Synthesis and supramolecular assembly of clicked anionic dendritic polymers into polyion complex micelles†‡

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Remarkably stable polyion complex micelles with narrow size distribution result from the supramolecular assembly of clicked anionic PEG-dendritic block copolymers with oppositely charged polymers.

Biocompatible anionic polymers have recently attracted a great deal of attention in biotechnology and drug delivery thanks to the possibility of forming functional electrostatic complexes, such as micelles, nanoparticles, and layer-by-layer assemblies.[1,2] In addition, various anionic polymers have been reported as mimetics of glycosaminoglycans (GAGs) with the ability to potentiate fibroblast growth factor and to inhibit Alzheimer amyloid aggregation, as well as demonstrated microbicide, anti-inflammatory, and anticoagulant activities.[3–5] Among anionic polymers, those with dendritic structure are especially attractive due to their monodisperse nature, and the possibility of controlling their size and the number of anionic groups at the periphery. However, as the preparation of anionic dendrimers often relies on inefficient amide linkages and sulfation procedures, non-homogeneous decoration patterns result, limiting their applicability.[4,5]

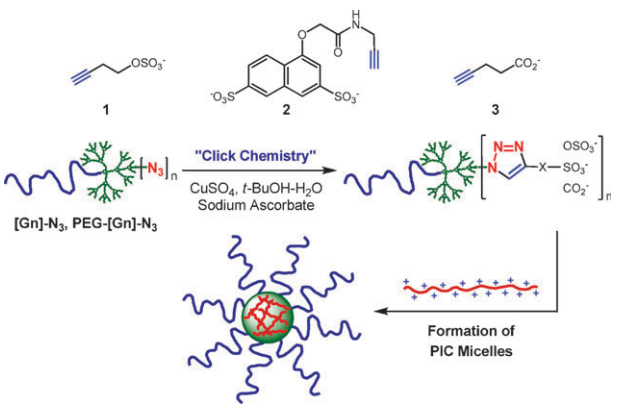
Herein, we report an efficient click approach to anionic dendritic polymers, potential mimetics of GAGs, as well as their supramolecular assembly into physiologically stable polyion complex (PIC) micelles, attractive drug delivery systems of small size and remarkably narrow dispersity (Scheme 1).

With this purpose, we have relied on the Cu(I)-catalyzed azide–alkyne [3 + 2] cycloaddition, an extremely powerful technology recently exploited by us and others for the ready and complete surface functionalization of dendrimers in high yields.[6,7] As dendritic partner, GATG (gallic acid-triethylene glycolazide) dendrimers (**[Gn]–N_3**) and PEG-dendritic block copolymers (**PEG–[Gn]–N_3**, synthesized from MeO-PEG-OH, M_n 5055.5, M_w 5087.8 by MALDI-TOF)[8] were selected because of their terminal azides, good solubility properties, and easy structural modification (Scheme 2).[6] The incorporation of PEG into the block copolymers is expected to confer them further solubility and biocompatibility, as well as better biodistribution. An alkyne-derived series of sulfate (**1**), sulfonate (**2**), and carboxylate (**3**) was selected based on their potential biomedical applications (Scheme 1). Interestingly, sulfonate **2** is the active species present in VivaGel™, a dendritic vaginal microbicide under development for the prevention of sexually transmitted infections, such as genital herpes (HSV-2) and HIV.[5]

Indeed, when **[G1]–N_3** was treated with sulfate **1** in the presence of $CuSO_4$ and sodium ascorbate (*t*-BuOH–H_2O, 24 h, rt), pure **[G1]–1** was obtained with complete regioselectivity in 80% yield, after purification by ultrafiltration (Scheme 2). Incorporation of three sulfate residues in **[G1]-1** was clearly established by ^{1}H NMR (D_2O) thanks to the disappearance of the characteristic signal of the methylene protons adjacent to the azide groups (3.40 ppm). Also, the presence of two singlets at 7.88 and 7.86 ppm (1 : 2 ratio), corresponding to three triazol protons, and two triplets at 3.04 and 2.99 ppm (1 : 2 ratio, J = 6.2 Hz), due to the methylene protons β to the sulfate groups, denoted complete functionalization.

Likewise, when **[G2]–N_3** and **[G3]–N_3** were treated with **1** under identical reaction conditions, pure **[G2]–1** and **[G3]–1** incorporating 9 and 27 sulfate groups were obtained in excellent yields (91–92%, Scheme 2). Gel permeation chromatography of the resulting sulfated dendrimers confirmed their purity, and the good performance of the generation growth-surface decoration procedure (Fig. 1).

Similarly, this click anionic decoration proceeded in excellent yields when dealing with three generations of the more demanding block copolymers **PEG–[Gn]–N_3**, or when sulfonate (**2**) and carboxylate (**3**) were employed (90–100%, Scheme 2). Complete functionalization was again confirmed in these examples by ^{1}H NMR (Fig. 2a and b) and IR (complete disappearance of the intense characteristic azide signal at 2107 cm^{-1}).



Scheme 1

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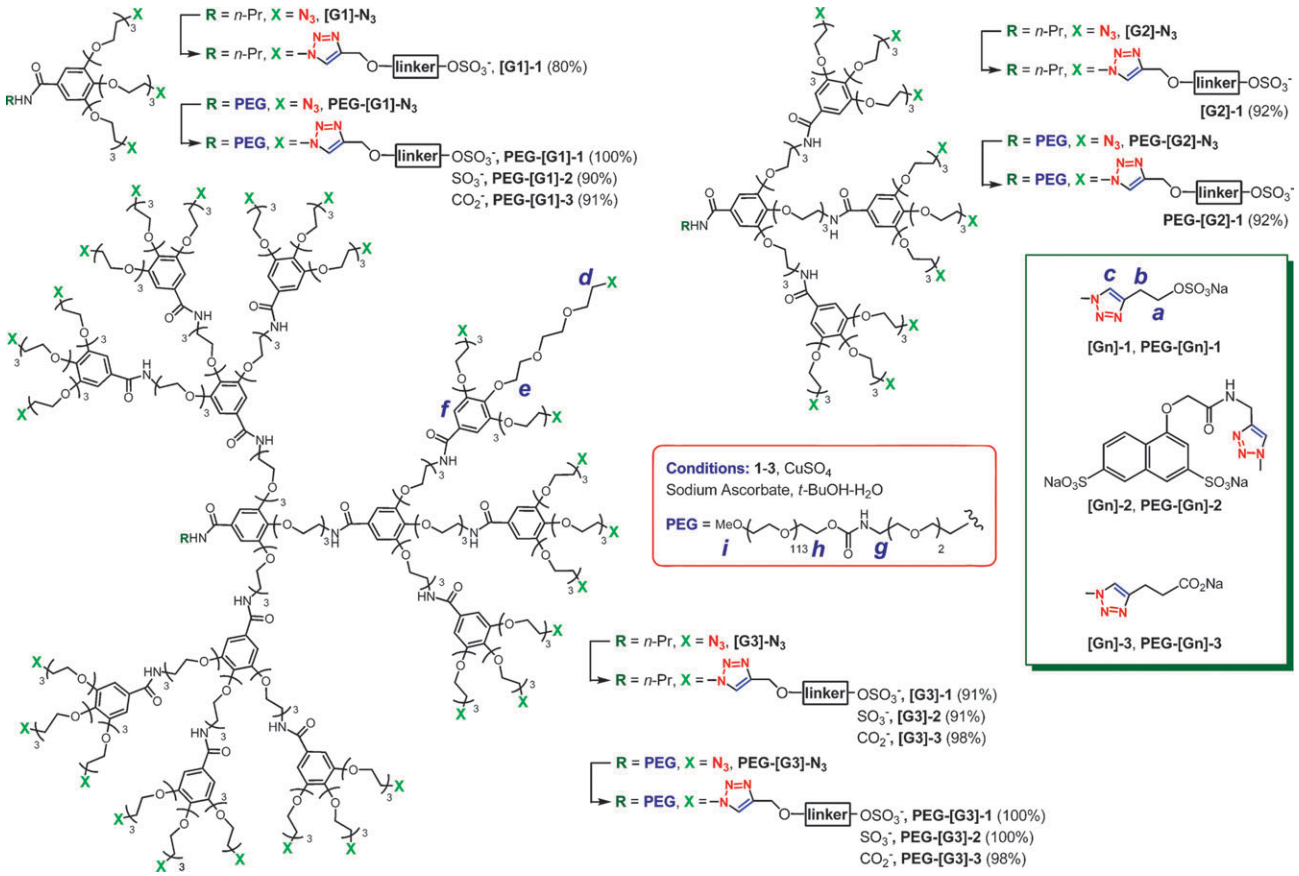
‡ Dedicated to Prof. Antonio Mouriño on the occasion of his 60th birthday.



Scheme 2 Click decoration of **[Gn]–N_3** and **PEG–[Gn]–N_3**.

With a reliable method for the anionic decoration of PEG–dendritic block copolymers in hand, we envisioned their assembly with oppositely charged polymers as an attractive route to produce stable PIC micelles.

PIC micelles are considered smart delivery systems due to their electrical neutrality, small sizes, and fairly narrow size distribution.[2] Their formation is driven by electrostatic attraction, and therefore they are salt-sensitive and fall apart as the ionic strength reaches a critical value of 150–200 mM. Interestingly, Kataoka and co-workers have recently reported PIC micelles with an unusual higher stability as a result of their more rigid dendritic architecture.[9]

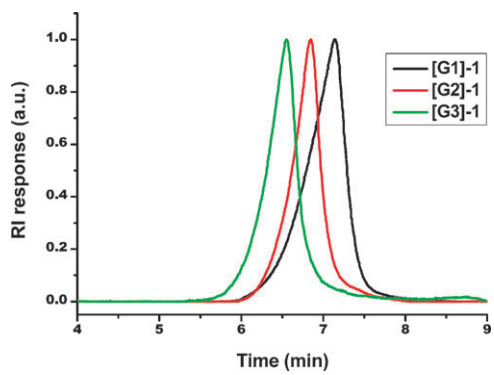


Fig. 1 GPC traces for **[G1]–1**, **[G2]–1**, and **[G3]–1** (CH_3CN–H_2O, 1 : 1).

Our approach incorporating PEG–dendritic block copolymers instead of dendrimers should benefit from the additional advantages:

1. The straightforward synthesis of PEG–dendritic block copolymers that are easily purified by precipitation, avoiding solvent extractions and tedious chromatographies usually associated to the synthesis of dendrimers.

2. The attachment of PEG to the dendritic partner allows the use of oppositely charged ''off the shelf'' polyions rather than non-commercially available PEG–polyion copolymers.

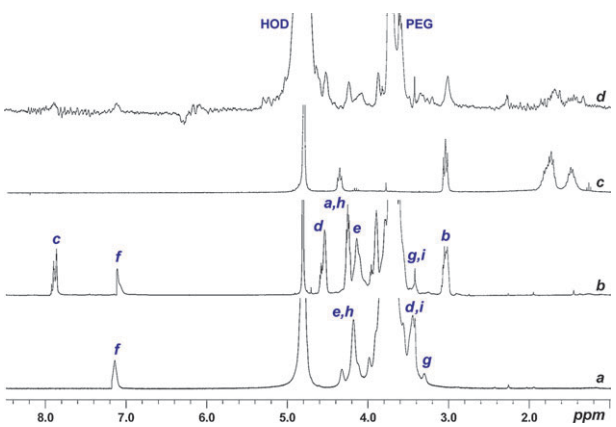


Fig. 2 ^{1}H NMR spectra (D_2O, 500 MHz) of **PEG–[G3]–N_3** (a), **PEG–[G3]–1** (b), PLL (c), and PIC micelles in 80% H_2O–20% D_2O (d). The localization of positions ***a–i*** is shown in Scheme 2.

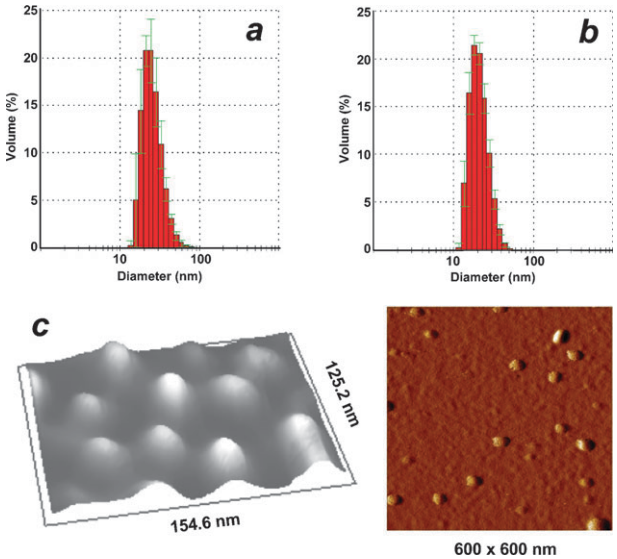


Fig. 3 DLS histograms (detection angle 170°) of PIC micelles in H_2O at 25 °C (a), and in 10 mM PBS pH 7.4, 150 mM NaCl, 37 °C (b). Tapping-mode AFM images (c).

Indeed, when stoichiometric ratios of sulfated **PEG–[G3]–1** and poly-L-lysine (PLL, M_n 12 400, M_w 16 100, DP = 77) were mixed in H_2O at 25 °C, spherical PIC micelles with a remarkably narrow size distribution were obtained. The dynamic light scattering (DLS) histogram of the micelles as prepared revealed a hydrodynamic size of *ca.* 25 nm and a low polydispersity index (0.12), without any sign of larger aggregates (Fig. 3a). The zeta-potential was close to zero, in agreement with the expected charge neutrality. Interestingly, the size of the micelles remained quite unaffected when prepared in 10 mM PBS pH 7.4, even after the addition of 150 mM NaCl and at 37 °C, conditions of special interest for possible *in vivo* applications (Fig. 3b).

The remarkable tolerance of these micelles at high ionic strengths was confirmed by DLS, with no variation in size and polydispersity up to 0.5 M NaCl (H_2O, 25 °C). These micelles were also revealed to be quite stable through time. Thus, neither sign of precipitation nor cluster formation was observed after five months at 4 °C in both H_2O and PBS pH 7.4. Similarly, dilution of the original PIC solution (0.98 mg mL^{-1} in H_2O) down to 0.2 mg mL^{-1} did not result on any effect on the size. Further proof of their stability was obtained after freeze drying, the method of choice to gain stability and suitable shelf-life to labile therapeutic systems. Thus, resuspended micelles showed only a small reduction in size (to *ca.* 20 nm) by DLS, with no effect on dispersity.

Visualization of the micelles was possible by AFM, revealing a spherical shape and a mean diameter of 28 ± 4 nm, in complete agreement with DLS measurements (Fig. 3c).

The core–shell architecture of these PIC micelles, with a segregated core (network of polyions) surrounded by a palisade of flexible and hydrophilic PEG, was confirmed by ^1H NMR (Fig. 2). Thus, the dendritic block signals in the micelles showed only an intensity of about 20% [normalized to the terminal methoxy (PEG) signal] when compared to the free block copolymer, suggesting more dense packing and decreased solvation within the core. The steric stabilization imparted by the PEG corona, along with the size and narrow distribution of the PIC micelles, should result in longer circulation times, improved biocompatibility (stealth property), and enhanced ability to extravasate into the disease sites.

In conclusion, click chemistry has been revealed as a straightforward technology for the preparation of anionic dendritic polymers, potential mimetics of GAGs. Supramolecular assembly of anionic PEG–dendritic block copolymers with PLL led to spherical PIC micelles of *ca.* 25 nm, low dispersity, and remarkable stability against freeze drying, dilution, and ionic strength. These micelles are envisioned as attractive delivery systems of low molecular weight drugs, proteins, nucleic acids, and imaging agents.

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