Wydawnictwo UR 2020
ISSN 2544-1361 (online); ISSN 2544-2406
doi: 10.15584/ejcem.2020.4.4

http://www.ejcem.ur.edu.pl/en/
European Journal of Clinical and Experimental Medicine
Eur J Clin Exp Med 2020; 18 (4): 281–285

ORIGINAL PAPER

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Low generation polyamidoamine dendrimers (PAMAM) and biotin-PAMAM conjugate – the detailed structural studies by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy

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ABSTRACT

Introduction. The concept of targeted drug delivery is nowadays based on nanoparticle transporters. Such drug delivery systems for cancer cells should follow the requirements like: efficient drug release, selective binding and internalization to cancer cells. The anticancer drug selectivity can be achieved by attachment of cancer cell-recognizing molecules, like biotin. Among nanosized carriers the PAMAM dendrimers are tested intensely, especially they can be modified by covalent attachment of prodrug molecules and biotin as targeting molecule.

Aim. We aimed at construction and characterization of a conjugate formed between PAMAM and biotin (Biot). The nuclear magnetic resonances is powerful tool to determine both the structure and stoichiometry of the conjugate.

Material and methods. PAMAM G0 has been synthesized and functionalized with biotin by reaction with N-hydroxysuccinimide ester of biotin to obtain G0 double-substituted with biotin. All the compound were thoroughly characterized by the NMR spectroscopy.

Results. The conjugate of PAMAM G0 dendrimer with two amide-bonded biotin molecules was obtained and fully characterized by NMR spectroscopy.

Conclusion. N-hydroxysuccinimide ester of biotin spontaneously reacts with PAMAM G0 to obtain the conjugate of 2:1 biotin:G0 stoichiometry. The latter was designed as a targeting molecule in formation of megameric multidrug delivery system.

Keywords. biotin conjugate, nuclear magnetic resonance, polyamidoamine dendrimer

Introduction

Based on WHO data from 2018 cancer was second major cause of death worldwide leading to nearly 9.6 million deaths. Afterwards being leading cause of death cancer is also remarkably harmful to global economy. Direct medical costs for cancer in United States was estimated to $80.2 billion in 2015 which shows range of a problem.[1,2]

Systemic chemotherapy is one of the main approaches in treatment of cancer. In spite of its utility there are some limitations in efficacy of conventional chemotherapy caused by unsatisfying solubility of che-

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

Received: 6.12.2020 | Accepted: 17.12.2020
Publication date: December 2020

Wróbel K, Wołowiec S. *Low generation polyamidoamine dendrimers (PAMAM) and biotin-PAMAM conjugate – the detailed structural studies by 1H and 13C nuclear magnetic resonance spectroscopy.* Eur J Clin Exp Med. 2020;18(4):281–285. doi: 10.15584/ejcem.2020.4.4

motherapeutics, lack of adequate selectivity of therapy, low membrane permeability of drugs and multidrug resistance of cancer cells. To overcome this challenges, conceptions of using nanomaterials as a drug carriers have emerged.[3,4]

Poly(amidoamine) dendrimers are class of hyperbranched nanopolymers tested as potential carriers for drug delivery. PAMAM are characterized by having ethylene diamine core and alkylamido structure of branches terminated by amine groups in case of full generation and carboxyl groups in half generation.[5] Number of terminal groups in full generation PAMAMs doubles with every next generation starting from 8 for generation zero (G0). The main advantages of PAMAM dendrimers are their good water solubility, flexibility, relatively low cytotoxicity and rapid cellular uptake. These properties of PAMAM are used to increase drug bioavailability by conjugation with dendrimers or drug encapsulation. It was shown that conjugation with PAMAM significantly increased water solubility and transepithelial transport of propranolol and naproxen (poorly water-soluble drugs). Moreover, multitude of terminal groups of PAMAM provides an opportunity to conjugate them with multidrug combination or functionalize with other compound such as α-*D*-glucoheptono-1,4-lactone to decrease their toxicity which otherwise is stimulated by free terminal amine groups.[5,6]

There are evidences that some cancer cells overexpress sodium-dependent multivitamin transporter (SMVT) which is responsible for biotin, pantothenic acid and lipoic acid cellular uptake. From certain point of view it also plays a role of biotin receptor. It was demonstrated by *Vadlapudi et al.* that expression of SMVT in T47D breast cancer cell line is significantly higher in comparison to normal mammary epithelial cells (MCF-12A).[7-9]

Overexpression of biotin receptors by specific cancer cells offered the opportunity to increase selectivity of nanoparticle drug delivery systems by biotinylation of nanocarriers. In case of dendrimers such approach seems to be reasonable as enhanced cellular uptake of biotinylated dendrimer conjugates and less cytotoxicity for normal cells in comparison to specific cancer cells lines were shown in several studies.[10-13]

Aim

The aim of our studies was to synthesize biotinylated G0 derivative and then characterize it using NMR spectroscopy. Biotinylated G0 will be used further as substrate in synthesis of megamer of mixed generations G3-G0 PAMAM dendrimers which will be covalently conjugated with anticancer drugs. Such nanocarrier-attached multidrugs will be evaluated *in vitro* studies. We assume that presence of biotinylated G0 in structure of megamer will provide a significant preference in targeting cancer cells overexpressing biotin receptors.

Material and methods

Materials

Solvents, NHS-biotin, ethylenediamine (en), methylacrylate were purchased from Merck (KGaA, Darmstadt, Germany). The polyamidoamine dendrimers of low generation; $G_{-0,5}$ and G_0 were synthesized accordingly to the published procedure.[14]

Synthesis of biotinylated G_0 Dendrimer

Solid biotin N-hydroxysuccinimide ester (NHS-biotin, 690 mg, 2021μmoles) was added in portions to G_0 solution in MeOH (4 ml, 1272 mg, 2460 μmoles) with magnetic stirring. Then 4 ml MeOH and 4 ml of water were added and stirring continued until all reagents dissolved. After 7 hrs gel precipitate was formed which left at 5 °C overnight. The precipitate was filtered off and washed with 30 ml of cold water and dried 12 hours under vacuum. The compound was identified as G_0 substituted with two biotin equivalents (G_0-Biot) by ^{1}H NMR spectrum in dmso-d_6. Yield: 440 mg (45 %). The remaining part of biotin substrate was recovered and identified as G_0 – monosubstituted with biotin contaminated with unreacted G_0.

NMR Spectroscopy

The 1-D ^{1}H and ^{13}C NMR spectra as well as 2-D ^{1}H-^{1}H correlations spectroscopy (COSY), ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation spectra (HMBC) were recorded in deuterated water using Bruker 300 MHz (Rheinstetten, Germany) and worked up with TopSpin 3,5 software at College of Natural Sciences, University of Rzeszów.

Results

The ^{1}H NMR spectra of substrates and G_0-Biot

Polyamidoamine dendrimers were synthesized starting from en core. The methyl acrylate was added to en to obtain generation -0,5 dendrimer ($G_{-0,5}$), which was further converted into zero-generation PAMAM dendrimer, G_0. The dendrimer G_0 was substituted with biotin by reaction with N-hydroxysuccinimide ester of biotin (NHS-biotin) to obtain G_0-Biot conjugate. All the compounds were characterized by the ^{1}H and ^{13}C NMR spectroscopy. The ^{1}H NMR spectra of $G_{-0,5}$, G_0, and G_0-Biot are presented at Figure 1. The ^{1}H NMR spectral assignments of the compounds were performed according to the atom numbering presented at Figure 2. Additionally the 2-D COSY ^{1}H-^{1}H experiments enabled to join the 3d to 4d triplets into pairs for all compounds by scalar coupling peaks (COSY spectrum for G_0-Biot is shown at Figure 3). Thus, the most upfield shifted triplet

in all derivatives was assigned to 4d methylene protons, at: 2.28 for $G_{-0,5}$, 2.18 for G_0, and 2.18 G_0-Biot. Scalar coupled 3d methylene proton triplets were observed at 2.65 for $G_{-0,5}$, 2.62 for G_0, and 2.62 for G_0-Biot. The 4d methylene proton resonances in all cases showed strong long-range coupling (via two bond) with carbon nucleus (5d) next to 4d (full ^{1}H-^{13}C HSQC and HMBC maps for G_0-Biot are presented at Figure 4).

The addition of en into $G_{-0,5}$ led to amine-terminated G_0 dendrimer. In the ^{1}H NMR spectrum of G_0 additional proton multiplets were observed at 2.53 (t) and 3.01 (q) (Figure 1), which showed scalar coupling in COSY spectrum. Additionally, the scalar coupling cross-peak was observed in COSY spectrum of G_0 between 3.01 quartet and NH (6d) triplet at 7.89 ppm (Figure 1).

Similar pattern of ^{1}H resonances was observed in case of G_0-Biot (Figure 1, upper trace). Based on integral intensity of the biotin resonances and 4d resonances, the latter of intensity corresponding to 8H, we determined the level of G_0 substitution with biotin, which was nearly two. This means that two of four terminal amine groups were converted into amide-linked biotin. In fact we have observed three amide proton resonances in the 7.85-8.00 ppm region, from 6d proton of unsubstituted arm, another one from 6d of substituted arm and 9d' from newly formed amide group (for numbering see figure 2). Simultaneously, the 8d methylene protons of substituted arm shifted from 2.53 ppm into 3.05 ppm as could be determined by integral intensity of overlapped resonances from 7d and 8d'. Such a dramatic chemical shift was due to a change of chemical vicinity of the 8d methylene group from amine ended in G_0 to amide ended 8d', thus similar to 7d which is next to amide.

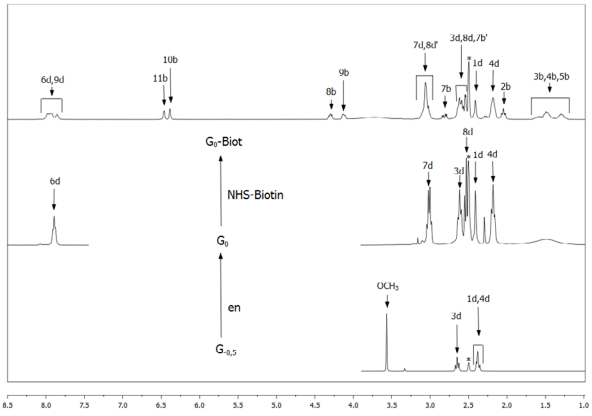

Fig. 1. The relevant fragments of ^{1}H NMR spectra of $G_{-0,5}$ (1), G_0 (2), full spectrum of G_0-Biot (3). The resonances from dendrimer are labeled as d, while resonances of biotin in G_0-Biot are labeled as b and numbered in accordance with figure 2. The residual resonance from dmso-d_6 is labeled with asterisk. The insert illustrates the conversion scheme starting from $G_{-0,5}$ to final G_0-Biot. The structures of compounds are drawn at Figure 2

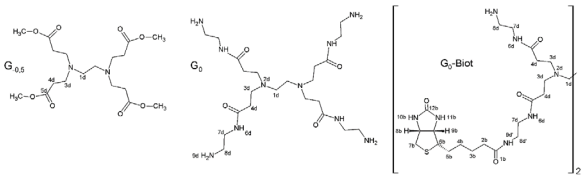

Fig. 2. The formulas of studied compounds with atom numbering.

The homonuclear ^{1}H-^{1}H COSY spectrum is presented at Figure 3. The experiment allowed to assign the resonances of 3d and 4d protons, which are scalar coupled (peak a) and signals of 7d and 8d protons, which are scalar couple (peak b), while 7d and 8d' protons showed scalar coupling with neighboring amide NH protons, namely 6d and 9d (group of cross-peaks labeled as c at Figure 3). The biotin proton cross-peaks are shown in left-upper part of the COSY map at Figure 3. The triplet of 2b methylene proton is coupled (cross-peak e) with 3b methylene protons, while 4d and 5d methylene proton resonances are overlapped within 1.2-1.6 ppm and coupled (groups of d peaks at Figure 3). The resonance of 6b proton is hidden under high-intensity resonance from PAMAM core 7d and 8d' resonances, nevertheless it could be identified through revealed cross-peak f between 6d and 5d protons. Two protons at C-7 are not magnetically equivalent, and the multiplets (dd) of 7d and 7d' are coupled via g cross-peak. The cross-peak j allowed to assign 8b resonance, while coupling between 6b proton and 9b unambiguously assigned the latter by cross-peak h. Consequently the cross-peaks m and n enabled to assign the resonances of 11b and 10b protons coupled with 9b and 8b, respectively. The group of cross-peaks (l) illustrated the three-bond scalar coupling between 7d and 6d of unsubstituted G_0 arm as well as 8d' and newly formed amide proton 9d'.

The ^{13}C NMR spectra of substrates and G_0-Biot

With assigned ^{1}H NMR resonances of G_0-Biot in hand we have performed the 2-D heteronuclear HSQC and HMBC experiments for ^{13}C signal assignments. The combined 2-D map of cross-peaks is presented at Figure 4. The red-yellow contour peaks (obtained in HSQC experiment) correspond to one-bond couplings between hydrogen nuclei and carbon nuclei and are not labeled. They enabled to straightforward assign the ^{13}C resonances of hydrogen-bonded carbon atoms (see Table 1). The magnetization transfer from ^{1}H nuclei to two-bond distant ^{13}C nuclei observed as cross-peaks in the HMBC experiment are shown as black-grey contours at Figure 4. Some of this peaks are crucial for detailed ^{13}C signal assignment. The 12d ^{13}C resonance at 162.5 ppm was then identified by cross-peaks q and s corresponding to magnetization transfer from protons 10b and 11b (q) and from 8b and 9b (s) to 12d carbon nucleus. Another

crucial cross-peaks enabled to assign the amide group carbon resonance 1d (coupled with 2d; cross-peak t), while cross peaks p and r correspond to magnetization transfer from 10b and 11b protons to 8b and 9b carbon nuclei, and from 6d and 9d' protons to 1b carbon nucleus. The entire picture of heteronuclear ^{1}H-H-H

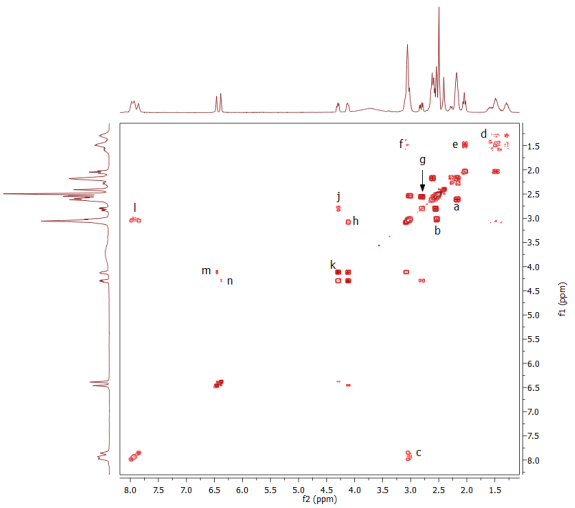

Fig. 3. ^{1}H-^{1}H COSY spectrum of G_0-Biot in dmso-d_6. The relevant off-diagonal peaks are labeled in right-lower part for dendrimer and left-upper part for biotin

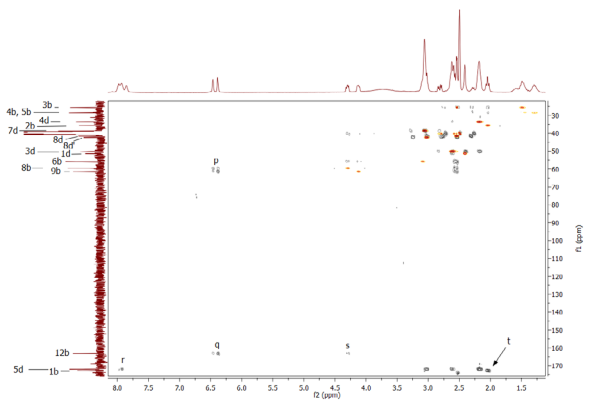

Fig. 4. The combined map of heteronuclear ^{1}H-^{13}C HSQC (red – yellow contours) and HMBC (black – gray)

^{13}C experiments is in precise agreement with ^{1}H spectral assignments described above.

The total assignment of relatively simple molecular system like G_0-Biot is necessary step to construct more composed macromolecules and characterize them structurally by the same set of spectral techniques, which were successfully used here. The ^{1}H and ^{13}C resonance assignments are collected in Table 1.

Discusssion

Despite the starting 1.2:1 G_0:NHS-Biotin molar stoichiometry of the starting mixture, the double-Biotin-substituted dendrimer was the main product isolated from the mixture. We have observed this before for non-steroidal antyinflamatory drug, Nimesulide.[15] It seems that hydrophobic interaction between incoming substituents is a driving force for double substitution instead mono-substitution of G_0. Nonetheless, the isolated conjugate still is equipped with two free amine terminal groups, which can be used for further derivatization.

Table 1. The assignment of ^{1}H and ^{13}C resonances of the conjugate G_0-Biot, G_0 substrate and $G_{-0,5}$ precursor. The upper case letters encounter the cross-peaks observed in HMBC spectrum of G_0-Biot (as specified in Figure 4), which are combining the ^{1}H signals with ^{13}C resonances of two-bond distant carbon-13 nuclei

Compound®	$G_{-0.5}$		G_0		G_0-Biot	
Locant -	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1d	51.8	2.38 (s, [4H])	51.4	2.41 (s, [4H])	51.4	2.42 (s, [4H])
3d	49.7	2.65 (t, [8H])	50.3	2.61 (t, [8H])	50.2	2.61 (t, [8H])
4d	32.5	2.38 (t, [8H])	33.7	2.18 (t, [8H])	33.7	2.18 (t, [8H])
5-OCH$_3$	51.6	3.57(s,[12H])	-	-	-	-
5d	172.9	-	171.8	-	172.0^{r}, 171.9^{r}	-
6d	-	-	-	7.90 (t, [4H)	-	7.93 (bs)^{r}; 7.85 (bs)^{r}
7d			42.7	3.01 (q, [8H])	38.8	3.06
8d			41.8	2.53 (t, [8H])	41.7	2.54
8d′					42.4	3.03
9d′					-	7.98 (bs, [1H])^{r}
1b					172.7^{t}	-
2b					35.8	2.05 (t, [2H])^{t}
3b					25.7	1.44-1.51 (m, [2H])
4b					28.5	1.44-1.57 (m, [2H])
5b					28.7	1.21-1.36 (m, [2H])
6b					55.8	3.06 (m, [2H])
7b, 7b′					40.4	2.81 (dd, [1H]), 2.56 (dd, [1H])
8b					59.7^{p}	4.30 (m, [1H])^{s}
9b					61.6^{p}	4.12 (m, [1H])^{s}
10b					-	6.39 (s, [1H])^{p,q}
11b					-	6.46 (s, [1H])^{p,q}
12b					163.2^{q,s}	-

The detailed NMR spectral characterization of obtained G_0-Biot compound is necessary step to obtain multidrug anticancer conjugates. The conjugates will be obtained as megamers of mixed-generation PAMAM dendrimers. The core dendrimer of third generation G_3^{OH} will be the PAMAM dendrimer totally covered with polyhydroxy substituents. Then terminal hydroxyl groups will be functionalized with NPCF (*para*-nitro-

phenylortochloroformate) and used to bind low-generation G_0 dendrimers containing combination of two anticancer drugs: G_0-nimesulide or G_0-celecoxib and G_0-daunorubicin or G_0-doxorubicine. The third component of the megamer will be G_0-Biot. The latter will play the role of cancer cell targeting molecule. The protocol to obtain such megamer-attached anticancer drug has been already elaborated and published.[15] The megameric conjugates are the macromolecules of an average molecular weight ca 20 kDa which can be characterized by NMR spectroscopy in solution to determine the composition of multicomponent macromolecule. Also the size and zeta potential of the megamers will be determined by Dynamic Light Scattering method in solution.[15] In the solid state Atomic Force Microscopy will be used for imaging the macromolecules deposited on mica, while Differential Scanning Calorimetry will be used to elucidate the flexibility of megameric macromolecules as before.[6,15] Finally, the megameric multidrug associates will be tested *in vitro* on glioblastoma cells and other lines. The PAMAM G3-based multidrug delivery system (MDDS) was already elaborated and applied combination of F-Moc-L-Leu and celecoxib was demonstrated as *in vitro* effective MDDS against glioma.[16] The megameric MDDS is the novel concept which is next to execute. Structurally characterized G0-Biot is part of this MDDS.

Conclusion

Two biotin conjugated with PAMAM dendrimer of generation 0 (G_0-Biot) was characterized by 1-D and 2-D homo- and heteronuclear magnetic resonance spectroscopy. The unambiguous ^{1}H and ^{13}C signal assignments of this molecule will enable to construct high molecular weight associates containing G_0-Biot and combination of anticancer drugs with NMR spectral control of the multicomponent macromolecules composition, which will be used as targeting drug delivery systems.

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