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Polymer Interactions with Nucleic Acids Under Various Physiological Conditions

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Honors Research Project

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Abstract/Executive Summary

Problem statement

The goal of this project is to improve our understanding of nucleic acid interactions with cationic polymers with the theory that the polymers could protect the nucleic acids from degradation caused by biological enzymes. We seek to understand what the limitations of the cationic polymers are which, in this case, is mainly polymer-DNA compatibility. This experiment utilized peptide-dextran hybrid polymers with differing functionalizations to condense anionic nucleic acids into nanometer-sized polyplexes. Techniques of dynamic light scattering and zeta-potential were utilized to determine the particle sizes and surface charges of polyplexes.

Results and Conclusions

In this experiment, dextran with a molecular weight of 20 kDa was used. The dextran was then functionalized in four combinations: R_3H_3C or R_5H_5C conjugations each with and without CB-functionality. Additionally, N/P ratios of 0, 1, 5, 10, 20, and 30 were tested for each combination. The results, quantified in Tables 1 to 4, and summarized in Figure 10 and Figure 11 near the end of this document, indicate dextran polymer compatibility with DNA improves with the addition of CB-functionality, using the larger R_5H_5C peptide over R_3H_3C, and increasing N/P ratios.

Implications and Recommendations

This project could potentially be used in future gene therapy projects as we have taken steps towards understanding what tweaks can be made to improve nucleic acids interactions with cationic polymers. We have also gained experience in properly using lab equipment, a much better insight into the difficulties of synthesizing materials, especially when working with extremely small quantities, and we have observed the detrimental effects time constraints have on data quality. In light of knowledge gained from dealing with the difficulties

encountered, it is recommended that this experiment be repeated under less time pressure to obtain better data.

In the future, we recommend trying different molecular weights of dextran in addition to varying the degrees of CB-substitution, and possibly using a greater variety of peptides (i.e. lysine in place of arginine). Performing titrations are also highly recommended as the zeta-potentials are highly impacted by pH.

Advice to Students

My advice to students looking to undertake similar work is to be aware of the difficulties of dealing with extremely small quantities of reagents, especially regarding the sensitivity of the results. Additionally, expect to repeat synthesis steps for it is rare for things to go right on the first try. A good rule-of-thumb is to take the estimated time required and multiply it by three.

1. Introduction

The purpose of this project is to develop a better understanding of nucleic acid interactions with cationic polymers. The theory driving this project is that the polymers could possibly act as a "bodyguard" by protecting the nucleic acids from degradation caused by biological enzymes. Therefore, it is crucial to understand the limitations of the cationic polymers under various physiological conditions (i.e. the amount of DNA the polymers are capable of holding, the tenacity that the polymer hangs on to the DNA, polymer-DNA compatibility, etc.).

This experiment utilizes cationic (positively charged) polymers to condense anionic (negatively charged) nucleic acids into nanometer-sized polyplexes. In order to investigate the interactions of the nucleic acids with the polymer, we utilize techniques of dynamic light scattering to determine the particle sizes and surface charges of polyplexes in addition to agarose gel electrophoresis to determine the particle sizes, surface charges, and the binding strength of the polyplexes formed under different physiological pH conditions.

This project has the potential to be utilized in future projects regarding gene therapy as the cationic polymers promote nucleic acids to enter cells. Such applications are of significance due to the proven ability of gene therapy to cure hereditary diseases, such as SCID-X1[1], and some forms of cancer. Utilizing the polymers as a non-viral vector for gene therapy, the costs and risks could potentially be lowered thereby increasing the accessibility of gene therapy. By undertaking this project, we hope to determine the possibility and feasibility of such outcomes with a better understanding of how nucleic acids interact with cationic polymers.

2. Background Information

By definition, gene therapy is "the transfer of new genetic material to the cells of an individual in order to produce a therapeutic effect".[1] On paper, gene therapy is rather simple and can be summed up as manipulating deoxyribonucleic acid (DNA) in the genome using

vector carriers, analogous to replacing defective parts on a piece of machinery where the DNA is the part, the genome is the machinery, and the vector is the tool that gets the job done. However, the reality of gene therapy was discovered to be much more complicated in practice.

Such evidence demonstrating the complexity of gene therapy was observed in the only documented gene therapy success in humans that treated children with severe combined immunodeficiency disease (SCID), or more specifically, the SCID-X1 form. Children with SCID are required to be isolated in a sterile environment because they will receive fatal infections otherwise, hence their nickname of "bubble babies". This is due to inheriting a faulty gene that encodes the γc cytokine receptor that is essential for the immune system to function properly. The cure involved incubating bone marrow cells with a viral vector that replaced the defective γc cytokine receptor gene with a functional one then returning the incubated bone marrow cells into the body. While this treatment restored the function of their immune systems, however, two of the children developed leukemia resulting from of this treatment. Mishaps such as these occur due to the use of viral vectors that indiscriminately integrate the genetic material into the genome.[1]

As mentioned previously, there are plenty of issues confronting gene therapy. One of the largest issues is that the "surgical strike" is rather elusive and is rarely clean and effective as the theory suggests.[2] In addition to precision, the success of gene therapy also depends on safe and efficient gene delivery systems that offer: DNA protection, cellular uptake, endosomal escape, and low toxicity.[3] Of the myriad of delivery systems available, all of them can be narrowed down into two groups: viral vectors and non-viral vectors,[3] each with their advantages and disadvantages.

The main problem with non-viral vectors is lower efficiency in comparison to viral vectors, but they are usually safer because they avoid problems associated with viruses such as immunogenicity, "insertional mutagenesis", which is what occurred with two of the SCID-

X1 patients to cause development of T-cell acute lymphoblastic leukemia as determined by U.S. and French investigations,[2] and complexity of production.[3] On the other hand, non-viral vectors, which can induce high gene transfection in vitro, are typically comprised of synthetic materials such as cationic lipids, polymers, and dendrimers. As a result, non-viral vectors usually suffer from additional problems of toxicity, non-biodegradability, and often exhibit poor biocompatibility when used in vivo.[3]

In light of problems faced by non-viral vectors, previous research indicates that a dextran-peptide hybrid polymer may be a suitable non-viral vector in the future for a safe and efficient gene therapy delivery system.[3] Considering the historical applications of dextran by itself, it should come as no surprise that dextran would be suitable for gene therapy.

For example, one of the best-known historical applications of dextran was as a blood plasma volume expander during the Korean War. In 1952, dextran was approved by the Surgeon General for use on battle casualties in the Korean War and it was here that dextran was first administered intravenously as a substitute for blood plasma and became credited with saving thousands of American lives during the war.[4] Prior to this, it was pioneered by the Swedish in an investigation during 1944 and 1945. Shortly afterwards, a 6% solution of a dextran fraction was approved for clinical use in Sweden in 1947[5] and an estimated 20,000 units had been administered to Swedish patients by 1949.[6] Also, in addition to being used as a blood plasma volume expander, dextran was also used as an anti-coagulant which came about as a result of observations during blood plasma substitution trials where dextran was determined to be the cause for a bleeding tendency upon its administration. From this observation, dextran was used as a means to prevent arterial and venous thrombosis.[7]

3. Materials and methods

3.1. Materials

N,N-Dimethylglycine ethyl ester (DMGEE, CAS 33229-89-9, M_W=131.17, ρ=0.93) manufactured by Tokyo Chemical Industry Co., LTD. (6-15-9 Toshima, Kita-Ku, Tokyo, Japan).

Sodium hydroxide (NaOH, CAS 1310-73-2, M_W=40.00, bp=1,390°C, mp=318°C, ρ=2.1300) manufactured by Sigma-Aldrich Co. (3050 Spruce Street, St. Louis, MO 63103 USA).

Dextran (Dex20, CAS 9004-54-0, M_R=15-25 kD) manufactured by Sigma-Aldrich Co. (3050 Spruce Street, St. Louis, MO 63103 USA).

Epichlorohydrin (ECH, CAS 106-89-8, M_W=92.53, Fp=32°C(89.6°F), bp=115-117°C, mp=-57°C, ρ=1.18) manufactured by Sigma-Aldrich Co. (3050 Spruce Street, St. Louis, MO 63103 USA).

Glycidyl methacrylate (GMA, CAS 106-91-2) manufactured by Sigma-Aldrich Co. (3050 Spruce Street, St. Louis, MO 63103 USA).

Methylsulfoxide (DMSO, CAS 67-68-5, M_W=78.14) manufactured by EMD Chemicals Inc. (480 S. Democrat Rd., Gibbstown, NJ 08027 USA).

4-Dimethylaminopyridine (DMAP, CAS 1122-58-3, M_W=122.17) manufactured by Oakwood Products Inc. (1741 Old Dunbar Rd., West Columbia, SC 29172 USA).

Hydrochloric acid (HCl, CAS 7647-01-0, 1.0M Solution) manufactured by Ward's Science Plus (5100 West Henrietta Rd., Rochester, NY 14692-9012 USA).

Spectra/Por®3 Dialysis membrane standard RC tubing (M_WCO=3.5 kD, Nominal flat width=54 mm, Diameter=34 mm, Vol/Length=9.3 mL/cm).

Water purified using a Millipore Milli-Q Direct 8 Ultrapure Water system (Billerica, MA, USA) and filtered through a 0.22 μm Millipak® filter.

The R_3H_3C and R_5H_5C peptides were synthesized by GenScript (Piscataway, NJ).

pCMV-Luc DNA was manufactured by Elim Biopharmaceuticals (Hayward, CA).

Methacrylate functionalized dextran was synthesized in a previous work in May 2013.

3.2. Synthsis of CB-functionalized dextran (Dex-CB)

Dimethylglycine ethyl ester (DMGEE) was hydrolyzed prior to its addition to dextran. DMGEE was hydrolyzed in an aqueous solution containing an equimolar amount of sodium hydroxide overnight at 50°C. The ethanol byproduct was then separated by means of vacuum evaporation using a Heidolph Hei-VAP Value "The Collegiate" rotary evaporator (roto-vap). Afterward, this dried product was dissolved in water and briefly placed under sonication prior to reacting with dextran and epichlorohydrin. This reaction was allowed to proceed over the course of two days under elevated temperature at 55°C. The CB-functionalized dextran product was then isolated by means of dialysis against water through a cellulose membrane with a 3.5k cut-off over the course of three days with manual daily water change cycles.

Upon completion of the dialysis, the product was then lyophilized (freeze-dried) by first freezing at -80°C in a VWR freezer unit prior to transferring to a Labconco FreeZone 4.5 freeze-dryer vacuum. The product remained under vacuum until all the water sublimated which took about 2-4 days.

In order to increase the degree of CB substitution, the above procedure was repeated where freeze-dried CB-functionalized dextran was used in place of pure dextran after the roto-vap step.

3.3. Synthsis of MA-functionalized dextran-CB (Dex-CB-MA)

The next segment involved adding a methacrylate (MA) group to the CB-functionalized dextran (Dex-CB) polymer backbone. This MA functional group provides the double bonds that allow the cysteine residue on the peptides to bond.

First, the CB-functionalized dextran product was placed in a 2-neck flask and completely dissolved in dimethyl sulfoxide (DMSO) under a nitrogen atmosphere at 90°C. Upon the completion of the dissolution, the solution was cooled to 33°C where 62.5 mg per 250 mg dextran of 4-Dimethylaminopyridine (DMAP) catalyst and 0.6 equivalents of glycidyl

methacrylate (GMA) were added. This reaction was then allowed to proceed under a nitrogen atmosphere for two days prior to undergoing dialysis against water.

3.4. Synthesis of dextran-peptide hybrids (Dex[-CB]-R_xH_xC)

In order for dextran to "grab" the DNA, it needs a positively charged moiety such as that provided by the arginine and histidine residues in the peptide. The first step for functionalizing dextran with a peptide (either R_3H_3C or R_5H_5C) involves dissolving the dextran polymer in water. In the next step, water and TCEP are added to the peptide and incubated for 5 minutes at room temperature. After incubation, NaOH solution is added to the water/TCEP/peptide mixture until the pH is around 6 (it is initially around 2). Afterward the water/TCEP/peptide mixture is added to the dissolved polymer, and NaOH is added until a final pH between 7.5 and 8 is reached.

3.5. Creating the dextran-peptide-DNA polyplexes

In order to determine the interactions between positively charged polymers and negatively charged DNA, the N/P ratios were varied where N is the number of moles of a positively charged peptide residue (arginine) and P is the number of moles of a negatively charged phosphate group on a DNA residue. Samples were prepared for N/P molar ratios of 0, 1, 5, 10, 20, and 30 (40 is incomplete due to time constraints).

The preparation involved creating a 10 mg per mL working solution of the polymer and diluting 1 mg per mL working solution of DNA with saline solution at a ratio of 1:4 respectively by volume. The polymer working solutions were then further diluted with a pre-calculated amount of saline solution and combined with the saline-diluted DNA working solution. These blends were allowed to sit for 20 minutes at room temperature prior to analysis.

3.6. Determining degree of substitution (DS)

Degree of substitution determination was carried out via 300MHz ^{1}H NMR. To prepare the sample for nuclear magnetic resonance (NMR) analysis, approximately 10 mg of

the freeze-dried product was dissolved in deuterium oxide (D_2O) in a capillary tube. The NMR results were then analyzed to determine degree of substitution.

NMR spectroscopy works by recording the energy difference between +1/2 and -1/2 spin states, which are directly proportional to applied magnetic field strength. The NMR spectrum is generated by irradiating a specimen with a certain frequency radiation (300 MHz for the purposes of this project) and slowing increasing the field strength. Eventually the nuclei of the molecules in question will absorb RF energy, thus creating a resonance signal that is recorded and shown in the spectra.[8]

3.7. Size and Zeta-potential measurements

To prepare the samples for zeta-sizer analysis, the polymer-DNA blends were further diluted by adding 800 μL of water to 95 μL of polymer-DNA blend to create the final analysis solution. This final analysis solution was first placed in a disposable cuvette for the Malvern ZetaSizer Nano ZS (Red badge) for obtaining particle size data. After obtaining the particle sizes, the sample was then transferred to a zeta-potential cuvette and the zeta-potentials were measured. In total, five particle size measurements and four zeta-potential measurements were obtained per N/P ratio for each polymer-DNA blend.

The Zeta-sizer measures particle size by a method called Dynamic Light Scattering (DLS). DLS works by measuring the intensity fluctuations of diffracted laser light caused by Brownian motion (motion induced via particle-particle collisions) and correlating it to particle size via the Stokes-Einstein equation. If a particle is small, the movement will be much greater than if the particle is large.[9]

Zeta-Potential is the charge that exists at the plane between the Stern layer (layer of ions bound to the surface of the particle) and the diffuse region. The Zeta-potential is determined by measuring the electrophoretic mobility and applying the Henry equation. The electrophoretic mobility is determined using Laser Doppler Velocimetry (LDV) to measure the particle velocity when a potential is applied to the system. In short, the Zeta-potential tells

us how stable the system is and it is heavily affected by pH. The system is most unstable when the Zeta-potential is zero (isoelectric point) and is considered stable when the Zeta-potential is outwith ±30 mV.[9]

4. Results and Discussion

Dextran with a molecular weight of 20 kD was first functionalized with a carboxyl (CB) group by DMGEE hydrolysis to yield Dex20-CB(33%) product as determined via NMR analysis shown in Figure 1. This Dex20-CB(33%) product was further functionalized with MA by transesterification of GMA to yield Dex20-CB(46%)-MA(99%) product as determined by NMR analysis shown in Figure 2. The reaction schematic for these steps can be seen in Scheme 1.

Upon review of lab notes, it was found that the reason for the excessive degree of MA substitution was due to a calculation mistake resulting in the addition of GMA to Dex-CB reactant at a molar ratio of 1.5:1 of GMA to dextran. Consequently, this reaction was redone using a molar ratio of 0.6:1 as originally intended. The result yielded Dex20-CB(35%)-MA(90%) as determined by NMR analysis shown in Figure 3. The reason for the repeat of "impossibly high" DS was due to improper pipette usage – specifically, pushing the plunger all the way down during the draw step resulting in a larger-than-indicated draw – discovered later on when preparing the zeta-sizer solutions for Dex20-CB(35%)-MA(90%)-R_5H_5(80%).

Higher DS CB-functionalized dextran synthesis yielded a product with a DS no higher than the Dex20-CB(33%) reactant (NMR not shown), so the procedure for increasing CB DS was re-done. The re-done synthesis ended up yielding a lower DS of 25% as determined by NMR analysis shown in Figure 4. Excessive NaOH addition is suspected for this enigmatic result as the NaOH facilitates CB fall-out.

Given the difficulties encountered up to this point and considering deadlines, MA-functionalized dextran from a previous study was utilized in order to save time by eliminating

the steps required for the synthesis thereof. An NMR analysis was performed on this compound and shows that the compound is Dex20-MA(33%) as can be seen in Figure 5.

R_3H_3C and R_5H_5C proteins were conjugated to the Dex20-CB(35%)-MA(90%) and Dex20-MA(33%) polymers. The NMR analysis for the resulting dextran-peptide hybrids can be seen in Figure 6, 7, 8, and 9. The NMR analysis suggests final products of Dex20-CB(33%)-MA(90%)-R_3H_3(90%), Dex20-CB(35%)-MA(90%)-R_5H_5(80%), Dex20-MA(40%)-R_3H_3(40%), and Dex20-MA(20%)-R_5H_5(40%) respectively.

After running the NMR analysis, the peptide-functionalized dextran hybrids were conjugated with DNA at N/P ratios (molar ratio of peptide arginine to DNA phosphate) of 0, 1, 5, 10, 20, and 30. Each sample was run in the Zeta-sizer at a temperature of 25°C and at physiological pH around 8.0. The results of the Zeta-sizer analysis are shown in Figure 10 for polyplex size and Figure 11 for polyplex zeta-potential. Difficulties were encountered due to small particles in the ethanol used to wash the Zeta-sizer cuvettes between samples.

Additionally, time and material constraints limited data collection. Titrations and N/P ratios of 40 were cut and measurement data for R_5H_5-functionalized polyplexes were limited to 1 repetition whereas the R_3H_3-functionalized polyplexes were measured in 3 repetitions.

5. Conclusions

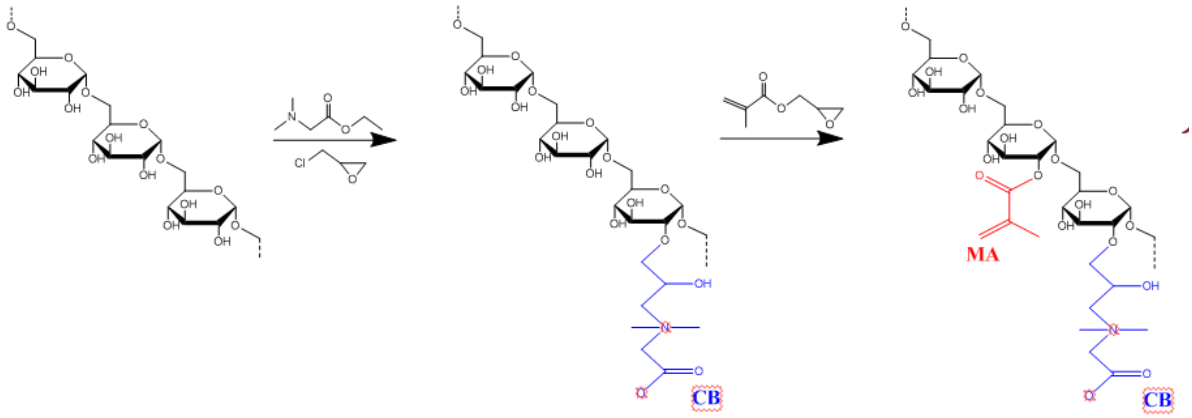
The data suggests that CB-functionalization of dextran leads to greater stability with DNA as indicated by the zeta-potential of CB-functionalized samples in addition to smaller polyplex sizes as indicated by the particle size data. Additionally, the data suggests that larger peptides (more arginine residues) also increase polyplex stability with DNA and generally decrease the polyplex sizes – the exceptions are non-CB-functionalized dextran at N/P ratios of 5, 10, and 20.

The author recommends proceeding with this study to gather information on the effects of dextran molecular weight and degrees of substitution (DMGEE, MA, and peptides). The author also recommends repeating this particular study to achieve better results as we

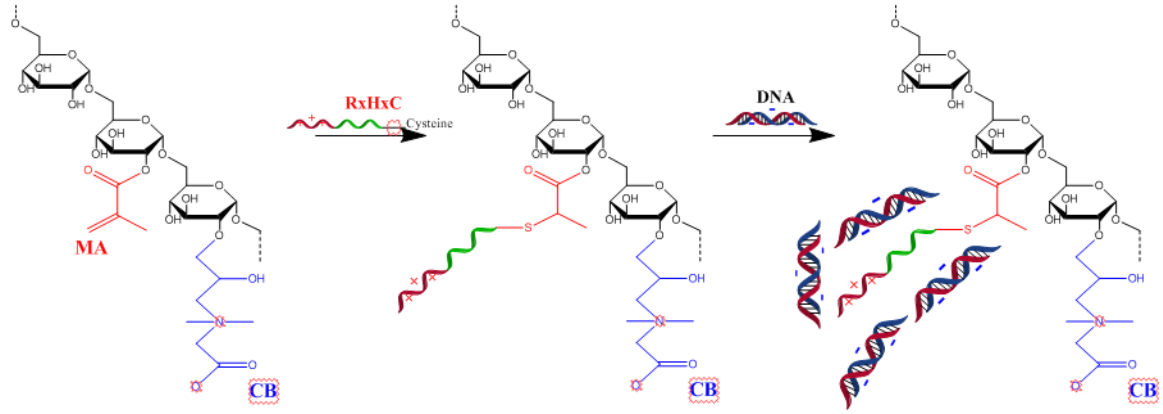
now have a better understanding of the difficulties encountered during this experiment (contaminated EtOH wash, improper pipette usage, and procedure intuition).

6. Acknowledgements

Background material research assisted by Matthew Mileusnich. Project oversight provided by Xia Lei and Dr. Gang Cheng.



Scheme 1. Synthetic route of Dex-CB-MA.



Scheme 2. Conjugation of peptide and DNA.

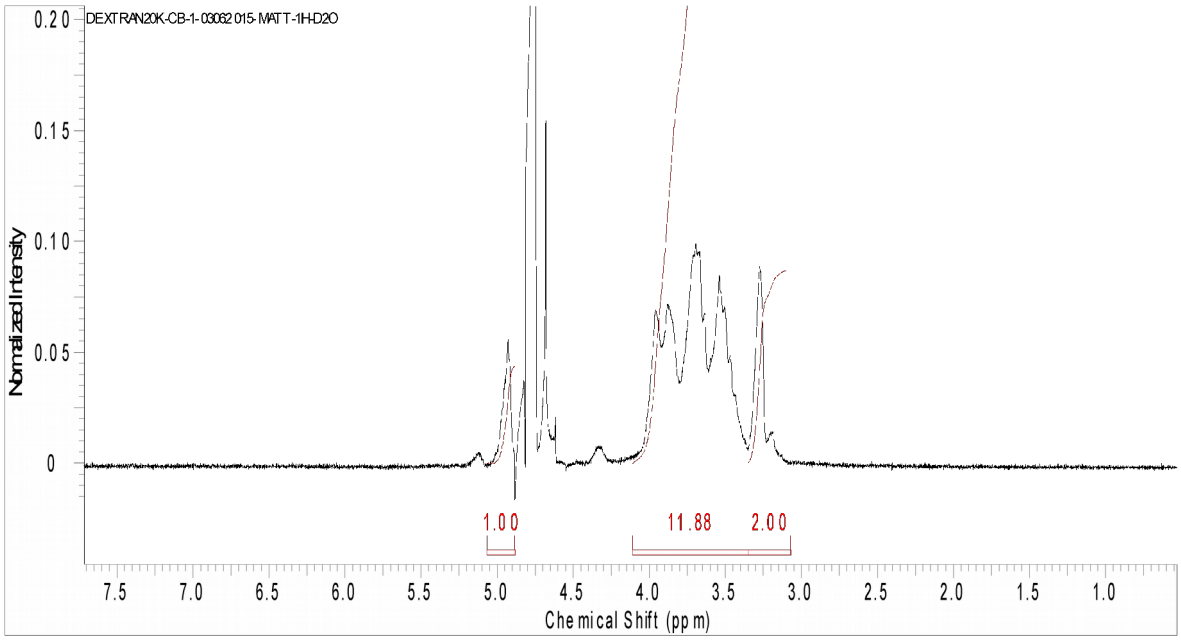


Figure 1. 300MHz $_1$H NMR spectrum of Dex20-CB(33%) in D_2O.

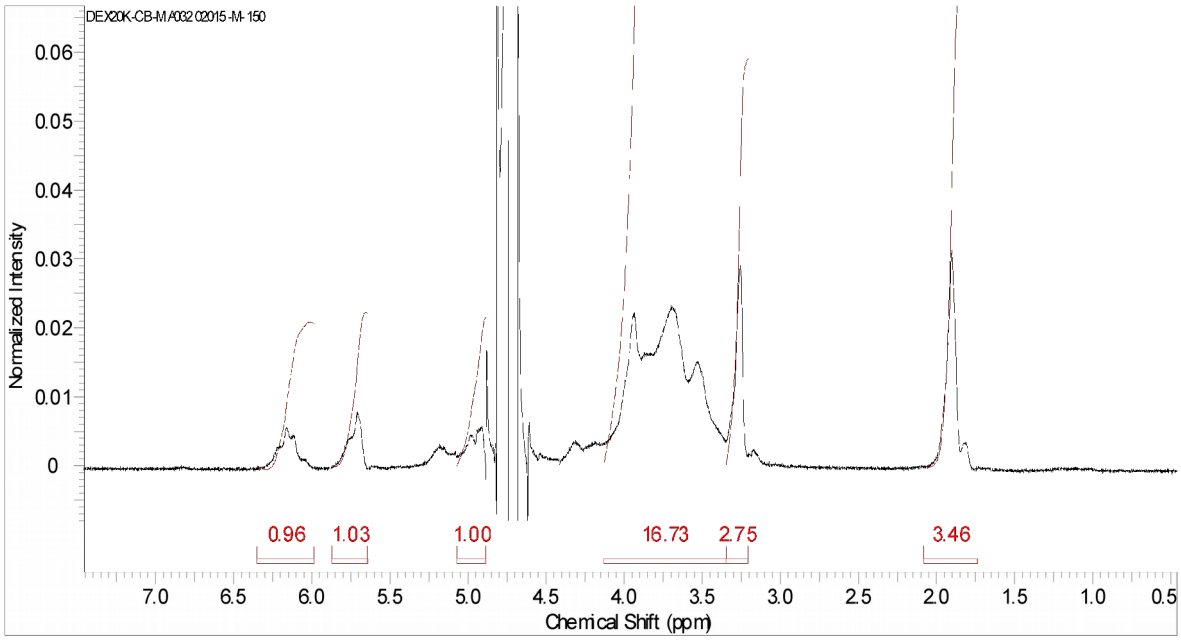


Figure 2. 300MHz $_1$H NMR spectrum of Dex20-CB(46%)-MA(99%) in D_2O.

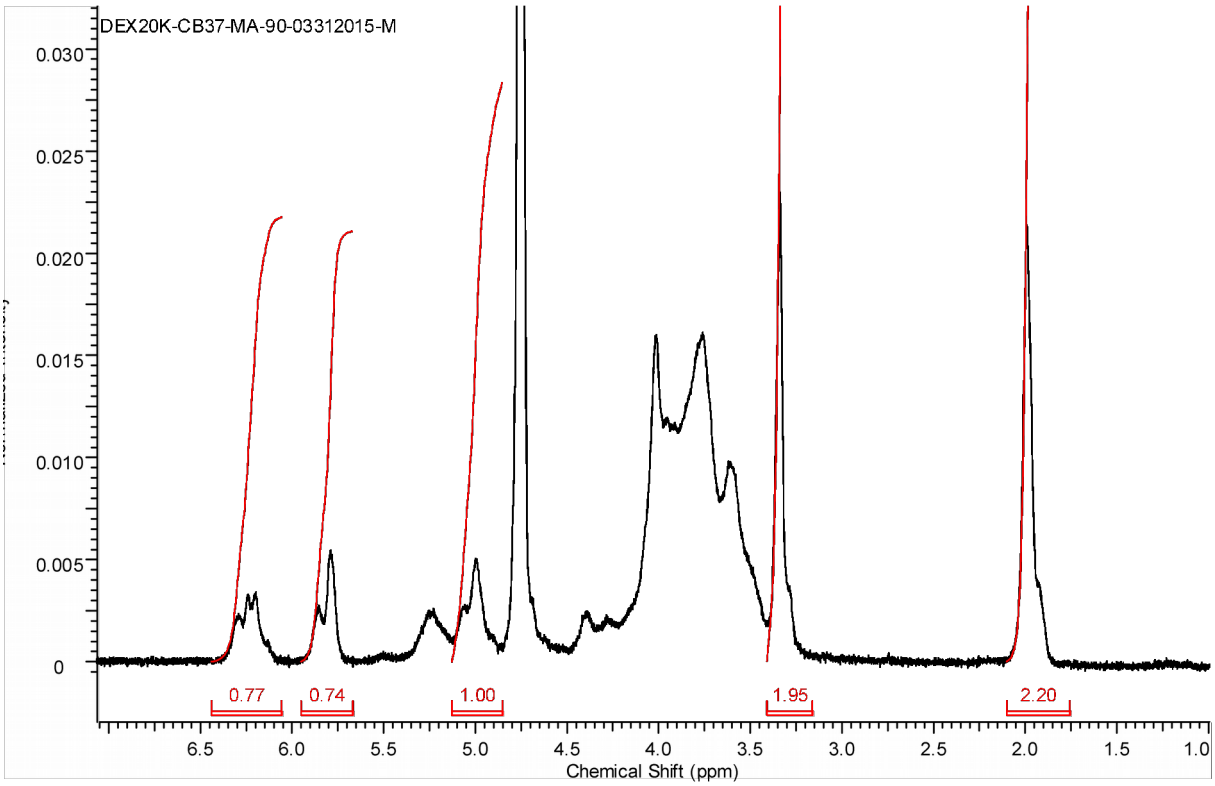


Figure 3. 300MHz $_1$H NMR spectrum of Dex20-CB(35%)-MA(90%) in D_2O.

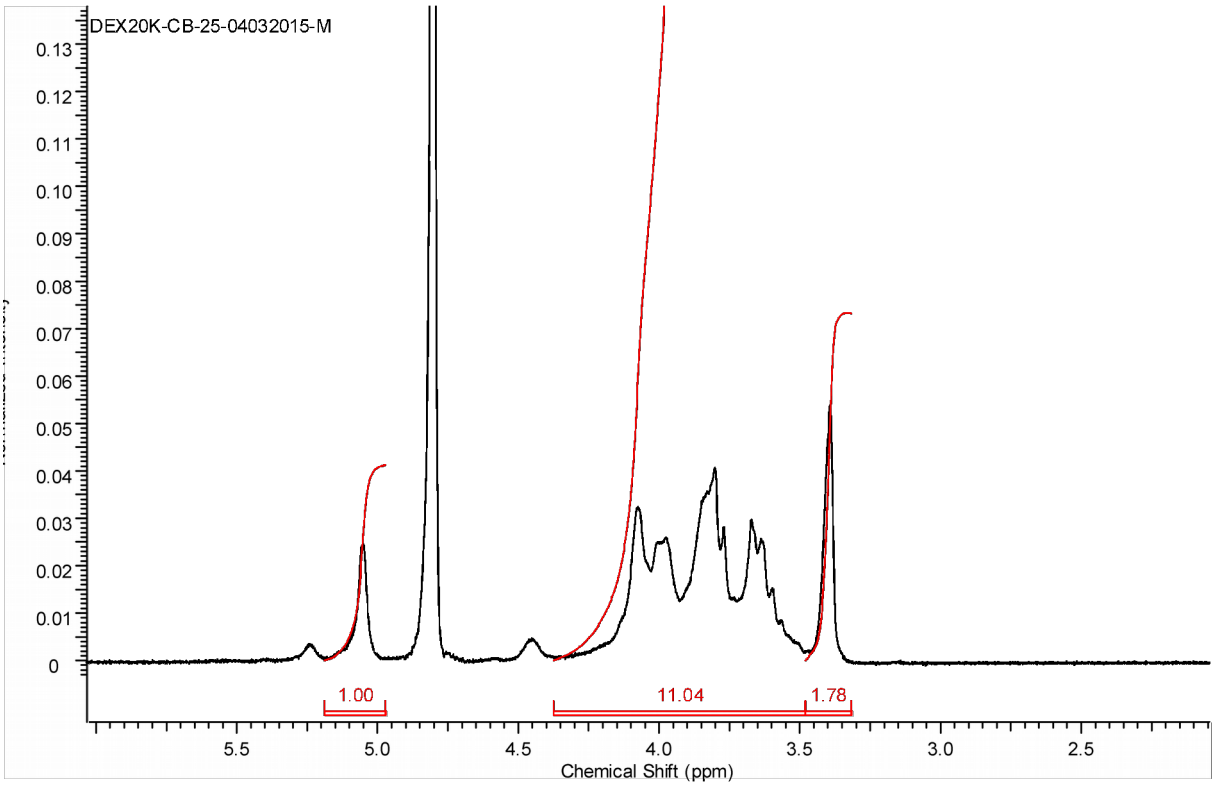


Figure 4. 300MHz $_1$H NMR spectrum of Dex20-CB(25%) in D_2O.

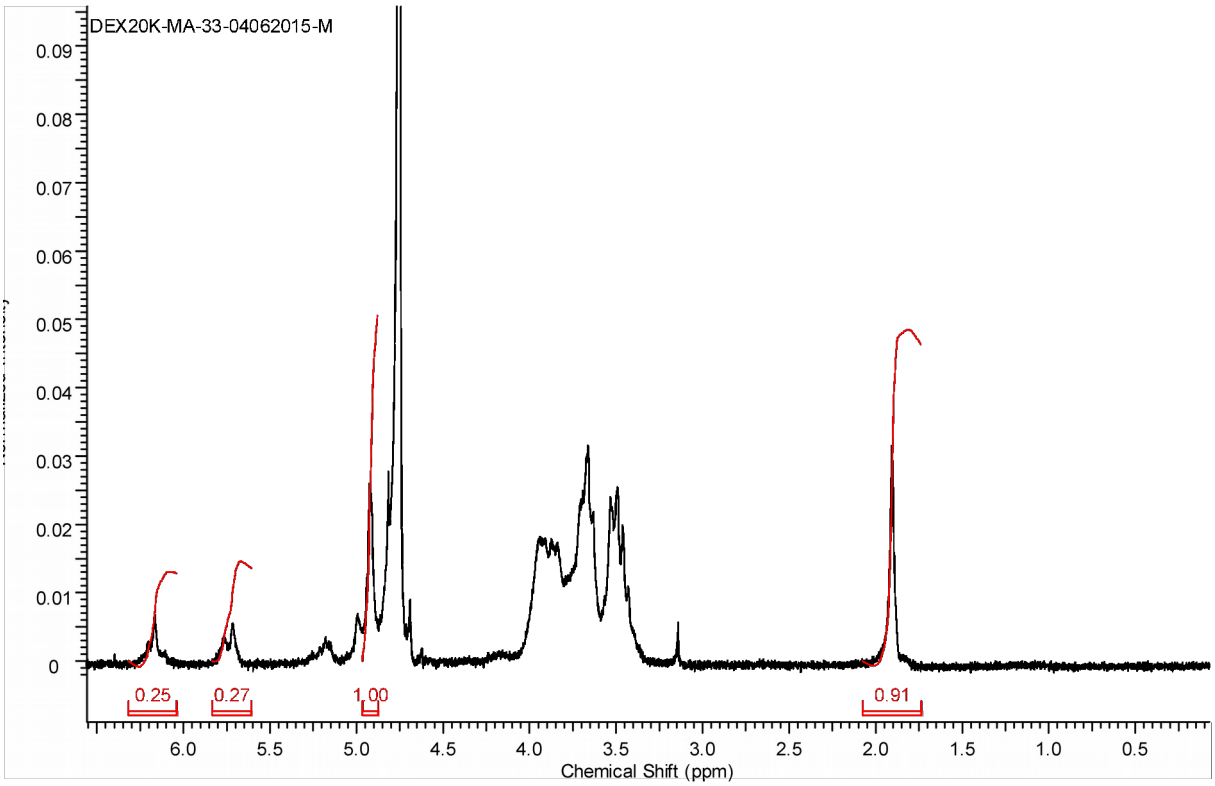


Figure 5. 300MHz $_1$H NMR spectrum of Dex20-MA(33%) in D_2O.

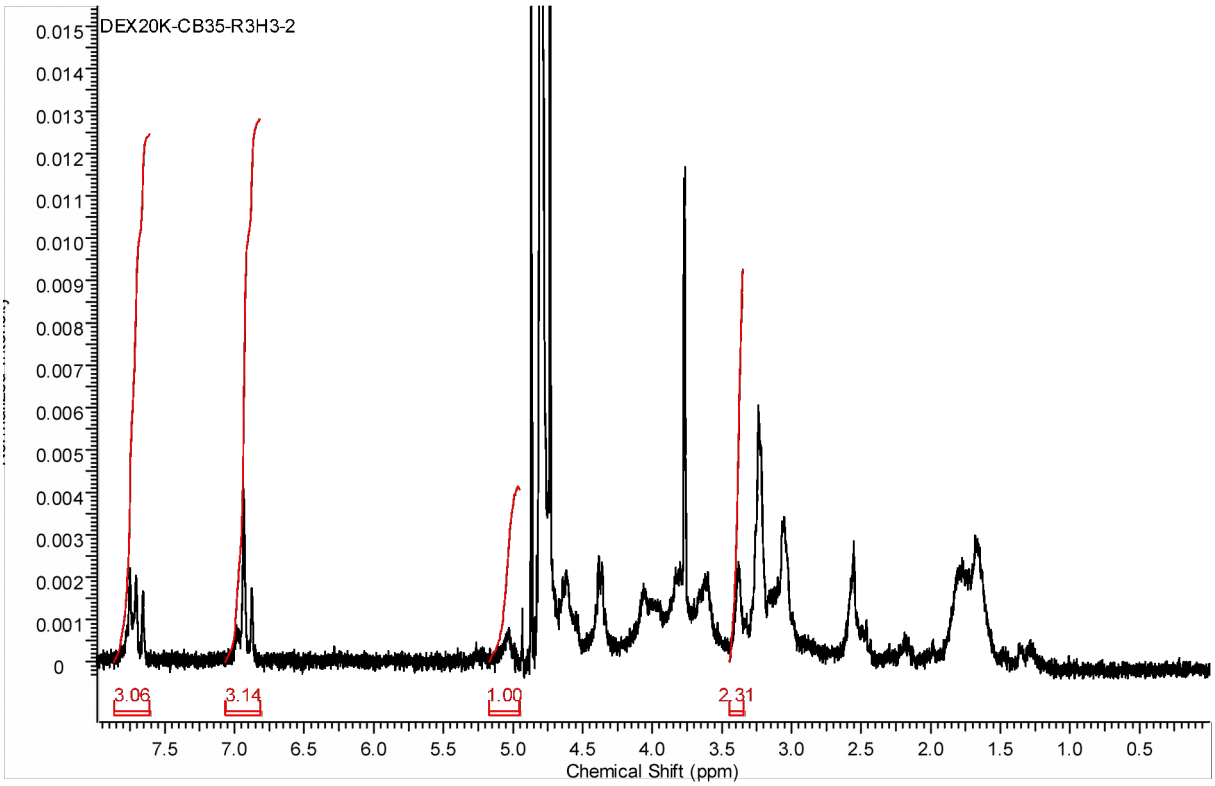


Figure 6. 300MHz $_1$H NMR spectrum of Dex20-CB(33%)-MA(90%)-R_3H_3(90%) in D_2O.

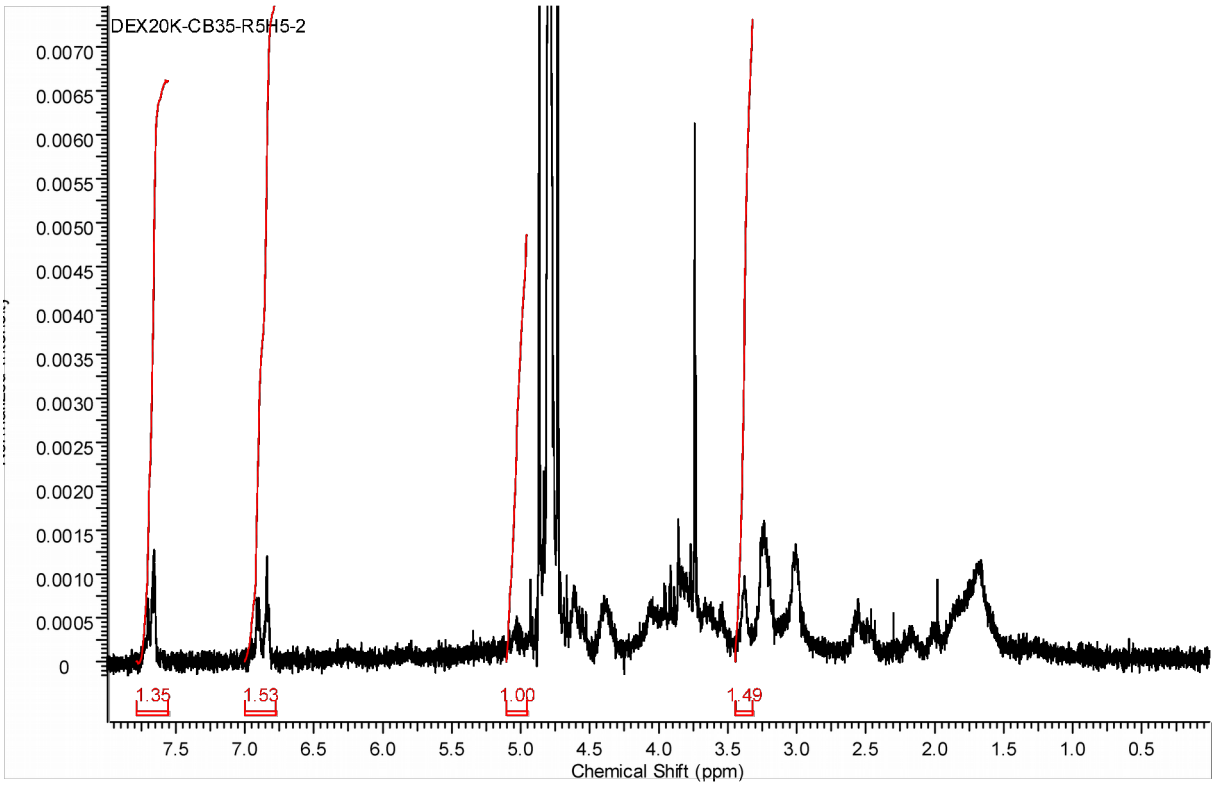


Figure 7. 300MHz $_1$H NMR spectrum of Dex20-CB(35%)-MA(90%)-R_5H_5(80%) in D_2O.

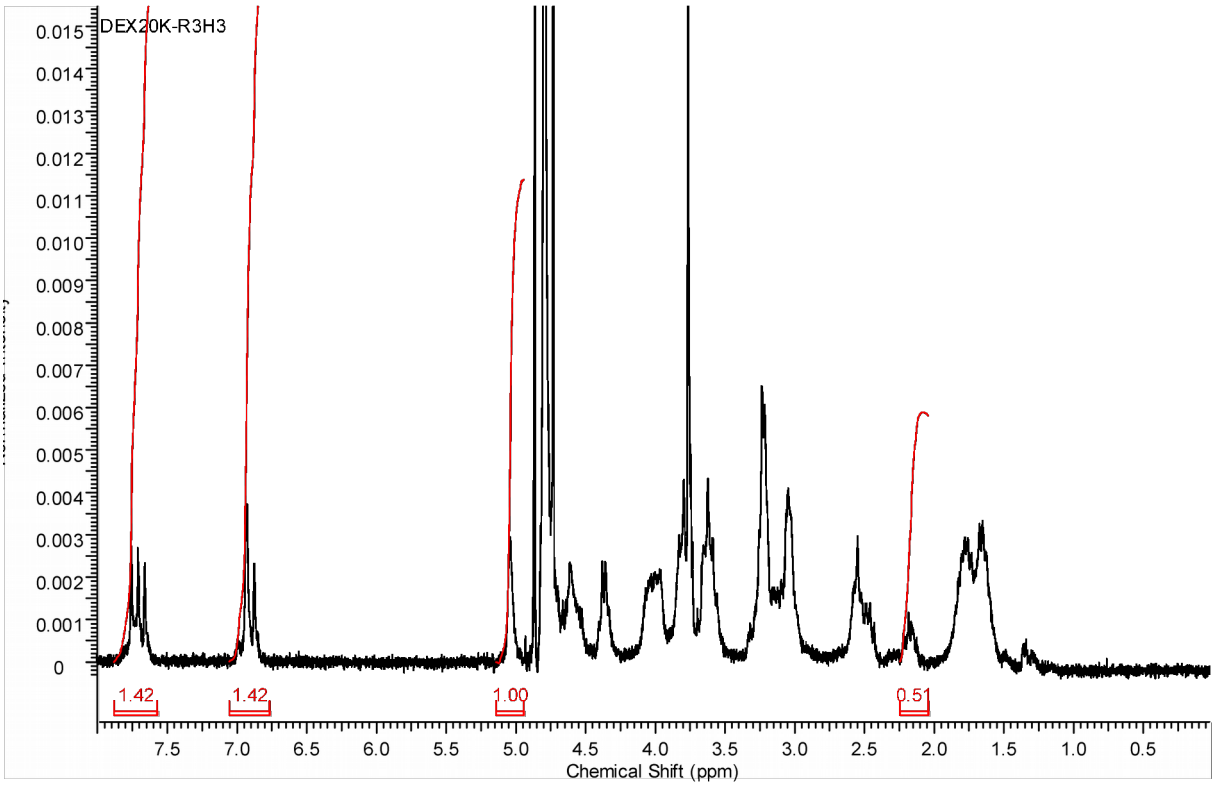


Figure 8. 300MHz $_1$H NMR spectrum of Dex20-MA(40%)-R_3H_3(40%) in D_2O.

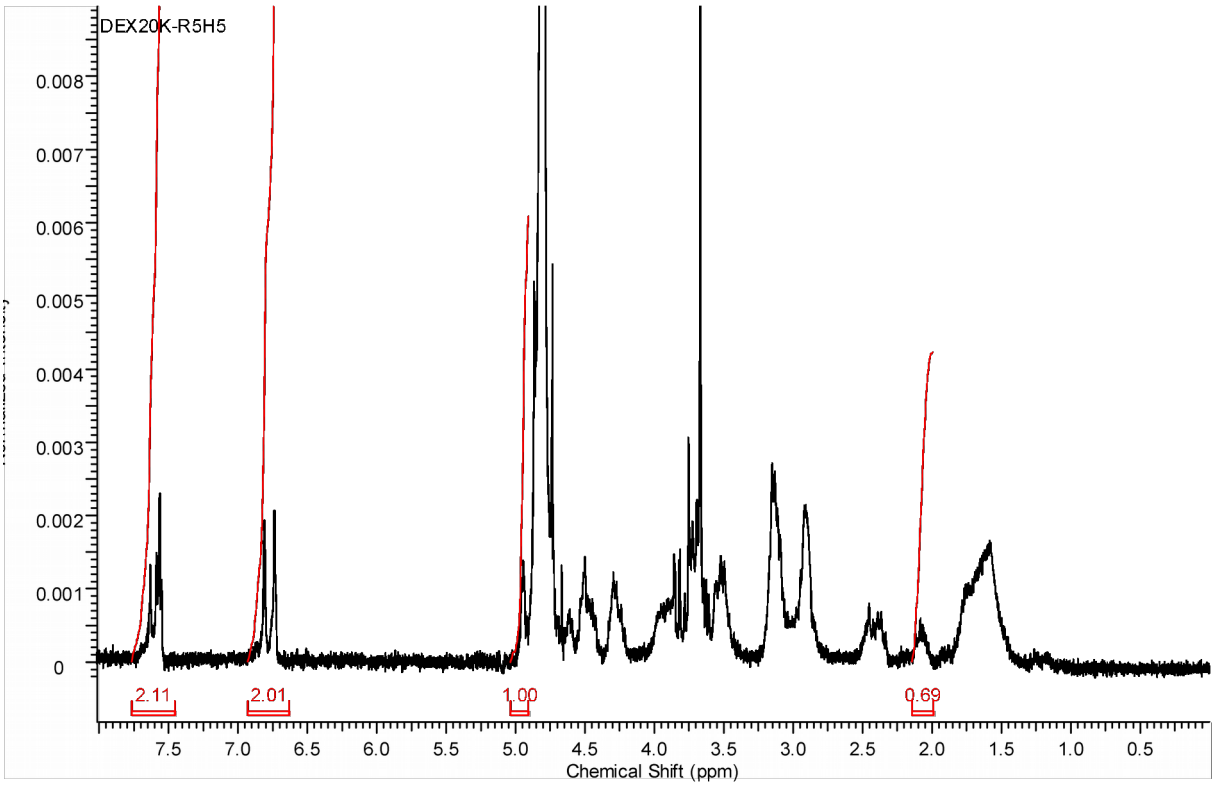


Figure 9. 300MHz $_1$H NMR spectrum of Dex20-MA(20%)-R_5H_5(40%) in D_2O.

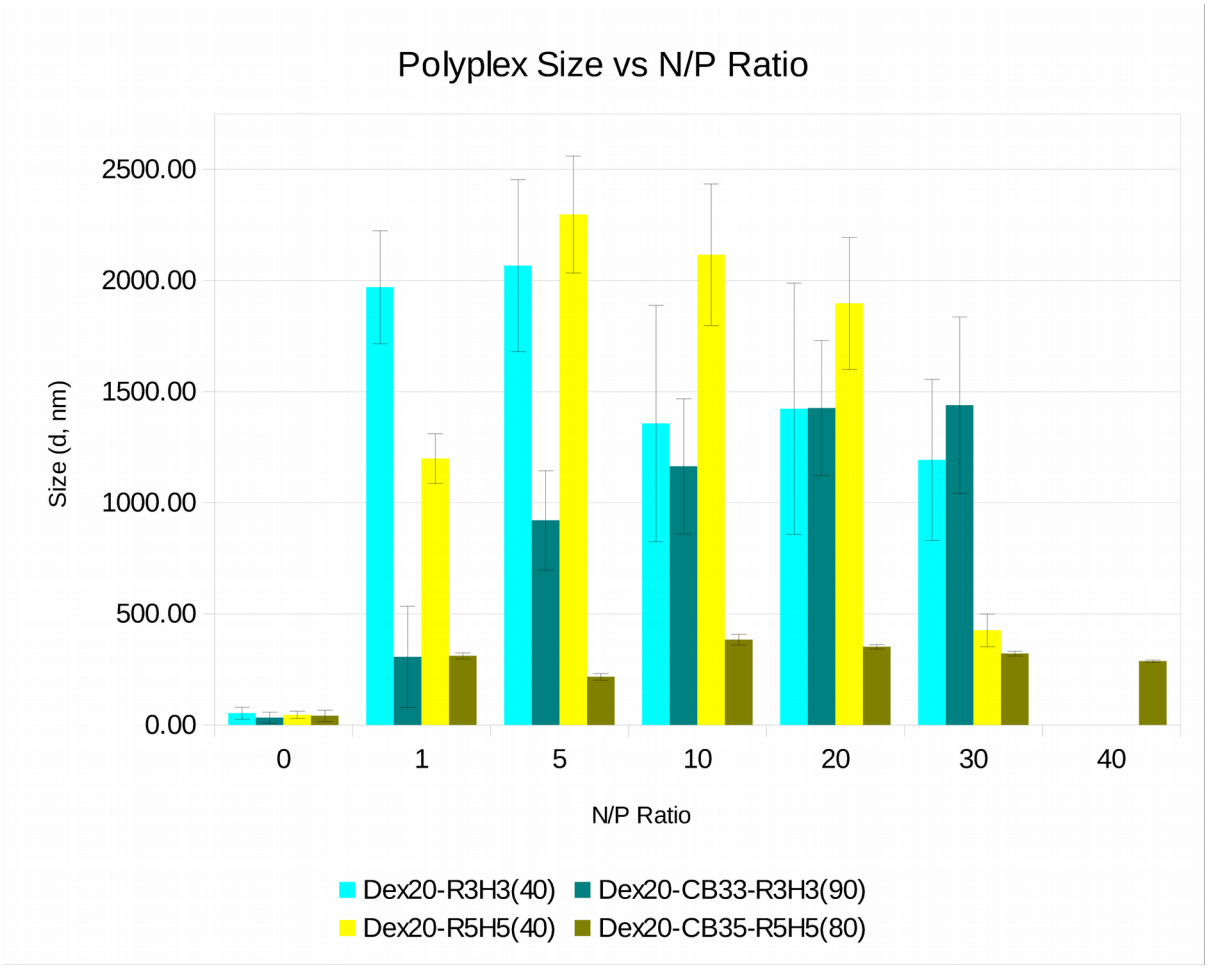


Figure 10. Zeta-Sizer data for polyplex size.

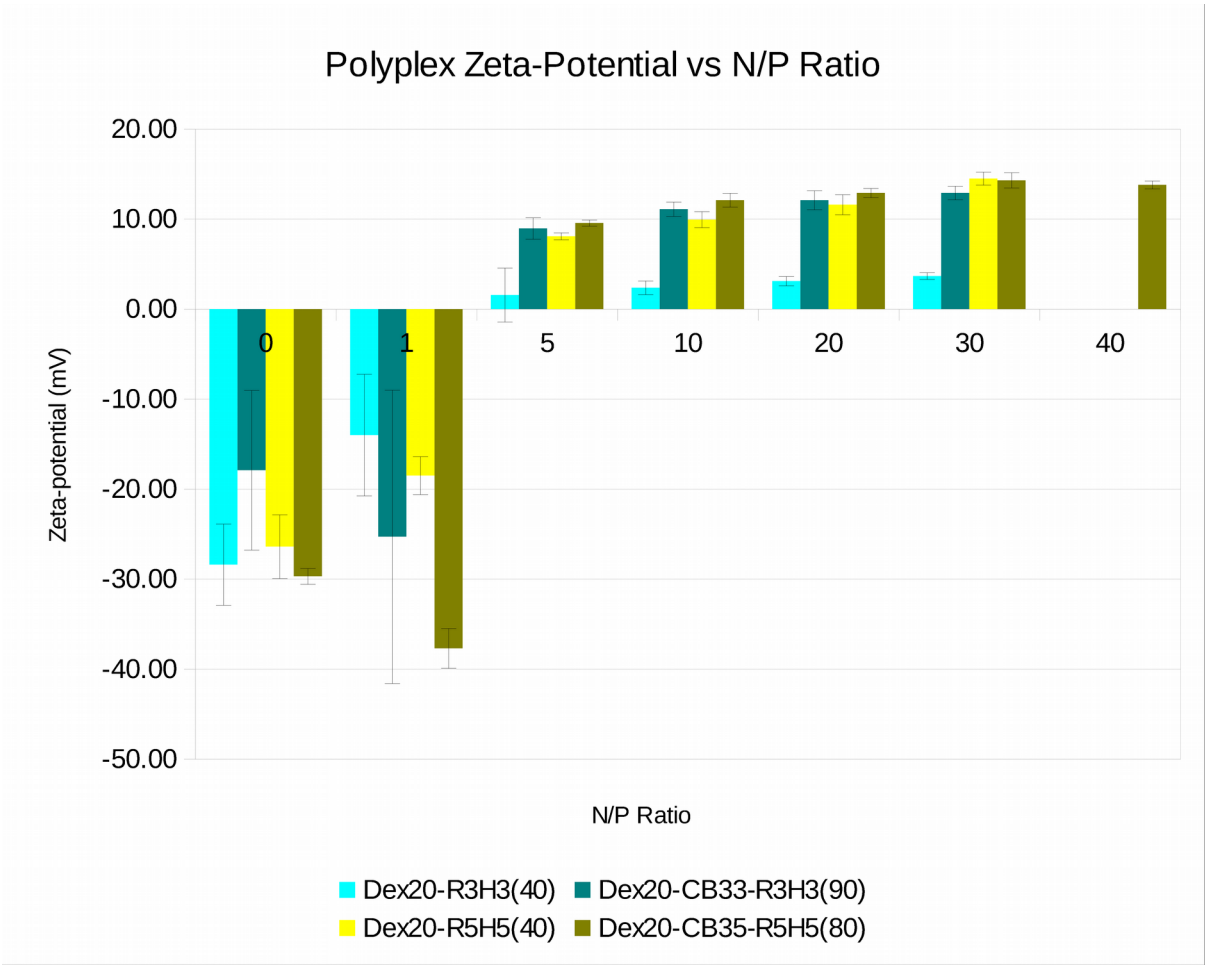


Figure 11. Zeta-Sizer data for polyplex Zeta-potential.

Dex20-R3H3(40)				
N/P	Size	StDev	Zeta Potential	StDev
0	53.29	26.60	-28.40	4.51
1	1970.00	254.20	-14.00	6.76
5	2067.00	387.20	1.57	3.00
10	1357.00	532.00	2.37	0.76
20	1423.00	565.30	3.11	0.52
30	1193.00	362.40	3.67	0.37

Table 1. Tabulated size and zeta-potential data for Dex20-R3H3(40).

Dex20-CB33-R3H3(90)				
N/P	Size	StDev	Zeta Potential	StDev
0	32.86	25.00	-17.90	8.87
1	306.60	227.40	-25.30	16.30
5	920.70	223.30	8.96	1.19
10	1164.00	304.30	11.10	0.79
20	1426.00	303.30	12.10	1.05
30	1439.00	397.10	12.90	0.75

Table 2. Tabulated size and zeta-potential data for Dex20-CB33-R3H3(90).

Dex20-R5H5(40)				
N/P	Size	StDev	Zeta Potential	StDev
0	45.35	16.76	-26.40	3.54
1	1199.00	111.80	-18.50	2.11
5	2297.00	263.20	8.09	0.38
10	2116.00	318.50	9.94	0.88
20	1897.00	296.80	11.60	1.11
30	426.10	73.71	14.50	0.72

Table 3. Tabulated size and zeta-potential data for Dex20-R5H5(40).

Dex20-CB35-R5H5(80)				
N/P	Size	StDev	Zeta Potential	StDev
0	41.58	25.49	-29.70	0.87
1	311.10	13.34	-37.70	2.21
5	216.90	14.84	9.56	0.34
10	383.70	24.57	12.10	0.77
20	352.10	10.41	12.90	0.51
30	321.50	10.90	14.30	0.85
40	287.20	4.97	13.80	0.44

Table 4. Tabulated size and zeta-potential data for Dex20-CB35-R5H5(80).

7. References

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