Copper-catalyzed Click Chemistry for polyether-based dendronized multi-amphiphilic polymers and their applications

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ABSTRACT

Click chemistry and bio-orthogonal chemistry stands out as dynamic tools for chemists and biologists to snap together chemical entities as well as chemical and biological building blocks with just a click to construct a variety of intricate molecular structures with ease, rapidity, and selectivity. The ground-breaking work that contributed to developing click chemistry and bio-orthogonal chemistry has been acclaimed for its biocompatibility in biological environments, high reaction rates, and simplicity. Much interest has been shown in these reactions across several disciplines, but especially in the creation of DDS and pharmaceuticals. The utilization of "click" functionalization of enzymatically synthesized polyether-based polymeric scaffolds to generate dendronized polymers and their applications in biomedicine are summarized in this mini-review article. This compilation outlines the key techniques used to click functionalize linear polymers with dendrons to create amphiphilic denpols, with a focus on drug encapsulation and delivery. Considering the findings reported from 2013 to the present, prospects of polyether-based denpols have been highlighted.

Keywords: Polyether, Novozyme-435, click chemistry, dendronized polymers, nanocarriers
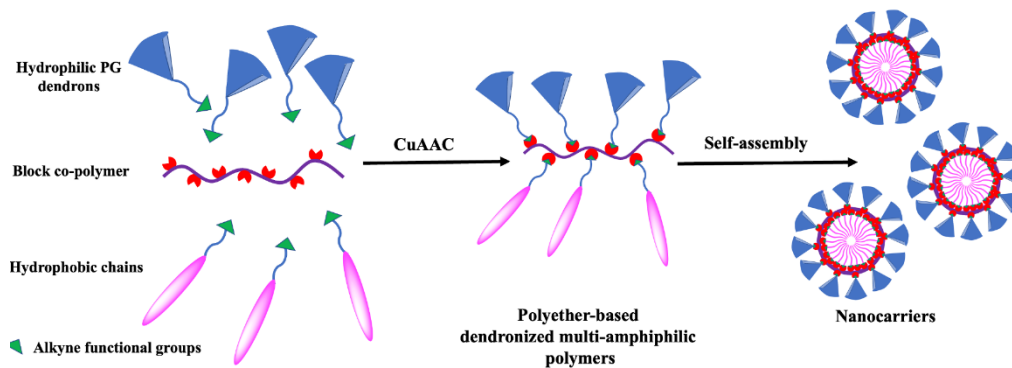
INTRODUCTION

"Click chemistry" was the term first introduced by Nobel laureate K. Barry Sharpless[1] in 1999 at the 217th American Chemical Society annual conference, and it later gained popularity among the scientific community and is still the most researched chemical reaction to date and won the 2022 Nobel prize in Chemistry.[2,3] The criteria set forth by Sharpless for reactions to be classified as "click reactions" include the chemical reaction to be modular, simple to perform, have a wide substrate scope, require mild reaction conditions, high reaction yields, easy isolation/purification of final products (preferably without chromatography), harmless or no by-products

formation, regioselective, stereospecific (may or may not be enantioselective), the ready availability of synthons/reagents and it should be possible to carry out the reaction in solvent-free environments or with solvents that are environmentally benign, like water.[1]

The above criteria for a reaction being considered as a "click reaction" fit perfectly with the CuAAC reaction. The prominent name for CuAAC thus became "Click chemistry", and it is one of the most efficient, adaptable, dependable, and modular methods for the quick and regioselective preparation of 1,4-disubstituted derivatives of 1,2,3-triazole molecules. This synthetic protocol involves the addition of organic azides to terminal alkynes and is catalyzed by copper(I).[4]

With its beginning in 1893, Michael described the first 1,3-dipolar cycloaddition reaction that results in 1,2,3-triazoles by reacting organic azides with terminal alkynes.[5] Huisgen conducted extensive additional research on the thermal 1,3-dipolar AAC reaction in the 1960s and found that it required high-temperatures of around 110ºC, had slow reaction kinetics, required long reaction times and was non-regioselective giving 1,4- and 1,5-regio-isomers in equal proportions as the end products (Scheme 1).[6–8]
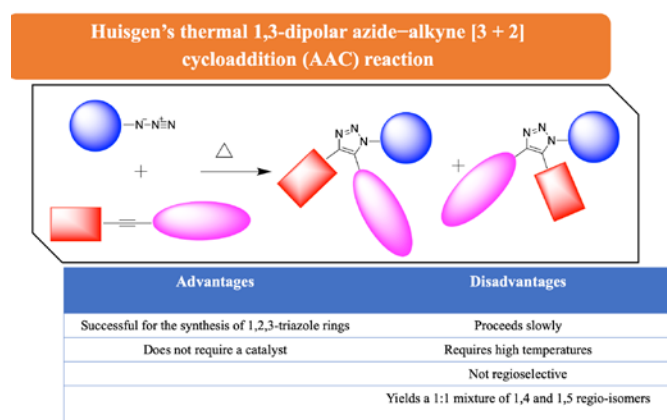
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Scheme 1. Huisgen's thermal AAC reaction and its merits and demerits.

The modified form of this reaction, which was conducted at room temperature using a copper catalyst, was separately and independently reported by Sharpless[9] and Meldal[10] in 2002. It was found that using a Cu(I) catalyst causes the reaction to exclusively produce the 1,4-regioisomer, significantly speeds up the reaction rate (second-order rate constant, k = 10-100 $M^{-1}s^{-1}$ with 20 μM Cu(I)) and removes the need for high temperatures (Scheme 2). The Cu(I) catalyst reduces the activation energy barrier for the generation of the triazole ring, leading to dramatically improved kinetics and a high second-order rate constant.[9–12] Many procedures have been established to use Cu as a catalyst for CuAAC.[13] The three main strategies include either the use of Cu(I) salts directly;[14] or oxidation of Cu(0);[15] or conversion of Cu(II) salts into the active catalyst by the addition of reducing agents such as sodium ascorbate etc.[11]
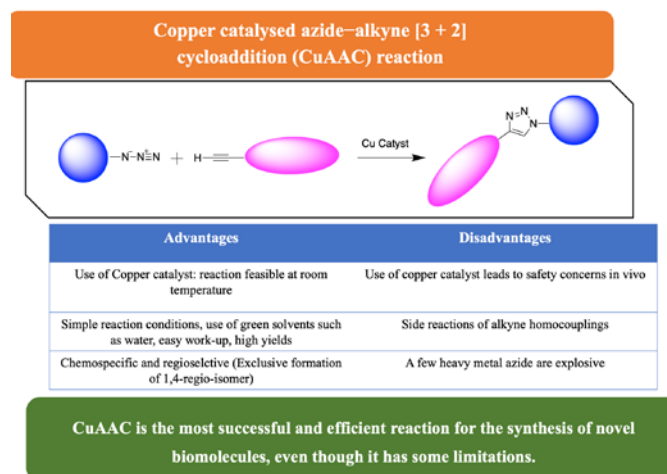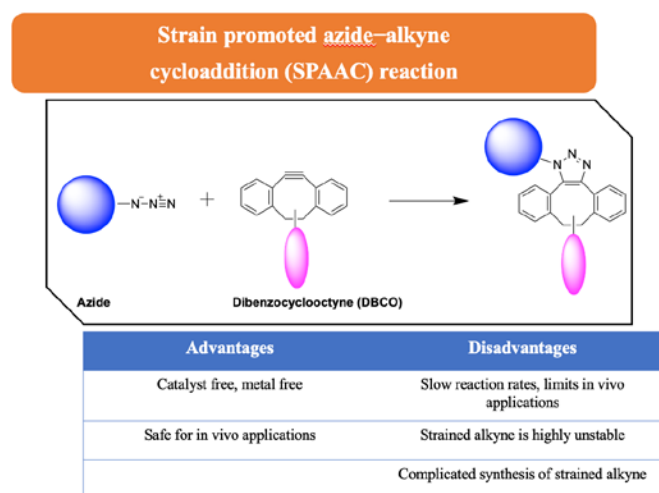

Scheme 2. CuAAC reaction and its merits and demerits.

The reaction thus can be performed with readily available azides and terminal alkynes, is simple and modular way, has a broad substrate scope, provides very good yields, gives no by-products, is highly regioselective, stereospecific and feasible in environmentally benign solvents like water under mild reaction conditions. However, the inclusion of a cytotoxic copper catalyst raised some issues with this adaptable reaction, limiting its utility *in vitro* and *in vivo* applications. Despite this, it has been described as the most effective and successful "click reaction" to date, and it has been discussed in several recently published review articles.[4,12,16–18]

Further research to eliminate the use of cytotoxic metal catalysts led to the development of SPAAC reactions, where the alkynes were used as a part of strained ring systems such as cyclooctyne (Scheme 3).[19] However, the reaction kinetics was found to be much slower than the CuAAC, which led to the generation of modified strained alkynes such as cyclooctyne bearing fluorine atoms,[20] incorporation of sp^2 hybridized carbons in cyclooctyne rings,[21] cyclopropane rings fused to cyclooctyne,[22] and other strained systems such as DIFO,[20] BARAC,[23] TMTH[24] etc. Although the metal-free click reaction with moderate second-order reaction rates (1-60 $M^{-1}s^{-1}$) has been achieved by SPAAC, still a serious limitation arises from the use of large strained ring structures of alkynes, which are not just difficult to synthesize but also curtails its use in different synthetic applications, especially in polymer chemistry.[25] However, SPAAC has gained a lot of attention as the metal-free alternative to copper-catalyzed click reactions and offers a variety of applications.[26]

Scheme 3. SPAAC reaction and its merits and demerits.

Although further discussion is beyond the purview of this mini-review, recently published articles[27–29] and reviews[4,16–18,26,30–36] highlight the numerous additional alterations and adaptability of click chemistry in detail.

Polyether-based scaffolds

Polyether-based scaffolds have drawn a lot of interest among the numerous drug-delivery systems investigated.[37–42] Due to their great biocompatibility, hydrophilic polyether-based polymers are frequently utilised to improve the pharmacokinetic characteristics of drug carriers. PEG is regarded as the benchmark standard for drug delivery and has received FDA approval for a variety of pharmaceuticals.[38,39] It has prospective uses in the biomedical and pharmaceutical industries due to its unique qualities, which include chemical non-reactivity, solubility in aqueous media, well-established safety profile, reduced contact with blood, and biocompatiblilty.[40]

Additionally, polyols with an aliphatic dendritic polyether scaffold, with the possibility of multiple groups on the architectural periphery for functionalization, and a closely-packed, perfectly structured tree-like dendritic structure have grown in significance.[40–45] For example, OG and PG have outstanding characteristics such as numerous hydrophilic end-groups, and flexible and highly biocompatible polyether architecture.[41,42,45] These substances have the ability to alter the therapeutic efficacy of the active molecules. Numerous studies have been reported using these versatile polyether scaffolds for drug-delivery applications using click chemistry as an indispensable tool for the attachment of drugs, dyes and molecular probes.[40–45]

Dendronized Polymers

Block copolymers' design and synthesis have drawn a great deal of interest since they serve as the framework for materials that can be used for a variety of purposes.[46] Dendronized polymers (more commonly known as denpols), which have been the focus of intensive research over the past two decades, were introduced as a new branch in the field of block copolymer synthesis, primarily for their applications at nanoscale levels.[47,48] Denpols are highly intriguing because of the intrinsic topological differences between the linear and dendritic segments and the adjustable amphiphilicity that allows them to self-assemble into nanostructures with unusual morphologies.[49] Their distinctive framework is advantageous over spherically shaped dendrimers in particular for nanoscale applications where the classical dendrimers' spherical shape may make them inappropriate for use.[50,51] Similar in nature (but not in architecture) to the spherically shaped dendrimers, these denpols can be customized appropriately with precise structure, different molecular dimensions and shapes, rigidity at a higher degree of dendronization,[52–55] plus having multiple options for surface functionalization while retaining the properties of the linear backbone.[50,51,56,57] In view of these characteristics they may adopt cylindrical shapes[58] and thus have appropriately been referred to as "molecular cylinders".[47]

There are three primary methods for the generation of denpols as depicted in Figure 1. Method 1 involves the attachment of a preconstructed dendron to a polymer backbone that has suspended functional groups using a "graft to" technique (Figure 1a).[59–62] Convergent synthesis makes this technique appealing, however full coverage of the backbone employing high-generation dendrons (G3 or larger) is a difficult task.[63] Method 2 is a "graft from" approach (Figure 1b), which proceeds from the polymer backbone through a step-growth process.[64] Although the possibility of structural flaws makes this pathway seem difficult, it still offers a technique to attain the highest level of dendronization.[65] Recently, Fytas and Choi *et al*., reported the successful synthesis of up to sixth-generation denpols *via* this method using ROMP.[66] Dendrons are incorporated into the monomer in Method III, the "macromonomer" strategy (Figure 1c). The polymer's repeat units all have flawless pre-attached pendant dendrons, which is advantageous, and the highest dendronization is possible with

this method.[67–69] The drawback with this synthetic strategy is that it typically only achieves low degrees of polymerization due to high steric strain.[70] These macromonomers can only be polymerized using standard homopolymerization techniques such as step growth[71] or radical chain growth mechanisms.[68,72] To achieve this, a variety of techniques have been used, including radical,[73] living cationic,[74,75] ATRP,[76] ROMP,[77,78] Suzuki,[79] and Heck coupling.[80]
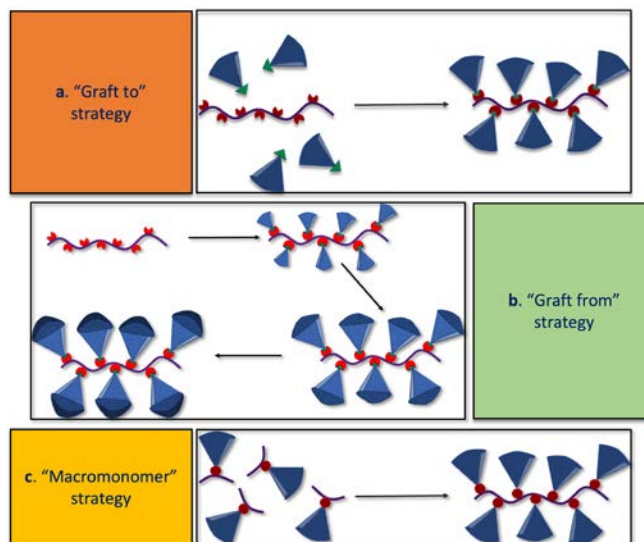

Figure 1. Different strategies for the generation of denpols; **a**) the "Graft to" approach, **b**) the "Graft from" approach and **c**) the "Macromonomer" approach.

Not just chemical synthesis but the biocatalytic "green" approach is an alternative for producing multifunctional polymers with fine composition and an established structure, polymerization employing enzymes as biocatalysts have gained popularity.[81–85] Lipases (hydrolytic enzymes), one of the several classes of known enzymes, have garnered a great deal of attention lately. Lipases (EC 3.1.1.3) are omnipresent in most living species. The synthesis or cleavage of ester linkages in triacylglycerols is a natural function of lipases. They are the most adaptable group of enzymes due to their attributes such as steadiness in organic solvents, high catalytic performance absent cofactors, wide specificity of the substances on which it acts, and excellent chemo-, regio-, and stereo-selectivity.[81,86,87] Recent reviews have highlighted the application of lipases in the synthetic transformation of small molecules as well as the development of polymeric systems for drug delivery applications.[81–88]

Considering the significance of polyether scaffolds and amphiphiles based on polyethers for DDS, this mini-review emphasises the versatility of the CuAAC as an effective method for the functionalization of enzymatically synthesised linear polyether-based copolymers to generate a variety of oligo glycerol-dendronized multi-amphiphilic polymers by "graft to" method.

CuAAC for the synthesis of Polyether-based Dendronized Multi-amphiphilic Polymers

N435 catalyzed block copolymers

Parmar and co-workers explored the field of lipase-catalyzed synthesis of PEG-based macromolecular systems utilizing pharmaceutically notable small molecules like polyphenols, amides, nucleosides, etc., and their uses as carriers for drugs and genes.[81] On the other hand, Haag and colleagues investigated dendritic PGs for biological and pharmaceutical applications.[41,43,44,89]

Further work by Sharma and co-workers explored a new area utilizing lipases for catalytic step-growth condensation polymerization of glycerol/glycerol derivatives (azido-glycerol/azido-tri-glycerol) and PEG-based linear block polymers. With the advancement in these block copolymers, dendronization with oligo glycerol (polyether dendrons) was carried out using highly efficient CuAAC reactions and different series of denpols were generated, which are going to be discussed in the following sections.

N435 catalyzed PEG$_{600}$-*co*-glycerol polymers (Block copolymer 1)

Regioselective synthesis of PEG$_{600}$-*co*-glycerol block copolymer was carried out by N435 (an immobilized enzyme) catalyzed transesterification polymerization of PEG$_{600}$bis(carboxymethyl)ether dimethyl ester with primary hydroxyl groups of glycerol (Scheme 4).[90]
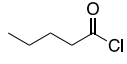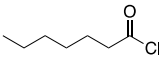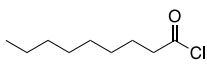
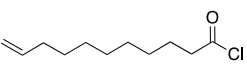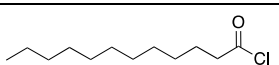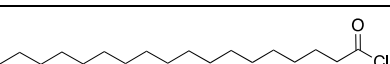**Acylation with C-5, C-7 and C-9 acyl chains**

The post-functionalization of the linear copolymer was done with hydrophobic acyl chains of different chain lengths i.e. C-5, C-7 and C-9 acyl chlorides (Table 1) *via* an acylation process onto the pendant secondary hydroxyl groups of glycerol moieties (Scheme 4, Table 5, entry 1.1).[90] In aqueous media, the polymers created amphiphilic micellar aggregates, as observed by SLS techniques. The nanocarriers were further assessed for encapsulation of the hydrophobic drug vitamin E.[90]
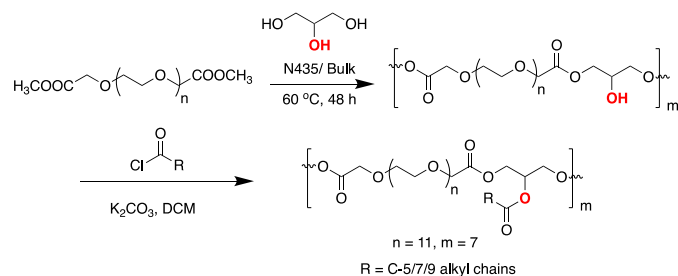
Scheme 4. Synthesis of PEG600-co-glycerol polymers and their acylation with different acyl chlorides (Table 1) to generate polymeric nanocarriers.[90]

This synthetic strategy created nano-architectures with fine-tuneable hydrophobic segments, however, the limitation of the pendant hydroxyl group is that complete functionalization is difficult to achieve and further fine-tuning of the hydrophobic-hydrophilic balance by attaching hydrophilic groups like OG/PG dendrons may have limited attachments through traditional chemical coupling methods.

Table 1. Hydrophobic acyl chlorides used for acylation in **Schemes 4** and **6**.

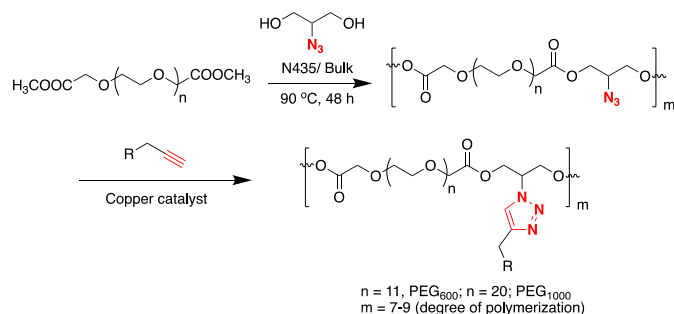| Hydrophobic R' groups |
|---|
| |
| C-5 acyl chloride (C$_4$H$_9$COCl) |
| |
| C-7 acyl chloride (C$_6$H$_{13}$COCl) |
| |
| C-9 acyl chloride (C$_8$H$_{17}$COCl) |
| |
| C-11 terminal alkenoyl chloride (C$_{10}$H$_{19}$COCl) |
| |
| C-12 acyl chloride (C$_{11}$H$_{23}$COCl) |
| |
| C-18 acyl chloride (C$_{17}$H$_{35}$COCl) |

N435 catalyzed PEG$_{600}$-*co*-azido-glycerol polymers (Block copolymer 2)

Exploring the chemistry for efficient functionalization and bio-orthogonal approach, Sharma and co-workers developed a novel class of polyether-based denpols by collaborating with Haag and colleagues to effectively functionalize these polymeric systems utilizing OG/PG dendrons as side chains *via* highly efficient CuAAC using the "graft to" strategy (Figure 1a). A new class of multi-amphiphilic polyether-based denpols[91] were designed by selectively functionalizing glycerol to azido-glycerol by N435 catalyzed reaction (protection of primary hydroxyl groups of glycerol, followed by azidation and deprotection of hydroxyl groups) and then transesterification polymerization of PEG$_{600}$bis(carboxymethyl)ether dimethyl ester with azido-glycerol's hydroxyl groups by the previously reported protocol[90] (Scheme 5).

Click functionalization with OG dendrons ([G1.0] and [G2.0]) and hydrophobic chains (C-18)

The PEG$_{600}$-*co*-azido-glycerol polymers (Scheme 5) enabled efficient CuAAC reactions *via* pendant azide functionality on the copolymer backbone with alkyne functionalized C-18 hydrophobic chains (Table 2) and [G1.0] and [G2.0]-OG dendrons as hydrophilic side chain appendages (Table 3) in different ratios to fine-tune the hydrophobic-hydrophilic balance (Table 5, entry 2.1).[91]

In aqueous media, the rearrangement of the exterior surface with hydrophilic dendrons and the interior core with hydrophobic alkyl chains created micelles (Figure 2) with

Scheme 5. Synthesis of PEG-co-azido-glycerol polymers and their click functionalization with various hydrophobic (**Table 2**) and/or hydrophilic R groups (**Table 3**) to generate non-dendronized / dendronized polymers.
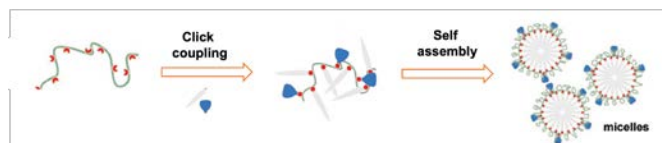

Figure 2. Schematic representation of (PEG$_{600}$-*co*-azido glycerol) polymers synthesis and their functionalization to self-assembled nano-micellar architectures. Reprinted from reference [91] copyright (2013) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.
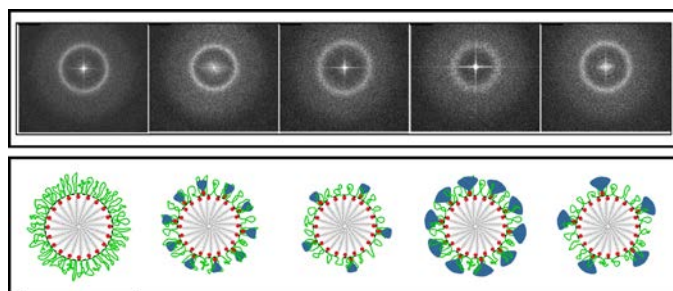

Figure 3. Comparison of the five FT of cyro-TEM images with five graphical models for the non-dendronized (1st) and the dendronized multi-amphiphilic polymers' (2nd – 5th) spatial micellar structure. The addendum proportions and the dendron generations influence the micellar dimensions and exterior patterns. Reprinted from reference [91]. Copyright (2013) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

diameters in the nanometer range and CAC values in the micromolar range (Table 5, entry 2.1). The FT of cryo-TEM images observed experimentally were compared with models created by graphical modelling (Figure 3). Both depict the effect of dendron generation and addendum proportion (both hydrophilic and hydrophobic) on the micellar dimensions and exterior patterns (Figure 3).[91]

In addition to assisting in preserving the amphiphilicity of micellar structures, OG dendrons also improved the robustness, biocompatibility, and transport capacity of the newly synthesized micellar denpols. These micelles' potential to serve as nanocarriers for drug transport systems was demonstrated with hydrophobic pyrene as a guest molecule and binding investigations with fluorescent dye 1-anilinonaphthalene-8-sulfonic acid (ANS) utilizing spectrofluorometric techniques revealed unimolecular binding properties in the micelle's hydrophobic domain, indicating that the guest molecules are primarily found inside the aggregates' hydrophobic cores.[91] The

higher guest solubilization efficiency, increased biocompatibility using polyether-based PEG and OGs and reduced exchange rates due to covalently linked amphiphiles as compared to unimolecular amphiphiles encouraged further research for more stable architectures to acquire controlled drug release capabilities by fine-tuning the structure of the denpols.

Table 2. Hydrophobic R groups used for click functionalization in Schemes 5 and 6.

Hydrophobic R groups
C-18 (~OC$_{18}$H$_{37}$)
C-14 (~C$_{11}$H$_{23}$)
C-12 (~C$_9$H$_{19}$)
C-8 (~OC$_8$H$_{17}$)
Fluoro-C-8 (~OC$_8$H$_4$F$_{13}$)
Azobenzene (~OC$_{16}$H$_{17}$N$_2$)

N435 catalyzed PEG$_{1000}$-*co*-azido-glycerol polymers (Block copolymer 3)

Kumari *et al*., later explored the influence of PEG chain length on supramolecular self-assembly and the physiochemical characteristics of this novel class of denpols by substituting a longer PEG$_{1000}$ chain for PEG$_{600}$ (Scheme 5).[92]

Click functionalization with OG dendrons ([G1.0] and [G2.0]) and hydrophobic chains (C-12 and C-14)

The hydrophobic tails (C-12 and C-14, Table 2) and the hydrophilic dendrons ([G1.0] and [G2.0]-OG, Table 3) were subsequently appended to the linear block PEG$_{1000}$-*co*-azido-glycerol polymers using an effective copper-catalyzed "Click chemistry" (Scheme 5, Table 5, entry 3.1).[92]

The resulting amphiphilic denpols in an aqueous solution self-assembled to generate distinct micelles. To study the transport behaviour of these polymers, Nile red was used as a fluorescent model dye. A few exemplary polymers' cytotoxicity profiles and biocompatibility were investigated in connection with the attachment of hydrophilic oligo glycerol dendrons and the length of hydrophobic alkyl chains. In comparison to their C-12 counterparts, the C-14 alkyl chain-attached non-dendronized polymers exhibited a better transport capacity.

Table 3. Hydrophilic R groups used for click functionalization in Scheme 5 and 6.

Hydrophilic R groups

[G1.0]-OG

[G2.0]-OG

hPG
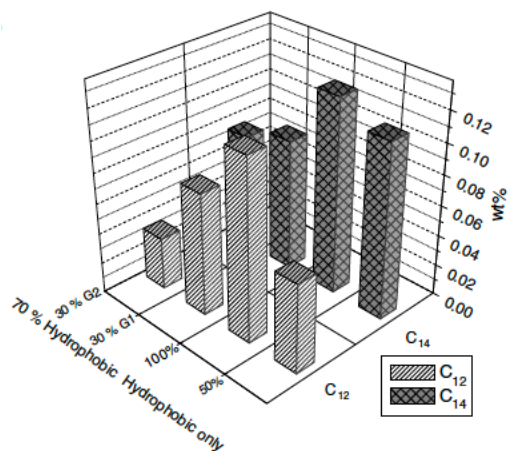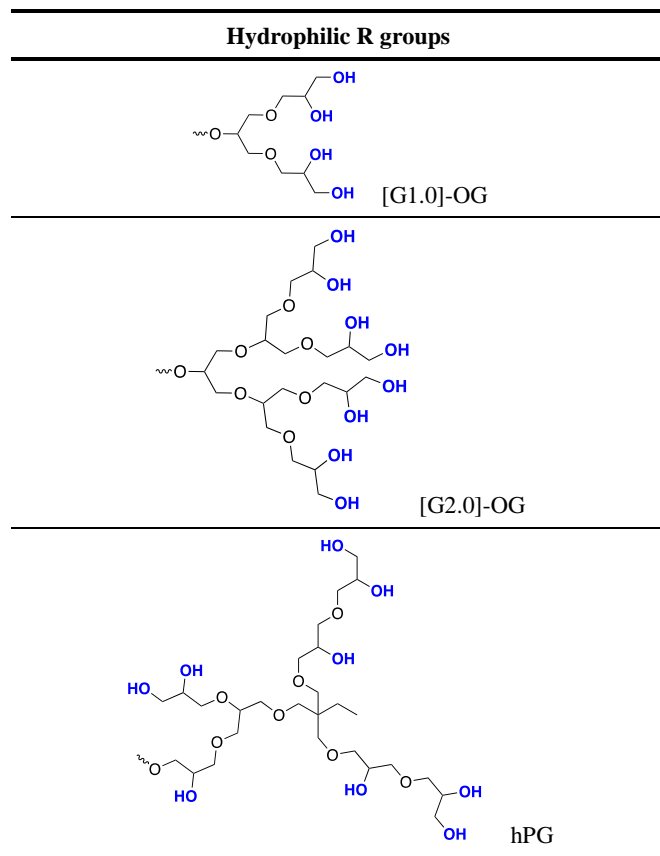
Figure 4. The transport capacity of Nile red encapsulated non-dendronized/dendronized polymers. Reproduced from reference [92]. Copyright 2014 John Wiley & Sons, Ltd.

Besides that, the transport capacity was reduced when OG dendrons were grafted to create denpols (Figure 4), however, the biocompatibility of the denpols was found to be better than the non-dendronized counterparts. [92] This study moved a step ahead in the search for fine-tuning the amphiphilic architecture and obtaining more biocompatible denpols.

Click functionalization with OG/PG dendrons ([G1.0], [G2.0] and hPG) and hydrophobic chains (C-18)

In a similar set of nano-micellar denpols with PEG_{1000} as monomers, an extra hPG (Table 3) as a hydrophilic addendum

via "click chemistry" was also explored for their transport behaviour and other properties (Table 5, entry 3.2). [93] The transport efficiency of the Nile red encapsulated non-dendronized, as well as dendronized polymers, were evaluated, and found to be maximum (4.60 mg/g) for 50% C-18 and 50% [G2.0]-OG grafted denpol, followed by the 70-50% C-18 and 30-50% hPG grafted denpols (2.73, 2.71 mg/g respectively). [93] Photographs of QGP-1 cells treated with denpols encapsulating the dye Nile red (Figure 5a), showed improved dye uptake using FACS analysis and cellular fluorescence microscopy compared to (i) dye-loaded PEG_{600} counterpart denpols (Figure 5b), (ii) [G2.0]-OG dendron - C-18 alkyl chain 'clicked' amphiphile (Figure 5c and 6) and (iii) free dye Nile red (Figure 5d). [93]
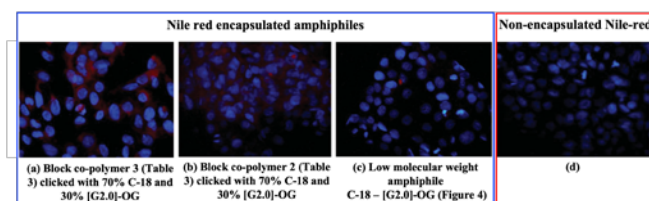
Figure 5. Fluorescence pictures of QGP-1 cells with red coloured Nile red and blue-stained DAPI nuclei for (**a**) PEG_{1000} and (**b**) PEG_{600}-based denpols, (**c**) C-18 – [G2.0]-OG amphiphile and (**d**) non-encapsulated Nile red (blank cells). Reproduced from reference [93]. Copyright 2015 Wiley-VCH Verlag GmbH & Co. KGaA.

Furthermore, even at concentrations as high as 500 (μg/mL), these micelles exhibited little cytotoxicity after 72 hours. The improved intracellular stability of supramolecular self-assembled denpols could explain why polymeric amphiphiles have a better transport potential than small amphiphiles (Figure 6).
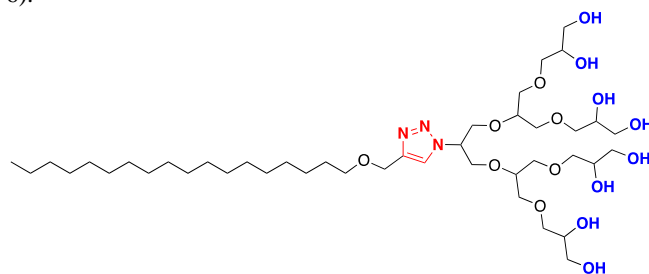
Figure 6. C-18 – [G2.0]-OG 'clicked' amphiphile.

The work however concluded the need for longer alkyl chains for the effective encapsulation of hydrophobic pharmaceuticals and the importance of maintaining a balance between hydrophilic and hydrophobic scaffolds to preserve biocompatibility. [93] With the purpose of creating the best possible drug/dye delivery systems, more modifications were needed to better understand the encapsulation phenomena.

Click functionalization with OG dendrons ([G2.0]) and hydrophobic chains (azobenzenes)

The photoresponsive behaviour of azobenzene moiety is well known in the literature as an essential component for the development and breakdown of supramolecular structures. [94–97] Also click chemistry has been a mainstay option for the conjugation of azobenzene within the polymer's main chain [95] or

grafted to the polymers as side appendages.[96] Yet, due to their distinct characteristics, polymers with pendant azobenzenes have garnered much consideration,[97] as opposed to the polymers containing azobenzenes in the main chain. Such polymers have the added benefit of exhibiting photoresponsivity even at lower azobenzene proportions as the block copolymers' segregation capacity improves because of nanoscale interactions between different azobenzene moieties, which produce well-defined structures. To introduce this photoresponsive behaviour in the amphiphilic nano-carriers, Kumari *et al*., generated photoresponsive non-dendronized polymers as well as denpols (Scheme 5) by grafting azobenzenes (Table 2) and/or [G2.0]-OG dendrons (Table 3) on PEG$_{1000}$-*co*-azido-glycerol base copolymer using "click" functionalization (Table 5, entry 3.3).[98] In the aqueous solution, supramolecular aggregation behaviour was displayed by both non-dendronized as well as dendronized polymers with azobenzene in the side chain as observed *via* DLS and cryo-TEM measurements (Figure 7).

Curcumin and Nile red were used to investigate the encapsulation capability of polymeric systems. Curcumin encapsulation was studied qualitatively, due to the superimposition of the curcumin and polymers' absorption spectra, while Nile red at a concentration of 0.4 mM per mg per ml of the polymer's solutions was quantitatively investigated. When compared to the non-dendronized analogue (70% azobenzene grafting only), the dendronized polymer (70% azobenzene and 30% [G2.0]-OG grafting) demonstrates superior transport potential with encapsulation efficiencies of 1.45 and 1.87%, respectively. When compared to earlier synthesized similar denpols with C-12 and C-14 alkyl chains and [G2.0]-OG grafting (Table 5, entry 3.1), the transport efficiency of these azobenzene grafted denpols was found to be 7 and 3 times significantly higher, respectively.[92] Plausible explanation for azobenzene functionalized denpols' higher encapsulation potential could be explained by the interactions between the π-electron cloud of azobenzene's aromatic rings with the ones in Nile red, both inside the hydrophobic nano-micellar core.

UV-vis spectroscopy was used to investigate the photo-isomerization of the polymers. The release profile of the polymers encapsulating Nile red was affected by isomerization. It was observed that photoisomerization (*cis-trans* and *trans-cis*) takes place at a faster pace at concentrations below CAC than at concentrations above CAC. Azobenzene-grafted non-dendronized polymers showed a higher discharge of Nile red as compared to the azobenzene and [G2.0]-OG grafted denpols, demonstrating the denpols ability to form stable micelles. The intensity of Nile red fluorescence decreased because of the *trans-cis* isomerization, while vice-versa was observed for *cis-trans* isomerization. On one entire *trans-cis-trans* photo-isomerization cycle, the intensity of Nile red fluorescence could not be fully recovered, indicating some net dye release. These results suggest that the azobenzene moieties mediate the photo-responsive release and that [G2.0]-OG dendrons increase the stability of the formed micelles and their potential for encapsulation.
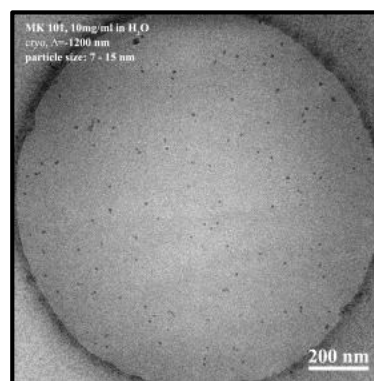

Figure 7. Spherical micelles with diameters ranging from 7 to 15 nm displayed by cryo-TEM measurement of the 70% azobenzene-grafted non-dendronized polymers. Reproduced from reference [98]. Copyright 2015 Royal Society of Chemistry.
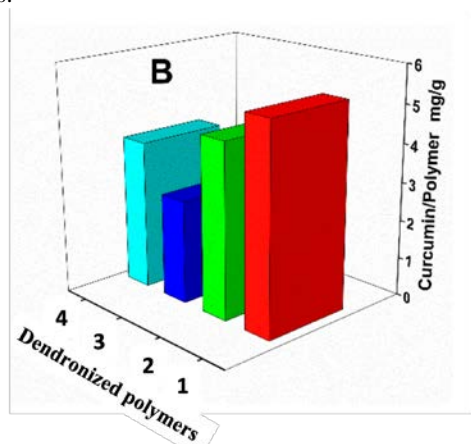
Click functionalization with OG dendrons ([G2.0]) and hydrophobic chains (C-8 and fluoro C-8)

In another study by Parshad *et al*., hydrophobic fluorinated alkyl tails were used to further modify the hydrophobic-hydrophilic balance of denpols (Scheme 5). It is well known that compared to the electron cloud surrounding hydrogen in alkyl chains, the electron cloud surrounding fluorine atoms in fluoroalkyl analogues is relatively more tightly held, and due to that the polarization is much reduced, thus replacing the alkyl tails with their fluorinated versions leads to diminished unwanted interactions among the pendant hydrophobic chains.[99,100] Further, the higher surface area of the fluorine atoms than the hydrogen atoms increases the overall hydrophobic character of perfluoroalkyl chains,[101,102] and would thus enable the formation of stiffer and neatly packed stable micellar aggregates with CMC values in micromolar range, suggesting that they would be more effective for applications in drug delivery.

Keeping in view these facts, PEG$_{1000}$-*co*-azido-glycerol (Scheme 5) was appended with hydrophobic fluoro C-8 chains or C-8 alkyl chains (Table 2) and hydrophilic [G2.0]-OG dendrons (Table 3) using highly efficient copper-catalyzed click chemistry (Table 5, entry 3.4) to generate a new series of denpols.[103] Using the hydrophobic drugs curcumin and dexamethasone, the resultant polymers were then evaluated for their self-assembly behaviour, the extent of hydrophobic drug solubilization, transport potential, and drug release profiles.

DLS and *cyro*-TEM measurements revealed smaller micellar dimensions (8-10 nm) for the perfluoroalkyl chain appendaged denpols compared to larger micelles (100-150 nm) formed by the alkyl grafted denpols. The transport potential of denpols was quantified by studying the absorption spectra of the denpols encapsulating curcumin (Figure 8). Denpols with fluoro C-8 chain and [G2.0]-OG dendron (denpol 1- 70% fluoro C-8 and 30% [G2.0]-OG grafting and denpol 2- 50% fluoro C-8 and 50% [G2.0]-OG grafting) had the highest level of curcumin encapsulation, with transport efficiencies of 5.3 and 4.5 mg drug/g denpol, respectively. However, denpols 3 and 4 (C-8 alkyl analogues of denpols 1 and 2), had quantitatively less drug transport efficiencies, respectively (Figure 8). A similar trend

was observed for the encapsulation of the highly hydrophobic fluorinated steroidal drug dexamethasone. Perfluoro-grafted denpols exhibited greater transport efficiencies for this drug as well, which may be explained by their stable multi-amphiphilic micellar structures, which are the result of the orderly packing of perfluoroalkyl chains in the hydrophobic core of the micelles.[103]

Figure 8. Transport efficiency of perfluoro alkyl chain grafted denpols compared to alkyl chain grafted denpols for hydrophobic drug curcumin in mg/g. Reproduced from reference [103]. CC4.0.

Dendronized polymers	Hydrophobic content	Hydrophilic content
1	70% fluoro C-8 chain	30 % [G2.0]-OG
2	50% fluoro C-8 chain	50 % [G2.0]-OG
3	70% C-8 alkyl chain	30 % [G2.0]-OG
4	50% C-8 alkyl chain	50 % [G2.0]-OG

Along with drug encapsulation, the enzyme-triggered controlled drug release also plays a crucial role to maintain the therapeutic concentrations under physiological conditions. Thus, N435-mediated release of the hydrophobic drug curcumin was investigated by incubating the drug-encapsulated nanocarriers with the enzymes in the dark. Figure 9 depicts a proposed drug release from amphiphilic polymers.[103] In order to conduct a comparative release study, N435 (200 wt%) was added to the curcumin-encapsulated denpols solution (fluorinated and alkyl analogues). Both fluorinated and non-fluorinated denpols showed up to 90% curcumin release triggered by the enzyme within 12 days; however, fluorinated denpols showed a faster release, which was presumed to be due to their enhanced fit with the enzymes' hydrophobic active site. Perfluorinated denpols also stabilised curcumin more effectively, as shown by the fluorescence intensity, which was retained even in the absence of an enzyme. Additionally, HeLa cells exposed to the perfluorinated denpols up to a concentration of 100 g/mL for 72 hours showed almost no toxicity. The study concluded that the efficient drug release under physiological conditions and good encapsulation potential of these denpols show their potential to function as efficient DDS.[103]
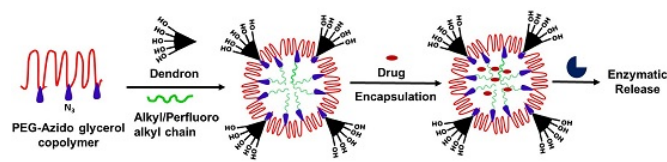

Figure 9. N435-mediated controlled release of hydrophobic drugs (red coloured) encapsulated in the denpols synthesized by click functionalization of PEG$_{1000}$-*co*-azido glycerol base polymers with alkyl/fluoroalkyl chains and [G2.0]-OG dendrons. Reproduced from reference [103]. CC4.0.

N435 catalyzed PEG$_{600}$-*co*-azido-tri-glycerol polymers (Block copolymer 4)

A series of supramolecular architectures with altered hydrophobic-hydrophilic balances were produced with the development in the synthesis of polyether-based PEG-*co*-azido-glycerol "clicked" denpols. These structures had high drug encapsulating efficiency and transport potentials, but to further fine-tune the supramolecular architecture another class of denpols were created by altering the base copolymer and adding azido-tri-glycerol as one of the monomers in the ongoing hunt for more precisely tuned structures. Azido-tri-glycerol is a versatile synthon which can be synthesized by several different methods reported in the literature.[104–107] This monomer offers different functional groups (primary hydroxyl, secondary hydroxyl, and azide functional groups) for orthogonal chemistry.

Acylation with C-12 hydrophobic chains and orthogonal click functionalization with OG dendrons ([G1.0 and [G2.0]) and hydrophobic chains (C-12)

Kumar *et al*., bio-catalytically differentiated the primary and secondary hydroxyl groups of azido-tri-glycerol monomer and utilized the former for polymerization with PEG$_{600}$ diethyl ester generating PEG$_{600}$-*co*-azido-tri-glycerol polymers having orthogonal functionalities (secondary hydroxyl and azide functional groups) for post-functionalization to generate advanced polyether-based denpols (Scheme 6).[108] The backbone's secondary hydroxyl groups were available as an acylation site to refine the physicochemical features by attaching hydrophobic C-12 acyl chains (Table 1) as reported in the earlier work,[90] whereas by using the click-functionalization, the C-12 alkyl chains (Table 2) or [G1.0] and [G2.0]-OG dendrons (Table 3) were further grafted onto the copolymer *via* the azide group to generate the new generation of non-dendronized/dendronized polymers (Scheme 6, Table 5, entry 4.1).

The transport capacity was evaluated using hydrophilic Cy3, a dye which emits strong fluorescence in the visible spectrum (540–570 nm), and is known to aggregate in aqueous solutions.[109,110] The dye was encapsulated in polymeric nanocarriers and subsequently evaluated for transport potential using UV absorption spectra and fluorescence emission spectra, which indicated a decreasing trend in transport potential with increasing hydrophilic content i.e. with an increase in OG grafting from [G1.0] to [G2.0]. The non-dendronized exclusively alkyl-grafted polymer had better transport capacity, while the acylated polymer without any click functionalization

i.e. with free pendant azide groups gave the best results with the highest transport capacity (19.15 mmol/mol). These results indicate that an appropriate hydrophobic core is essential for guest encapsulation depending on the nature of the guest molecule to achieve maximum loading.[108]
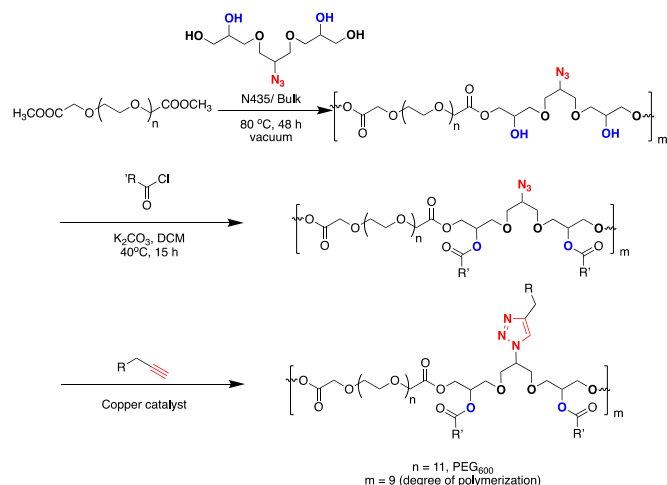
Scheme 6. Synthesis of PEG-*co*-azido-tri-glycerol polymers and their acylation and click functionalization with various hydrophobic (Table 1 (acyl chlorides) and Table 2 (R groups)) and hydrophilic R groups (Table 3) to generate dendronized/non-dendronized polymers.
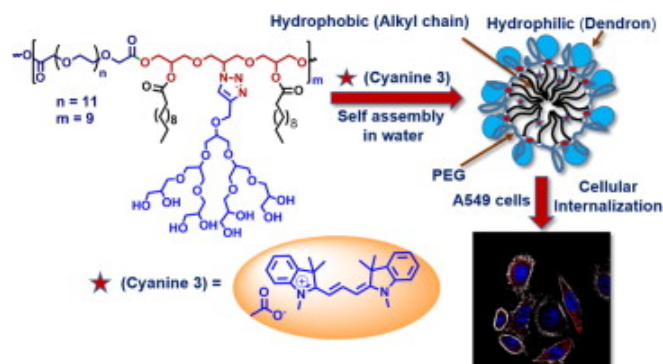

Figure 10. Schematic representation of denpol with C-12 acylation on secondary hydroxyl groups and [G2.0]-OG dendronization on azide group *via* click functionalization on azido-tri-glycerol monomer, Cyanine 3 dye encapsulation and self-assembly in aqueous solution and CLSM images of Cy3 internalization in A549 cells after an incubation period of 4 h. Cy3 loaded polymers appear red, nuclei are stained blue and cytoskeleton white. Reproduced from reference [108]. Copyright 2015 Elsevier.

Subsequently, DLS and cryo-TEM studies were used to analyse the aggregation behaviour and size of the amphiphilic denpols in an aqueous media, with most of the polymers exhibiting stable micelles, except for exclusively C-12-functionalized non-dendronized polymer, which demonstrated the formation of vesicles of various sizes in addition to spherical micelles. The cytotoxicity evaluation of the denpols was conducted using A549 cell lines and dox as a reference drug with and without Cy3 loading. Both kinds were non-toxic upto a concentration of 100 μg/mL, while at 500 μg/mL the denpols irrespective of Cy3 encapsulation were found to be less-toxic

than the partially and fully alkyl grafted non-dendronized polymers, which indicated that a fragile hydrophilic/hydrophobic balance is essential to minimize cytotoxicity. To investigate the internalisation of these synthetic denpols inside the cells, Cy3 was again utilised. According to CLSM and FACS measurements, the [G2.0] grafted denpols demonstrated greater cellular internalisation of Cy3 than the [G1.0] grafted denpols, which demonstrated insignificant uptake (Figure 10). These denpols' micellar size and CAC values have been compared to the analogues previously reported (Table 5, entries 2.1, 3.1 and 3.2).[91–93] This study offered fresh perspectives on the imaging of cancer cell lines using polyether-based denpols.
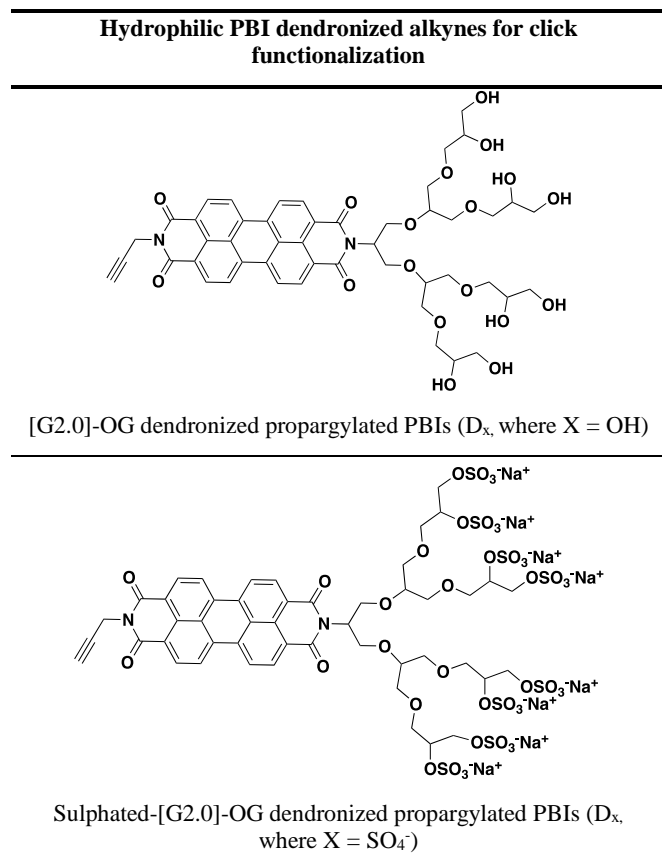
Acylation with C-11 terminal alkene hydrophobic chains and orthogonal click functionalization with OG dendrons ([G1.0 and [G2.0]) and hydrophobic chains (C-12)

In a subsequent study by Kumar *et al*., utilizing the same PEG$_{600}$-*co*-azido-tri-glycerol polymers, the post-polymeric functionalization was carried out with hydrophobic C-11 terminal alkenoyl chloride (Table 1) *via* acylation of secondary hydroxyl group and orthogonally by hydrophilic [G1.0] and [G2.0]-OG dendrons (Table 3) *via* highly efficient click chemistry to generate denpols (Scheme 6).[111] The idea behind the introduction of a terminal double bond in the hydrophobic side chains was to achieve better stabilization of micellar aggregates by π-π interactions among the peripheral double bonds. The same was observed by the reduced CAC values in the micromolar range (Table 5, entry 4.2)[111] of the denpols compared to the previous reports with similar denpols lacking the olefinic bonds in the hydrophobic side chains (Table 5, entry 4.1).[108]

Additionally, the internalisation of Cy3 in these denpols was significantly reduced[111] than in denpol analogues without π-bonds,[108] and the plausible explanation relate to the stable aggregate formation achieved by π-π interactions, and preventing foreign guest molecules from entering easily into the hydrophobic core of the denpols. So, further development of a polymeric system with better capacity for encasing hydrophobic guest molecules and the ability to form stable aggregates was required.[111]

Acylation with C-18 hydrophobic chains and orthogonal click functionalization with dendronized (hydroxylated/sulphated)-[G2.0]-OG PBIs

Bio-imaging studies were further reported by Huth *et al*., by generating denpols (having PBI moieties) complexes with SWCNTs. PBIs (dye **D**) were chosen for this study as they have exceptional fluorescence characteristics, a long emission wavelength, and high photophysical and chemical stability, making them ideal for bio-imaging applications.[112,113] They have been used in a variety of ways, such as fluorescent tags,[114–117] membrane markers,[118,119] or anti-inflammatory drugs.[120] PEG$_{600}$-*co*-azido-tri-glycerol-based block copolymers were functionalized with hydrophobic C-18 acyl chains (Table 1) *via* acylation procedure to generate non-dendronized polymers (**P**$_{alkyl}$) and further functionalized orthogonally *via* efficient click chemistry with [G2.0]-OG dendronized propargylated

Table 4. Different hydrophilic R groups used in Scheme 6 to generate PBI-dye labelled denpols (**P**$_x$, where X = OH/SO$_4^-$)

Hydrophilic PBI dendronized alkynes for click functionalization
[G2.0]-OG dendronized propargylated PBIs (D$_x$, where X = OH)
Sulphated-[G2.0]-OG dendronized propargylated PBIs (D$_x$, where X = SO$_4^-$)

PBIs (**D**$_x$, X = OH, Table 4) or negatively charged sulphated-[G2.0]-OG dendronized propargylated PBIs (**D**$_x$, X = SO$_4^-$, Table 4) to generate denpols (**P**$_x$) (Scheme 6, Table 5, entry 4.3).[121] Each part of this intricate system served certain functions, such as the base copolymer solubilizes the toxic SWCNTs; the polyhydroxy glycerol dendron generates hydrophilicity and inhibits the quenching of the dye; the dendronized PBIs are fluorescent marker; and SWCNTs constitute the fundamental immobilisation framework. Further, a straightforward sulphation method was adopted to generate negatively charged denpols with the aim of further preventing fluorescence quenching agglomeration and enhancing receptor-mediated cellular uptake.[121] Figure 11 schematically depicts the idealized wrapping of the polymers around the SWCNTs in a helical fashion after sonication in aqueous media.

Although SWCNTs have great potential for biomedical applications, still their utilisation is restricted due to their poor biocompatibility and cytotoxicity, which are brought on by their high aspect ratio and surface area.[122–124] The use of commercially available surfactants (SDS/Triton X-100) promotes more decrease in the cytocompatibility of SWCNTs[125,126] and thus, it was anticipated that the wrapping of the denpols around the SWCNTs (Figure 11) would be a better alternative than large amounts of high CMC surfactants. To investigate this, the **P**$_{alkyl}$-wrapped SWCNTs were studied for toxicity studies with HeLa cell lines and the results indicated a considerable increase in cell viability of polymer-wrapped SWCNTs compared to immaculate tubes from 66% to ≥98%, on the other hand, a decrease in viability was observed with surfactant-wrapped tubes by ≤20%. Compared to cells treated with surfactant-solubilized SWCNTs, which exhibit an elevated rate of cell death, cells treated with **P**$_{alkyl}$-wrapped SWNTs were robust with good cell vitality.

Cellular uptake studies were performed *in vitro* on HeLa cells using the non-dendronized (**P**$_{alkyl}$)/dendronized (**P**$_x$)-SWCNT complexes. Qualitative and quantitative analysis was performed *via* CLSM (Figure 12A) and flow cytometry respectively (Figure 12B). The free dyes **D**$_x$ (neutral/charged i.e. X = OH or
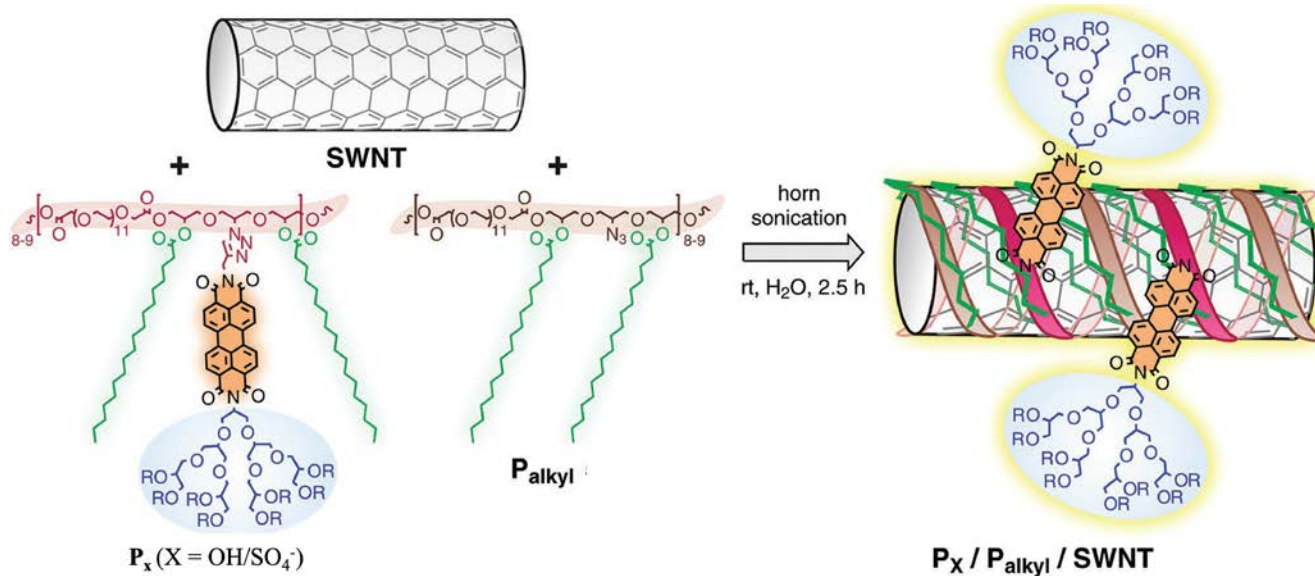

Figure 11. Schematic representation of polymer (**P**$_x$/**P**$_{alkyl}$)-SWCNT complexes formed by sonication in an aqueous solution (X=OH/SO$_4^-$) and their idealized wrapping around in helical patterns over the surface of nanotubes. Reproduced from reference [121]. Copyright 2018 Wiley-VCH Verlag GmbH & Co. KGaA.

Table 5. Progression of polyether-based denpols using the versatile click chemistry and their applications for the delivery of active components.

S. No.	Hydrophobic R groups (% composition in final polymers)	Hydrophilic side chains	Coupling strategy	CMC/CAC (M)	Molecular weight M_n (Da) by GPC	Applications of denpols	Ref.
Block copolymer 1	**Poly(PEG$_{600}$-*co*-glycerol)**						
1.1	C-5, C-7, C-9 acyl chlorides (100%)	-	**Acylation** reaction K$_2$CO$_3$, DCM, rt, 6 h	2.4 x 10^{-2}	5.5 x 10^3 5.7 x 10^3 5.9 x 10^3	Hydrophobic drug Vitamin E encapsulation	90
Block copolymer 2	**Poly(PEG$_{600}$-*co*-azido-glycerol)**						
2.1	C-18 (50-70%)	[G1.0], [G2.0]-OG dendrons (30-50%)	**CuAAC:** CuBr, DMF, rt, 24 h	2.0 x 10^{-5} -2.94 x 10^{-5}	2.9 x 10^3 – 5.1 x 10^3	Hydrophobic guest pyrene encapsulation and ANS binding studies	91
Block copolymer 3	**Poly(PEG$_{1000}$-*co*-azido-glycerol)**						
3.1	C-12 and C-14 (50-100%)	[G1.0], [G2.0]-OG dendrons (30-50%)	**CuAAC:** CuSO$_4$.5H$_2$O, Sodium ascorbate, THF:H$_2$O (3:1), 40ºC, 48-60 h	1.4 x 10^{-5} - 3.4 x 10^{-5}	1.4 x 10^3 -8.8 x 10^3	Hydrophobic dye Nile red encapsulation and cytotoxicity studies	92
3.2	C-18 (50-100%)	[G1.0], [G2.0]-OG hPG dendrons (0-50%)	**CuAAC:** CuSO$_4$.5H$_2$O, Sodium ascorbate, THF:H$_2$O (3:1), 40ºC, 48-60 h	6.7 x 10^{-1} - 34 x 10^{-6}	1.1 x 10^3 – 6.5 x 10^3	Hydrophobic dye Nile red encapsulation by denpols and their significant uptake by QGP-1 cells, cytotoxicity profiles of denpols	93
3.3	Azobenzene (70%)	Without/with [G2.0]-OG dendrons (0-30%)	**CuAAC:** [Cu(PPh$_3$)$_3$]Br, DIPEA, DCM/DMF, rt, 48 h	1.07 x 10^{-5} - 1.66 x 10^{-5}	4.3 x 10^3 – 4.7 x 10^3	Hydrophobic dye Curcumin and Nile red encapsulation by amphiphilic micelles and subsequent study of photoresponsive release of Nile Red.	98
3.4	Fluoro C-8 (50-70%)	[G2.0]-OG dendrons (30-50%)	**CuAAC:** [Cu(PPh$_3$)$_3$]Br, DIPEA, DCM/DMF, rt, 48 h	8.9 x 10^{-6} -7.8 x 10^{-6}	3.2 x 10^3 – 4.1 x 10^3	Solubilization and transport capacity evaluation of poorly water-soluble drugs curcumin and dexamethasone	103
	C-8 (50-70%)			1.2 x 10^{-5} – 4.5 x 10^{-5}	1.4 x 10^3 – 2.0 x 10^3		
Block copolymer 4	**Poly(PEG$_{600}$-*co*-azido-triglycerol)**						
4.1	C-12 acyl chloride (R') (66%)	[G1.0] and [G2.0]-OG dendrons (33%)	**Acylation:** K$_2$CO$_3$, DCM, 35-40ºC, 12 h	3.29 x 10^{-5} – 4.56 x 10^{-5}	1.8 x 10^3 – 10.4 x 10^3	Cytotoxic evaluation of denpols in A549 cell lines. CLSM and FACS measurements demonstrated greater cellular internalisation of Cy3 by the [G2.0]-OG grafted denpols.	108
	C-12 acyl chloride (R') - acylation and/or C-12 (R) - click reaction (66-100%)		**CuAAC:** CuSO$_4$.5H$_2$O, Sodium ascorbate, THF:H$_2$O (3:1), 50ºC, 24 h				
4.2	C-11 terminal alkenoyl chloride (R') -acylation (66%)	[G1.0] and [G2.0]-OG dendrons (33%)	**Acylation:** K$_2$CO$_3$, DCM, 35-40ºC, 12 h	1.9 x 10^{-5} – 4.23 x 10^{-6}	8.0 x 10^2 – 15.7 x 10^3	Non-toxicity of denpols upto a concentration of 500µg/mL in A549 cell lines and Cy3 encapsulation in sufficient amounts.	111
	C-11 terminal alkenoyl chloride (R') -acylation and/or C-12 (R) -click reaction (66-100%)		**CuAAC:** CuSO$_4$.5H$_2$O, Sodium ascorbate, THF:H$_2$O (3:1), 50ºC, 24 h				
4.3	C-18 acyl chloride		**Acylation:** EDC, DMAP, DCM/DMF 0-35ºC, 12 h	0.53 x 10^{-3} – 0.63 x 10^{-3}	2.03 x 10^3 – 54.5 x 10^3	Water-soluble, fluorescent, and debundled SWCNT complexes were obtained with PBI grafted denpols that were found appropriate for bioimaging studies.	121
		[G2.0]-OG PBI/[G2.0]-sulphated-OG dendronized PBI	**CuAAC:** Sodium ascorbate, CuSO$_4$.5H$_2$O, Cu Wire, DMF/H$_2$O, 50ºC, 24 h				

SO$_4$$^-$) were not visible in the cells after a 4 h incubation period. In contrast, as evidenced by the fluorescent signals of the PBI dye, the dye-conjugated denpols (**P$_x$**) and the complex formed by **P$_x$/P$_{alkyl}$**/SWCNT demonstrated an active uptake. These results combined with the evidence that no signal was observed when free dye (**D$_x$**)/free dye-alkyl grafted polymer (**D$_x$/P$_{alkyl}$**) mixtures were used, concluded the necessity of covalent bonding of the dye with polymer backbone for cellular uptake.

Intracellular uptake studies of the complexes demonstrated an impressive performance of the denpol-SWCNT complex as a probe for bioimaging (Figure 12). The charged SWCNT-complex (sulphated denpols-complex) outperformed its non-charged analogue in biological and photophysical investigations in terms of cellular uptake, intracellular staining, and optical characteristics. Due to their nanosize dimensions, outstanding fluorescence, and great cytocompatibility, the denpol-SWCNT

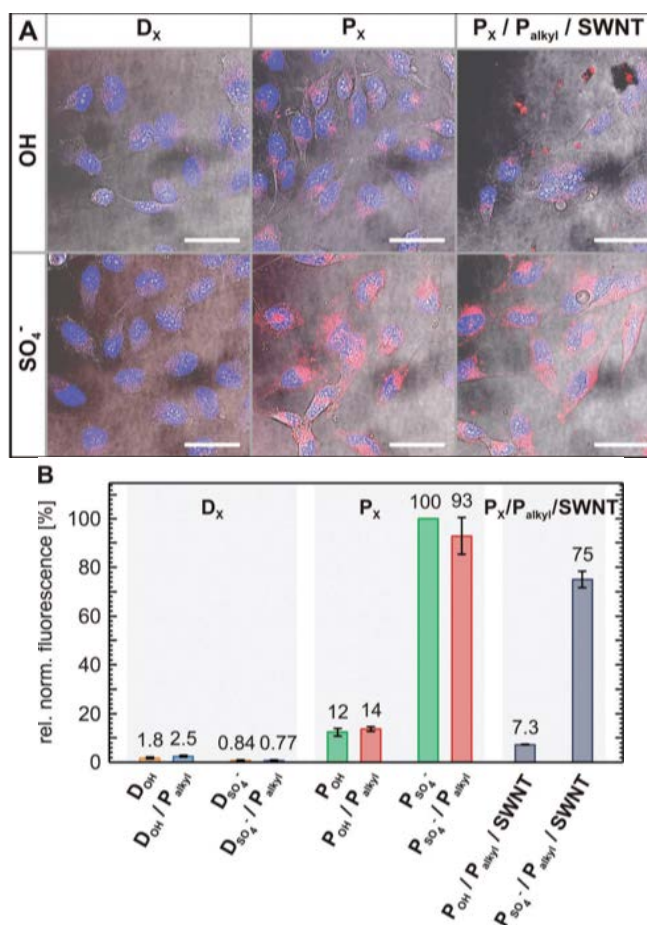complexes proved to be an important tool for fluorescent bioimaging with a wide range of applications.[121]

Figure 12. Cellular uptake studies *in vitro* on HeLa cells with free dye **D$_x$** (X = OH/SO$_4^-$), free dye-polymer mixtures (**D$_x$/P$_{alkyl}$**) and SWCNT complexes formed with PBI-dendronized/non-dendronized polymers (**P$_x$/P$_{alkyl}$/SWCNT**). **A**) After 4 hours of incubation, live-cell microscopy pictures of HeLa cells (cell nuclei stained blue) treated with the respective PBI-based compounds (stained red) are shown. Scale bar, 50 μM; **B**) Analysis of flow cytometry data from HeLa cells treated with the above-mentioned compounds show that sulphated analogues outperform the hydroxylated denpols and SWCNT-denpol complexes in terms of uptake behaviour and staining efficiency. Reproduced from reference [121]. Copyright 2018 Wiley-VCH Verlag GmbH & Co. KGaA

CONCLUSION

The versatility of "click chemistry" in various fields has already been comprehensively surveyed recently,[127] whereas herein another research area exploring this highly adaptable reaction has been reported. Despite its early stages of research, polyether-based dendronized multi-amphiphilic polymers have demonstrated remarkable potential as supramolecular self-assembled nano-transporters for the delivery of active pharmaceuticals, much work is still required to get over these dendronized nanocarriers' constraints and explore them in clinical settings. Future research must also focus on strategies for bio-orthogonal click chemistry utilizing the many available end-groups for functionalization to attach molecular tags, drugs and dyes for *in vivo* studies. Further advancements in this field would open up new avenues for enhancing the flexibility of denpols with polyether scaffolds and developing next-generation DDS.

ABBREVIATIONS

A549, Adenocarcinoma human alveolar basal epithelial cell lines; AAC, Azide-alkyne cycloaddition; ATRP, atom transfer radical polymerization; BARAC, Biarylazacyclooctynones; CAC, Critical Aggregation Concentration; CLSM, Confocal laser scanning microscopy; CMC, Critical Micelle Concentration; Cryo-TEM, Cryogenic Transmission Electron Microscopy; Cu(n), Copper with oxidation state n (n=0,I,II); CuAAC, Copper-catalyzed azide-alkyne cycloaddition; Cy3, Cyanine dye; DDS, Drug Delivery Systems; Denpols, Dendronized polymers; DIFO, Difluorocyclooctyne; DLS, Dynamic Light Scattering; Dox, Doxorubicin; FACS, Fluorescence-Assisted Cell Sorting; FDA, Food and Drug Administration; FT, Fourier Transform; [Gn.0], nth Generation dendron (n=1,2,3 etc.); GPC, Gel Permeation Chromatography; hPG, hyperbranched polyglycerol; N435, Novozym 435; OG, Oligoglycerol; PBIs, Perylene bisimides; PEG, Polyethylene glycol; PG, Polyglycerol; ROMP, Ring-Opening Metathesis Polymerization; SDS, Sodium dodecyl sulphate; SLS, Static Light Scattering; SPAAC, Strain-promoted azide-alkyne cycloaddition; SWCNT, Single-Walled Carbon Nanotubes; TMTH, Tetramethylthiacycloheptyne; UV-vis spectroscopy, Ultraviolet-visible spectroscopy.

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