme 1). To obtain the appropriate dendrons necessary for second generation synthesis diverse modifications were carried out for compound **5**. The benzyl group of compound **5** was eliminated by hydrogenolysis resulting in dendron **6** with a carboxylic acid at the focal point and the surface amine groups Boc-protected. On the other hand elimination of Boc amino protecting groups of compound **5** by treatment with 4 M HCl in dioxane gave dendron **7**, in which the amino groups are free and the focal point is a benzyl ester. The best results in the preparation of **11** were obtained by reacting compound **7**, with four units of dendron **6** with the carboxylic acid activated as a pentafluorophenyl ester. The multifunctional and well-defined AB_{16} dendron was obtained in a 48% yield and high purity (97%) and was suitable for the derivatization of the carboxylic acid or amino groups.

Starting from the first and the second generation (**5** and **11**), a family of dendrons was synthesized (Table 1) by modifying the surface group and the focal point derivatization, thereby demonstrating the versatility of the dendritic platform. Selective elimination of Boc and benzyl protecting groups was performed following standard methods (see the Experimental section). The acetylation of the amino groups of the first generation dendron **7** was achieved using acetic anhydride and DIEA in CH_2Cl_2, and the fully acetylated product **8** was obtained after 1 h of reaction. Benzyl group elimination yielded compound **9**. Nevertheless, the acetylation of the second generation dendron **13** was performed in water because of the poor solubility of the deprotected dendron in organic solvents. Also, to complete this reaction, the consecutive addition of an excess of acetic anhydride over two days was necessary. All the products were obtained as unique compounds with exact molecular weight, as demonstrated by MALDI-TOF, HRMS and NMR techniques.

First and second generation dendrons with free or acetylated surface amines (**G1-NH_2-Bn**, **G1-Ac-Bn**, **G2-NH_2-Bn** and **G2-Ac-Bn**) were chosen from the family as model compounds of this dendritic platform to evaluate the influence of dendron generation and terminal groups on the biocompatibility, stability, and size of the final system. These two surface group modifications were selected as representative models of this dendron ability to act as: (1) drug conjugated delivery systems *via* covalent union between the drug and the dendron (**G1-Ac-Bn** and **G2-Ac-Bn**); or (2) drug/gene delivery systems *via* ionic interaction between terminal ammonium group dendrons with drug or gene material (**G1-NH_2-Bn** and **G2-NH_2-Bn**).

Size and ζ-potential

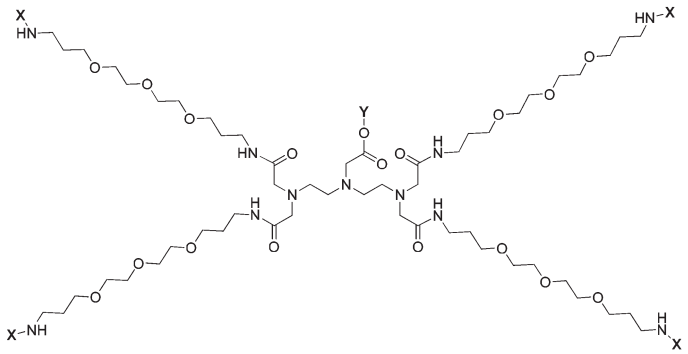
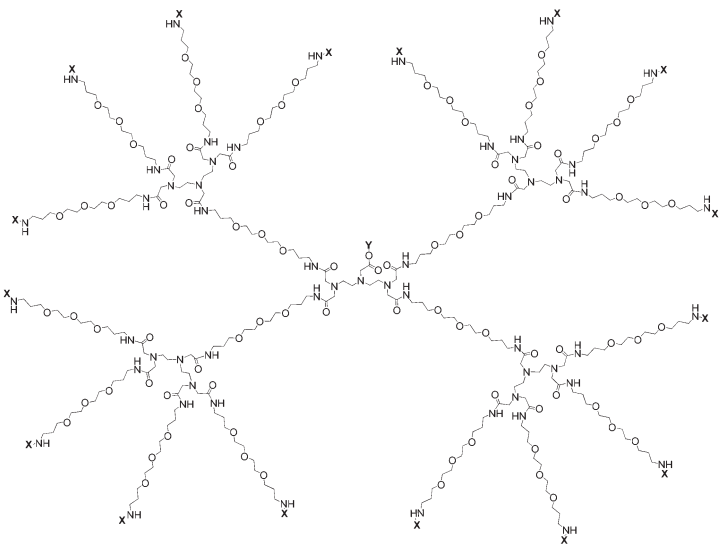
The size (hydrodynamic diameter) of second generation dendrons was measured by dynamic light scattering (Table 2 and see also Fig. 1 in ESI†). Due to the threshold of the DLS equipment, the lower concentration used for the measurement of the size and zeta-potential was the higher concentration used in the cytotoxicity and hemocompatibility experiments. Lower dendron concentrations used in these experiments were not optimum for the measurement by DLS. However, in order to confirm that the size and zeta-potential were concentration-independent, higher sample concentrations were also used. As can be observed in size distribution profiles, **G2-Ac-Bn**, containing the acetylated amino-terminal groups, measured



Scheme 1 Synthesis of first and second generation dendrons. (a) H_2, Pd/C; (b) 2,3,4,5,6-pentafluorophenol, EDC, DMAP; (c) dendron **7**, DIEA.

4.1 nm. This result is consistent with the previously reported sizes of G2 and G3-PAMAM (poly(amido amine)) dendrimers.[18–24] It is worth noting that the size of the G3-PAMAM dendrimer, which had 32 terminal groups and a higher molecular weight than our dendron (6909 compared with 6721), was slightly less than **G2-Ac-Bn**. This difference is

Table 1 Library of synthesized dendrons

Dendron structure	Compound	X,Y substituent
	Dendron **5** [**G1-Boc-Bn**]	X=  Y= 
	Dendron **6** [**G1-Boc-COOH**]	X=  Y= H
	Dendron **7** [**G1-NH_2-Bn**]	X= H Y= 
	Dendron **8** [**G1-Ac-Bn**]	X=  Y= 
	Dendron **9** [**G1-Ac-COOH**]	X=  Y= H
	Dendron **10** [**G1-NH_2-COOH**]	X= H Y= H
	Dendron **11** [**G2-Boc-Bn**]	X=  Y= 
	Dendron **12** [**G2-Boc-COOH**]	X=  Y= H
	Dendron **13** [**G2-NH_2-Bn**]	X= H Y= 
	Dendron **14** [**G2-Ac-Bn**]	X=  Y= 
	Dendron **15** [**G2-NH_2-COOH**]	X= H Y= H

attributable to the superior swelling capacity of the triethylene glycol branches, thus resulting in a higher hydrodynamic diameter.

Interestingly, when measurements were performed with **G2-NH_2-Bn**, the size increased dramatically to about 189.4 nm, and higher size dispersion was also detected. This observation

Table 2 Size and ζ-potential of second generation dendrons in PBS

	Size (nm)		ζ-Potential (mV)	
Conc. (mg mL^{-1})	**G2-NH$_2$-Bn**	**G2-Ac-Bn**	**G2-NH$_2$-Bn**	**G2-Ac-Bn**
10	189.4 ± 87.7	4.1 ± 0.7	41.5 ± 11.5	5.66 ± 6.4
5	189.6 ± 87.6	3.9 ± 0.9	43.2 ± 9.6	5.08 ± 5.2
1	168.9 ± 61.3	4.4 ± 0.8	43.8 ± 11.0	5.61 ± 4.7

highlights that dendron aggregation occurred under our measurement conditions.

The electrical potential at the dendron surface was determined by ζ-potential measurements. As expected, at physiological pH, the ζ-potential of **G2-NH$_2$-Bn** was positive (about 42 mV) while **G2-Ac-Bn** was practically neutral (about 5 mV) (Table 2 and see also Scheme 1 in ESI†). These results are again consistent with previously reported ζ-potential data of G3-PAMAM dendrimers containing amino terminal groups.[24]

First generation dendrons were also measured by dynamic light scattering (data not shown) but their small size (around 1 nm) precluded obtaining an accurate value. Although an aggregation behavior for **G1-NH$_2$-Bn** would be expected – perhaps to a lesser extent than **G2-NH$_2$-Bn** – it was not observed under our measurement conditions, indicating that the aggregation does not take place in systems with few amino terminal groups.

Stability in serum

Given the potential biomedical applications of these dendritic platforms, we studied their stability in human serum, cytotoxicity and hemocompatibility, in order to evaluate their biocompatibility, in particular in the case of intravenous delivery.

Chemical stability in serum was tested for a period of 120 h (Fig. 1). Comparative results showed degradation of **G1-Ac-Bn** and **G1-NH$_2$-Bn** already after 24 h of incubation; with 85% of products remaining intact after 120 h. Surprisingly, **G2-Ac-Bn** was not degraded after 120 h of incubation. The major stability of the second generation dendrons could be attributed to a minor exposure of the amide-dendritic skeleton to the hydrolytic serum enzymes. As dendrimer generation increases, the dendron interior becomes more protected by the surrounding environment. In addition, increasing the generation would also increase the amount of triethylene glycol units and thus, the "stealth" effect, typical of PEG, prevents interactions between amide-dendron and hydrolytic components.[25]

The stability of **G2-NH$_2$-Bn** could not be determined by this methodology due to a strong interaction between the dendron and serum proteins. This results in precipitation of **G2-NH$_2$-Bn** with serum proteins when prior to HPLC analysis CH_3CN is added to clean the sample from proteins, removing the dendrimer from the sample.

This phenomenon was observed in these experiments and corroborated by previous studies, where the interaction between amino-terminated PAMAM dendrimers (G3- and G4-PAMAM dendrimers containing 32 and 64 primary amines) and bovine serum albumin was described.[26–28] However, the dendron–albumin interaction was not observed with **G2-Ac-Bn** (**14**), suggesting that this phenomenon is directly associated with the number of amines exposed on the dendrimer surface. Therefore, the conjugation of a drug to the dendron amino-surface groups or the complexation with an oligonucleotide (forming a dendriplex) will reduce the serum proteins interaction resulting in a suitable drug delivery system. Further future investigations will be conducted toward these issues.

Cytotoxicity

Cytotoxicity was tested by MTT assay using HT-29 and SK-BR-3 human tumor cell lines. Cell lines were incubated for 72 h with the different dendrons, and viability was then measured. SK-BR-3 cell viability was not affected by any of the compounds tested, even at high dendron doses (100 μM) (Fig. 2). In the HT-29 cell line, only the highest concentration of **G2-NH$_2$-Bn** (100 μM) displayed significant toxicity, but viability was not reduced at lower doses. On the basis of these results, and in agreement with the reported literature,[18,19,21,29–32] our data confirm that *in vivo* toxicity increases with the number of free amino-terminal groups on the dendron surface.

Microscopic evaluation of the cells after exposure to the dendrons corroborated the viability assays. No changes were observed regarding the morphology of SK-BR-3 cells for any of the dendrons tested, compared with control cells (Fig. 3). However, slight morphological differences were observed when HT-29 cells were treated with **G2-NH$_2$-Bn**, compared with the rest of the samples (Fig. 3d).

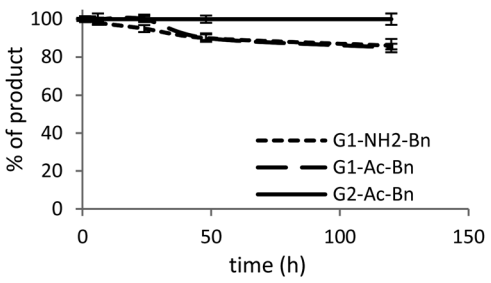


Fig. 1 Stability of dendrons **7**, **8** and **14** (**G1-NH$_2$-Bn**, **G1-Ac-Bn** and **G2-Ac-Bn**) in human serum at 37 °C. Percentages are referred to the remaining products that are not degraded after the corresponding incubation time.

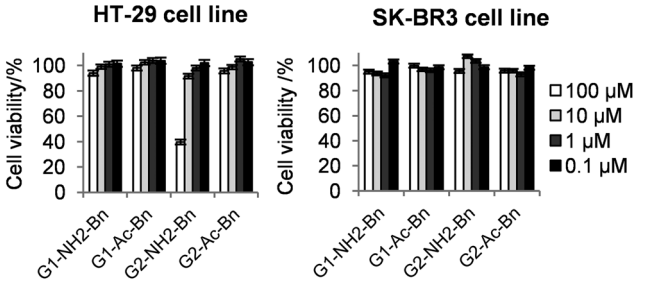


Fig. 2 Cell viability of HT-29 and SK-BR-3 cell lines treated with dendrons at 72 h. The assay was performed when the cells reached 90% of confluence.

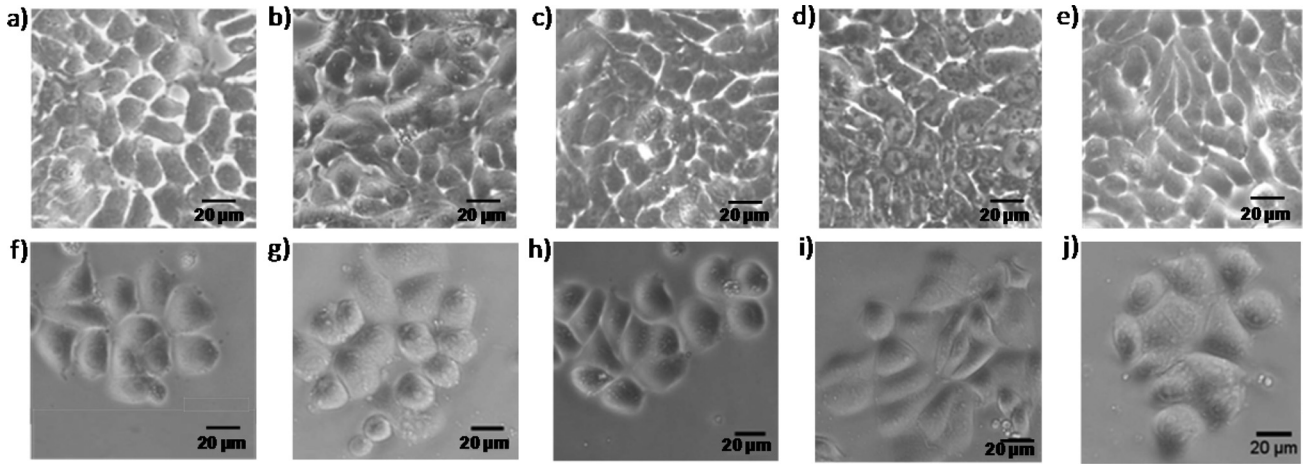


Fig. 3 Cell morphology observed at 20× using inverse phase microscopy. (a–e) HT-29 cell lines. (f–j) SK-BR-3 cell line. (a) and (f) control cells. (b) and (g) cells incubated with 100 μM of **G1-NH_2-Bn**. (c) and (h) cells incubated with 100 μM of **G1-Ac-Bn**. (d) and (i) cells incubated with 100 μM of **G2-NH_2-Bn**. (e) and (j) cells incubated with 100 μM of **G2-Ac-Bn**. The assay was performed when the cells reached 90% of confluence.

Although no examples of cell viability experiments with HT-29 or SK-BR3 cells with low generation dendrimers have been found, some cytotoxicity studies with other cell lines have been reported.[29,33,34] For example, cytotoxicity experiments using B16F10 melanoma cells were described. Experiments realized with second generation diaminoethane (DAE) dendrimers displaying also 16 amino terminal groups showed 50% of viability when cells were incubated with 60 μM of the dendrimer.[29] Another study performed with the same cell line resulted in a reduction of viability to 40% when cells were incubated with 20 μM of second generation polyamidoamine (PAMAM) dendrimers containing 16 amino terminal groups.[33] Cytotoxicity of lower generations of non-cationic OEG based dendrimers was also estimated using B16F1 and HaCat cell lines showing a cell viability around 100% for second generation dendrimers and reduction of viability below 50% for B16F1 and 80% for HaCat, respectively, for first generation dendrimers.[34] Regarding these results, our dendrons seem to be less toxic than other reported dendrimers containing the same number of cationic groups on their surface; even our dendrons have higher molecular weight. Indeed, the influence of this OEG-based scaffold could lead to a reduction of toxicity compared to other dendrimer platforms.

Hemocompatibility

Dendron hemocompatibility was evaluated by studying hemolysis, the morphology of blood cells, complement activation (C3a), and coagulation activation, through the extrinsic and intrinsic pathways.

We performed a hemolytic test by incubating whole blood with dendron samples for 15 min. Following ASTM (Standard Practice for Assessment of Haemolytic Properties of Materials), all dendrons were non-hemolytic (hemolysis < 2%) within the concentrations tested (Table 3). The interaction between polycations and serum proteins can neutralize the positive charges of polycations and prevent the interaction between them and RBCs in a competitive way.[35–37] To evaluate the influence of plasma proteins in hemolysis, the hemolytic assay was also performed with RBCs previously washed with PBS to discard serum proteins. No significant hemolytic activity was observed for any of the dendrons tested; even avoiding the serum proteins interaction, thereby suggesting that the cationic surface does not affect the integrity of erythrocytes in this case (Table 4).

Table 3 Percentage of hemolysis after incubation at 37 °C of the dendrons in whole blood

% Hemolysis				
Conc. (μM)	**G1-NH_2-Bn** (**7**)	**G1-Ac-Bn** (**8**)	**G2-NH_2-Bn** (**13**)	**G2-Ac-Bn** (**14**)
0.1	1.00 ± 0.56	1.19 ± 0.00	1.44 ± 0.06	1.07 ± 0.37
1	1.06 ± 0.25	1.12 ± 0.00	1.38 ± 0.31	1.19 ± 0.25
10	1.38 ± 0.12	1.44 ± 0.06	1.25 ± 0.06	1.18 ± 0.19
100	1.13 ± 0.00	0.75 ± 0.81	1.19 ± 0.25	1.13 ± 0.00

Hemagglutination (erythrocyte aggregation) occurs when attractive interactions between positively charged molecules and negatively charged RBCs overcome repulsive forces between negatively charged cellular surfaces.[38] Polycations in contact with blood can cause hemagglutination, a

Table 4 Percentage of hemolysis after incubation at 37 °C of the dendrons in blood. The blood was washed with PBS before the incubation

% Hemolysis				
Conc. (μM)	**G1-NH_2-Bn** (**7**)	**G1-Ac-Bn** (**8**)	**G2-NH_2-Bn** (**13**)	**G2-Ac-Bn** (**14**)
0.1	0.00 ± 0.04	0.00 ± 0.06	0.15 ± 0.07	0.08 ± 0.03
1	0.02 ± 0.05	0.01 ± 0.02	0.07 ± 0.04	0.23 ± 0.05
10	0.00 ± 0.03	0.01 ± 0.07	0.00 ± 0.07	0.07 ± 0.08
100	0.00 ± 0.07	0.05 ± 0.05	0.3 ± 0.06	0.01 ± 0.05

phenomenon that can induce severe circulatory disorders and even lethal toxicity.

Therefore, blood morphological changes are a key parameter that should be considered.[39] Moreover, foreign macromolecules can activate white blood cells and platelets and induce their aggregation, leading to changes in the number and size distribution of blood cells. Most cationic dendrimers showed haemotoxic activity because of their positively charged surface.[40] Recent studies have confirmed that large cationic PAMAM dendrimers (G4, G5, G6 and G7) induce platelet aggregation by disturbing the integrity of cell membranes[24] and altering their morphology.[41] Micrographs of blood smear (see Fig. 2 in ESI†) confirmed the results obtained in hemolysis tests, since neither morphological changes in cells nor hemagglutination were observed. In addition, the count and size distribution of red blood cells, platelets and white blood cells were not altered after incubation with dendrons (see Fig. 3 in ESI†).

This observation supports the notion that our first and second generation dendrons do not interact significantly with blood cell elements, even displaying amino terminal groups. Haemoreactivity differences between our dendrons and high generation cationic PAMAM dendrimers can be associated to an increasing charge-density effect associated to higher dendrimer generations.

The interaction with blood cell elements of both dendrimers type with similar or equivalent charge-density should be studied to establish a clear comparison between them.

The complement system is a mechanism of immune response to eliminate certain pathogens from the body. Activation of the complement system by systemically administered drugs can be responsible for hypersensitivity reactions and anaphylaxis.[42]

Moreover, complement activation mediated by nanoparticles can result in rapid removal of these particles from systemic circulation by mononuclear cells *via* receptor-mediated phagocytosis of complement.[42] No significant activation of the complement system was detected in the range of dendron concentrations tested compared with untreated blood, even for the second generation dendron containing amino terminal groups at high concentrations (100 μM) (Fig. 4).

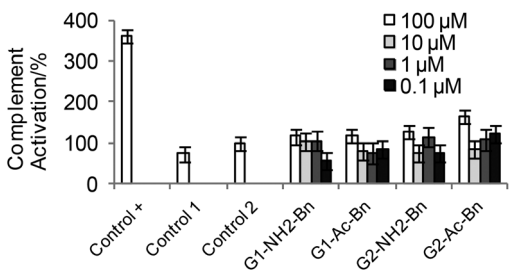


Fig. 4 Activation of the complement system after incubation of blood at 37 °C with the dendrons for 15 min. Complement activation is expressed as a % of C3a concentration, taking control 2 as 100%. Control +: blood incubated with Zymosan. Control 1: blood not exposed to dendrons and not incubated. Control 2: blood incubated but not exposed to dendrons.

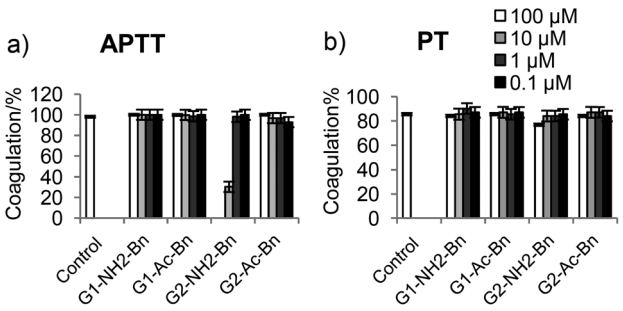


Fig. 5 Analysis of the coagulation cascade after incubation with dendrons for 15 min either through (a) an intrinsic pathway (APTT assay), or (b) an extrinsic pathway (PT assay). Control: blood incubated but not exposed to dendrons. APTT: activated partial thromboplastin time; PT: prothrombin time.

The effect on hemostasis control was determined by coagulation assays, both through the extrinsic pathway (PT assay) and the intrinsic pathway (APTT assay). Clot formation was determined after blood incubation with dendrons. The extrinsic coagulation pathway was not affected by any of the dendrons, while the intrinsic pathway was significantly inhibited at the highest concentrations of **G2-NH_2-Bn** (100 and 10 μM) (Fig. 5). This inhibition could result from an interaction between the positively charged **G2-NH_2-Bn** and proteinic factors associated with the intrinsic coagulation pathway (several of them with an acidic pI), thus leading to a blocking or denaturation of these factors.[39,43] The lack of reactivity of the more neutral **G2-Ac-Bn** supports this assumption.

Conclusions

By means of an original chemical strategy, we have successfully synthesized multifunctional and monodisperse dendritic structures with a satisfactory yield and purity. Using triethylene glycol units as dendritic branches, we developed an oligoethylene glycol-based scaffold, thus avoiding the polydispersity problems that arise from the use of large PEG polymers. Consistent with previous studies,[18–21,24,29–33,40,41,44–46] the cytotoxicity and hemocompatibility of our compounds strongly depended on the groups on the dendron surface as well as on the dendron generation, the second generation cationic dendron being the most toxic and the least hemocompatible. However, all the first generation dendrons and the second generation ones with acetylamide surface groups did not present cytotoxicity or hemoreactivity. Based on our prescreening tests, the biocompatibility of our dendritic platforms highlights them as very interesting candidates for future biomedical applications, such as drug delivery systems, diagnosis, bioassays, and imaging agents.

Experimental section

Materials

All solvents, coupling reagents and other reagents were purchased from commercial suppliers at the highest purity

available and used without further purification. *N*-Bromosuccinimide (NBS, from Sigma Aldrich) was purified by crystallization following *Purification of Laboratory Chemicals* (Armarego, W. and Chai, C.; Elsevier; 2003). *tert*-Butyl bromoacetate and ethanolamine were purchased from Sigma Aldrich and 1-(*tert*-butyloxycarbonyl-amino)-4,7,10-trioxa-13-tridecanamine (Boc-TOTA) was purchased from Iris Biotech. McCoy's 5A medium was purchased from Invitrogen and human serum (from human male AB plasma) from Sigma Aldrich. Trypsin-EDTA 0.25% and penicillin/streptomycin were purchased from Biological Industries and fetal bovine serum from Lab Clinics. MTT, Drabkin's reagent (cyanmethemoglobin) and bovine hemoglobin were purchased from Sigma-Aldrich. The human C3a ELISA Kit for quantification of Human C3a-des-Arg was from Becton Dickinson (Erembodegem, Belgium). Thromborel® S (human thromboplastin, containing calcium for prothrombin time) was acquired from Dade Behring Siemens (1070 Bruxelles, Belgium). STA®-C.K. Prest® 2 (kaolin, for determination of the activated partial thromboplastin time) was purchased from Roche Diagnostic Belgium (Vilvoorde, Belgium). Phosphate buffer (PBS) 100 mM was prepared by dissolving one tablet of phosphate salts (Sigma) in 100 mL of Milli-Q water.

Synthesis of 4-benzyloxycarbonylmethyl-1,1,7,7-tetra(*tert*-butyloxycarbonylmethyl)-1,4,7-triazaheptane (3). $KHCO_3$ was added (6.18 g, 61.8 mmol, 2.5 eq.) to a solution of *tert*-butyl bromoacetate (8.05 mL, 54.5 mmol, 2.2 eq.) in 50 mL of anhydrous DMF. The suspension was cooled at 0 °C, ethanolamine (1.48 mL, 24.5 mmol, 1 eq.) was added *via* a syringe over a 5 min period, and the solution was stirred at 0 °C for 30 min. The reaction mixture was then allowed to warm to room temperature and stirred for 22 h. After this time, most of the DMF was evaporated under vacuum and 50 mL of saturated $NaHCO_3$ was added. The resulting mixture was extracted with diethyl ether (3 × 50 mL), and the combined organic phases were washed with 50 mL of brine, dried with Na_2SO_4, and evaporated to afford the crude product *N*,*N*-bis[(*tert*-butyloxycarbonyl)methyl]-2-hydroxyethylamine (**1**) as an oil (7.41 g). This crude product was dissolved in CH_2Cl_2 and solid triphenylphosphine (8.66 g, 33.0 mmol, 1.3 eq.) was added to the solution, which was cooled at 0 °C. After that, solid NBS (5.87 g, 33.0 mmol, 1.3 eq.) was added over 5 min and the mixture was stirred for 2 h at 0 °C. After this time, the solvent was evaporated and the semisolid residue obtained was triturated with diethyl ether. The solid was filtered, washed with diethylether and discarded, and the ether phase was evaporated to afford an oily residue, which was purified by flash chromatography on silica (5→30% diethyl ether in hexane), yielding 6.18 g (17.5 mmol, yield: 71%) of 2-[bis-(*tert*-butyloxycarbonylmethyl)amino]ethyl bromide (**2**). 50 mL of phosphate buffer (2 M, pH 8) was added to a mixture of the tosylate salt of glycine benzyl ester (2.60 g, 7.7 mmol, 1 eq.) and bromoderivative **2** (6.14 g, 17.0 mmol, 2.2 eq.) in 50 mL of CH_3CN. The resulting mixture was stirred vigorously for 24 h at rt. Then CH_3CN was evaporated and the product was extracted with CH_2Cl_2 (100 mL). The phosphate buffer phase was re-extracted with CH_2Cl_2 (50 mL) and all the organic phases were combined and washed with brine (100 mL), dried with Na_2SO_4 and evaporated. The resulting crude product was purified on basic alumina (5→30% ethyl acetate in hexane), affording 3.16 g of dendron core **3** (4.46 mmol, yield: 58%). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 1.44 (s, 36 H), 2.82 (s, 8 H), 3.42 (s, 8 H), 3.59 (s, 2 H), 5.12 (s, 2 H), and 7.35 (m, 5 H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 28.13, 52.25, 52.75, 54.87, 56.02, 65.92, 80.79, 128.17, 128.23, 128.47, 135.87, and 170.60. Analytical HPLC: 50→100% CH_3CN in water over 8 min, t_R = 6.69 min (purity 92% determined at λ = 210 nm). Calcd mass for $C_{37}H_{61}O_{10}N_3$: 707.4; Mass found by HPLC-MS: 708.4 (M + 1), 354.9 (M + 2)/2.

Synthesis of 4-benzyloxycarbonylmethyl-1,1,7,7-tetra(carboxymethyl)-1,4,7-triazaheptane (4). Compound **3** (2.16 g, 3.05 mmol, 1 eq.) was placed in a round-bottomed flask and dissolved in 5 mL of dioxane. 4.0 M HCl in dioxane (20 mL, 80.0 mmol, 26 eq.) was added to the mixture, and the resulting solution was stirred overnight at rt. HCl–dioxane was then removed by evaporation. The resulting crude product was co-evaporated twice with dioxane, affording compound **4** quantitatively as a white solid, which corresponds to the hydrochloride salt of the tertiary amines (1.99 g). This crude product was used in the next step without further purification. ^{1}H NMR (400 MHz, D_2O, 25 °C): δ = 3.13 (t, ^3J(H,H) = 4.97 Hz, 4 H), 3.49 (t, ^3J(H,H) = 5.90 Hz, 4 H), 3.69 (s, 2 H), 3.74 (s, 8 H), 5.23 (s, 2 H), and 7.45 (m, 5 H). ^{13}C NMR (100 MHz, D_2O, 25 °C): δ = 49.57, 53.41, 54.22, 55.66, 66.72, 128.88, 129.06, 135.35, 169.29, and 172.27. Analytical HPLC: 5→100% CH_3CN in water over 8 min, t_R = 3.49 min (purity 91% determined at λ = 210 nm). Calcd mass for $C_{21}H_{30}O_{10}N_3$: 484.1931; Calcd mass for $C_{21}H_{29}O_{10}N_3Na$: 506.1750; Mass found by MALDI-TOF: 484.2; Mass found by HPLC-MS: 484.2 (M + 1); Mass found by HRMS: 484.1920, 506.1746 (M + Na).

Synthesis of first generation dendron (G1-Boc-Bn, 5). Solid PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, 7.51 g, 14.4 mmol, 4.8 eq.) and Boc-TOTA (1-(*tert*-butyloxycarbonyl-amino)-4,7,10-trioxa-13-tridecanamine, 6.62 g, 14.4 mmol, 4.8 eq.) dissolved in 10 mL of CH_2Cl_2 were added to a solution of compound **4** (2.0 g, 3.1 mmol, 1 eq.) in 200 mL of anhydrous CH_2Cl_2–DMF (7 : 3, v : v). The pH was adjusted to 8 with DIEA (*N*,*N*-diisopropylethylamine) and the reaction mixture was stirred for 1.5 h at rt. After this time, the solvents were evaporated to dryness. The resulting crude product was dissolved in 100 mL of CH_2Cl_2 and washed with 5% $NaHCO_3$ (3 × 100 mL). The organic phase was dried with Na_2SO_4 and evaporated. The crude product was transferred to a 50 mL tube, dissolved in CH_2Cl_2 (5 mL), and hexane (45 mL) was added. The mixture was stirred vigorously and centrifuged. The supernatant was discarded and the remaining orange oily precipitate was purified by reverse phase chromatography (C_{18} column, 50→80% CH_3CN in aqueous 20 mM NH_4HCO_3 solution), affording 3.62 g (2.14 mmol, yield: 72%) of the first generation dendron (**5**). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 1. 43 (s, 36 H), 1.76 (m, 16 H), 2.68 (m, 8 H), 3.18 (m, 16 H), 3.32 (m, 8 H), 3.42 (s, 2 H), 3.52 (m, 16 H), 3.58 (m, 16 H), 3.62 (m,

16 H), 5.13 (s, 2 H), 7.35 (m, 5 H), and 7.66 (bs, NH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 28.27, 29.30, 29.50, 36.80, 38.22, 52.31, 53.30, 55.29, 58.91, 66.29, 69.08, 69.24, 69.96, 70.27, 70.29, 78.63, 128.11, 128.28, 128.45, 135.23, 155.88, 170.52, and 171.11. Analytical HPLC: 5→100% CH_3CN in water over 8 min, t_R = 7.02 min (purity 99% determined at λ = 210 nm). Calcd mass for $C_{81}H_{149}O_{26}N_{11}$: 1692.0670; Calcd mass for $C_{81}H_{148}O_{26}N_{11}Na$: 1714.0489; Calcd mass for $C_{81}H_{148}O_{26}N_{11}K$: 1730.0228; Mass found by MALDI-TOF: 1693.1 (M + 1), 1715.1 (M + Na) 1731.1 (M + K); Mass found by HPLC-MS: 1694.2 (M + 1), 847.4 (M + 2)/2; Mass found by HRMS: 1692.0672, 1714.0483 (M + Na), 1730.0114 (M + K).

Synthesis of dendron 6 (G1-Boc-COOH). Compound **5** (200 mg, 0.12 mmol, 1 eq.) was dissolved in 5 mL of MeOH, and 10% Pd/C (20.2 mg, 10% w/w) was added to this solution. The resulting suspension was stirred for 1 h under an H_2 atmosphere at rt. The catalyst was removed by filtration through Celite, and the solvent was evaporated to afford dendron **6** as a waxy solid (176 mg, 0.11 mmol, yield: 92%). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 1.43 (s, 36 H), 1.77 (m, 16 H), 2.75 (bs, 8 H), 3.22 (m, 16 H), 3.32 (m, 10 H), 3.53 (m, 16 H), 3.59 (m, 16 H), 3.63 (m, 16 H), and 7.82 (bs, NH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 28.40, 29.28, 29.65, 36.99, 38.26, 52.88, 53.59, 58.98, 69.30, 69.98, 70.05, 70.35, 70.39, 78.86, 139.12, 156.11, and 170.68. Analytical HPLC: 5→100% CH_3CN in water over 8 min, t_R = 6.45 min (purity 97% determined at λ = 210 nm). Calcd mass for $C_{74}H_{143}O_{26}N_{11}$: 1602.0206; Calcd mass for $C_{74}H_{142}O_{26}N_{11}Na$: 1624.0025; Mass found by MALDI-TOF: 1625.3 (M + Na); Mass found by HPLC-MS: 1604.2 (M + 1), 802.4 (M + 2)/2; Mass found by HRMS: 1602.0211, 1624.0020 (M + Na).

Synthesis of dendron 7 (G1-NH$_2$-Bn). Totally protected dendron **5** (200 mg, 0.12 mmol, 1 eq.) was dissolved in 2 mL of dioxane and 4.0 M HCl in dioxane solution was added (5 mL, 20 mmol, 166 eq.). The mixture was vigorously stirred for 1 h at rt and the resulting suspension was evaporated to obtain the semi-solid product **7**. The product was dissolved in 1 mL of 20 mM NH_4HCO_3 and the salts were removed by reverse phase chromatography using a PoraPak column and 20% of CH_3CN in water as an eluent, obtaining 134 mg of dendron **7** (0.10 mmol, yield: 87%). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ = 1.80 (m, 8 H), 1.98 (m, 8 H), 3.09 (m, 4 H), 3.13 (t, ^3J(H,H) = 7.07 Hz, 8 H), 3.27 (t, ^3J(H,H) = 6.90 Hz, 8 H), 3.41 (m, 12 H), 3.56 (t, ^3J(H,H) = 6.41 Hz, 8 H), 3.69 (m, 40 H), 4.35 (s, 2 H), 5.37 (s, 2 H), and 7.49 (m, 5 H). ^{13}C NMR (100 MHz, D_2O, 25 °C): δ = ^{13}C NMR (100 MHz, D_2O, 25 °C): δ = 26.73, 28.60, 36.57, 37.89, 50.76, 53.30, 53.48, 57.89, 68.49, 68.55, 68.71, 69.56, 69.62, 69.76, 69.79, 128.49, 129.14, 129.21, 135.05, 168.02, and 172.31. Analytical HPLC: 5→100% CH_3CN in water over 8 min, t_R = 3.44 min (purity 92% determined at λ = 210 nm). Calcd mass for $C_{61}H_{117}O_{18}N_{11}$: 1291.8573; Calcd mass for $C_{61}H_{116}O_{18}N_{11}Na$: 1314.8470; Mass found by MALDI-TOF: 1292.9 (M + 1), 1314.9 (M + Na); Mass found by HPLC-MS: 1294.0 (M + 1), 647.1 (M + 2)/2, 431.8 (M + 3)/3, 324.0 (M + 4)/4; Mass found by HRMS: 1291.8570.

Synthesis of dendron 8 (G1-Ac-Bn). Dendron **7** (60 mg, 0.046 mmol, 1 eq.) was dissolved in CH_2Cl_2 and mixed with acetic anhydride (26.3 μL, 0.28 mmol, 6 eq.) and DIEA (97 μL, 0.56 mmol, 12 eq.). After stirring for 1 h, the mixture was evaporated and the crude product was purified by reverse phase chromatography (C_{18} column, 25→60% CH_3CN in aqueous 20 mM NH_4HCO_3 solution), affording the waxy dendron **8** (37 mg, 0.025 mmol, yield: 51%). ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ = 1.77 (m, 16 H), 1.95 (s, 12 H), 2.97 (bs, 4 H), 3.10 (bs, 4 H), 3.31 (m, 16 H), 3.38 (s, 2 H), 3.50 (t, ^3J(H,H) = 6.02 Hz, 8 H), 3.53–3.60 (m, 32 H), 3.60–3.65 (m, 16 H), 5.15 (s, 2 H), 7.34 (m, 5 H), and 7.98 (bs, NH). ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ = 23.35, 28.90, 29.30, 37.04, 37.90, 51.14, 51.79, 57.65, 67.04, 69.15, 69.91, 69.98, 70.06, 70.35, 70.41, 70.44, 128.44, 128.64, 128.69, 135.00, 169.42, and 170.39. Analytical HPLC: 5→100% CH_3CN in water over 8 min, t_R = 4.26 min (purity 97% determined at λ = 210 nm). Calcd mass for $C_{69}H_{125}O_{22}N_{11}$: 1459.9001; Calcd mass for $C_{69}H_{124}O_{22}N_{11}Na$: 1481.8820; Mass found by MALDI-TOF: 1460.9 (M + 1), 1482.9 (M + Na); Mass found by HPLC-MS: 1461.0 (M + 1), 731.3 (M + 2)/2; Mass found by HRMS: 1459.8999, 1481.8816 (M + Na).

Synthesis of dendron 9 (G1-Ac-COOH). Dendron **8** (31 mg, 0.021 mmol, 1 eq.) was dissolved in 5 mL of MeOH, and 10% Pd/C (3 mg, 10% w/w) was added to the solution. The resulting suspension was stirred for 1 h under an H_2 atmosphere at rt. The catalyst was removed by filtration through Celite, and the solvent was evaporated to afford dendron **9** as a waxy solid (23 mg, 0.017 mmol, yield: 84%). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 1.77 (m, 16 H), 1.97 (s, 12 H), 3.15 (bs, 4 H), 3.31 (m, 16 H), 3.37 (bs, 4 H), 3.50 (t, ^3J(H,H) = 5.78 Hz, 8 H), 3.53–3.60 (m, 48 H), 4.00 (s, 2 H), and 8.00 (bs, NH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 22.94, 28.81, 29.04, 37.06, 37.75, 51.01, 52.33, 53.40, 57.67, 69.01, 69.67, 69.84, 69.88, 70.24, 70.31, 169.43, and 171.06. Analytical HPLC: 5→100% CH_3CN in water over 8 min, t_R = 3.61 min (purity 99% determined at λ = 210 nm). Calcd mass for $C_{62}H_{119}O_{22}N_{11}$: 1369.8531; Calcd mass for $C_{62}H_{118}O_{22}N_{11}Na$: 1391.8351; Mass found by MALDI-TOF: 1370.9 (M + 1), 1392.9 (M + Na); Mass found by HPLC-MS: 1370.9 (M + 1), 686.1 (M + 2)/2; Mass found by HRMS: 1369.8502, 1391.8330 (M + Na).

Synthesis of dendron 10 (G1-NH$_2$-COOH). Dendron **6** (55 mg, 0.034 mmol, 1 eq.) was dissolved in 2 mL of dioxane and 4.0 M HCl in dioxane solution was added (5 mL, 20 mmol, 588 eq.). The mixture was vigorously stirred for 1 h at rt and the resulting suspension was evaporated, obtaining the semi-solid product **10**. The product was dissolved in 1 mL of 20 mM NH_4HCO_3 and the salts were removed by reverse phase chromatography using a PoraPak column and 20% of CH_3CN in water as an eluent, obtaining 34 mg of dendron **10** (0.028 mmol, yield: 83%). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ = 1.83 (m, 8 H), 1.97 (m, 8 H), 3.13 (t, ^3J(H,H) = 6.64 Hz, 8 H), 3.23 (bs, 4 H), 3.33 (t, ^3J(H,H) = 5.98 Hz, 8 H), 3.45 (bs, 4 H), 3.59 (t, ^3J(H,H) = 6.07 Hz, 8 H), 3.65–3.73 (m, 40 H), 3.77 (bs, 2 H), and 3.97 (s, 8 H). ^{13}C NMR (100 MHz, D_2O, 25 °C): δ = 29.84, 31.56, 39.94, 40.97, 53.81, 55.83, 56.94, 60.08, 71.59,

71.61, 72.66, 72.73, 72.87, 72.89, 170.70, and 175.59. Analytical HPLC: 5→100% CH_3CN in water over 8 min, t_R = 3.04 min (purity 98% determined at λ = 210 nm). Calcd mass for $C_{54}H_{111}O_{18}N_{11}$: 1201.8109; Calcd mass for $C_{54}H_{110}O_{18}N_{11}Na$: 1223.7928; Mass found by MALDI-TOF: 1202.8 (M + 1), 1224.8 (M + Na); Mass found by HPLC-MS: 1202.6 (M + 1), 602.2 (M + 2)/2; Mass found by HRMS: 1201.8104.

Synthesis of second generation dendron (G2-Boc-Bn, 11). 2,3,4,5,6-Pentafluorophenol (96.6 mg, 0.53 mmol, 1.5 eq.), EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 100.6 mg, 0.53 mmol, 1.5 eq.), and DMAP (4-dimethylaminopyridine, 21.4 mg, 0.18 mmol, 0.5 eq.) were added to a solution of dendron **6** (600 mg, 0.35 mmol, 1 eq.) in anhydrous CH_2Cl_2. The solution was then stirred for 3 h at rt. Dendron **7** (103.1 mg, 0.08 mmol, 0.22 eq.) dissolved in a minimal amount of CH_2Cl_2 was then added to the resulting active ester, and the pH was adjusted to 8 with DIEA. The mixture was stirred overnight at rt. After that, the solution was washed three times with 5% $NaHCO_3$ (3 × 50 mL), and the organic phase was dried with Na_2SO_4 and evaporated. The crude product was transferred to a 50 mL tube and dissolved in 5 mL of CH_2Cl_2, and 45 mL of hexane was added. The mixture was stirred vigorously and centrifuged. The supernatant was discarded, and the remaining orange oily precipitate was purified by reverse phase chromatography (C_{18} column, 60→90% CH_3CN in aqueous 20 mM NH_4HCO_3 solution), affording second generation dendron **11** (292.3 mg, 0.038 mmol, yield: 48%). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 1.43 (s, 144 H), 1.77 (m, 80 H), 2.66 (bs, 40 H), 3.18 (m, 80 H), 3.32 (m, 48 H), 3.42 (s, 2 H), 3.53 (m, 80 H), 3.59 (m, 80 H), 3.63 (m, 80 H), 5.13 (s, 2 H), 7.35 (m, 5 H), and 7.79 (bs, NH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 28.39, 29.33, 29.47, 29.54, 29.64, 36.76, 38.29, 53.08, 59.07, 66.42, 69.03, 69.10, 69.17, 69.32, 70.02, 70.05, 70.07, 70.33, 70.37, 70.40, 78.81, 156.04, 170.75, and 170.85. Analytical HPLC: 5→100% CH_3CN in water over 8 min, t_R = 7.04 min (purity 97% determined at λ = 210 nm). Calcd mass for $C_{357}H_{681}O_{118}N_{55}$: 7627.8978; Calcd mass for $C_{357}H_{680}O_{118}N_{55}Na$: 7649.8798; Calcd mass for $C_{357}H_{679}O_{118}N_{55}Na_2$: 7671.8617; Calcd mass for $C_{357}H_{678}O_{118}N_{55}Na_3$: 7692.8358; Mass found by MALDI-TOF: 7643.7; Mass found by HPLC-MS: 1273.0 (M + 6)/6, 1091.2 (M + 7)/7, 955.0 (M + 8)/8; Mass found by HRMS: 7627.8948, 7649.8713 (M + Na), 7671.8518 (M + 2Na), 7692.8318 (M + 3Na).

Synthesis of dendron 12 (G2-Boc-COOH). Dendron **11** (57 mg, 0.0075 mmol, 1 eq.) was dissolved in 5 mL of MeOH, and 10% Pd/C (14 mg, 20% w/w) was added. The resulting suspension was stirred for 2 h under an H_2 atmosphere at rt. The catalyst was removed by filtration through Celite, and the solvent was evaporated, affording dendron **12** as a waxy solid (39 mg, 0.0052 mmol, yield: 69%). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 1.43 (s, 144 H), 1.77 (m, 80 H), 2.70 (bs, 40 H), 3.21 (m, 80 H), 3.31 (m, 50 H), 3.53 (m, 80 H), 3.59 (m, 80 H), 3.63 (m, 80 H), and 7.81 (bs, NH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 28.43, 29.25, 29.67, 37.06, 38.35, 58.39, 69.17, 69.37, 70.05, 70.10, 70.39, 70.43, 78.86, 156.09, and 170.59. Analytical HPLC: 5→100% CH_3CN in water over 8 min, t_R = 7.21 min (purity 95% at λ = 210 nm). Calcd mass for $C_{350}H_{675}O_{118}N_{55}$: 7537.8509; Calcd mass for $C_{350}H_{674}O_{118}N_{55}Na$: 7559.8328; Calcd mass for $C_{350}H_{673}O_{118}N_{55}Na_2$: 7581.8148; Calcd mass for $C_{350}H_{672}O_{118}N_{55}Na_3$: 7597.7498; Calcd mass for $C_{350}H_{671}O_{118}N_{55}Na_4$: 7619.7317; Mass found by HPLC-MS: 2514.9 (M + 3)/3, 1886.6 (M + 4)/4, 1509.6 (M + 5)/5; Mass found by HRMS: 7559.8138 (M + Na), 7581.8126 (M + 2Na), 7597.7803 (M + 3Na), 7619.7565 (M + 4Na).

Synthesis of dendron 13 (G2-NH_2-Bn). Compound **11** (45 mg, 0.0059 mmol, 1 eq.) was dissolved in 3 mL of dioxane, and 4.0 M HCl in dioxane solution was added (2 mL, 1356 eq.). The mixture was vigorously stirred for 2 h at rt, and the resulting suspension was evaporated to obtain the solid product **13**. The product was dissolved in 1 mL of 20 mM NH_4HCO_3, and the salts were removed by reverse phase chromatography using a PoraPak column and 20% of CH_3CN in water as an eluent, obtaining 28 mg of dendron **13** (0.0047 mmol, yield: 79%). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ = 1.83 (m, 48 H), 1.98 (m, 32 H), 3.14 (t, ^3J(H,H) = 6.92 Hz, 32 H), 3.22 (bs, 16 H), 3.32 (t, ^3J(H,H) = 6.95 Hz, 54 H), 3.39 (bs, 16 H), 3.57 (m, 80 H), 3.65–3.75 (m, 208 H), 4.03 (s, 2 H), 5.31 (s, 2 H), and 7.49 (m, 5 H). ^{13}C NMR (100 MHz, D_2O, 25 °C): δ = 26.69, 28.46, 28.57, 36.59, 36.86, 37.84, 51.07, 53.03, 57.87, 68.46, 68.51, 69.54, 69.59, 69.73, 69.75, 129.11, 166.53 and 171.35. Analytical HPLC: 5→100% CH_3CN in water over 8 min, t_R = 3.27 min (purity 98% determined at λ = 210 nm). Calcd mass for $C_{277}H_{553}O_{86}N_{55}$: 6027.0589; Calcd mass for $C_{277}H_{552}O_{86}N_{55}Na$: 6050.0482; Mass found by MALDI-TOF: 6043.6 (M + $NH4^+$), 6060.2 (M + Na); Mass found by HPLC-MS: 1509.06 (M + 4)/4, 603.98 (M + 10)/10, 549.22 (M + 11)/11, 503.50 (M + 12)/12; Mass found by HRMS: 6027.0877.

Synthesis of dendron 14 (G2-Ac-Bn). Dendron **13** (60 mg, 0.01 mmol, 1 eq.) was dissolved in 5 mL of water, and acetic anhydride (76 μL, 0.8 mmol, 80 eq.) was added. Solid $NaHCO_3$ was added to the solution to achieve pH 6–7. The mixture was stirred for 24 h, and 80 eq. more of acetic anhydride were added. The pH was again increased to 6–7 with $NaHCO_3$. After 24 h, 80 eq. more of acetic anhydride was added to the mixture and the pH was adjusted to 6–7. The solution was then stirred for a further 15 h and freeze-dried. The crude product was purified by reverse phase chromatography (C_{18} column, 30→70% CH_3CN in aqueous 20 mM NH_4HCO_3 solution), affording the dendron **14** (32.4 mg, 0.0048 mmol, yield: 48%). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ = 1.78 (m, 80 H), 1.98 (s, 48 H), 2.67 (s, 40 H), 3.19 (s, 2 H), 3.26 (m, 128 H), 3.56 (m, 80 H), 3.61–3.71 (m, 160 H), 5.22 (s, 2 H), and 7.43 (m, 5 H). ^{13}C NMR (100 MHz, D_2O, 25 °C) δ: 22.0, 28.42, 28.74, 28.87, 36.40, 36.64, 52.83, 53.17, 57.96, 58.72, 67.05, 68.58, 68.57, 68.70, 69.54, 69.62, 69.79, 69.80, 128.37, 128.87, 129.05, 135.77, 160.39, 173.20, 173.26, and 173.96. Analytical HPLC: 5→100% CH_3CN in water over 8 min, t_R = 3.98 min (purity 88% determined at λ = 210 nm). Calcd mass for $C_{309}H_{585}O_{102}N_{55}$: 6699.2280; Calcd mass for $C_{309}H_{584}O_{102}N_{55}Na$: 6721.2099; Mass found by MALDI-TOF: 6718.2 (M + Na); Mass found by HPLC-MS: 2235.4 (M + 3)/3, 1676.9 (M + 4)/4, 1341.6 (M + 5)/5, 1118.0 (M + 6)/6,

958.5 (M + 7)/7, 838.8 (M + 8)/8; Mass found by HRMS: 6699.2392, 6721.2303 (M + Na).

Synthesis of dendron 15 (G2-NH_2-COOH). Dendron **12** (32 mg, 0.0042 mmol, 1 eq.) was dissolved in 1 mL of dioxane, and 4 M HCl in dioxane solution was added (1 mL, 0.25 mmol, 60 eq.). The solution was stirred for 2 h and evaporated to dryness. The resulting crude product was dissolved in water and freeze-dried. Solid product **15** was obtained quantitatively as a hydrochloride salt (24.7 mg, 0.0041 mmol, yield: 99%). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ = 1.83 (m, 48 H), 1.97 (m, 32 H), 3.13 (t, ^3J(H,H) = 6.79 Hz, 32 H), 3.32 (m, 88 H), 3.58 (t, ^3J(H,H) = 6.17 Hz, 32 H), 3.70 (m, 208 H), 3.74–3.86 (bs, 48 H), and 3.89 (s, 2 H). ^{13}C NMR (100 MHz, D_2O, 25 °C): δ = 26.62, 28.41, 36.65, 37.75, 51.68, 51.99, 57.21, 68.38, 68.41, 69.46, 69.51, 69.66, 69.68, and 168.91. HPLC: 5→100% CH_3CN in water over 8 min, t_R = 3.17 min (purity 85% determined at λ = 210 nm). Calcd mass for $C_{270}H_{547}O_{86}N_{55}$: 5937.0115; Calcd mass for $C_{270}H_{545}O_{86}N_{55}Na_2$: 5981.9832; Mass found by MALDI-TOF: 5980.0 (M + 2Na); Mass found by HPLC-MS: 743.5 (M + 8)/8, 660.9 (M + 9)/9, 594.9 (M + 10)/10, 540.9 (M + 11)/11, 495.9 (M + 12)/12, 457.9 (M + 13)/13. Mass found by HRMS: 5937.0063.

Dynamic light scattering (DLS) and ζ-potential experiments

For dendron size determination, DLS measurements were performed on a Brookhaven Instruments 200 SM laser light scattering goniometer using an He–Ne 125 mW633 nm laser. Single scans of 10 min exposure were performed, and particle sizes were estimated using the CONTIN multiple-pass method of data analysis at angles of 60°, 90°, and 120°. Measurements for ζ-potentials were made with a Malvern ZetaSizer Nano ZS instrument. Dendrons dissolved in PBS at different concentrations (10, 5, and 1 mg mL^{-1}) and passed through a 0.2 μm filter were placed in a specific cuvette, and certain parameters of the refractive index and the absorption coefficient of the material and the viscosity of the solvent were introduced into the software.

Stability experiments in serum

Stability assays in human serum were carried out by incubation of dendrons (dendrons **7**, **8** and **13**) with serum (diluted 9 : 1 in HBBS buffer) at 37 °C. Compounds were used at a final concentration of 0.8 mg mL^{-1} for dendrons **7** and **8** (**G1-NH_2-Bn** and **G1-Ac-Bn**) and 1.25 mg mL^{-1} for dendron **14** (**G2-Ac-Bn**). Aliquots of 60 μL were periodically taken at 0 to 120 h and mixed with 200 μL of CH_3CN in order to precipitate the serum proteins. The mixture was cooled to 4 °C and centrifuged at 13 000 rpm for 15 min at 4 °C. The supernatant was passed through a 0.45 μm filter, and 20 μL was injected in an analytical HPLC system using a C_{18} reverse phase column and CH_3CN–water 0.05% of TFA as eluents. The gradient used was 5→100% of CH_3CN in water over 8 min. Two blank samples were also performed in parallel following the same procedure reported above. In the first blank sample, water was used instead of human serum, whereas in the second blank the serum solution did not contain dendrons.

Cell culture

HT-29 and SK-BR-3 cell lines were cultured in McCoy's 5A medium supplemented with 10% (v/v) fetal calf serum and 100 mg mL^{-1} of penicillin and streptomycin. Cells were subcultured routinely using 0.02% (w/v) trypsin-EDTA.

Antiproliferative activity *via* MTT assay

HT-29 and SK-BR-3 cell lines were seeded in standard 96-well plates at a density of 5×10^3 and 10^4 cells per well respectively and grown for 24 h in McCoy's 5A culture medium. When the HT-29 and SK-BR-3 cell monolayers reached 90% of confluence they were treated for 72 h with 100 μM, 10 μM, 1 μM, or 0.1 μM of the different dendron solutions in a fresh culture medium. A control experiment was also performed by incubating the cells with PBS instead of dendrons. Viable cell density was then assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, both treated and untreated cells were washed thoroughly with PBS and incubated in MTT solution (0.5 mg mL^{-1} MTT in PBS, 100 μL per well of a 96-well plate) for 2 h at 37 °C in a humidified atmosphere of 5% CO_2. Dehydrogenase activity in the cells reduces MTT, forming a purple formazan salt, which was eluted from the cells using isopropanol (100 μL per well of a 96-well plate). After dissolution of the formazan salt, the optical density at 540 nm was measured using a plate reading spectrophotometer (ELx800 Bio-Tek Instruments, Inc). The percentage of viability was expressed taking the control experiment as 100% of viability.

Microscopy

The HT-29 and SK-BR-3 cell lines were seeded in 24-well plates at a density of 10^4 and 5×10^4 cells per well respectively and grown for 24 h in McCoy's 5A culture medium. Cells were incubated with 100 μM of the different dendron solutions for 72 h. Cell morphology was assessed using a Nikon TE200 inverse phase microscopy (Japan) equipped with a 40× objective Nikon LWD 0.52 NA (Japan).

Blood sample collection

The studies involving donor blood were approved by the Ethics Committee of the Medical Faculty of Liège. Human blood was obtained from the Red Cross Transfusion, University Hospital of Liège. Blood was collected from healthy donors in 4.5 mL tubes containing 3.2% sodium citrate as an anticoagulant. Experiments were done 15 min after collection.

Blood smears for the control of the RBC (red blood cell) morphology

Dendrons were dissolved in PBS at a range of concentrations, and a volume of sample solution was diluted in 9 volumes of whole blood, to obtain final concentrations of 100, 10, 1, and 0.1 μM of dendron solutions. Samples were incubated for 15 min at 37 °C under lateral agitation (250 rpm). After blood incubation, 5 μL of the blood was withdrawn and spread on a microscopy glass slide. Blood cells were observed with an

Olympus Provis microscope at 20× and 50× magnification in transmission mode. At least two representative pictures were acquired per sample with a digital camera (VisiCam (5 megapixels), VWR International).

Hemolytic test

Dendron solutions and blood were prepared and incubated as described above. The hemolytic test was performed following Standard Practice for Assessment of Haemolytic Properties of Materials (ASTM designation F 756-00). Briefly, after incubation, the samples were centrifuged at 600*g* for 5 min at rt, and supernatants were collected and mixed with the cyanmethemoglobin reagent. The hemoglobin released was measured at 540 nm in a microplate reader (Anthos HT III, type 12600, Anthos, Salzburg, AU). A calibration curve was established using bovine hemoglobin as the standard. Saponine (0.8 mg mL^{-1}) and PBS were used as positive and negative controls, respectively. Hemolysis was expressed as the percentage of hemoglobin released to total content, taking the positive control as 100% of hemolysis. Three independent experiments (with distinct blood donor samples) were performed, and the tests were done in triplicate. In view to assess the influence of plasma protein on the hemolytic action of the dendrons this test was also performed using washed RBCs (4% RBC suspension) instead of whole blood.

Count and size distribution of RBCs, platelets and white blood cells

Dendron solutions and blood were prepared and incubated as described before. After 15 min of incubation, blood cells were analyzed with an automated CELL-DYN 18 Emerald (Abbott Diagnostics). Three analyses were conducted per sample without agitation of the tube before each run.

Complement activation

Complement activation was estimated using the Human C3a ELISA kit for quantification of Human C3a-des-Arg (Becton Dickinson). After a 15 min incubation of blood and dendron mixtures, EDTA (1 mM final) was added to stop complement activation. Samples were centrifuged at 2000*g* for 5 min at rt, and supernatants were used for the analysis of complement activation following the kit protocol (BD OptEIA, Human C3a ELISA, Instruction Manual. Cat. No. 550499). Absorbance was measured at 450 nm with a microplate reader (Anthos HT III, type 12600). Plasma containing 2 mg mL^{-1} of Zymosan was taken as a positive control and plasma without additives as a negative control. The concentration of C3a was expressed as a percentage of activation, taking the value of the negative control incubated as a 100% of complement activation. Measurements were done in duplicate.

Coagulation experiments

Whole blood and dendrimer solutions were mixed and incubated as described before. Samples were centrifuged at 2000*g* for 5 min at rt, and the supernatants were collected, recalcified to reverse the effect of citrate anticoagulant, and supplied with the specific activators of coagulation (thromboplastin). Prothrombin time (PT), to evaluate the extrinsic pathway, and activated partial thromboplastin time (APTT), to evaluate the intrinsic pathway, were measured directly with a Dade Behring Coagulation Timer analyzer (BCT) (Siemens Healthcare Diagnostics NV/SA, Belgium). Kaolin reagent was used as a positive control and PBS as a negative control. Clotting time was measured for each sample, and coagulation capacity was expressed as a percentage, taking the value of standard human plasma (Dade Behring/Siemens) as 100%. Two independent experiments were performed, and measurements were done in duplicate.

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