INVESTIGATING THE GENE-DELIVERY CAPABILITIES OF HYPERBRANCHED POLY BETA AMINO ESTERS OF DIFFERENT MOLECULAR MASS

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ABSTRACT

This study aims to determine the ideal molecular mass of the hyperbranched poly beta amino ester (HPBAE) as a non-viral gene-delivery vector for the most effective gene-delivery, with the maximum amount of genes delivered into the target organism. The objective of this study is successfully synthesising HPBAE and evaluate their effectiveness by varying their molecular mass. This is in hopes that a cure for *cystic fibrosis*, a genetic disorder, can be found via this non-viral gene-delivery vector. The HPBAE was synthesised via the Michael Addition reaction by adding tris(aminoethyl)amine to 1,3-butanediol diacrylate, with dimethyl sulfoxide (DMSO) as a solvent to vary concentrations. By varying the concentrations of both chemicals, different molecular masses of HPBAE were obtained. The HPBAE were then bound with a plasmid coding for luciferase and introduced to *C. elegans*, after which luciferin, which glows due to an oxidative reaction in the presence of the luciferase produced as a catalyst, is added. The amount of plasmid successfully delivered is measured by measuring the amount of luciferase produced by the plasmids over a period of 3 days. With the addition of luciferin, the light emitted via the reaction between luciferin and luciferase is measured to determine the amount of plasmid delivered. Hence, it can be concluded that the ideal molecular mass of HPBAE for most effective gene-delivery peaks at 1231.7g/mol as the light intensity peaked at that point before dipping.

1. INTRODUCTION

Gene therapy, which is used to cure genetic disorders like *cystic fibrosis*, could be achieved via gene delivery by using hyperbranched poly beta amino esters (HPBAE) (Ramamoorth and Narvekar, 2015). Current methods for gene delivery include viral and non-viral gene-delivery vectors. The danger in gene delivery comes when a viral vector is used to transport genes into lung cells (Ginn *et al*. 2016) as viral vectors transduce dividing cells and integrate the viral genome to the genome of host cells, thus infecting and killing the host cells (Dziaková *et al*. 2016).

The dangerous use of viral vectors in gene therapy is also used in the cure of *cystic fibrosis.* According to Milla, Carlos, Moss and Richard (2015), it is a life-shortening autosomal recessive disease caused by mutations in the gene encoding the *cystic fibrosis* transmembrane conductance regulator. This genetic disorder causes persistent lung infections and limits breathing. More than 30000 people in the US suffer from the disease, with about 1000 new cases diagnosed every year. The delivery of healthy genes to the lung cells to replace mutated genes (Milla et al. 2015) is required to cure it. Past research has shown that current treatments are either dangerous or ineffective (Milla *et al.* 2015). Effective corrective therapy and drugs containing viral vectors are not approved, while approved drugs are shown to be ineffective (Milla *et al*. 2015).

Recently, non-viral gene-delivery vectors have shown to be promising delivery agents as they have many potential benefits including ease of production, low immunogenicity and toxicity to the organism (Ledley, 1995). A promising group of positively-charged, non-viral gene-delivery vectors are the hyperbranched poly beta amino esters (HPBAE) as they have shown to be superior to the commercial well-studied reagent, Superfect, and reached levels of gene-delivery that surpasses hyperbranched polyethylenimine (Akinc *et al*, 2003) which is currently being used to treat cystic fibrosis, as it can achieve ultrahigh gene transfection efficiency.

According to Cutlar *et al.* (2015), a HPBAE is a safe and efficient non-viral vector shown to be a possible gene-delivery alternative that has robust gene transfection efficiency, which makes the HPBAE superior to its counterparts (Cutlar *et al*. 2015). Furthermore, HPBAE can be easily synthesized via the Michael addition reaction.

HPBAE can be widely applied to treat cystic fibrosis (Davies, Geddes and Alton, 2001) by introducing the CFTR gene into the patient's lung cells. Its effectiveness is seen through the research conducted by Patel *et al.* (2019) where the HPBAE of 150 nm was tangled with the plasmid that contains luciferase, which was used as a substitute for the CTFR gene. This plasmid-bound HPBAE was then delivered to mice as an inhalable mist, and the luciferase reporter assay suggested that the gene delivery was successful, efficient and effective as light was emitted through the reaction with luciferin (Naylor, 1999).

The research of HPBAE and their gene-delivery capabilities is an uncommon field of research. This study aims to determine the optimal molecular mass of the HPBAE for it to function most efficiently and effectively as a non-viral gene-delivery vector.

2. OBJECTIVES AND HYPOTHESES

This study aims to:

- Investigate the molecular mass of the hyperbranched poly beta amino esters with regards to the reaction time before the end-cap amine is added.
- Investigate the uptake of the plasmid of the *Caenorhabditis elegans* with different molecular masses of hyperbranched poly beta amino esters as vectors.

Hypotheses

1. HPBAE can be successfully synthesised with tris(2-aminoethyl)amine and 1,3-butanediol diacrylate, with dimethyl sulfoxide (DMSO) as a solvent.
2. As the concentration of the reactants increases, the molecular mass of HPBAE formed increases.
3. Larger HPBAEs will be more effective gene-deliverers, delivering an increased amount of gene into an organism.

3. MATERIALS AND METHODS

3.1 Materials

The starting reagents tris(2-aminoethyl)amine, 1,3-butanediol diacrylate and luciferin were procured from Sigma Aldrich, while pSLGCV plasmid was purchased from Addgene and *Escherichia coli (OP50)*, *Caenorhabditis elegans* and DMSO were obtained from the school Biology lab.

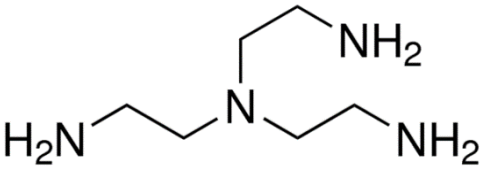

Figure 1: Molecular structure of tris(2-aminoethyl)amine

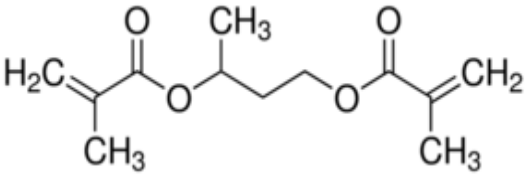

Figure 2: Molecular structure of 1,3-butanediol diacrylate

3.2 Methodology

3.2.1. Synthesis of HPBAE

In Figure 3, 5 samples of HPBAE with different concentrations of tris(2-aminoethyl)amine and 1,3-butanediol diacrylate, with dimethyl sulfoxide as an organic solvent, were synthesised via the Michael A2B2BB'2 Reaction so as to obtain different molecular masses of

Sample	Tris(2-aminoethyl)amine/µl	1,3-butanediol diacrylate/µl	DMSO/µl
A	500	500	500
B	500	600	500
C	600	500	500
D	600	600	500
E	700	700	500

Figure 3: Concentrations of starting reagents in the synthesis of HPBAE

HPBAE. Within 5 minutes, the reactants formed a gel-like solid. The 5 solid samples underwent FTIR and mass spectrometry to determine the different molecular masses of HPBAE formed by the differing concentrations. The solids had to be melted down in a water bath at less than 100°C as the plasmid was unable to bind to the solid HPBAE and introduced to the *C. elegans*. Plasmid purchased from Addgene coding for the protein luciferase was then added and bound to the liquid HPBAE.

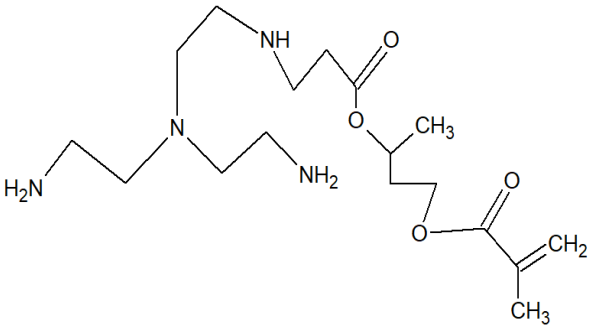

Figure 4: Bonding between ONE amine and ONE acrylate functional group

HPBAE is synthesized from the 2 starting reagents, 1,3-butanediol diacrylate and the tris(2-aminoethyl)amine. As shown in Figure 4, the C double-bond from the acrylate group breaks up to form a C-C bond between the 2 functional groups. This is known as the Michael Addition reaction, which is the nucleophilic addition of a carbanion. In this case, the amino group acts as the electron donor, resulting the structure reducing from NH_2 to NH. Figure 4 depicts the reaction between one amino and acrylate group. Since all amino and acrylate groups are able to bond with each other, it will result in a chain of molecules bound to one another in a hyperbranched structure, and the resultant polymer formed is known as the HPBAE. This study aims to vary the concentrations of starting reagents to form different molecular masses of HPBAE.

3.2.2. Introduction of plasmid-bound HPBAE to *C. elegans*

5 plates of *C. elegans* were cultured in agar plates with *E. coli (OP50)* as food for each of the 5 samples of HPBAE. 4.00 µl of plasmid-bound HPBAE were then introduced to 3 regions on the corresponding plate of *C. elegans*, with another 4.00 µl of luciferin added on top of it. The luciferase produced by the plasmid catalyses

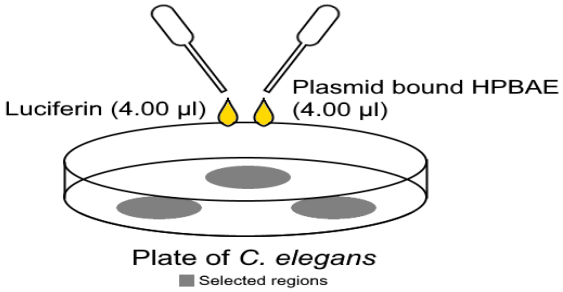

Figure 5: Illustration of introduction of plasmid-bound HPBAE to *C. elegans*

the oxidation reaction of luciferin, allowing the luciferin to produce light in the presence of luciferase. The successful delivery and uptake of plasmid will enable the *C. elegans* to produce luciferase after the plasmid has been incorporated into their cells. The presence and varied intensity of light measured from the *C. elegans* will show the success and extent of plasmid uptake by the *C. elegans* as it represents the amount of luciferase present to catalyse the reaction of luciferin. However, the luciferin is found to emit light without reaction, hence control set-ups with only 4.00 µl of luciferin added were necessary. The difference in light intensity between the set-ups and control will determine the extent of plasmid uptake of the *C. elegans*.

3.2.3. Light Intensity Measurements

To facilitate accurate measurements of light intensity, all samples including the control set-up were incubated for 3 days to allow the gene-delivery to occur before light intensity was measured. The *C. elegans* were washed off the agar plate by adding 1.00 ml of amine broth to the plate, removing it after shaking and then centrifuging it twice, with the supernatant removed after each centrifuge, leaving a pellet of *C. elegans* at the bottom of the centrifuge tube.

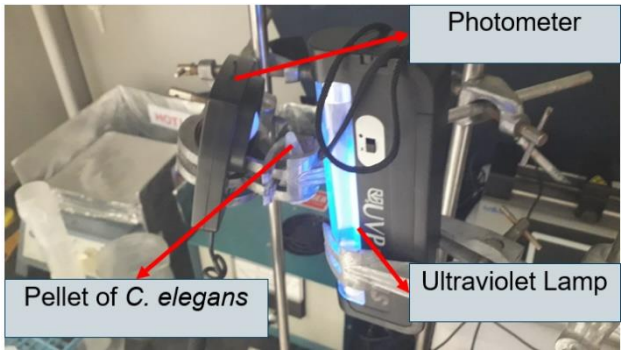

Figure 6: Light intensity measurement set-up

Ultraviolet light of wavelength 350 nm was shone at the pellet to bring out the glow of the *C. elegans* and a photometer was used to measure the respective light intensities of the samples in a dark location. Extra care was taken to ensure the photometer did not detect any ultraviolet light or light from any other light source, thus ensuring there would not be any abnormality in the readings. Data collected underwent the Mann-Whitney U-Test to check for significant differences against the control, to determine the success and extent of plasmid uptake by the *C. elegans*. The samples of HPBAE of different molecular masses were then compared against one another to determine the ideal molecular mass and concentration of reactants of HPBAE for maximum gene-delivery efficiency.

4. RESULTS AND CHARACTERISATION

4.1. Fourier Transform Infra-red Spectrometry (FTIR)

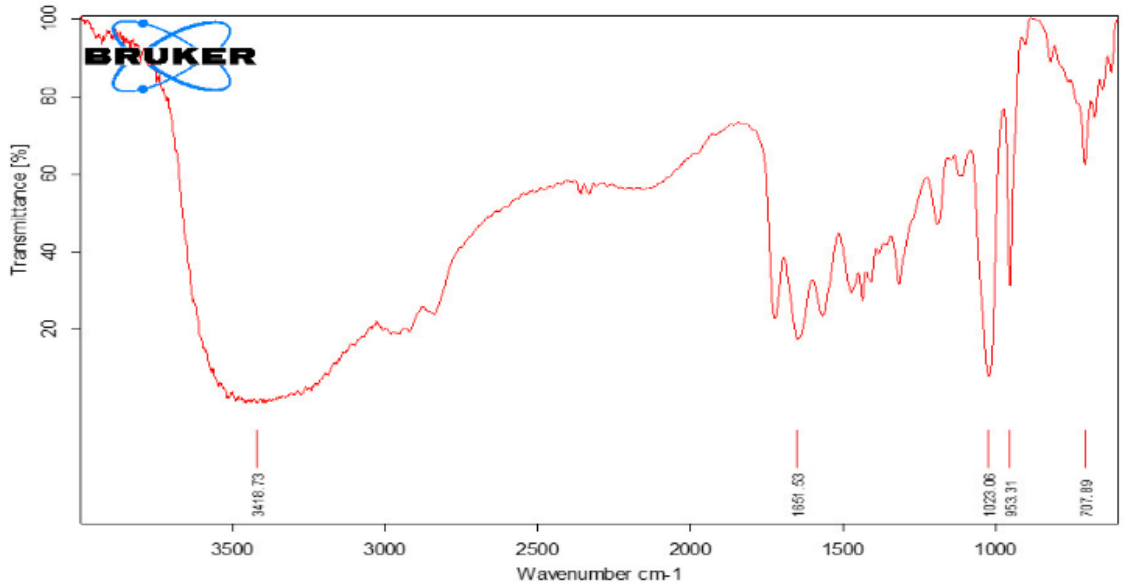

Figure 7: Fourier Transform Infra-red spectrum of HPBAE

To test whether the samples of HPBAE have been successfully synthesised, FTIR was used to scan for the presence or absence of the amino and acrylate functional groups used in the reaction.

According to Figure 7, the HPBAE is expected to have the ester and amino functional groups, with the initial acrylate group being completely reacted and hence absent in the final result. According to the IR Table from Sigma Aldrich, there are multiple distinct peaks on Figure 7, of which the peak at 3418.73 corresponds to the amino functional group and the peak at 1651.53 corresponds to the ester functional group, which is a result from the successful reaction. The peak of the acrylate functional group, however, is not detected and thus it is concluded that the acrylate peak has fully reacted with the amino group to form the ester group, leaving only amino groups in excess, suggesting the successful synthesis of the hyperbranched poly beta amino ester.

4.2. Mass Spectrometry

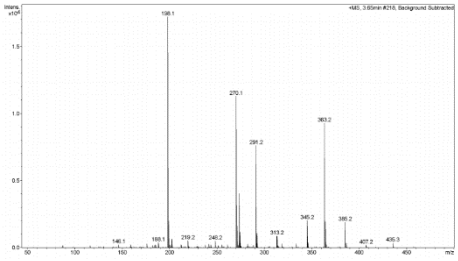

Figure 7: Mass spectrum of Sample A, right-most peak of 436.3

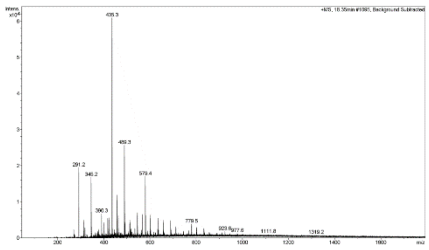

Figure 8: Mass spectrum of Sample B, right-most peak of 1319.2

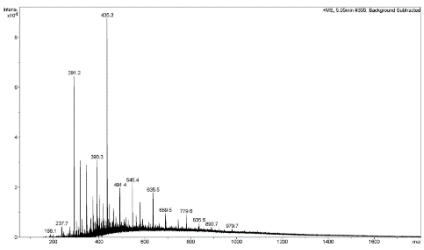

Figure 9: Mass spectrum of Sample C, right-most peak of 979.7

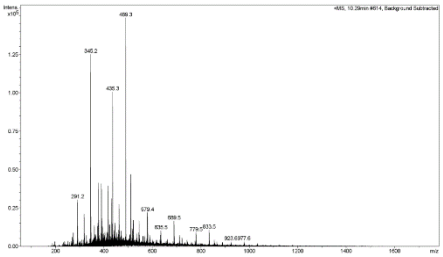

Figure 10: Mass spectrum of Sample D, right-most peak of 977.6

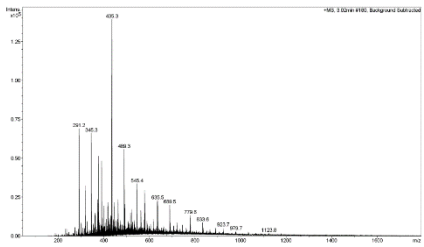

Figure 11: Mass spectrum of Sample E, right-most peak of 1123.8

Mass spectrometry had to be carried out on the samples of HPBAE to determine the respective molecular masses of polymers formed. Electrospray ionization (ESI) was used instead of electron ionization (EI) and matrix-assisted laser desoprtion/ionization (MALDI) because electrospray ionization uses a high voltage to create a liquid aerosol to fragment the molecules. ESI is better than both other methods as it produces multiple-charged ions, extending the mass spectrum to detect larger fragments of polymer present, while MALDI is unable to. EI uses highly energetic electrons to bombard the sample and is usually used to determine the individual components of a sample. As the molecular mass of the whole HPBAE, a polymer, has to be measured and not its individual components, ESI is most suitable as it is just strong enough to overcome the forces between the polymer, while not fragmenting the polymer itself.

The value of the right-most peak can be reasonably assumed as the relative molecular mass of the HPBAE because as the sample passes through the mass spectrometer, an electron is knocked off the polymers, resulting in half the readings on the spectrum to be either knocked off electrons or fragmented polymers, leaving the molecular mass of the in-tact polymers at the right-most peak.

4.3. Light Intensity Data of *C. elegans*

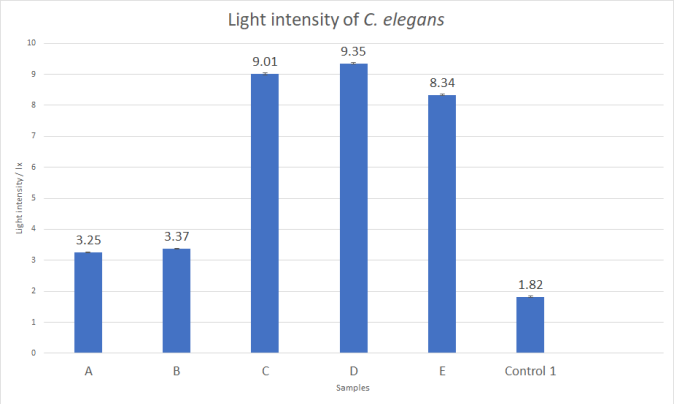

Figure 12: Light intensity of *C. elegans* with different samples of HPBAE

As shown in Figure 7, although the light intensities of Samples C, D and E are significantly greater than that of A and B, all samples show significant difference from the control set-up, suggesting that the gene-delivery was least successful for all molecular masses of HPBAE. Sample D showed the greatest light intensity, while samples A showed the least. This shows that the molecular mass of the HPBAE in Sample D is the most effective for gene-delivery. Although the molecular mass of HPBAE in D was not the highest, it proved to be the most effective gene-deliverer, which suggested that the most ideal molecular mass of HPBAE is between 977.6g/mol to 1123.8g/mol . This could be due to HPBAE of higher molecular mass being able to incorporate and carry more plasmid due to its increased size and area for the plasmid to be bound within the HPBAE. However, as the results have shown, HPBAE with molecular mass greater than 1737.0g/mol has shown to be less effective, which could be due to the HPBAE getting tangled up itself, leaving less space for the plasmid to be bound.

The light intensities highlight the effectiveness of HPBAE in gene delivery. The success rates of direct injection of a gene into a cell is extremely low. When the HPBAE was used as a gene-delivery vector, it can successfully proliferate the nuclear pore with the plasmid and the plasmid will be able to introduce their DNA into the nucleus of the infected cell (US National Library of Medicine, 2019). The mRNA of the cell will successfully be altered, changing the proteins that are being produced (US National Library of Medicine, 2019). Thus, by the varying molecular mass of HPBAE used, different amounts of plasmid will be delivered to the nuclear pore. Thus, different light intensities could be measured and confirming the light intensity emitted is proportional to the gene delivery capabilities of the hyperbranched poly beta amino ester.

5. CONCLUSION

After measuring the light intensity of the *C. elegans*, it can be concluded that all samples show that regardless of the molecular mass, the plasmid bound HPBAE is still capable of successful gene-delivery as all samples show significant difference against the control. Although the HPBAE of 977.6g/mol showed to be the most effective gene-deliverer, it was not the HPBAE with the highest molecular mass, which suggests that the effectiveness of HPBAE in gene-delivery peaks at around 977.6g/mol before steadily decreasing. As seen from Figure 13, there was a sharp increase in light intensity from HPBAE of 436.3g/mol to 977.6g/mol, where that in Sample A yielded significantly less results than that of D. The graph also showed a steady decrease after 1123.8g/mol, suggesting that any HPBAE with molecular mass lower than 436.3g/mol or above 1123.8g/mol can be deemed as significantly less efficient and effective in gene-delivery.

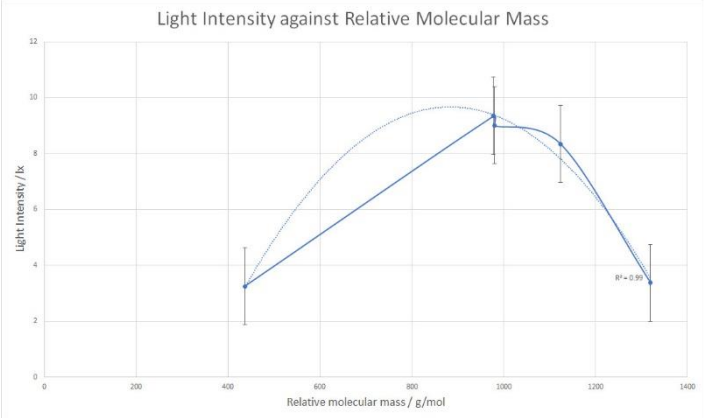

Figure 13: Graph of light intensity against relative molecular mass of HPBAE

6. FURTHER STUDIES

By using the results from this study, this study will be able to determine the sample that is most efficient in gene delivery. Complete characterisation could be done to determine the boiling point, stability and the molecular mass of the HPBAE by using different types and modes of mass spectrometry (EI/MALDI) to see which fragments the polymer the least to give the most accurate molecular mass of HPBAE. With this new information, different types of HPBAE could be synthesised with different starting reagents to investigate if they possess similar characteristics of HPBAE from this study and thereby determine the most effective starting reagents and ideal molecular mass for gene-delivery.

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