# The Effects of Epigallocatechin Gallate on Amyloid Fibrils Formed From Beta-Lactoglobulin Aggregation and Engineering Epigallocatechin Gallate Cocrystals For Better Bioavailability

## PURPOSE

- To test the disaggregation properties of EGCG on aggregates formed from Beta-Lactoglobulin aggregation
- To increase the bioavailability of EGCG by forming different cocrystals

## INTRODUCTION

Protein aggregation is a hallmark for a plethora of human diseases. Protein aggregation is where misfolded proteins clump together and form aggregates that are usually correlated to diseases. There are three main types of protein aggregates: amorphous aggregates, oligomers, and amyloid fibrils. Proteins that form amyloid fibril aggregates are known as amylomes and are responsible for neurodegenerative diseases such as Alzheimers or Parkinsons. In Alzheimer's, amyloid fibrils are formed from the protein Amyloid Beta 42(Aβ-42). This protein is cleaved from the Amyloid Precursor Protein by β-secretase and γ-secretase and forms plaques that disrupt neuronal function by interfering with connection between neurons. In response to the buildup of plaques, glial cells, specifically microglia, attempt to clear up the debris of plaques but fail to do so. This causes a buildup of microglia that release a chemical, causing inflammation and inducing toxicity in neurons. Currently there is no cure for Alzheimer's and the first step toward this cure should be focused on Aβ-42 aggregates.

β-Lactoglobulin (β-lg) is the main protein in whey and represents 0.2–0.4% (w/v) of skim milk. It is a small globular protein(18.3kDa) and has two disulfide bridges and a free thiol group. β-lg forms aggregates and folds in certain ways depending on various factors such as ionic strength, pH, protein concentration, and reaction time. β-lg is capable of forming amyloid fibrils when heated to a certain temperature and incubated under its isoelectric point(pH 5.1). These amyloid fibrils are similar to the amyloid fibrils formed from Aβ-42 in Alzheimer's disease and therefore β-lg can be used to model protein aggregation in Alzheimer's. If aggregation of β-lg is successfully prevented or reduced, then it could have implications regarding Alzheimer's disease.

In recent years, polyphenols have been of increasing interest for their ability to reduce health risks due to their antioxidant properties. They are a class of mostly naturally occurring compounds and are characterized by phenol structural units. Flavonoids are a type of polyphenols and account for approximately 60% of all polyphenols. Green tea is rich in polyphenol flavonoids including catechins. Catechins are a type of polyphenolic compounds that are abundant in tea and have potent antioxidant properties. Epigallocatechin 3-gallate (EGCG) is the most abundant and potent green tea catechin and has been extensively studied for its beneficial health effects. In the case of Alzheimer's disease, effects such as the inhibition of reactive oxygen species accumulation, promotion of beta amyloid degradation, reduction in the production of beta amyloid, lower levels of beta and gamma secretase activity, higher levels of alpha secretase activity and suppression in phosphorylation of tau protein have been noted. On the other hand, an area of concern regarding therapeutic advances is poor oral bioavailability of EGCG. Bioavailability is one of the principle pharmacokinetic properties of drugs and it refers to the fraction of an administered drug that reaches circulation and is able to have an effect. A poor oral bioavailability results in low efficacy of the drug and can cause the drug to fail in clinical trials. Solubility and membrane permeability are important factors in bioavailability. Having low solubility or permeability causes a drug to have a low bioavailability and results in the drug being inefficient. According to the Biopharmaceutical Classification System (BCS), EGCG is a Class III drug which means it has a high solubility but a low membrane permeability. Membrane permeability is important because a low membrane permeability will cause the drug to not be able to cross barriers such as the gastrointestinal tract, and render the drug ineffective at reaching the intended site.

Cocrystals are solids composed of two or more different compounds joined together in a stoichiometric ratio. The compounds are not solvates or simple salts. Cocrystals have received increasing attention due to their ability to modify the physicochemical properties of the starting materials. The different physicochemical properties is a result of the different crystal structure of the resulting cocrystal. At first, cocrystals have been utilized to change the solubility of compounds, but they are now being used to change a plethora of other properties. Taking advantage of cocrystals offers limitless possibilities to enhance drugs so that they can be made safer or more effective for treatment.

Nicotinamide is a component of NAD+, which is a coenzyme involved in multiple processes throughout your body and is central to metabolism. Isonicotinamide is an isomer of nicotinamide, and the amide form of isonicotinamide acid. The membrane permeability of isonicotinamide acid has been reported to be high.

## HYPOTHESIS

- EGCG will reduce amyloid fibril aggregation of β-lg significantly. The reason is because EGCG is known for its anti amyloidogenic properties and a high dietary intake of EGCG has shown to reduce the risk of neurodegenerative diseases.
- The cocrystals of EGCG will have a higher permeability rate than pure EGCG. The cocrystals consist of EGCG;isonicotinic acid cocrystal, EGCG;isonicotinamide cocrystal, and EGCG;nicotinamide cocrystal. Isonicotinic acid is highly permeable and combining it with EGCG will produce a cocrystal that will have a higher membrane permeability than just EGCG. Isonicotinamide is the amide form of isonicotinic acid and an isomer of nicotinamide.

## RESULTS/DATA

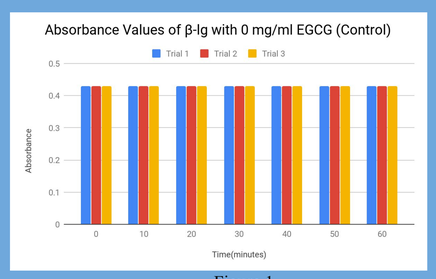
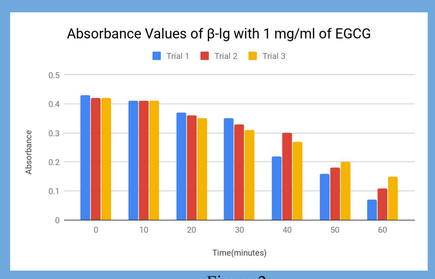



Figure 1

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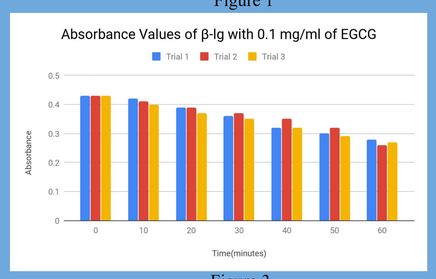


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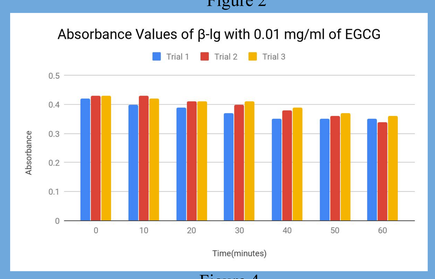


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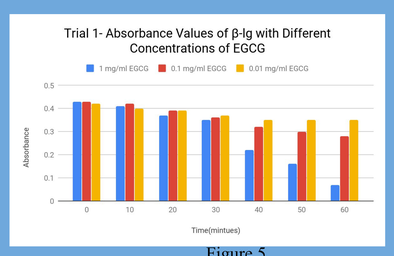


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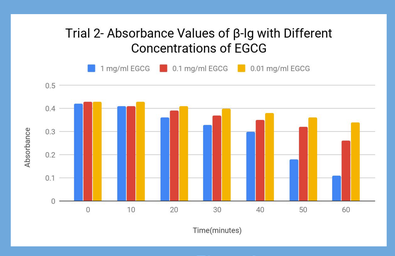


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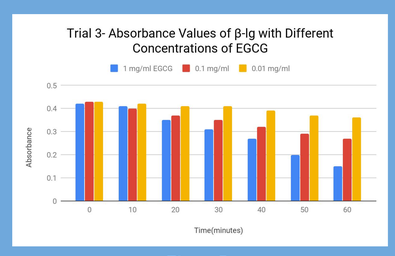


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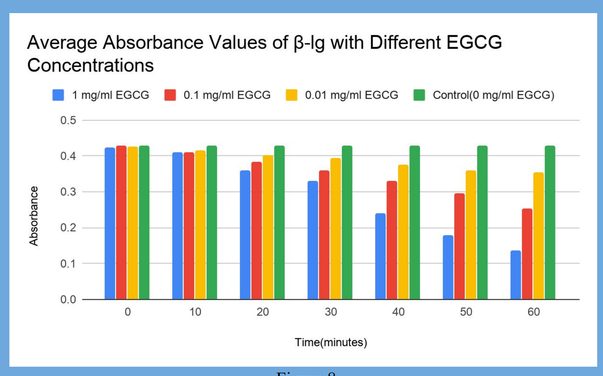
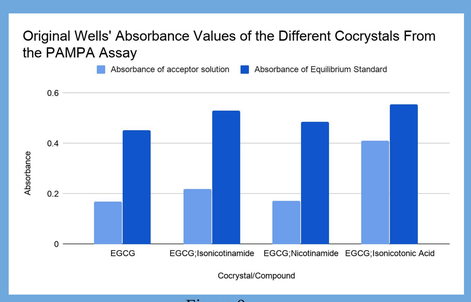



Figure 8

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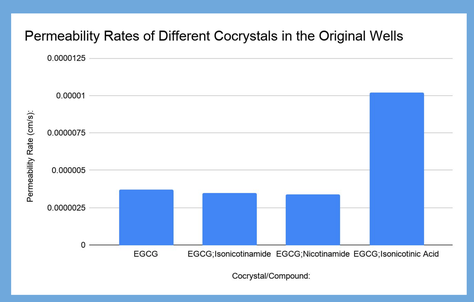
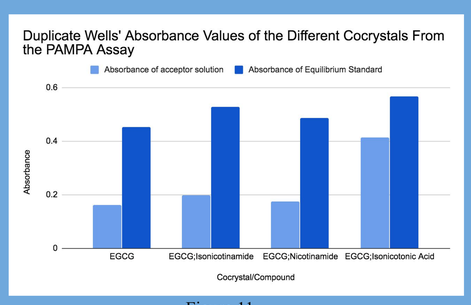



Figure 10

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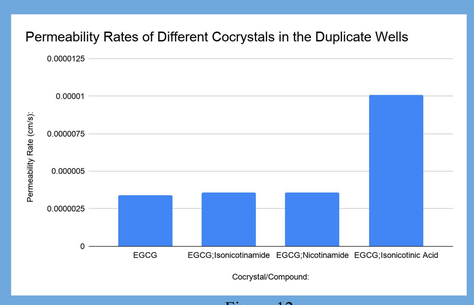


Figure 12

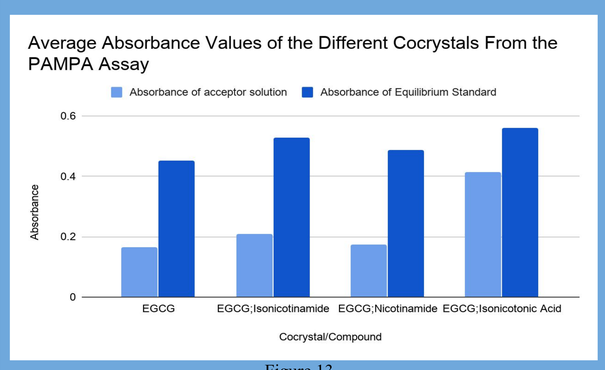


Figure 13

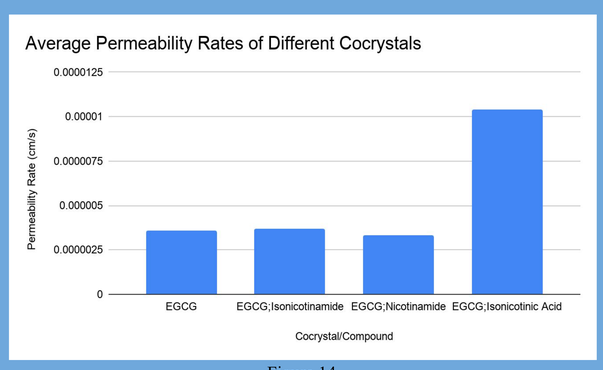


Figure 14

All figures and tables were created by Akshanth Bandla. Absorbance values in figures 1-8 were measured at wavelength of 340 nm. Absorbance values in figures 9,11, and 13 were measured at wavelength of 242 nm.

## PROCEDURE

Protein Aggregation

1. Add 8.3 mL of pH 2 HCL solution to a flask with 25 mg of Beta-Lactoglobulin to get a protein concentration of 3 g/L
2. Heat the flask at 80° C for approximately 4-5 hours.
3. Measure aggregation in spectrophotometer at 340 nm and record the absorbance value.

Making EGCG Concentrations

1. Extract EGCG powder from capsules and place in container.
2. - Dissolve 40 mg of EGCG in 40 mL of distilled water to get a concentration of 1 mg/ml EGCG.
   - Dissolve 40 mg of EGCG in 400 mL of distilled water to get a concentration of 0.1 mg/mL.
   - Dissolve 40 mg of EGCG in 4 L of distilled water to get a concentration of 0.01 mg/mL.

Experiment

1. Add 0.6 mL of aggregated protein and 1.8 mL of appropriate concentration of EGCG into cuvettes.
2. Place cuvette in spectrophotometer and measure absorbance values every 10 minutes for an hour.
3. Repeat for 3 trials for every EGCG concentration.
4. Perform a control trial by adding 0.6 mL of aggregated protein and 1.8 mL of distilled water into a cuvette and measure in spectrophotometer at wavelength of 340 nm.

Cocrystal Formation

1. EGCG;isonicotinamide cocrystal - Dissolve 45 mg of EGCG and 12 mg of isonicotinamide in 5 mL of distilled water. Crystals should form in 5-10 minutes and should be block-like in appearance.
2. EGCG;nicotinamide cocrystal - Dissolve 45 mg of EGCG and 12 mg of nicotinamide in 5 mL of distilled water. Crystals should form after three days and should be in a cluster of crystals.
3. EGCG;isonicotinic acid cocrystal - Dissolve 45 mg of EGCG and 12 mg of isonicotinic acid in 5 mL of distilled water. Crystals should form in one day and be needle-like in appearance.

PAMPA

1. Dissolve cocrystals in 0.01 M HCL solution, then adjust pH to 7.4 with bicarbonate buffer(models the GI tract).
2. Prepare 10 mM stock solutions of EGCG, EGCG;isonicotinamide, EGCG;nicotinamide, and EGCG;isonicotinic acid
3. Mix 25 µL 10 mM of each compound in DMSO + 475 µL PBS.
4. Mix 80 µL of 500 µM of each compound with 120 µL PBS.
5. In a separate tube, mix 5 µL DMSO + 245 µL PBS to prepare the Blank Control. This is to make sure that the peak absorbances are due to the compounds and not the DMSO.
6. Set aside the Equilibrium Standards and Blank Control for analysis the next day.
7. Add 5µL of 4% Lecithin in Dodecane to the well membranes of the donor plate
8. Add 300 µL of PBS to wells in the acceptor plate
9. Add 200 µL of each 500 µM compound to duplicate wells of the donor plate.
10. Carefully place the donor plate into the acceptor plate wells. Incubate at 37 C and leave overnight.
11. Carefully remove donor plate and collect the liquid in acceptor plate wells for analysis.
12. Add 100 µL of Acceptor Solution and Equilibrium Standards for each compound to UV plate. Also add 100 µL Blank Control to wells of UV plate.
13. Read Absorbance spectrum and determine peak absorbance.
14. Determine the permeability rate using this formula: $P_e = C \times -\ln\left(1 - \frac{OD_A}{OD_E}\right) cm/s$

## RESULTS

The effect of EGCG on Beta-Lactoglobulin aggregates was measured for an hour at each concentration. Absorbance refers to how much of the light is being absorbed by the protein/aggregates. The lower the absorbance, the smaller or fewer the aggregates. As shown in Figure 8, on average, EGCG concentration of 1 mg/ml was the most effective in reducing Beta-Lactoglobulin aggregates. According to figure 2, by the end of the 60 minutes, 1 mg/ml EGCG reduced the absorbance from 0.43 to below 0.15 in all three trials. As shown in figure 8, on average, 0.1 mg/ml EGCG and 0.01 mg/ml EGCG reduced absorbance from 0.43 to around 0.27 and 0.36 respectively. According to Figures 2, 3, and 4, the absorbance values for all three trials of each concentration were similar to each other. According to figures 5, 6, and 7, 1 mg/ml of EGCG was more effective than the other two concentrations in each trial. 1 mg/ml EGCG was the most effective, followed by 0.1 mg/ml EGCG and finally 0.01 mg/ml EGCG. A PAMPA assay was performed and absorbance values for each cocrystal and compound were obtained. A higher absorbance value corresponds to a higher concentration of the compound.The permeability rate was determined based on the formula in step 14. The absorbance values at the wavelength with the highest peak in absorbance were used. According to figures 9 and 11, the absorbance values of the original wells are similar to the absorbance values in the duplicate wells. According to figure 13, on average, the absorbance values for the EGCG;isonicotinic acid cocrystal were significantly higher than the other cocrystals, indicating a higher permeability and a higher permeability rate as shown by figure 14. According to figures 10, 12, and 14, the permeability rates of the EGCG;isonicotinamide cocrystal and the EGCG;nicotinamide cocrystal are close to the permeability rate of EGCG and did not improve permeability of EGCG.

## CONCLUSION

Both hypotheses were supported. 1 mg/ml of EGCG significantly reduced aggregates and the EGCG;isonicotinic acid cocrystal increased membrane permeability. Based on these results, EGCG can have therapeutic applications. EGCG, by either reducing aggregates or by reducing the size of aggregates, can reduce the cytotoxic effects of Aβ-42 in Alzheimer's and ultimately reduce the severity of Alzheimer's and prevent progression of the disease. By increasing membrane permeability, the EGCG;isonicotinic acid cocrystal ultimately improves the oral bioavailability of EGCG. The EGCG;isonicotinic acid cocrystal could be implemented in treatments for Alzheimer's instead of EGCG when bioavailability is an issue.