Supplementary Material (ESI) for Analytical Methods

Acetonitrile Shortage: Use of Isopropanol as an alternative elution system for Ultra/High Performance Liquid Chromatography

Ankur M Desai,[a] Mark Andreae,[a,] Douglas G Mullen[a,b], Mark M Banaszak Holl [a,c] and James R Baker Jr.,*[a]

[a] *Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan, 9220 MSRB III, 1150 W. Medical Center Dr., Ann Arbor, MI 48109-5648.*

[b] *Program in Macromolecular Science and Engineering,* [c]*Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109.*

***E-mail:jbakerjr@umich.edu; Fax: +1 734 936 2990; Tel: +1 734 647 2777**

Supplemental Information:

(A) Experimental Conditions

A previously developed HPLC protocol to characterize and analyze PAMAM dendrimer conjugates was transferred to a UPLC protocol by using an Acquity UPLC column calculator, version 1.1.1 (Waters Corporation, Milford, MA). The software estimates peak capacity for the gradient separation based on the HPLC parameters input. It provides a choice of UPLC conditions that offers high resolving power. Our UPLC conditions were transferred and derived based on this software.

(1) Ultra Performance Liquid Chromatography using Acetonitrile as an organic modifier.

, UPLC analysis was carried out on a Waters Acquity Peptide Mapping System equipped with a Waters photodiode array detector, a column manager that facilitates 4 column housing, and a sample manager. The instrument is controlled by Empower 2 software. For characterization, calibration and quantitation studies, G5 PAMAM dendrimer, its conjugates, free folic acid and methotrexate were ran on an Acquity BEH C4 column (100 x 2.1 mm, 1.7 µm). The analysis was carried out using a gradient elution beginning with 99:1 (v/v) water/acetonitrile (ACN) reaching 20:80 water/acetonitrile (ACN) in 13.40 minutes. Trifluoroacetic acid (TFA) at 0.14 wt % concentration was added in water as well as in ACN as a counter ion to make the dendrimer surfaces hydrophobic. The gradient was then reequilibrated back to starting conditions in the next 1.0 minute. Flow rate was maintained at 0.208 mL/min. and The software is equipped with three different injection options. A 3 µL of sample was injected using one such option ("partial loop with needle overfill"), The column temperature was maintained at 35 C. The concentration of G5 PAMAM dendrimer and dendrimer-ligand conjugate were maintained at 1 mg/mL.

(2) Ultra Performance Liquid Chromatography using Isopropanol as an organic modifier.

For experiments using isopropanol as a part of the eluent system, the analysis was carried out using the same gradient as mentioned above, except that the organic modifier, acetonitrile, was replaced with isopropanol. Briefly, the gradient elution began with 99:1 (v/v) water/isopropanol reaching 20:80 water/isopropanol in 13.40 minutes. Trifluoroacetic acid (TFA) at 0.14 wt % concentration was added in water as well as in ACN as a counter ion to make the dendrimer surfaces hydrophobic. The gradient was then reequilibrated back to starting conditions in the next 1.0 minute. Flow rate was maintained at 0.208 mL/min. The software is equipped with three different injection options. A 3 µL of sample was injected using using one of these options ("partial loop with needle overfill"), The column temperature was maintained at 35 C.

(3) (Semi-preparative) High Pressure Liquid Chromatography using Isopropanol as an organic modifier.[1]

HPLC isolation was carried out on a Waters Delta 600 HPLC system equipped with a Waters 2996 photodiode array detector, a Waters 2707 auto sampler, and Waters Fraction collector III. The instrument was controlled by Empower 2 software. For analysis of the conjugates, a C5 silica-based RP-HPLC column (250 x 21.20 mm, 10µ 300 Å) connected to a C5 guard column (50 x 21.20 mm) was used. The mobile phase for elution of the conjugates was a modified linear gradient beginning with 100:0 (v/v) water/isopropanol and ending with 60:40 (v/v) water/isopropanol over 25 min at a flow rate of 10 mL/min. The system was reequilibrated back to the starting conditions. Trifluoroacetic acid (TFA) at 0.14 wt % concentration in water as well as in isopropanol was used as a counter ion to make the dendrimer surfaces hydrophobic. Elution traces of the dendrimer-ligand conjugate were obtained at 210 nm.

(4) Calibration curves for free methotrexate and free folic acid

Calibration curves for free folic acid and methotrexate were generated by using a serial dilution protocol. A stock solution for both samples at a concentration of 10 mM was made in DMSO. The stock solution was then diluted to the 10 µM using PBS buffer. This served as a working stock solution. This was serially diluted to generate various dilutions ranging from approximately 156 nM to 10 µM. A 3 µL sample was injected using a "partial loop with needle overfill", an inbuilt sample loop option within the software and ran on a similar column and both the UPLC conditions as mentioned above. The samples were detected at a λ_{max} of 285 nm. For quantitative assessment, 3 µL of a 10 µM of nanodevice was injected to calculate amounts of free folic acid and methotrexate within the nanodevice.

(5) Synthesis of dendrimer-ligand samples[2]

The Azide Ligand (29.0 µg, 0.146 µmole) in anhydrous DMSO (14.6 µL), was added to a solution of partially acetylated dendrimer **3** (4.4 mg, 0.14 µmole) in anhydrous DMSO (0.978 mL). N,N-diisopropylethylamine (1.1 mg, 1.5 µL, 8.6 µmole) was added to the reaction mixture and the resulting solution was stirred for 30 minutes. A solution of PyBOP (74.0 µg, 0.143 µmole) in anhydrous DMSO (13.8 µL) was added in a dropwise

manner (0.1 mL/min) to the dendrimer solution. The resulting reaction mixture was stirred for 24 hrs under nitrogen and then purified using 10,000 MWCO filters (Amicon Ultra). Purification consisted of one cycle with 1x PBS (w/o magnesium or calcium) and five cycles with DI water. Each cycle was 10 minutes at 5,000 rpm. The purified product, sample, was lyophilized for three days to yield a white solid (3.7 mg, 84%). ^{1}H NMR integration determined the mean number of Azide Ligands per dendrimer to be 0.4

(6) Stability of the samples and the repeatability of the developed method

As the dendrimer and its conjugates are further subjected to purification using semi-prepartive HPLC. The usual time of collection of fractions to its confirmation with either UPLC or analytical HPLC is about 48 hours. To ascertain that the collected fractions and in general our dendrimer samples do not degrade over this incubation time, we tested these samples by incubating in the UPLC eluent. The dendrimer sample was made in 99/1 Water/Isopropanol with 0.14% Trifluroacetic acid and incubated for a period of 48 hours at room temperature. The sample was then injected twice at Day 0 followed by injections at the end of 48 hours incubation. Figure S1 shows an overlaid chromatogram for G5 PAMAM ran at time 0 and at the end of incubation. Based on the data obtained, we did not see any significant degradation for the sample.

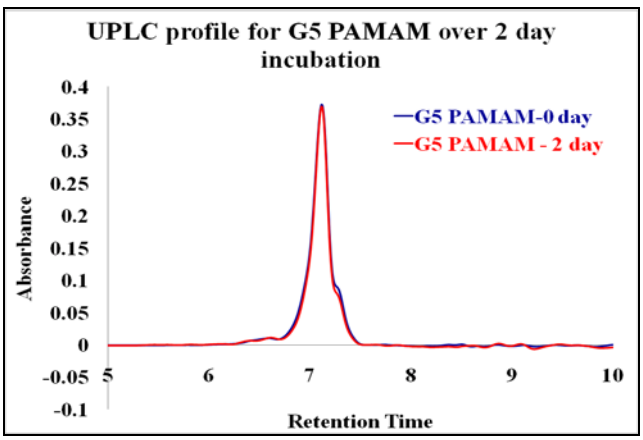

Fig S1: Overlaid UPLC chromatogram for G5 PAMAM dendrimer incubated in Water/Isopropanol/Trifluoroacetic acid eluent over two days at room temperature.

To ascertain that the developed method is also repeatable, the sample was run thrice within a day and five times after the 2 day incubation. The retention times were repeatable with a mean retention time of 7.11087 $\pm$ 0.00212 minutes with a standard deviation of 0.00601. Figure S2 shows the plot of retention times for G5 PAMAM dendrimer against number of injections over the two day incubation period.

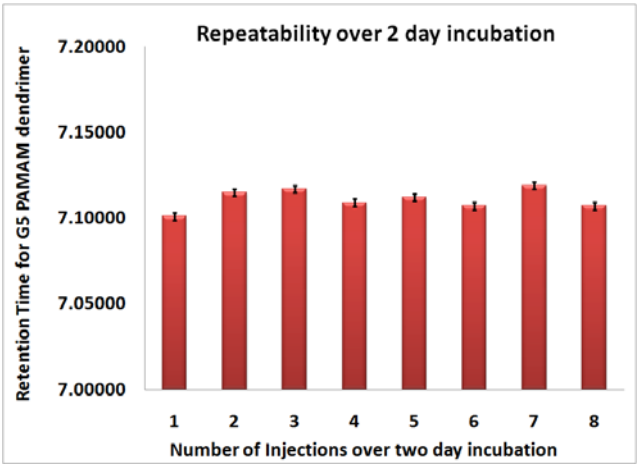

Fig S2 Plot indicating the repeatability of the developed method over a two day incubation period

References:

1. Isolation and characterization of dendrimer with precise numbers of functional groups; Douglas G. Mullen, Emilee L. Byrne, Ankur M. Desai, Mallory van Dongen-Soher, Mark Barash, James R. Baker Jr. and Mark M. Banaszak Holl; *Chemistry – A European Journal, Volume 16, Issue 35, pages 10675–10678.)*

2. The effect of mass transport in the synthesis of partially acetylated dendrimer: Implications for functional ligand-nanoparticle distributions; Douglas G. Mullen, Emilee L. Bryne, Ming Fang, Daniel Q. McNerny, Ankur Desai, James R. Baker Jr., Bradford G. Orr, Mark M. Banaszak Holl *Macromolecules*, 2010, 43 (16), pp 6577–6587*)*