The Effect of Nanodiamonds on the Antioxidant Activity of Tiopronin

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Abstract

In 2019, 65.2 million people suffered from cataracts. Cataracts form when reactive oxygen species (ROS) damage proteins in the lens of the eye, resulting in loss of transparency. High levels of endogenous antioxidants remove most ROS, but the abundance of these antioxidants decreases with age. It is hypothesized that antioxidant drugs may be able to prevent or slow cataract formation when endogenous antioxidant defenses are insufficient. Tiopronin (MPG), an FDA-approved antioxidant drug, has demonstrated some anti-cataract effects in live-cell and animal models. Its effectiveness may be limited by its uptake in the lens, but it could be improved with the use of nanodiamonds (NDs). To investigate how ND effects MPG’s antioxidant activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging kinetics and cupric reducing antioxidant capacity (CUPRAC) were analyzed for solutions of MPG and suspensions of MPG adsorbed to ND. Methodology was also developed to overcome challenges due to the turbid suspensions of ND. The information gathered from this study will aid in the optimization of ND as a drug delivery platform.

Introduction

Free radicals are defined as atoms or molecules that have unpaired electrons increasing the reactivity of the species. These highly reactive species can then wreak havoc on different biological processes leading to many disorders and common illnesses. The harmful consequences of these free radicals on the body falls under the category of oxidative stress. The formation of these species can be due to number of biological imbalances in the various system. However free radicals are not the only matter that attributes to these issues. Reactive oxygen species (ROS) are defined as highly reactive oxygen containing species. According to Auten and Davis, “ROS can also provoke damage to multiple cellular organelles and processes, which can ultimately disrupt normal physiology”. Luckily, the human body contains natural antioxidants that are used to combat the roaming of ROS. One important antioxidant is Glutathione (GSH). Low GSH levels are associated with diabetes, Parkinson’s disease, Alzheimer’s disease, and cataracts. Since GSH levels lower with age, the need for an external antioxidant treatments increase. According to the FDA, Tiopronin has been used since 1988 to treat homozygous cystinuria. Because of MPG’s history with treating other cysteine related illnesses, it has become the ideal antioxidant for external drug testing. In order to fully investigate MPG bounded to ND’s ability to halt the progression of ROS, it is important to examine how while MPG is able to scavenge radicals (DPPH assay) and reduce oxidized species (CURPAC assay) alone and in the presence of ND.

As previously stated, oxidative stress occurs when free radicals in the body disrupt biological processes. Cataracts is caused by the denaturing of proteins within the eyes’ lens due to their exposure to free radicals. Once these proteins denature, they lose their transparency, cloud the lens, and lead to loss of vision. This is a major issue because 65.2 million people world-wide suffer from this condition and treatments are limited. Cataract surgery, the removal of the patients’ lens to be replaced with an artificial lens, is the most common treatment to combat the condition. While this surgery has been normalized there are still many drawbacks. These disadvantages include the formation of secondary eye conditions due to the removal of the eyes’ nature antioxidant supplier and socioeconomic factors that leaves some without the option to take part in surgery. For these reasons, another option needs to be presented. Hopefully by researching and investigating a possible eyedrop solution, its outcome will be able to counter the issues found in lens removal surgery.
Materials, Instruments, and Software

The following materials were bought from ThermoFisher or Millipore Sigma unless otherwise stated: ammonium hydroxide (NH$_4$OH), copper(II) chloride (CuCl$_2$), DPPH, ethanol, glacial acetic acid, methanol, MPG, nanodiamonds as receive (ND-AR, from Daicel), and neocuproine (NC). For the UV-Vis photospectrometer the Bio Rad Semimicrovolume Cuvettes with a pathlength of 1cm were used. The Thermo Science Nunc 96 well optical plates were used for the platereader. The Shimadzu UV-1700 PharmaSpec was the UV-Vis photospectrometer utilized in this lab, and it ran the UV Probe 2.43 software. The Platereader used in this project is the BMG Labtech FLUOstar Omega running Omega v1.3. All centrifuged materials used the Hettich Zentrifugen Universal 32 R centrifuge.

Experimental Procedure

DPPH Procedure

When approaching this challenge of comparing MPG alone to MPG bounded to NDs, it was found that the techniques used in the paper, “Kinetics and Mechanisms of Antioxidant Activity using the DPPH Free Radical Method” by V. Bondet, W. Brand-Williams and C. Berset best suited our goal. This method compares the Activation Energy ($E_a$) or energy required to move a reaction forward, of MPG to that of MPG-ND. By observing a decrease in $E_a$, it can be inferred that the NDs did accelerate the rate at which the MPG was able to react. In experiment studies the rate at which MPG is able to scavenge the free radical DPPH∙. This rate is determined by the chemical kinetic equation below.

$$Rate = k[DPPH \cdot]^x[MPG]^y$$

Eqn. 1

From finding the order of this equation (the x and y values), the rate constant can be determined and used in the Arrhenius equation, which relates k, $E_a$, and temperature(T).

$$k = Ae^{-\frac{E_a}{RT}}$$

Eqn. 2.1

By simply modifying Eqn. 2.1, we develop a linear relationship that relates the ln(k) to the inverse of temperature, where the slope equals $-\frac{E_a}{R}$.

$$ln(k) = -\frac{E_a}{R} \frac{1}{T} + ln(A)$$

Eqn. 2.2

In order to deter the x and y values of Eqn. 1, the EC$_{50}$ is used to find the stoichiometric ratio between DPPH∙ and MPG. Since the reaction rate and reaction constant depends on the temperature at which the data was collected, trials have to be ran at various temperatures to utilize Eqn. 2.2. Unfortunately, this procedure could not be studied pass preliminary testing because a temperature control cuvette jack was unable to be obtained due to the Covid pandemic backorders. It can be suggested, however, after further examination of using the platereader in place of the photospectrometer (see in later section), it may be a possible option for continuing this portion of the project.

CUPRAC Procedure: MPG Alone

The CUPRAC procedure applied in this project involved some alterations to the procedure found in, “Comparative evaluation of antioxidant capacities of thiol-based antioxidants measured by different in vitro methods” by Nilay Güngör, Mustafa Özyürek, Kubilay Güclü, Sema Demirci
Cekic, and Resat Apak. In their procedure, they added 1mL of CuCl₂, NC, ammonium acetate (NH₄Ac), and 1.1 mL their various antioxidant solution in a 1:1:1:1.1 volumetric ratio. Since the cuvettes in our lab only allowed for a maximum of 1.5 mL of solution, the amounts were scaled back to 250 µL of each reagent and changed to a 1:1:1:1 volumetric ratio for simplification. The 1.078x10⁻² M CuCl₂ was created in an aqueous solution. The 1 M NH₄Ac buffer solution was synthesized via ammonium hydroxide and glacial acetic acid reaction. Finally, in ethanol 7.78x10⁻³ M of neocuproine and 1x10⁻³ M of a MPG stock solution were made, separately. In a cuvette 250 µL of the CUPRAC reagents were added in amounts indicated on Table 1 and allowed to react for 30 minutes. Once the reaction took place the photospectrometer was set to read at 450 nm and zeroed with distilled water. The cuvettes were loaded into the instrument and the data was collected.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Water Added (µL)</th>
<th>MPG Stock added (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>225</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
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<td>75</td>
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<td>8</td>
<td>50</td>
<td>200</td>
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<tr>
<td>9</td>
<td>25</td>
<td>225</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>250</td>
</tr>
</tbody>
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While more in-depth comparison of why the platereader presented itself as an improved system compared to it UV-Vis predecessor will be analysis further in a later section, the procedure for the platereader remained consistent except the 1 mL of sample yielded 3-4 data points. This was done because the 1mL of sample was reacted and then using a micropipette 250-300 µL of the sample was pipetted into the plate cells and placed in the platereader that was set to read the absorbance of the sample at 450 nm.

**CUPRAC Procedure: MPG-ND-AR**

The original objective of this project is to determine how the ND affect the antioxidant capability of MPG. Once the platereader became the choice method for CUPRAC analysis the following procedure was established. An additional 1x10⁻³M MPG solution was made then 16.5mg of ND-AR were added to 4.11mL of the MPG solution making a 4mg/ml ND-AR antioxidant suspension. In centrifuge tubes 500 µL of each reagent was added and 500 µL of the MPG-ND-AR solution was added according to Table 2 and allowed to react for 18 minutes. After 18 minutes the tubes were added to the centrifuge and spun at 1200g for 12 minutes. The tubes were then removed and using a micropipette the supernatant was loaded into the plate. Each place was carefully labeled and taking to the instrument room where the platereader was set to 450 nm. After collecting the data, the material was properly disposed. It is also important to note none of the material needed special disposal process outside of normal chemical procedure.
Table 2

<table>
<thead>
<tr>
<th>Trial</th>
<th>Water Added (µL)</th>
<th>MPG-ND-AR Stock added (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>450</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
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<tr>
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<td>50</td>
<td>450</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>500</td>
</tr>
</tbody>
</table>

Every sample has 500µL of 1.078x10^{-2} CuCl₂, 500µL of 7.78x10^{-3} Nc, 500µL of 1 M NH₄Ac and Water/1x10^{-3}M MPG in 4mg/mL ND-AR stock mix. The control consist of 500µL of Water.

Results and Discussion

In order to create a side-by-side comparison of the UV-Vis photospectrometer to the platereader one must understand what to search for in the data. Now as the concentration of MPG increase it is expected that the absorbance of the sample would also increase, leading to a positive slope. The R² of the line will showcase how linear the relationship between is between MPG and its reducing power. The closer to 1 the R² is the better relationship and data reading. One can see on Graph 1 the R² value of the platereader is higher, but its slope is not the same as the UV-Vis photospectrometer. This is slightly discouraging because the entire point of utilizing the platereader is to similar data to the UV-Vis photospectrometer by using less material. It is important to look at the drawbacks of using the platereader and one of the major issue is that the instrument is not greatly precise, in the fact that the same sample or sample composition will not read the same absorbance. This issue can be dampened by including a blank and control in every sample plate.
The disadvantages of using the UV-Vis photospectrometer was that the instrument can only read 6 cuvettes at a time. As one can see it would require 12 cuvettes (including controls) to get one set of data. In order to reduce error multiple data points for each trial was needed and the instruments low-capacity increased analysis time. Another issue is the fact the instrument required 1 mL of each sample to run. In order to run six sets of sample it would require 72 mL of material (18 mL of each reagent). While this is not an exuberant amount of material usage there is always a need for a more cost-efficient procedure which is why the platerader was introduced into this project. When working with the platerader, six sets of sample only requires about 24 mL of material. There is also a time aspect that must be considered. In the platerader all six sets of samples can be ran at once, whereas the UV-Vis photospectrometer takes about 12 times the amount of time since only 6 samples can be loaded at a time. It is for these reasons the issue with the platerader were overlooked and preferred compared to the UV-Vis photospectrometer.

Once a preferred method was chosen a deeper investigation on how ND-AR affect MPG’s antioxidant capacity. Since ND does not have molar mass their concentration is measured in mg per volume. For the purpose of comparing MPG’s reducing abilities alone and in bounded to ND-AR, it is important to note how much antioxidant is loaded on each ND. According to data supplied by Dr. Pfaff, 3.94x10^-4 mg of MPG is absorbed to 1 mg of ND. Using this information and molecular weight of MPG a comparison of MPG alone and in the presence of ND can be seen on Graph 2 and Graph 3.

Graph 2

![Absorbance vs MPG alone](image1)

Graph 3

![Absorbance vs MPG-ND-AR](image2)

As previously mentioned, the slope is an indicator of the relation between MPG and how much the of the solution it was able to reduce. The increases when it takes less MPG to reduce the same amount of solution. One can see that while the correlation or the R^2 value decreased, the ability for MPG to reduce the oxidized material increased by about 7100%. This is exciting because this data coincides with the hypothesis that MPG bound to ND would have a synergistic affect. Now to address the issue with the decreased R^2 value. There are a number of reasons that could be causing this dilemma, but it is believed to be simple human error. The reason why the material needed to be centrifuged was because the ND suspension is turbid and affects the instruments’ ability to scan the sample. In the case of Graph 3 it is possible that NDs did get into the supernatant altering the absorbance the instrument read.

While this data is promising, there is always room for more investigation. Naturally more trials need to be ran to see if this data is reproducible. Also finishing the DPPH assay to get a larger picture of MPG antioxidant abilities would be significantly useful and researching other antioxidant assays would really being together this argument and hypothesis.
**Nomenclature**

- [X] – shorthand notation for the concentration of X
- Activation Energy ($E_a$) – the energy curve that the reaction needs to overcome to move forward
- Denature – this takes place when a protein unfolds and loses its normal conformation. The protein cannot be fold back and cannot function properly
- EC$_{50}$ – the amount of antioxidant needed (MPG) to reduce the radical (DPPH$^\cdot$) by 50%
- Free radical (radical for short)$^i$ – an atom, molecule, or chemical species that has an unpaired electron. Highly reactive, denoted by a ($\cdot$) beside its chemical name.
- Order – How much of said components is needed to produce that rate of reaction
- Oxidative stress – the consequence to different chemical imbalances in the body, including that of free radicals
- Oxidized – the loss of an electron to another species
- Rate – the pace at which a reaction takes place
- Rate Constant (k) – a temperature dependent constant that varies for every chemical reaction
- Reduce – the gaining of an electron from an oxidized species
- Scavenge – the act of reducing a radical

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