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Copper-free click conjugation of methotrexate to a PAMAM dendrimer platform

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Abstract

The synthesis of a generation 5 (G5) poly(amidoamine) (PAMAM) dendrimer platform having cyclooctyne ligands that were subsequently be used for a copper-free Huisgen 1,3-dipolar cycloaddition (click reaction) with azido modified methotrexate is described. The G5 PAMAM dendrimer was first partially (70%) acetylated and then coupled with 20 cyclooctyne ligands through amide bonds. The remaining primary amine groups on the dendrimer surface were neutralized by acetylation. The platform was then “clicked” with different numbers (5, 10, and 17) of γ -azido functionalized methotrexate. The copper-free click reactions were stoichiometric with excellent yields.

Keywords

PAMAM Dendrimer; Copper-free click reaction; Methotrexate

1. Introduction

The poly(amidoamine) (PAMAM) dendrimer has been an attractive drug delivery platform for several decades due to biocompatible, low polydispersity¹ and the ability to make a wide variety of surface modifications, including direct conjugation to small molecules such as folic acid², antibodies³ and therapeutic drugs⁴. The synthesis of a functionalized PAMAM dendrimer is generally carried out using a sequential synthesis strategy, with purification and characterization steps required after the conjugation of each type of small molecule.^{5,6} While these processes yield multifunctional PAMAM dendrimer conjugates that are effective both in vitro⁷ and in vivo⁸, we found that it was difficult to consistently replicate the number of small molecules conjugated to the surface of the dendrimer, both within and between batches.

In this communication, we report the development of a novel G5 PAMAM platform that is surface-modified with cyclooctyne groups and then further functionalized with small molecules through copper-free click chemistry. Cu(I)-catalyzed alkyne azide 1,3-dipolar cycloaddition (CuAAC) has been widely used for bioconjugation due to several criteria including high yields, tolerance toward functional groups, and virtually no side-reactions.⁹

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The cytotoxicity of copper, however, is a potential drawback of the CuAAC reaction for the synthesis and/or functionalization of nanomaterials such as dendrimers for biomedical applications, and the copper is difficult to remove from the dendrimer after conjugation.¹⁰ Although only recently developed, copper-free click chemistry has already been widely used in biological applications¹¹. The unusually high ring strain associated with cyclooctyne allows it to react selectively and rapidly with azide groups without byproducts and without the addition of a copper catalyst.¹² Due to the high efficiency of the reaction, we hypothesized that this synthetic approach should enable us to achieve better batch-to-batch reproducibility of our functionalized G5 PAMAM dendrimers. As a proof of concept, a γ -azido modified methotrexate (MTX) was synthesized and successfully conjugated to the dendrimer platform via copper-free click reaction.

2. Results and discussion

The synthesis of the G5 PAMAM dendrimer with 20 cyclooctyne molecules on the surface is shown in Scheme 1. Briefly, G5 PAMAM dendrimer (112 primary amine groups on surface based on potentiometric titration results)¹³ was first partially acetylated with acetic anhydride (75 equivalents) in methanol in the presence of triethyl amine (TEA). This step enhances the solubility of dendrimer in organic solvents. After purification using a 10K MW cut-off ultrafiltration membrane, compound **2** yielded a white solid after lyophilization. The cyclooctyne ligand **3** was synthesized by a method from recent reports^{12, 14}. Partially acetylated dendrimer **2** and compound **3** (20 equivalents) were then dissolved in anhydrous dimethyl sulfoxide (DMSO) with diisopropyl ethylamine (DIPEA). PyBop¹⁵ reagent in DMSO was added and the reaction was allowed to stir overnight at room temperature. The remaining primary amine groups were neutralized by acetylation to minimize nonspecific binding. Compound **5** was purified via membrane filtration as described above. The overall recovery yield of **5** is 81%. (**2** to **5**, two steps)

The average number of conjugated molecules per dendrimer was determined by ¹HNMR (Figure 1). Based on a literature method, the number of acetamide groups for compound **2** was calculated to be 72¹³. The integration of methyl groups (NHCOCH₃) (1.84 ppm) was set to 216 and this peak was used as an internal reference to determine the number of ligands (Figure 1, a). Based on the NMR integration values, 40 primary amine groups remained on the dendrimer surface that could be used to conjugate the cyclooctyne ligand. After the conjugation, the methyne proton signal (4.42 ppm) in the eight-membering of compound **3** was well separated from the dendrimer proton peaks in NMR spectrum that allowed us to integrate this peak and determine the total cyclooctyne numbers. (Figure 1, b) Based on the ¹HNMR, a total of 20 cyclooctyne molecules were attached per dendrimer molecule. The remaining amino groups were then completely acetylated to give compound **5**. In this case, if the integration of the methyne proton (4.42 ppm) is set to 20, the integration of the acetamide is 270 (Figure 1, c), which is close to the theoretic number 276 (92 acetamide groups).

MTX is a well-known and extensively characterized antineoplastic agent that is active when conjugated to a dendrimer¹⁶. Using a modified literature procedure¹⁷, γ -azido MTX was synthesized. (Scheme 2) Briefly, 4-amino-4-deoxy-*N*¹⁰-methylpteroic acid (APA) was first reacted with Glu- α -OtBu using BOP¹⁵ reagent to give α -OtBu-MTX (**6**). Then the γ carboxylic acid was coupled with 3-azido-propyl amine in the presence of HATU¹⁵. After exposure of γ carboxylic acid functional group with trifluoroacetic acid (TFA), compound **8** was obtained in excellent yield. All compounds were characterized with ¹HNMR and electrospray mass spectra. Compound **8** was further analyzed with HPLC (data not shown).

Using a copper-free click conjugations protocol, compound **8** was then clicked to the newly synthesized cyclooctyne dendrimer platform. (Scheme 3) Because MTX has a much lower affinity toward the alpha folate receptor than folic acid,¹⁸ different stoichiometries of MTX were conjugated to the platform in order to eventually use this material to test therapeutic effects. This was based on the hypothesis that more MTX molecules on dendrimer surface will enhance the internalization of the nanoparticle because of multivalent binding, thus increases cytotoxicity. Initially, the dendrimer platform **5** was dissolved in methanol (2.0mM), variable equivalents of γ -azido MTX **8** (5, 10, and 18 equ.) in DMSO (40mM) was added. The reactions were stirred for 24 hours at room temperature shielded from light. After the removal of organic solvent, the residues were redissolved in phosphate buffer saline (1×PBS buffer, pH=7.4) and purified by 10K centrifugal filters (PBS×2, DI water×6) and then lyophilized to give compounds **9**, **10**, and **11**. The recovery yields of **9** and **10** were excellent (>85%). Compound **11** has a relatively poor solubility in water and PBS buffer, which resulted in a low recovery yield (30%).

The average numbers of MTX molecules in each conjugates were determined by ¹HNMR. (Figure 2) The aromatic proton in the pteridine ring has a chemical shift at 8.61ppm and it is well separated from other proton peaks. Comparison of the integration of this peak to the internal acetamide proton integration will determine the numbers of MTX attached. In this case, the integrations of the internal reference peaks were set to 276 which represent 96 acetamide groups. The numbers of MTX are 4.78, 9.88, and 16.87 for compounds **9**, **10**, and **11**, respectively. These results indicate that the copper-free click reactions are highly efficient with >90% of γ -azido MTX conjugated. HPLC profiles of these three compounds have more than 98% purity. (data not shown)

3. Conclusion

We have synthesized a G5 PAMAM dendrimer platform attached with 20 cyclooctyne molecules with excellent yield (90%). Selective γ -azido-MTX also has been synthesized, and the copper-free click conjugations of this azido modified therapeutic drug with the dendrimer platform have been carried out with different numbers (5, 10, and 17) of MTX conjugated on dendrimers. The copper-free click reactions were very efficient and had high yields (compound **11** has low recovery due to poor solubility). These results indicate the feasibility of this non-copper based strategy for reproducibly synthesizing dendrimer conjugates with varied functionalities. The *in vitro* cytotoxicity studies of these compounds towards various cancer cell lines are currently under investigation. The conjugation of different functionalities, such as azido modified targeting molecules (folic acid², RGD peptide¹⁹, and glutamate ureas²⁰) and azido modified drugs onto the same dendrimer platform molecule are also in progress.

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Figure 1.
 ^1H NMR spectra of compound **2** (a), **4** (b), and **5** (c)

Figure 2. ^1H NMR of **9** (a), **10** (b), and **11** (c). Inserts are the enlarged portion around pteridine ring proton peak at 8.61ppm.

Scheme 1.

Synthesis of G5 PAMAM dendrimer platform with cyclooctyne ligands for copper-free click conjugation.

Scheme 2.
Synthesis of γ -azido-MTX **8**.

Scheme 3.

Copper-free click reaction of the dendrimer platform with γ -azido-MTX (only one isomer of the triazole ring is shown).