2016

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<https://doi.org/10.15760/honors.260>

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Synthesis, structure and contrast efficacy of bismuth (III) chelated with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetramethylene phosphonate and its incorporation into nano-assembled capsules

by

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An undergraduate honors thesis submitted in partial fulfillment of the

requirements for the degree of

Bachelor of Science

in

University Honors

and

Biochemistry

Thesis Adviser

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2016

ABSTRACT

Medical imaging now plays an integral role in the diagnosis and treatment of illness. The use of contrast agents in molecular imaging has been instrumental in the diagnosis of cancer and similar pathologies. The incorporation of lanthanide-based contrast agents employed in magnetic resonance imaging (MRI) into nano-assembled capsules (NACs) have shown potential utility for improved contrast in MRI by slowing the tumbling of the polyanionic paramagnetic metal ions. The utility of NACs was herein extended to computed tomography (CT) imaging by the incorporation of electron dense bismuth (III)-based chelates into NACs in order to provide a high payload of agent in relatively small volumes. Bismuth (III) was chelated with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetramethylene phosphonate (DOTP) and incorporated into a NAC encapsulated with a spherical silica nanoparticle shell at varied concentrations, solvent systems and temperature. The size distribution of these NACs was characterized and compared to gadolinium-based NACs. The formation of bismuth containing NACs was confirmed; the reproducibility in size distribution was not as reproducible as expected, and did not adhere to the same trends as lanthanide containing capsules. Although aging time may be directly related to size distribution of the capsules, the solvent system and temperature studies did not yield a consistent relationship.

INTRODUCTION

The importance of inflammation in the progression of cancer and cardiovascular disease has recently become apparent.¹ The ability to monitor how inflammation progresses *in vivo* may provide a more complete understanding of the development and treatment of these diseases. In order to accomplish this, it is necessary to employ methodologies that allow the different stages of

inflammation to be mapped. For practices such as magnetic resonance imaging (MRI), lanthanide(III) ion-based CAs have been shown to provide optimal effects on the relaxation rates of water protons in order to differentiate between tissues and aid in more meaningful diagnostics. However, lanthanide ions, such as gadolinium(III), are toxic *in vivo* and must therefore be chemically altered in order to be clinically useful.² The advantage chelates offer for the modification of xenobiotic metal ions in MRI are twofold: a reduction in toxicity and the possibility of an increase in relaxivity to provide superior contrast. A main aim in the development of contrast agents (CAs) is to design structures specifically to differentiate various inflammation types, which are characterized by the localization of macrophages that can conveniently be targeted with nano-scale particulate agents that are then endocytosed.³ Thus, the design of chelates suitable for the coordination of lanthanide ions has become a prevalent field of study, seated at a junction between inorganic coordination chemistry and organic synthesis in an overarching aim to diagnose and treat cancer.

Clinical contrast agents (CAs) for computed tomography (CT) imaging include high atomic number (Z) ions as X-ray attenuating agents; clinically they are largely comprised of small iodinated molecules such as iopamidol, iohexol and iodixanol.⁴ Although iodine is relatively electron dense and effective in X-ray attenuation, current CAs have limited utility in their capacity to target tissues.⁵ Bismuth nanoparticles (Bi-NPs) have become an increasingly attractive candidate for CT contrast agents due to low toxicity, high electron density, stability and surface modification capability.^{6,7} Synthesis of the nanoparticles published typically range from 30 to 80 nm in size, formed largely by the pH dependent reduction of bismuth(III) cations.

The comparison of two bismuth-based nano-systems with a potential utility in computed tomography imaging was herein investigated: dense and compact bismuth nanoparticles and Bi^{3+}

containing nano-assembled capsules similar to those that have been developed to enhance MRI contrast agents by slowing the anionic tumbling of lanthanide-based CAs.⁸ The NAC model has been of interest due to the ability to deliver a large payload of agent in small doses with surface modification potential. The synthetic protocol for lanthanide containing NACs has been adapted to determine whether NAC formation is possible for the incorporation of electron dense, diamagnetic Bi³⁺ complexes to provide a comparison of contrast efficacy of capsules versus nanoparticles.

Figure 1. A schematic representation of the preparation of BiDOTP⁵⁻ containing, SiO₂-NP coated NACs.

The nano-encapsulation process first involves synthesizing a polyanionic metal complex. Then, as shown in Figure 1, an aggregate of the chelate with a cationic polymer, in this case polyallylamine hydrochloride (PAH), is formed and encapsulated by a silica nanoparticle shell. The average size of gadolinium-based NACs was found to increase proportionally with the ratio of the concentration of charges on the anionic complex and cationic polymer; a term expressed as the *R* value (Eqn 1).

$$R = \frac{[chelate] \times Z^-}{[polymer] \times Z^+} \quad (1)$$

As the value of *R* was increase from 0.3 to 1.25 for Gd-DOTP⁵⁻ and Gd-TTHA³⁻ based NACs prepared in a solvent system of 3:2 v/v acetonitrile/water the capsule size increased correspondingly.⁹ Bismuth complexes of the same ligands (DOTP and TTHA) were anticipated to

facilitate capsule formation in much the same way as the Gd^{3+} chelates and to exhibit a similar trend in the relationship between the R value to the size of particle in a similar solvent system.

Figure 2. Structures of ligands investigated in this report.

Fig. 2 shows the ligands used to complex Bi^{3+} to conduct size comparisons of capsules with previously reported lanthanide NACs. The formation of these BiTTHA^{3-} and BiDOTP^{5-} and their synthetic protocols have been previously published.^{10,11} However, the BiTTHA^{3-} complex when synthesized was found to be insoluble in water and therefore could not be utilized in this study. BiDOTP^{5-} was employed to test the feasibility of preparing Bi^{3+} containing NACs and for size comparison experiments. DOTP is known to form a stable, soluble anionic complex with the trivalent ion of interest, Bi^{3+} . The size comparison study was extended to include various solvent systems including various solvents, R values, temperatures and aging times involved in the aggregate formation of polymer and complex.

METHODS

Reagents and Solvents

All water used in synthesis and instrumental testing was deionized water with a resistivity of 18.2 MΩ. H₆TTHA, PAH and Bi₂O₃ were purchased from the Sigma-Aldrich corporation. The molecular weight quoted by Sigma-Aldrich (St Louis, MO, USA) of the PAH used in this study is 56,000, not as previously reported by the manufacturer 70,000. H₈DOTP was purchased from Macrocyclics (Dallas, TX, USA). Silica nanoparticles Snowtex O-type were purchased from Nissan Chemicals USA (Houston, TX, USA) as a 20.2 w/w suspension in water (pH 3.5).

Bi-DOTP Synthesis

The synthesis of Bi³⁺ DOTP complexes was carried out by the addition of Bi₂O₃ at pH 0 in nitric acid portion wise to a solution of H₈DOTP at pH = 4 - 5 with heating. The progress of the chelation reaction was then monitored by potassium iodide tests to indicate whether any free bismuth was left in solution. If any free ion was detected, more ligand was added. Once all bismuth was complexed, the solution was freeze-dried to remove solvent. The resulting powder was weighed and dissolved in a small amount of water to create a stock solution from which all experiments were conducted.

NAC Preparation

A 8.9 μM stock solution of PAH was prepared by dissolving PAH (0.0498 g, 0.89 μmol) in a mixture correspondent to the solvent system being analyzed (10 mL). The pH of the chelate stock solution was adjusted to 9 by NaOH. A stock 2% suspension of SNPs was prepared by adding 1 mL of a commercially available 20.2 wt% suspension of SNPs in water to 9 mL of MeCN. Upon

addition of the chelate stock solution the reaction became turbid indicating the formation of an aggregate. Aggregates were aged for 10 minutes before addition of the 2% SNP stock solution (100 μ L). The reaction was vortexed for a further 10 seconds at medium speed and then allowed to age without agitation for 30 minutes. Excess SNPs were removed by filter centrifugation using 10 kDa MWCO filter centrifuge tube at 6,000 rpm. The NACs retained in the filter centrifuged tube were washed with water and filtered by centrifugation for 30 minutes at 14,000 rpm a total of six times. The NACs were then taken up into water (1 mL) and recovered into a sample vial.

Microscopy

Scanning electron microscopy (SEM) was performed on a FEI (Hillsboro, OR, USA) Sirion FEG electron microscope equipped with an energy dispersive X-ray (EDX) detector. A droplet of NACs suspension was placed on the aluminium stub and dried in air. The sample was then sputter coated with gold for 55 seconds. Secondary electron images were taken at 5 kV with a working distance between 5-10 mm.

Dynamic Light Scattering

Dynamic light scattering was performed on a Horiba (Irvine, CA, USA) LB-550 dynamic light scattering instrument. For these measurements freshly syringe filtered samples were dispersed in water and measured at four dilutions to ensure size distributions were independent of concentration effects. Samples were regularly agitated to guard against settling of larger particles.

RESULTS/DISCUSSION

Initial preparations of NACs containing BiDOTP⁵⁻ were attempted employing the most successful reaction conditions from the preparation of Gd³⁺ containing NACs⁹: 3:2 v/v MeCN and H₂O as solvent at pH 9. The formation of capsules was confirmed by electron microscopy (Figure 3) which shows spherical particles were produced in the reaction. The images also suggest chemical debris was not completely removed and more extensive centrifugation and cleaning should be used in order to remove all excess polymer. Significantly, the SEM images show similar polydispersity in the BiDOTP⁵⁻ containing NACs that was observed under the same conditions for the Gd-based NACs, although reproducibility was largely variable and often formed statistically different sized populations of NACs under the same conditions. Dynamic light scattering techniques depicted in Fig. 4 were consistent with the polydispersity shown in the SEM images.

Figure 3. SEM images of BiDOTP NACs formed in 3:2 v/v MeCN/water under room temperature conditions with 10 minute aging.

To determine whether Bi-DOTP interacted similarly with the PAH polymer as Gd-DOTP, the R value of Bi-DOTP NACs was varied to conclude whether the linear relationship with size distribution exists for the novel NACs in comparison to Gd-NACs, displayed in Fig. 4.

Figure 4. a) Chart of average size distributions in Bi-DOTP and Gd-DOTP NACs formed with 3:2 v/v MeCN/water under room temperature conditions and 10 minute aging. Error bars indicate the averaged standard deviation of the distribution population. **b)** Average size distributions over each synthetic attempt of Bi-NACs formed with 3:2 v/v MeCN/water under room temperature conditions and 10 minute aging.

BiDOTP NACs did not appear to follow the same linear relationship in size distribution with an increase in R value as those previously reported for Gd-based NACs. Fig. 4 depicted size distributions for Bi-NACs that were smaller and did not follow a linear trend, coupled with their irreproducibility, although data showed a slight Gaussian trend with a mean occurring at $R = 0.75$. In order to attempt manipulation of the size of the NACs, solvent systems of 1:1 v/v MeCN/water and 1:1 v/v EtOH/water were attempted at temperatures ranging from 0°C to ambient with aging times between 1 and 10 minutes.

Figure 5. Average size distributions of Bi-NACs formed with a solvent system of 1:1 v/v MeCN/water at ambient temperature with varied aging times. Error bars indicate the averaged standard deviation of the distribution population.

Fig. 5 showed an apparent linear trend with increased aging times and suggested that size manipulation for these conditions may be possible by a function of how long complex interacts with the polymer. However, the size distributions of these NACs were almost 5 times larger than those formed with 3:2 v/v MeCN/water solvent systems and were too large for a reasonable

comparison to the average sizes of Bi-NPs, which are typically 30-80 nm in diameter. Temperature was reduced during synthesis in further experiments to establish whether a decrease in kinetic energy would allow for smaller NAC formation.

Figure 6. a) Average size distributions of Bi-NACs with $R = 0.75$ formed with a solvent system of 1:1 v/v MeCN/water at varied temperature and aging times. b) Average size distributions of Bi-NACs formed with a solvent system of 1:1 v/v EtOH/water at varied temperature and aging times. Error bars indicate the averaged standard deviation of the distribution population.

When the temperature was decreased to 0°C, there was not a decrease in the size distribution of the particles as expected, with the exception of aging times 3 and 5 minutes in 1:1 v/v MeCN/water solvent system. Generally, the average size of capsule tended to increase with a decrease in temperature in the EtOH/water solvent system and the magnitude of the sizes remained too large for a viable comparison with Bi-NPs.

In conclusion, the formation of NACs containing Bi-DOTP complexes was confirmed. However, the size distribution of the particles was highly inconsistent. As a result the contrast efficacy was not established. Moving forward, further research should focus on additional chelates to determine

whether the size distributions are consistent in bismuth-based NACs in an effort to interpret the disparity in the ability of bismuth complexes to interact with the polymers in a similar manner as Gd-based counterparts. Furthermore, varied cationic polymers could also be studied to determine whether PAH is unique in its interactions with Bi-DOTP. Flow cytometry studies could potentially be utilized to compare contrast on a per-NAC basis with Bi-NPs of similar size.

CONCLUSIONS

Bismuth was complexed with DOTP to investigate the feasibility to form bismuth-based NACs in order to assess their contrast efficacy relative to bismuth nanoparticles for CT imaging. However, upon their formation, it was evident that the irreproducible nature of size distributions in bismuth-based NACs did not allow for a meaningful comparison. Thus, varied experimental conditions were investigated in an effort to control for the size dispersity of the Bi-NACs. However, varied solvent systems, synthesis temperature and aging time did not prove to have a significant effect on their size distribution.

Further studies in this work will focus on a method to separate the particles into more narrow size distributions. Once this is accomplished and a method of comparing Bi-NACs with Bi-NPs on a per-particle basis is established, series of phantoms containing Bi-NACs and Bi-NPs at different concentrations should be imaged to assess the effects on particle size and Bi^{3+} density on the ability to generate contrast in CT images. These experiments would allow for the identification of the agents suitable for CT contrast agents and once complete could lead to the modification of the particle synthesis to ensure adequate stability (and possible functionalization) necessary. Additional work could begin to examine how different genotypes of macrophage respond to each different nano-particle. In particular, the investigation into the extent to which one nano-particle may be favored for endocytosis by one genotype over another.

ACKNOWLEDGEMENTS

I would like to thank Drs. Mark Woods and Andrea Goforth for the opportunity to pursue this project; their help and guidance was fundamental in this work. The graduate students of the Woods and Goforth labs were also supportive and helpful in training me on instruments and helping to analyze data. I would especially like to thank Annah Farashishiko for her help with bismuth complex and NAC syntheses as well as SEM imaging. Lastly, I would like to thank all of the Chemistry Department and University Honors faculty at PSU who facilitated my undergraduate education.

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