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A Study of Dextran as a Macromolecular Crowding Agent

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A STUDY OF DEXTRAN AS A MACROMOLECULAR CROWDING AGENT

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DEDICATION

This thesis is dedicated to my family, friends, and everyone who has encouraged me throughout my educational journey while at the University of Mississippi. Thank you.

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ABSTRACT

Deoxyribonucleic acid (DNA) is the hereditary material in all living organisms. According to the Watson and Crick model, DNA exists as two long strands coiled into a double helix. However, DNA has been found to have the potential to exist in secondary forms known as G-Quadruplexes and i-motifs. I-motifs (intercalated-motif or iM) are formed under acidic conditions when sets of cytosines pair with protonated cytosines and are looped around each other. The pKa value for iMs in dilute solutions is approximately 6.5, but iMs can potentially exist at a higher, physiological pH value under crowded conditions. Within the interior of a cell, a large percentage of total cellular volume is a dense mixture of macromolecules and solutes. A typical environment for an *in vitro* biochemical experiment is a dilute solution however what is actually present in cells is crowded. To best mimic this "crowding" in an *in vitro* environment, crowding agents like Ficoll, Polyethylene Glycol (PEG) and Dextran can be used. Over the years, crowding agents have been studied primarily PEG because it is cost efficient and synthetically made. Dextran is a natural polymer that has been explored little because of the high cost. In this study, Dextran was explored to try and understand if it could be an effective crowding agent in the formation of i-motifs. However, a contaminate was found in some sources of Dextran which became the basis for further studies to determine its cause and solution which became the subject of this thesis. We found that Dextran from Alfa Aesar was high contaminated with a potential microbe, but Dextran from alternative manufacturers was not.

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LIST OF ABBREVIATIONS

Dextran 1.5	Dextran 1,500
Dextran 3	Dextran 3,000
Dextran 5	Dextran 5,000
Dextran 6	Dextran 6,000
Dextran 10	Dextran 10,000
DSS	Dextran Sodium Sulfate
DTT	Dithiothreitol
iM	I-motif
MES	2-(N-morpholino) ethanesulfonic acid
PEG	Polyethylene glycol

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INTRODUCTION

Deoxyribonucleic acid (DNA) is the hereditary material in all living organisms that is made up of nitrogenous bases (Adenine [A], Thymine [T], Guanine [G], and Cytosine [C]), a sugar (deoxyribose), and a phosphate group (1). These bases are paired according to base pairing rules (A with T and C with G). Adenine bonds to Thymine forming two hydrogen bonds, and Guanine bonds with Cytosine forming three hydrogen bonds (1). According to the Watson and Crick model that is shown in **Figure 1**, DNA exists as two long strands coiled into a double helix (1).

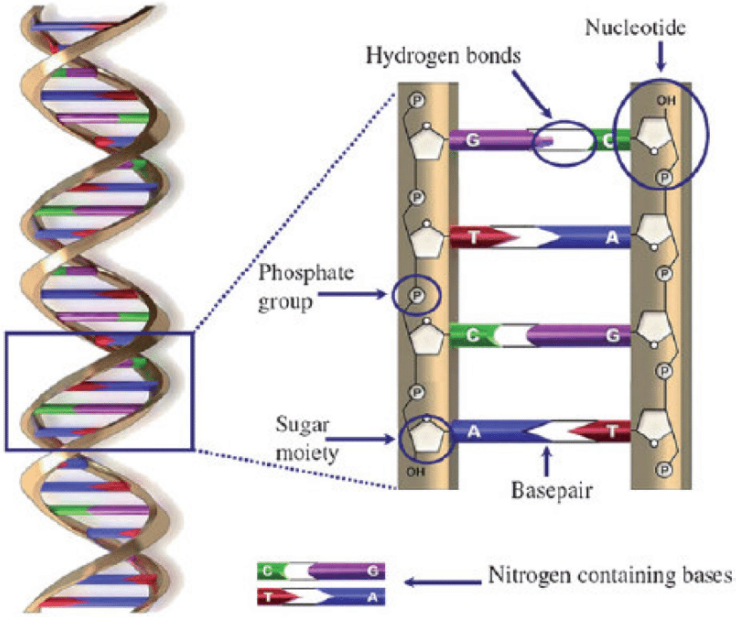

Figure 1: Double-helix DNA structure discovered by Watson and Crick (2).

At the end of eukaryotic chromosomes are repetitive sequences of non-coding DNA known as telomeres as seen in **Figure 2** (1,3). Each time a cell divides the telomeres become shorter and shorter eventually becoming so short that the cell no longer divides. Telomerase is an

enzyme that adds a TTAGGG sequence to the ends of each chromosome; however, this enzyme is not found at high concentrations in somatic cells (3). The result of this is aging cells which in turn means an aging body for us. The telomerase enzyme is found in high concentrations in germ-line cells and in cancer cells (3). This makes these cells essentially immortal so research surrounding telomeres and how telomerase could be used is of huge interest.

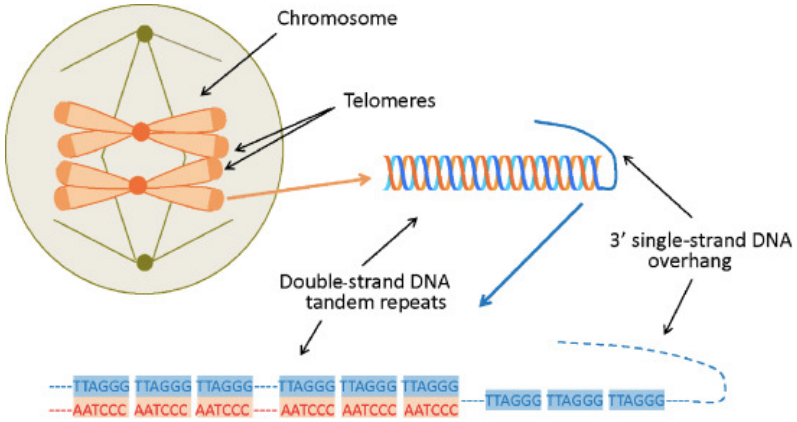

Figure 2: Representation of Telomeres and TTAGGG sequence (4)

Within telomeres, DNA has been found to exist in secondary forms from the double helix. Telomeric DNA sequences are asymmetric with one strand being guanine rich and the other cytosine rich. Displayed in **Figure 3**, the G-rich strand can form a four-stranded structure known as a G-quadruplex and the C-rich strand can fold into a four-stranded i-motif. The DNA forms a stacked guanine-rich tetrad, and a C-rich iM which is formed when sets of cytosines pair with other protonated cytosines (5,6). Under physiological conditions, telomeric DNA can be found as a double-helix, but if the pH is lowered and/or the temperature is raised, secondary

forms are found more often (3). These G-rich and C-rich telomeric strands have long been known to form *in vitro,* but more recently have been reported in DNA of human cells (3).

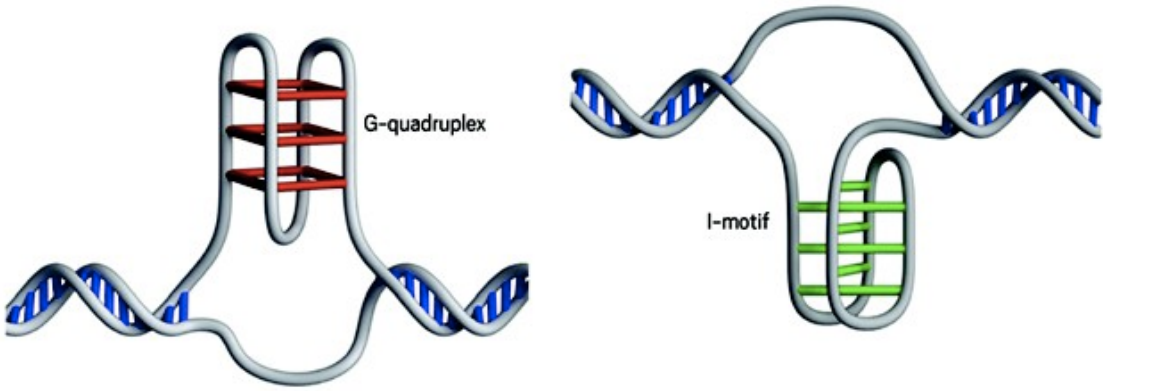

Figure 3: Structure of G-quadruplex (left) and i-Motif (right) DNA (7)

In dilute solutions, the pKa value for i-motif formation is around 6.5 but can potentially exist at a higher pKa under crowded conditions in a process that is not well understood (5,6,8). In a typical cell, a large percentage of total cellular volume is a dense mixture of macromolecules and solutes, including proteins, nucleic acids, organelles, carbohydrates, and other metabolites as displayed in **Figure 4** (9,10,11,12). Macromolecules typically take up 10-40% of total volume of a cell which limits space for other molecules (9,11).

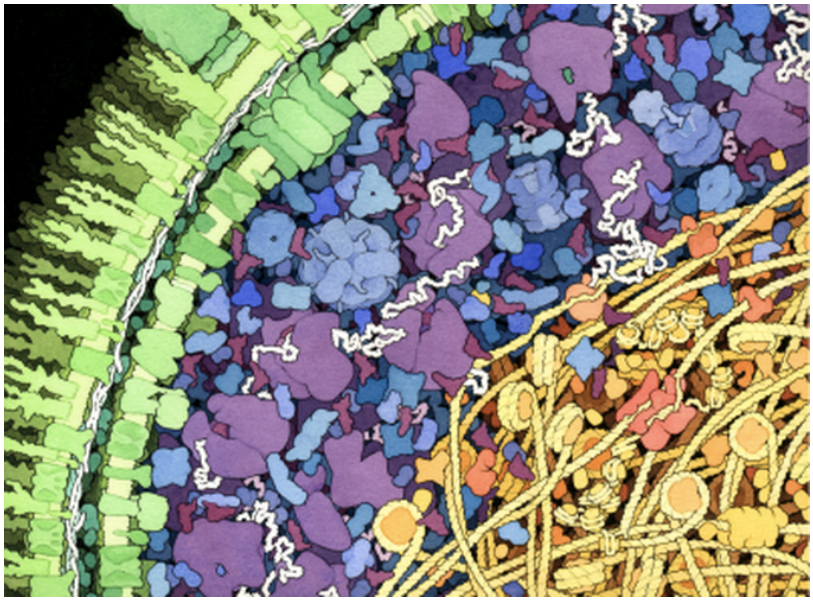

Figure 4: Cellular Crowding (12)

When performing biochemical experiments to study biochemicals, the best approach for getting the most accurate measurements would be to use highly concentrated cell environments. However, this is extremely complicated to do because of the biologically active molecules that would interact with what is being studied (10,11). Typically an *in vitro* biochemical experiment is used instead, and a typical environment for this type of experiment contains a diluted solution with less than 1 mg/mL of total macromolecular quantity (10). Substantial differences are observed for interactions in dilute solutions versus what is present in a crowded solution. These differences are due in part to two main factors: nonspecific interactions (repulsion and attraction) between molecules and exclusion effects, which happen when molecules cannot occupy the same space at the same time (12). To get the best and most accurate measurements of biochemicals, something is needed to mimic intracellular crowding.

To best mimic crowding in the intercellular environment in biochemistry studies, crowding agents like Ficoll, Polyethylene Glycol (PEG) and Dextran are used. These crowding

agents are large inert molecules that dissolve at high concentrations (12). It is well know that it is important to add a crowding agent to be able to stabilize enzymatic activities and occupy space so it is nearly identical to what is occurring in a cell (9,10,11,12). Over the years, various types of molecular crowding agents have been studied; primarily PEG because it is cost efficient and synthetically made. Dextran is a natural polymer that has been explored little, primarily because of the high cost (8).

Dextran is a sugar produced from the lactobacillus family (8). A species of lactic acid bacteria, *Leuconostoc mesenteroides*, when fermented, produces ethanol, lactate, and CO_2 but when it is grown in a sucrose solution, it converts the sugar to dextran (8). Dextran is a class of water-soluble polysaccharides that contains a D-glucose backbone linked mainly with an alpha-D-(1,6) bond (8,14). It is a very flexible polymer and can be obtained with well-defined molecular weights. Dextran is considered an ideal model crowding agent for iM formation because it is inert and an uncharged polymer (14). **Figure 5** shows the structure of Dextran.

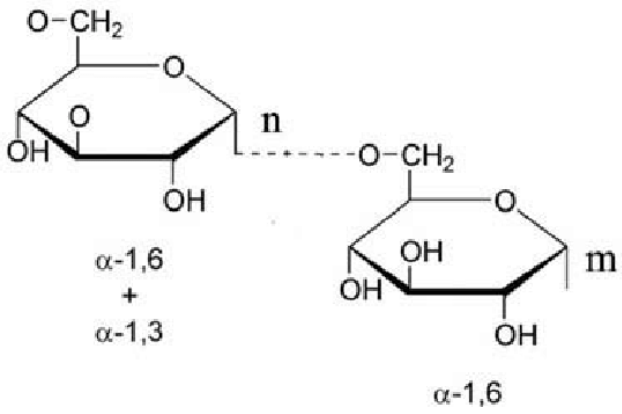

Figure 5: Chemical Structure of Dextran (15)

The original goal of this thesis was to determine if Dextran could be an effective crowding agent to study the formation of iMs. I used technical grade (used for laboratory experiments) Dextran (molecular weights 5,000 and 6,000) and Dextran Sodium Sulfate (molecular weight 40,000) as my crowding agents with C6T DNA, which is known to form i-motifs. The C6T sequence is 5' - TTC CCT ACC CTC CCC ACC CTA A - 3'. Dextran Sodium Sulfate is a derivative of Dextran that is synthetically made and contains sulfate groups that give it an overall negative charge (8). Because DNA is negatively charged, Dextran Sodium Sulfate has the potential to mimic this. For my buffer solutions, I primarily used Sodium Cacodylate, but MES and Bis-Tris were used as well. Sodium Cacodylate is an antimicrobial and contains Arsenic as its central element; therefore, it is extremely toxic to bacteria and an ideal buffer for biochemical studies.

While studying Dextran, my research shifted due to a possible contaminate I discovered while studying Dextran obtained from Alfa Aesar. I used multiple new Dextrans (MW 1,500, 3,000, 5,000, and 10,000) for comparison. I also introduced B-mercaptoethanol, Dithiothreitol, and Sodium Azide to try and eliminate the potential contamination to be able to study Dextran's effectiveness as a crowding agent in the formation of iMs. The goal of my thesis now was to determine the cause of the contamination I saw, and find a possible solution.

MATERIALS AND METHODS

As an alternative to Alfa Aesar, Dextran 1, 3.5, and 5 (MW 1,000, 3,500, 5,000) were purchased from Pharmacosmos (sourced form Denmark). Dextran 6 (MW 6,000) was purchased from Pharmacosmos (sourced from Denmark), Sigma Aldrich Chemical (sourced from China), and Alfa Aesar (sourced from Canada). Dextran 10 (MW 10,000) was purchased from Oakwood Chemical (sourced from the USA). Dextran Sodium Sulfate was purchased from Sigma-Aldrich Chemical (sourced from the USA). MES and Bis-Tris buffers were purchased from Fisher Biotech (sourced from the USA). Sodium Cacodylate was purchased from J.T. Baker (sourced from the USA). B-mercaptoethanol was purchased from Fisher Biotech, and Dithiothreitol (DTT) was purchased from ACROS (sourced from Belgium).

To begin my experiment, 30 mM solutions of MES, Sodium Cacodylate, or Bis-Tris buffer solutions were prepared using deionized water. 10, 20, 30, and 40% Dextran 5, 6, and Dextran Sodium Sulfate was dissolved in each of the buffer solutions at pH's between 5.5 and 8.0 at 0.2 unit increments. A clear solution was viewed in the Dextran 5 and Dextran Sodium Sulfate solutions. In the Dextran 6 solutions (Alfa Aesar), a caramelizing, cloudy solution was visualized a couple of days after the samples were made, and within a week, suspected microbial growth had formed. Sodium Cacodylate's antimicrobial abilities should have not allowed anything to grow in the solutions so it was thought that due to the caramel coloring that a chemical reaction of caramelization may have occurred since Dextran is a sugar. To prevent this chemical reaction from taking place, two reducing agents were introduced B-merceptoethanol and DTT.

A second set of samples were prepared with 20 and 40% Dextran 6 dissolved in Sodium Cacodylate or MES buffers at pH 6.0 and 8.0. Ten µmol of the reducing agents, B-mercaptoethanol and DTT were added to the individual samples. The presence of the thiol makes both good reducing agents because both reduce disulfide bonds and act as an antioxidant to prevent oxidation. However, the caramel color formed within a couple of days and potential microbial growth was seen within the week in these samples. After the reducing agents did not get rid of the caramel color, it was suspected that a microbe may be the contaminate - a contaminate Sodium Cacodylate was not able to kill.

A third set of samples was prepared with 20 and 40% Dextran 6 dissolved in Sodium Cacodylate and MES buffers at pH 6.0 and 8.0. Ten µmol of Sodium Azide was added to each of these samples to try and prevent microbial growth. Sodium Azide is toxic to bacteria and is similar to Cyanide in that both interfere with cellular respiration. After a couple of days, the caramel color had formed and later potential microbial growth was seen.

Since the Dextran 6 was the only strand to produce this caramelization and potential microbial growth, more Dextran was ordered from different companies for comparison. Samples with new crowding agents were prepared at 20 and 40% (Dextran 1, 3.5, and 6 sourced from Pharmacosmos, Dextran 6 sourced from Sigma Aldrich, and Dextran 10 sourced from Oakwood Chemical) at pH of 6.0 and 8.0. Each of these samples was dissolved in Sodium Cacodylate buffer. There was no caramelizing or microbial growth found in these samples.

RESULTS AND DISCUSSION

In the first set of samples, (Dextran 5, 6, and Dextran Sodium Sulfate dissolved in buffers) a caramelizing color was produced and later potential microbial growth formed in the Dextran 6 as seen in **Figure 6** (sourced from Alfa Aesar). No discoloring or growth was ever viewed in Dextran 5 or Dextran Sodium Sulfate.

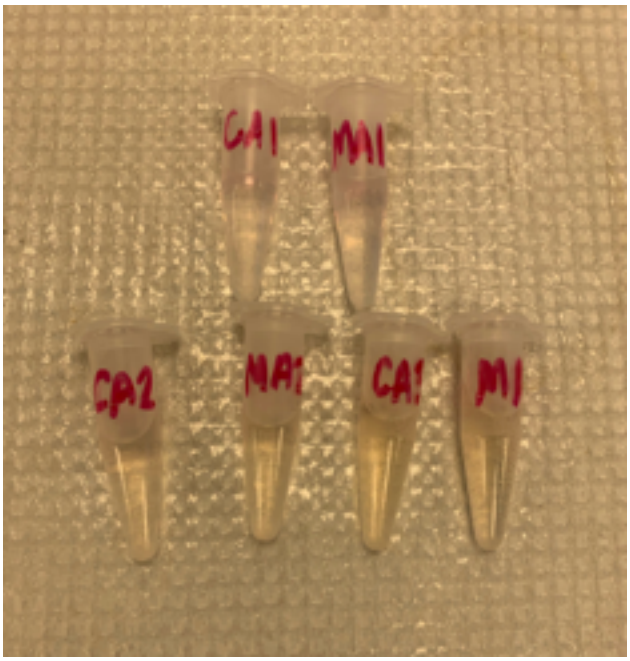

Figure 6: Samples of Dextran 6 from Alfa Aesar that produced caramel, cloudy solutions.

Since the Sodium Cacodylate buffer has antimicrobial properties, it was conflicting as to why any microbial growth could form. It was originally thought to have been due to a chemical reaction of caramelization since Dextran is a sugar. Two reducing agents were introduced, B-mercaptoethanol and DTT to prevent oxidation from occurring, yet the caramelizing and potential microbial growth still occurred (**Figure 7**).

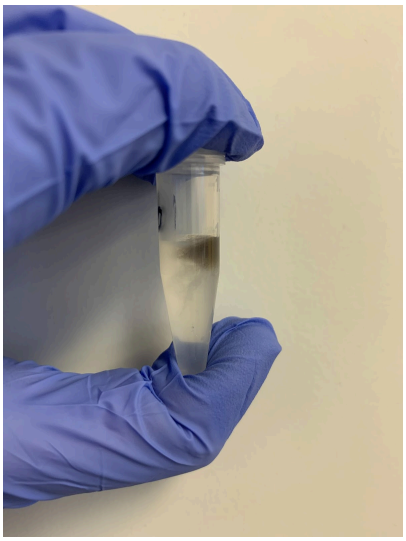

Figure 7: A Dextran 6 sample that produced potential microbial growth after a reducing agent was added.

After seeing that the reducing agents were not successful is getting rid of the contamination, it was suspected that microbial growth could be the answer. Sodium Azide was introduced in the next set of samples of Dextran 6. Sodium Azide is very toxic to bacteria because it interferes with cellular respiration and aerobic metabolism and prevents microbial growth from growing. However still, after introducing Sodium Azide, a caramel color formed, and within a week, what looked like microbial growth was viewed.

Since the Dextran 6 was the only strand that produced this potential microbial growth, it needed to be determined if it was a contaminate from the manufacturer Alfa Aesar. Dextran from various other companies (Pharmacosmos, Sigma Aldrich Chemical, and Oakwood Chemical) was purchased and studied to compare for similar results; however, typical clear solutions formed from these Dextrans as seen in **Figure 8**. Dextran 6 sourced from Alfa Aesar was the only Dextran to show caramel coloring and potential microbial growth.

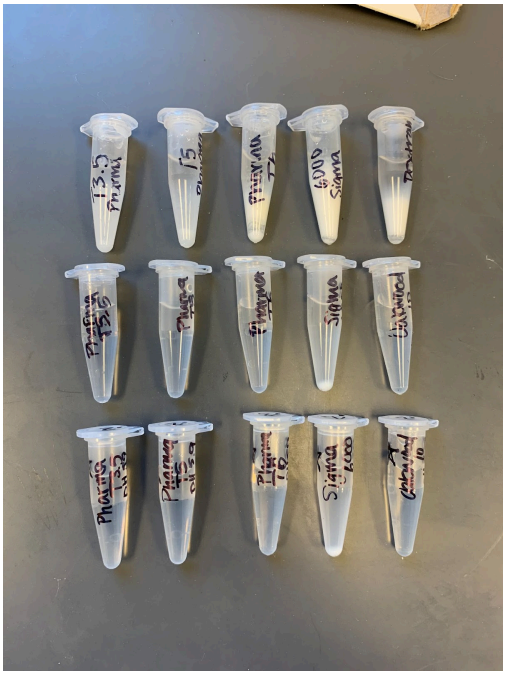

Figure 8: Dextran (obtained from Oakwood Chemical, Pharmacosmos, and Sigma Aldrich) samples dissolved in Sodium Cacodylate buffer with no caramelizing or microbial growth detected. (The apparent yellow tint in the top row is due to lighting.)

CONCLUSION

Originally, I was studying Dextran 5, 6, and Dextran Sodium Sulfate to see how effective they could be in forming iMs; however, after a caramel coloring and growth was viewed in many of my samples of Dextran 6, my study shifted. The results of my study showed that Dextran 1, 3.5, 5, and 6 from Pharmacosmos, Dextran 6 from Sigma Aldrich Chemical, and Dextran 10 from Oakwood Chemical produced clear and typical results for crowding agents. After introducing two reducing agents, B-mercaptoethanol and DTT, and a preservative, Sodium Azide to the Dextran 6 from Alfa Aesar, a caramel color and what appears to be microbial growth was viewed. Each step was taken to prevent discoloring and growth from forming but each step was ineffective in doing so.

From these findings, Dextran sourced from companies Pharmacosmos, Sigma Aldrich, and Oakwood Chemical do not seem to present any contamination and could be potentially good crowding agents in further studies. Dextran from Alfa Aesar needs to be studied further, but from the results of this study, it appears it may not be fit to be used as a crowding agent without first sterilizing to kill any potential contaminate. Sterilization may not be effective due to potential caramelization from exposure to heat so another lab student is currently studying this project. Further investigation is also underway to determine the type of microbial growth seen which we suspect could potentially be *Leuconostoc mesenteroides* which is where Dextran originates.

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