synthesis of sulfated polysaccharides for colitis mice model

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SYNTHESIS of SULFATED POLYSACCHARIDES for COLITIS MICE MODEL

by

QIANG MA

Under the Direction of Dr. Hamed Laroui
ABSTRACT

IBD (inflammatory bowel disease) is a pandemic chronic disease of the gastrointestinal tract inducing Ulcerative colitis (UC) and Crohn disease (CD). The inflammation of colon especially associated with the growing risk of developing colon cancer. To extend the research on IBD, chemical mice models are investigated on easy way to induce colitis in rodent. Dextran sulfate salt (DSS) 7 days, 3% in drinking water induce IBD on mice. One of the problem of DSS is that it requires a purification step of mRNA by LiCl. This step induce this mRNA harvest rate quantitatively and qualitatively.

In this study, we synthesized Pullulan Sulfate and Cyclodextrin Sulfate as alternative colitis inducers in mice. The specificity of sulfated polysaccharides in colon versus small intestine has been confirmed by common gastrointestinal method (pro inflammatory cytokines causes degradation of the epithelium, and the presence of blood in stool.)

INDEX WORDS: IBD, mice model, DSS, mRNA purification, Pullulan and Cyclodextrin.
SYNTHESIS of SULFATED POLYSACCHARIDES for COLITIS MICE MODEL

by

QIANG MA

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

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Georgia State University

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by

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I would like to dedicate this to my family for their love. I love them more than anything else in this world. They gave me the best childhood and take care of me on all the aspects. Thank you my mother, you teach me loyalty, give me an example of uprightness. And father, you show me how to be patient and careful. Because of you, my life is so splendid.
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LIST OF ABBREVIATIONS

Cyclodextrin (C)
Cyclodextrin sulfate (CS)
Inflammatory bowel disease (IBD)
Dextran sulfate salt (DSS)
Pullulan (P)
Pullulan sulfate I (PS I)
Pullulan sulfate II (PS II)
Immunohistochemistry (IHC)
Immunofluorescence (IF)
Hematoxylin & Eosin (H&E)
Interleukin 6 (IL6)
TNF alpha (Tumor necrosis factor alpha)
Tumor protein p53 (P53)
Running buffer (RB)
Transfer buffer (TB)
Washing buffer (WB)
Dimethyl sulfoxide (DMSO)
Nuclear magnetic resonance (NMR)
Infrared (IR)
Phosphate buffered saline (PBS)
Tris-buffered saline (TBS)
Diaminobenzidine (DAB)
Acid guanidinium thiocyanate-phenol-chloroform extraction (TRIzol)
Crohn’s disease (CD)
Ulcerative colitis (UC)
Polymerase chain reaction (PCR)
4, 6-diamidino-2-phenylindole (DAPI)
Degree of substitution (DS)
1 INTRODUCTION

1.1 A brief introduction of inflammatory bowel disease

Inflammatory bowel disease is a group of diseases occurring in the bowel system. Main one named Crohn’s disease (CD, occurring in all digestive track) and ulcerative colitis (UC, mainly colon) are the basic types of inflammatory bowel disease (1).

With a high morbidity and high mortality in developing area such as North Africa, China and India, IBD remains to be one of the most severe diseases around the world. (2) Patients suffer reduce intestine-stomach colic, long-term diarrhea, body weight losing and blood stools physically. Most of them also meet psychosocial problem like anxiety and depression on mental. This is especially a severe social problem which will lead patients to worry about their behavior and increase their anxiety disorder (1-3).

CD is an inflammatory bowel disease is a multifactorial disease (infection, heredity, cellular immunity) which mechanisms still keeps unknown.

IBD in Asia— during the past two decades, IBD reports and prevalence has a striking rising. According a study 2007, as a model of developed country, Japan has the prevalence of UC risen from 78 reports per million individuals to 636 reports per million individuals. Another studies on Singapore IBD prevalence, UC prevalence has increased from 60 reports per million individuals to 86 reports per million individuals, and the prevalence of CD has risen from 13 reports per million individuals to 72 reports per million individuals. As in Korea, there has also been a substantial rise in the prevalence of UC—from 76 reports per million individuals in 1997 to 309 reports per million individuals in 2007 (6,8).
Reasons that might responsible for the rising prevalence of IBD in Asia likely including industrialization of societies, deterioration of environmental factors (Westernization of lifestyle included), changes in diet (with the increasing economy, Asians eat more meat than mid 20th century), a huge advancement sanitation, and possibly, increasing using of antibiotics in medical treatment. In almost countries in Asia, antibiotics can now be extremely easily obtained from any pharmacy facility, in some areas, antibiotics are even not prescription drug. The assumption that antibiotic use earlier in childhood may augment the risk of IBD is hold by several case-control studies conducted in the West (9).

In Asia UC is over number CD, relatively much as it was in the other areas like Europe and north America (6.7) But, preliminary evidence also suggests that CD would overtake UC in developing countries over time (6.7.8).

**IBD in America**—An increase in the number of IBD patients is also continually shown, reflecting a rising need for more research to find a cure. In United states, about 1.6 million Americans are currently reported have IBD, An organization named Crohn’s and Colitis foundation of Americans (CCFA) is monitoring the IBD spreading and situation of IBD patients. According to their recent reports, a growth of Approximate 200,000 since the last time CCFA reported IBD population (in 2011). Every year, More than 70,000 new cases of IBD are diagnosed in the United States. What is worse? A significant part of them are children—thenre may be as many as 80,000 children in the United States with IBD in total.

Due to highly developed medical condition and clinic technology, circumstance of IBD carriers are getting better and better. Based on the publications of CCFA,
biochemists have identified over 160 genes related to IBD. Investigation of these genes will revolutionize our understanding of Crohn’s disease and ulcerative colitis mechanism and form the basis for investigating new drugs and equipment (10).

1.1.1 Different types of IBDs

CD and UC are the most common IBD. CD is more likely to be reported in western world, while UC is more common in Asia (11).

<table>
<thead>
<tr>
<th>Difference</th>
<th>UC</th>
<th>CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory disorder</td>
<td>Inflammation will be continuous throughout affected areas</td>
<td>Inflammation might periodically occur in patches</td>
</tr>
<tr>
<td>Position</td>
<td>Large intestine is basically the specifically affected site</td>
<td>Inflammation may attack anywhere along the gastrointestinal tract</td>
</tr>
<tr>
<td>exterior</td>
<td>Colon wall is thinner and shows continuous inflammation Mucus lining of large intestine may have ulcers, but they do not extend beyond the inner lining</td>
<td>Large intestine wall may be thickened and may have a rocky appearance Ulcers along the digestive track are deep and may extend into all layers of the bowel wall</td>
</tr>
<tr>
<td>Pain</td>
<td>Pain occurs in the lower left part of the abdomen</td>
<td>Pain is commonly taken place in the lower right abdomen</td>
</tr>
<tr>
<td>Bleeding</td>
<td>Bleeding from the rectum during bowel movements</td>
<td>Bleeding from the rectum during bowel movements is not common</td>
</tr>
</tbody>
</table>

1.1.1.1 Crohn’s disease

CD—it is named after Dr. Burrill B. Crohn that is first physician who described the disease in 1932. When talking about inflammatory bowel diseases, it is fundamental to realize that CD is not the same thing as UC, another type of IBD. These two IBDs share quite similar symptoms, but differ from areas affected in the gastrointestinal tract (GI tract).

The end of the small bowel (the ileum) and the beginning of the colon is mostly affected by Crohn’s, while it is able to affect any part of the gastrointestinal (GI) tract,
thus, from the esophagus to the anal opening. UC is just limited to the colon part, also called the large intestine, from cecum to rectum (14).

![Diagram of the digestive system](http://gastro.seoimportant.com/gi-diseases-and-conditions/crohns-disease)

**Figure 1: Crohn’s disease**, [http://gastro.seoimportant.com/gi-diseases-and-conditions/crohns-disease](http://gastro.seoimportant.com/gi-diseases-and-conditions/crohns-disease)

CD is also able to attack the whole thickness of the bowel wall, while UC only involves the innermost lining of the large intestine. Finally, in CD, the inflammation of the intestine can “skip”—leaving functional areas between patches of diseased intestine. In UC this does not occur.

The symptoms patients might experience:

*Common*—skin rash, slow growth, acute episodes, arthritis, depression, mouth ulcer, anal fissure, and bodyweight loss.

*Pain*—severe pain in the rectum, abdomen, low abdomen or joints.

*Body*—vomit, nausea and fever (13.15).

### 1.1.1.2 Ulcerative colitis

*Ulcerative colitis*—is a colon chronic disease, also known as a basic type of IBD, which is the lining of the large intestine becomes inflamed and develops extremely small out
sores, or ulcers, that generate pyaemia and mucous. The integration of inflammation and ulceration is able to cause gastrointestinal pain and periodic emptying of the colon. UC is the result of an abnormal response by your body's immune system. Normally, the cells and proteins that make up the immune system protect you from infection. In people with IBD, however, the immune system mistakes food, bacteria, and other materials in the intestine for foreign or invading substances. When this happens, the body sends white blood cells into the lining of the intestines, where they produce chronic inflammation and ulcerations (16).

Figure 2: ulcerative colitis, [http://www.ulcerativecolitis.net/colitis](http://www.ulcerativecolitis.net/colitis)

It's significant to tell the difference between ulcerative colitis and Crohn's disease. UC affects only the large intestine, on the other hand CD can affect any part of the Gastrointestinal (GI) Tract, from oral cavity to anus. Furthermore, while Crohn's disease is able to attack any layers of the bowel system, ulcerative colitis only affects the inner of the colon.

The symptoms patients might experience:
Common—scarring within the bile ducts or weight loss.

Pain—intermittent abdominal pain, joints or rectum pain.

Abdominal—tenderness or cramping.

Gastrointestinal—diarrhea, constipation, urgent need of defecation, colon tissue bloating, blood in stool, bowels empty inability, or leaking of stool (16.17).

<table>
<thead>
<tr>
<th>Symptoms of Ulcerative Colitis</th>
<th>Symptoms of Crohn's Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anemia caused by severe bleeding</td>
<td>Abdominal pain, cramping or swelling</td>
</tr>
<tr>
<td>Abdominal pain or discomfort</td>
<td>Persistent or recurrent diarrhea</td>
</tr>
<tr>
<td>Urgent bowel movements</td>
<td>Gastrointestinal bleeding</td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>Stomach ulcers</td>
</tr>
<tr>
<td>Rectal bleeding</td>
<td>Malabsorption</td>
</tr>
<tr>
<td>Bloody diarrhea</td>
<td>Weight loss</td>
</tr>
<tr>
<td>Malabsorption</td>
<td>Joint pain</td>
</tr>
<tr>
<td>Dehydration</td>
<td>Vomiting</td>
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<tr>
<td>Weight loss</td>
<td>Anemia</td>
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<tr>
<td>Fatigue</td>
<td>Fever</td>
</tr>
<tr>
<td>Joint pain</td>
<td></td>
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<tr>
<td>Fever</td>
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### 1.1.2 Research model.

Among the advantages to use mouse model, the most important is their striking similarity to humans in anatomy, physiology, and genetics. Over 95% of the mouse genome is similar to our own, making mouse genetic research particularly applicable to human disease.

Practically, mice are a cost-effective and efficient tool to speed research and the development of drug therapies. Mice are small and have a short generation time required to perform accelerated preliminary research experiments.
In addition, our ability to directly manipulate the mouse genome provides an incredibly powerful tool to model specific diseases for which the causative gene is known. For example, Ulcerative colitis can be induced by knocking down IL-10.

1.1.3 Common methods used to induce IBD mice model

![Figure 3: Semi-developed formula of 2, 4, 6-trinitro benzene sulfonic acid](image)

2, 4, 6-trinitro benzene sulfonic acid (TNBS), dextran sulfate sodium (DSS) and oxazolone are mostly research used inflammatory bowel disease inducers on mouse model.

2, 4, 6-trinitro benzene sulfonic acid (TNBS) model—The reason of TNBS induced colitis is because of susceptible strains of animals, for example rats, mice and rabbit. It will be delivered by instillation of the complex of TNBS and ethanol. Why ethanol is necessary is because it can break the mucosal barrier, so that TNBS can associated to large intestine autologous or microbial proteins having an interaction with the host immune system, which is supposed to be immunogenic.
As we know, CD 4+ T cells have already performed as a central role in TNBS induced colitis, this kind of colitis is usually used in studying T helper cell-dependent on the strain and independent modification of the concentrations of TNBS administered is necessary. Especially for generating colonic colitis.

Oxazolone colitis—oxazolone is another well-known reagents that research people widely used to induce inflammatory bowel disease on rodent animal like mouse. Mice skin will be painted with 2.5 percent oxazolone dissolved in 100 percent ethanol for first 2 days, then painted with 2.5 percent oxazolone dissolved in 50 percent ethanol in the following a week. Mice will be characterized by their daily body weight change rate, diarrhea, length of colon and bloody stools. In oxazolone colitis mice model, only distal colon and particularly mucosal layers will be attacked by the inflammatory disease. Just like what has been observed in human ulcerative colitis, histology test has shown that, Th2 cytokines, for example IL-6, TNF-alpha and P53 has dramatically increased compare to normal tissue. According to this discovery, this model will be a good choice for ulcerative colitis model.

![Figure 4: Semi-developed formula of oxazolone]
Dextran sulfate sodium colitis—in recent research, DSS is more used compared to TNBs and oxazolone. Because it is much more easy to use—directly dissolved into drinking water. No need to inject reagent into mice anal, or make an organic solution then smear it onto mice skin. Mice will be treated with 3 percent DSS into drinking water, and over a duration of 7 days, mice will have a severe inflammatory bowel disease.

![Semi-developed formula of dextran sulfate sodium.](image)

Figure 5: Semi-developed formula of dextran sulfate sodium.

The degree of substitution is 2. C2 and C4 is sulfated. The acute colitis DSS induced will be characterized by daily body weight change ratio or bloody diarrhea. Through the exactly reason of DSS is still keep unknown. There are 2 possible hypotheses is extensively accepted.

DSS may be directly toxic to gut epithelial cells of the colon crypts, which will affects the quantity of the mucosal barrier. The other assumption is that DSS will inhibit quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

Dextran sulfate sodium is also used to induce chronic inflammatory bowel disease. Mice will be treated with 1 percent DSS water solution in drinking water for the first 5 days, then just water is offered to mice for another 5 days. And this circle is
repeated for 135 days. A very severe IBD is induced, which can be characterized by the mice daily body weight change, bloody diarrhea and length of colon. Histology methods will be helpful to detect the severity of IBD. Especially, 45 days is slight inflammation, 90 days is more serious while 90 days tissue is deadly, the whole colon is inflammatory, and the villus crypt is destroyed.

1.1.4 Pullulan

Pullulan also named asα-1,4-;α-1,6-glucan, is unique linear polysaccharide, which is consisted with 3 glucose subunit named maltotriose. The so called moltotriose is make up with three glucose, which is bonded with α-1,4 glycosidic bonds. while the maltotrioses are connected of 3 glucoses, which is connected withα-1,4 glycosidic bonds. it has a high similarity to Dextran.

Figure 6: Semi-developed formula of pullulan
Cyclodextrin is a family of polysaccharides that made up of several sugar molecules bound in a ring. Beta-cyclodextrin is made of 7 glucose, which are connected with α-1, 4 glucosidic bond. This ring polysaccharide is generated by enzymatic conversion. They are often used in food, drug, chemical industry and pharmacy.

Beta-cyclodextrin is consisted with 7 members in a ring. Starch and cyclodextrin glycosyltransferase are involved in the synthesie of cyclodextrin.

2 EXPERIMENT

In this section we described, our methods including synthesis of pullulan sulfate (PS) and Cyclodextrin sulfate (CS), animal experiment, surgical procedures, western blot, immunofluorescence, immunohistochemistry, histology, gravimetric method, infrared and NMR.
2.1 Organic chemistry experiment

Pullulan sulfate and Cyclodextrin sulfate are synthesized, chlorosulfonic acid is used as sulfate agent.

$^1$H NMR and $^{13}$C NMR spectrums of pullulan sulfate are obtained with bruker 600M instrument.

Infrared spectrums of pullulan and pullulan sulfate are acquired by perkin elmer.

2.1.1 Synthesis of polysaccharide sulfate

Our idea to use pullulan sulfate and cyclodextrin as alternative to DSS is innovative as there is no sulfated pullulan or cyclodextran available on the market. Synthesis of PS and CS are achieved by pyridine- $\text{SO}_3^-$ complex.

\[
\text{Cl-SO}_3\text{H} + 2 \text{Pyridine} \rightarrow \text{Pyridine-SO}_3\text{H}^+ \quad \text{(secondary step)}
\]

**Figure 8: synthesis reaction.** Sulfonic acid is used to react with pyridine, pyridine-sulfate complex is generated, which will act as sulfate agent in the secondary step.

**Materials.**—Pullulan was purchased from Tokyo Chemical Industry CO., LTD, (MW=$2 \times 10^5$ g/mol). Beta-Cyclodextrin was obtained from ACROS ORGANIC. (MW=1134.98).

**Agents.**—all the agents used were of analytical grade. The formamide is obtained from ACROS ORGANIC. 99% for molecular biology.

**Preparation of the pyridine – $\text{-SO}_3^-$ complex** —40 ml pyridine was cooled in -20 °C for 30 min. Then the pyridine with 2-neck flask was submerged into 1: 3 ratio salt ice
mixture. Nitrogen was blowing into the flask as protection for 10 min, then the flask was isolated from air with rubber flask caps. 16 ml Chlorosulfonic acid was added to the flask drop by drop in the fuming cupboard in case of Chlorosulfonic acid smoke was made (8ml Chlorosulfonic acid is added drop by drop in order to get a low sulfate degree compound, named PS I. while the higher sulfate degree compound obtained by adding double equilibrium of Chlorosulfonic is named PS II. CS II is at high degree of substitution.). During adding the Chlorosulfonic acid, stirring was turn to medium level to break the pyridine –SO$_3^-$ complex (solid) for a better reaction efficiency. A constant stirring is applied. The complex was put in the ice-salt mixture for next step.

**Synthesis**—20ml of water free formamide was added into the flask for homogeneous reaction mixture. When all the pyridine –SO$_3^-$ complex solid was dissolved, 2g of pullulan or cyclodextrin was weight and slowly added into the flask with the stirring on. A balloon of nitrogen was connected to the isolated reaction system to block oxygen and water from air and balance pressure. Set the heater to 85 °C (65, 80, 85, 90 degree were used to obtain different degree of substitution). The reaction temperature was monitored. The sulfonation reaction took 4 h (continuous heating and stirring).

**Purification**—100 ml ultrapure water was frozen in -80 °C then break to pieces. Then all the mixture was decanted into 1: 1 ultrapure water ice mixture. The mixture was stirred for 30 min to make sure all the mixture was cool down and dissolved. Then the solution was poured to 400 ml methyl alcohol for precipitation. After that, it was separated by centrifuge (5000 rpm for 10 min). This step was repeated 3 times, then the precipitate was redissolved by water and transfer to salt form by adding 10ml 5 M NaOH
solution. The salt solution was dialysed against water for 1 days (Water was changed every 6 h). Then sample was dialysed against ultrapure water for 3 day with constant stirring to get rid of ion and small impurities. Sample was precipitated with methyl alcohol again, then dissolved with water (in order to increase yield, water volume 30 mL). Liquid nitrogen was used to quick freeze the whole flask. Then the flask was connected with lypholizer. Powder sample was obtained after 10 h. Dry sample was stored against air.

2.1.2 $^{13}$C NMR spectrum

$^{13}$C NMR spectrums were obtained by a 400M nuclear magnetic resonance spectrometer (bruker analytics) applying the Pulse Fourier Transform procedure. Deuterium (99.9 atom % D, alorich) oxide was used as solvent for $^{13}$C NMR. And deuterium dimethylsulfoxide (99.9 atom % D, alorich) was used as $^1$H NMR solvent. Factors: program zg. NS: 2048. DS: 4.

2.1.3 Infrared spectrum

Infrared spectrum was obtained by a Fourier transform infrared spectroscopy (Nicolet iS10 from Thermo Scientific). Water free samples were mixed with potassium bromide. The spectrometer worked in the wave range from 4000 to 650 cm$^{-1}$ in room temperature. The reference measurement was performed with a pure potassium bromide. Spectrometer’s Fourier self deconvolution function finished Final evaluation.
2.2 Biological experiment

PS I, PS II and CS was given to mice in drinking water (3%). DSS, Pullulan are used as reference groups (3%). Respectively for colitis inducer, mice body weight was measured daily.

2.2.1 Animal experiment

All mice were grouped by 5 mice per cage at our animal facility Georgia State University, under controlled conditions of light (12:12 hour dark/light cycle), 5% humidity and 25°C. All animal experiments were approved by the Institutional Animal Care and Use Committee of Georgia State University (Atlanta, GA), and in accordance with the guide for the Care and Use of Laboratory Animals by U.S. Public Health Service.

Wild type mice were from Deltagen (San Mateo, CA). 25 mice were evenly divided to 5 group: Pullulan group, Pullulan sulfate I group, Pullulan sulfate II group, DSS group, cyclodextrin group and cyclodextrin sulfate group. The concentration was fixed at 3% in drinking water.

Mice were sacrificed after 1 weeks. Large intestine length was recorded. Livers, small intestines, colons, spleens, hearts, lungs were collected and put in -80 °C.

2.2.2 PCR

2.2.2.1 TRIZOL RNA Isolation

Homogenization– tissue were homogenized in 2 ml of TRIZOL reagent per 100 mg of tissue using a glass-Teflon or power homogenizer (OMNI international LH96-Automatic homogenizer workstation). The sample volume should not exceed 10% of the volume of TRIZOL Reagent used for the homogenization.
Phase separation rinse—monolayer was cold with ice PBS for 5min. Lyse cells was directly put into a clean experiment plate, then 1 ml of TRIZOL Reagent was added to per 3.5 cm diameter dish and mixed with cell scraper. Sample was past to lysate several time through a micro pipette. Then the cell was homogenized through a vortex. Based on the area size of the dish used (96 holes), TRIZOL 10µL reagent was put into plate then completely mixed by the micro pipette. Otherwise, not enough amount of trizol reagent will lead to a contamination of DNA.

RNA precipitation—the RNA was precipitated from liquid phase through the mixture of cell and isopropyl ethanol. In addition, 0.45ml of isopropyl ethanol is added into per ml of TRIZOL reagent, mix well. Then used as initial homogenization. Samples was incubated in the incubator 25°C for 10 to 15 min (depend on the sample amount), then centrifuge at 11000 x g for 10 min at 4°C for pellet RNA.

RNA wash—after the centrifuge, supernatant was discarded completely, precipitate was collected, washed with gradient alcohol. Every ml of TRIZOL reagent was added more than 1ml of 75% alcohol. Vortex was used to mix these RNA precipitate and TRIZOL together. Then centrifuge again at 7000 x g for at least 5min, 4°C. Above washing steps might have to be repeat according to the purity of the RNA precipitate.

Spectrophotometric analysis—the RNA was diluted with DEPC-treated water at 1:40 ratio. 260nm and 280nm is used to detect sample concentration and purity. Once the result of A260/A280 is larger than 1.6, then the convention that 1 OD at 260nm equals 40µg /ml RNA.
2.2.3 mRNA purification.

Due to a strong inhibition effect of polysaccharide sulfate to both polymerase and reverse transcriptase, the mRNA cannot be used to make standard cDNA. So mRNA purification is necessary. According to the method published in 2013 by Dr. Viennois (33), LiCl can be used to precipitate polysaccharide sulfate molecular. the RNA were precipitated twice by 0.1 volume of 8 M LiCl, followed by a precipitation step in 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol. The RNA were then centrifuged, pellets were washed with 100 µL of 70% ethanol and RNAs were finally dissolved in 20-50 µl of RNase-free water.

2.2.3.1 Standard cDNA synthesis

Maxima First Strand cDNA Synthesis Kit for RT-qPCR from thermo scientific is used to make standard cDNA. In the beginning, an ice box is prepared. Then a sterile and RNAse-free tube is put on the ice. The reaction reagents were added into this order: 5x reaction mixture 4µL, maxima enzyme 2µL, template RNA 3µg, and nuclease-free water 20 µL. the tube was mixed on vortex for 10 min. then centrifuged at 5000 rpm 4degree 10min. Precipitate was disgarded. And the supernate was incubated for 10min at 25 ºC. And then incubated at 50 ºC for 15min.

Due to the primer sequence is GC rich sequence, which is with a large amount of secondary structure RNA template. The reaction temperature is increased to 65 ºC. The terminate reaction temperature is set as 85 ºC for 5min. The product is stored in -20 freezer.
2.2.4 Cell biology and histology

Paraffin embedding, OCT embedding, sectioning, H&E staining, immunofluorescence, immunohistochemistry were finished.

2.2.4.1 Paraffin embedding and sectioning.

Paraffin embedding—organs were dehydrated by being immersed into 50% ethanol alcohol for 90 min, 70% ethanol alcohol for 90 min, 85% ethanol alcohol for 90 min, 95% ethanol alcohol for 90 min, 100% ethanol alcohol for 60 min twice.

Embedding—Organs were washed with xylene ethanol alcohol 1:1 mixture for 60 min, then washed with xylene for 60 min twice. After that, they were permeated by xylene and paraffin 1:1 mixture for 90 min at 62 °C, paraffin alone for 90 min at 62 °C. Histology Cassettes was used to form organs with hot flowing paraffin. Then the organs included in paraffin solidified on the cold station for 5 h, then cassettes were put into -20 °C for 2 days.

Sectioning—Shandon™ Finesse™ 325 Manual Microtome (from thermo scientific) is used to section paraffin embedding organs. The organs buried in the paraffin were cut to 5 micrometer thick slices, then they were collected by ultraclear microscope slides (from denville) 5 pieces of organs in a row. Then the slides were soak in 42 °C water bath for 5 min to extend folded tissue. The slides were dried by a hotplate at 35 °C for 20 min.

2.2.4.2 Hematoxylin and eosin (H&E) staining.

Slides were washed with xylene for 5 min 3 times to remove paraffin. Then they were soaked into 100% ethanol alcohol for 10 min, 95% ethanol alcohol for 10 min, 70%
ethanol alcohol for 5 min. After that, slides were rinsed in distilled water for 10 min. Slides were stained in hematoxylin for 6 min with holder. Then they were washed with flowing tap water to wipe off attached hematoxylin then immersed in distilled water for 3 min. After washing, they were Decolorize in acidic alcohol just 1 tap or 1 second and rinsed in distilled water 3 min again. Then slides were immersed into Eosin for 10 seconds. After that, slides were dehydrated by 70% ethanol alcohol washing for 10 min, 95% ethanol alcohol washing for 10 min, 100% ethanol alcohol washing for 5 min twice. Sides were soaked into xylene 70% ethanol alcohol 1:1 solution for 1 min, and washed by xylene for 1 min as following. Cytoseal was used as mounting media to protect covered tissues. Finally, stained slides were observed under a microscope (M130 from denville).

2.2.4.3 Immunofluorescence

OCT slides were pre-observed and rinsed with ultrapure water for 10 min to eliminate OCT. Then the tissues were completely oxidized by hydrogen peroxide for 30 min and washed with 1x PBS with calcium and magnesium for 10 min again. Then slides were incubated with citrate buffer A (45 ml 0.1 M citrate buffer mixture with 205 ml 0.1 M sodium citrate buffer) for 10 min and cooled down. Washed again with 1x PBS with calcium and magnesium for 10 min. Then the slides were blocked with blocking buffer for 45 min at room temperature. After that, diluted primary antibody buffer was incubated with slides for an hour at room temperature. Next, the slides were washed again with 1x PBS for 3 min 3 times. Incubated with proper secondary antibody for 1 hour at room temperature as follows. Slides were covered with DAPI solution then protected by microscope cover slides.
### Table 3: buffers used for IF

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x PBS</td>
<td>mix the following reagents into 500mL ultrapure water</td>
</tr>
<tr>
<td></td>
<td>80.00g of NaCl</td>
</tr>
<tr>
<td></td>
<td>2.00g of KCl</td>
</tr>
<tr>
<td></td>
<td>14.40g of Na₂HPO₄</td>
</tr>
<tr>
<td></td>
<td>2.40g of KH₂PO₄</td>
</tr>
<tr>
<td></td>
<td>Adjust pH to 7.40</td>
</tr>
<tr>
<td></td>
<td>Adjust volume to 1L with additional distilled H₂O.</td>
</tr>
<tr>
<td></td>
<td>Then sterilize by autoclaving.</td>
</tr>
<tr>
<td>blocking buffer</td>
<td>To prepare 10 ml, add 0.5 ml BSA from the same species as the secondary antibody to 9 ml 1X PBS) and vortex for 5 min. add 30 µl Triton™ X-100 while stirring.</td>
</tr>
<tr>
<td>1° Ab</td>
<td>IL6, TNF alpha, P53 antibodies were used.</td>
</tr>
<tr>
<td></td>
<td>IL6 1µL to 1mL</td>
</tr>
<tr>
<td></td>
<td>TNF alpha 5µL to 1 mL</td>
</tr>
<tr>
<td></td>
<td>P53 2µL to 1 mL</td>
</tr>
<tr>
<td></td>
<td>Dilute antibody with blocking buffer at proper dilution ratio</td>
</tr>
<tr>
<td>2° Ab</td>
<td>Goat anti-rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate. Dilution ration: 1:500 in citrate buffer A</td>
</tr>
<tr>
<td>citrate buffer A</td>
<td>45 mL 0.1M citrate acid buffer mix with 205 mL 0.1M sodium citrate acid buffer.</td>
</tr>
<tr>
<td>DAPI</td>
<td>DAPI concentration was 0.5µg/mL 1xPBS.</td>
</tr>
<tr>
<td></td>
<td>Stored in 4 degree.</td>
</tr>
</tbody>
</table>

#### 2.2.4.4 Immunohistochemistry

Slides were firstly washed by xylene for 10 min, then washed by 100% ethanol alcohol washing for 5 min, 95% ethanol alcohol washing for 5 min, 70% ethanol alcohol washing for 5 min twice. Then the tissues on the slides were fixed by formalin for 10 min, then hydrogen peroxide was used to block the peroxidase. After washing with 1x PBS, the slides were incubated with citrate buffer B for antigen retrieval. Pressure cooker bottom was filled with distilled water, slides with citrate buffer was set in the container, then cooked in high pressure for 10 min cooled for 1 hour on bench. The slides were
completely cooled down, then washed with 1x TBS for 3 min 3 times. Then an immedge pen was used to circle the tissue on the slides.

Blocked with blocking buffer at room temperature for 45 min as follows. After blocking, those slides were incubated with primary antibody for 1 hour at room temperature. Then the rest primary antibody buffer was washed with 1x TBS for 3 min 3 times. Slides was incubated with secondary antibody for 1 hour at room temperature. Diluted DAB reaction buffer was dropped on the tissue circled for 5 min. Then DAB buffer was washed by 1x PBS with calcium and magnesium. Slides were stained with hematoxylin for 6 min and washed with 1x PBS. Finally, the tissues were protected with cytoseal and covered.

<table>
<thead>
<tr>
<th>Table 4: buffers used in IHC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>buffer</td>
<td>recipe</td>
</tr>
<tr>
<td>1x PBS</td>
<td>mix the following reagents into 500mL ultrapure water 80.00g of NaCl 2.00g of KCl 14.40g of Na2HPO4 2.40g of KH2PO4 Adjust pH to 7.40 Adjust volume to 1L with additional distilled H2O. Then sterilize by autoclaving.</td>
</tr>
<tr>
<td>1x TBS</td>
<td>Dissolve the following reagents into 500mL tap water 6.05 g Tris 8.76 g NaCl Adjust pH to 7.5 with 1 M HCl make volume up to 1 L with H2O. TBS is stable at 4°C for 3 mo.</td>
</tr>
<tr>
<td>blocking buffer</td>
<td>To prepare 10 ml, add 0.5 ml BSA from the same species as the secondary antibody to 9 ml 1X PBS) and vortex for 5 min. add 30 µl Triton™ X-100 while stirring.</td>
</tr>
<tr>
<td>1° Ab</td>
<td>IL6, TNF alpha, P53 antibodies were used. IL6 1µL to 1mL TNF alpha 5µL to 1 mL P53 2µL to 1 mL Dilute antibody with blocking buffer at proper dilution ratio</td>
</tr>
<tr>
<td>2°Ab</td>
<td>ABC kit from VECTOR LAB.</td>
</tr>
<tr>
<td>citrate buffer B</td>
<td>2.94g sodium citrate in 950mL dH2O Adjusted pH to 6.0, add 0.5mL Tween-20, add dH20 up to 1L. Mixed well</td>
</tr>
</tbody>
</table>
3 RESULTS

3.1 Synthesis analysis

3.1.1 $^{13}$C NMR

Fig. 9 shows the $^{13}$C NMR spectra of P and PS. Since PS is obtained from P and pyridine –SO$_3$ complex, there was no influence of the molar mass on the position of the peaks. In other words, sulfonation was the only factor that is able to differ $^{13}$C NMRs between P and PS. Due to a good solubility of P into water, D2O was chosen as the solution of $^{13}$C NMR spectra.

![Figure 9: Pullulan (P) and pullulan sulfate (PS) $^{13}$C NMR spectrum confirmed sulfate group location.](image)

Concentration: 0.030g/0.5ml D2O. Range from 55ppm to 105ppm. Green line—PS, Red line—P

As a classic linear alpha glucan, pullulan has the representative feature of ring carbohydrate. Thus the glucose ring is shown on the spectra. It could be seen that the C-1 glucose ring carbon absorbed at 97.93, C-2 at 71.71, C-3 at 73.43, C-4 at 69.47, C-
It has a high possibility that by branching at C-4, down field shifted resonances was not detected. Thus introduction of sulfate groups on pullulan might have a preference on taking place at the C-3. The ring carbons reactivity was in the order C-3>C-2>C-4. The substitution of a hydrogen atom by a sulfate group led to a down field shift of roughly 5 ppm of the appropriate C resonance and to a high field shift at the neighbouring C atoms, (26, 27) caused by magnetic anisotropy of the sulfate neighbouring groups. Since the local magnetic field was averaged over all segment movements and since these movements were slow, the line width was approximately 10 times larger than those of low-molecular materials. Due to the installation of the heavy sulfate group this effect was stronger for the sulfates than for the pure saccharides.

For the pullulan, no visible high field effect was observed at the C-6 and C-5 positions. The C-3 signal appeared at 77.58 ppm. Two additional smaller C-3 signals appeared at 82.88 and 79.57 ppm, describing the sulfates at C-2 and C-4. Besides the high field C-2 resonance at 71.87 ppm there was also a down field shifted signal of the sulfated C-2 at 75.51 ppm. The signals at 70.85 and 70.27 ppm arose from the C-5. This double split could be the result from a high field shift due to the sulfonation at the C-4. The down field shifted C-4 signal was negligible with respect to the resonance of the other signals. The 69.25 ppm peak was probably a high-field shifted resonance of the sulfated C-4 which occurred when a sulfate group was attached at the C-3.

### 3.1.2 FTIR spectrum

There were two obviously characteristic absorption bands showed on TFIR spectroscopy. On one hand, the peak at 1250 cm$^{-1}$ indicated a sulfur atom and oxygen
atom double band asymmetrical vibration. On the other hand, the one at 850 cm$^{-1}$ referred to a symmetrical C-O-S vibration associated to a C-O-SO$_3$ group. As for pullulan spectrum, the 920 cm$^{-1}$ band described alpha-(1,6) linkages. While alpha-(1, 4) linkages were observed at 970 cm$^{-1}$. Ring deformations and scaffold vibrations were showed at 710, 650 cm$^{-1}$. The absorptions at 850 and 765 cm$^{-1}$ showed that pullulan sulfate had a $^4$C$_1$ chair conformation.

As for pullulan sulfate, the 815 cm$^{-1}$ absorption band described a symmetrical C-O-S vibration, with C-O-C bending vibration at 755 cm$^{-1}$. Just like it is showed in Pullulan spectrum, the ring deformation and scaffold vibrations were observed at 700 cm$^{-1}$. There was no influence of the polymer molar mass and only a marginal influence on the degree of substitution. With increasing DS the intensity of the OH absorption band decreased slightly. A similar result was found by Miyaji (34).
3.2 Degree of substitution

Degree of substitution (DS) is detected gravimetrically. Accurate 1.0000g sample was weight, and dissolved with 10mL HCl (10%). Then the solution was precipitated with BaCl$_2$ (25%). The precipitate was obtained by filtration, then washed with DI water for an hour to make the solid ions free. Muffle oven was used to heat the solid to ash (800°C for 3h). The degree of substitution was calculated with following formula:

$$DS = \frac{163.1559 \times g \text{ BaSO}_4}{\text{mass of BaSO}_4}$$

<table>
<thead>
<tr>
<th>temperature/°C</th>
<th>65</th>
<th>80</th>
<th>85</th>
<th>85</th>
<th>90</th>
</tr>
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<tr>
<td>degree of substitution</td>
<td>1.31</td>
<td>2.02</td>
<td>0.71</td>
<td>2.09</td>
<td>Carbonization</td>
</tr>
<tr>
<td>acid used per gram polysaccharide/mL</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>ash weight/g</td>
<td>1.1419</td>
<td>1.4526</td>
<td>0.754</td>
<td>1.4775</td>
<td>NA</td>
</tr>
<tr>
<td>color</td>
<td>white powder</td>
<td>yellow solid</td>
<td>white powder</td>
<td>yellow solid</td>
<td>dark solid</td>
</tr>
</tbody>
</table>

3.3 Anatomy

Mice were treated with regular food and 3 percent PS II in drinking water for a span over 7 days, during the week, daily bodyweight change is recorded. Then those mice were sacrificed. Shorter colon and bloody stools were found.
Figure 11: PS II, CS and DSS induced colitis, confirmed by bodyweight change measurement. DSS—dextran sulfate sodium; PS II—pullulan sulfate II (DS=2.1); PS I—pullulan sulfate I (DS=0.7); P—pullulan; C—cyclodextrin; CS—cyclodextrin sulfate.

As shown in Fig. 11, PS II and DSS treated mice is keep losing weight over a week. On the other hand, PS I and Pullulan group mice body weight are not changing, even gain weight. Consider of those mice were 8 weeks old, and pullulan is a diet food, the body weight change is normal compared to the PS II and DSS group. This is a solid evidence that PS II induced IBD on those mice just ass DSS performed.

Figure 12: PS II induced colitis is confirmed by blood stools and diarrhea.
5 mice were put in this box for 20 min. It is white background. And bloody stools is everywhere.

Bloody stools is the classic feature of ulcerative colitis. Mice which is treated with dextran sulfate sodium will have bloody stools on day 5. In this pullulan sulfate II group, bloody stools was found on day 4. This support our hypothesis that pullulan sulfate has a similar effect on inducing IBD on mice model, which is ulcerative colitis not Crohn’s disease.

Figure 13: Colon length as colitis indicator compared to healthy colon.
7 centimeter long. No bleeding, no swelling is found. No erosion is found either.

Compare to normal colon from a healthy mouse. Inflammation colon is shorter. Because mice body immune system is keeping killing inflammation cells. The inflammation part is swelling and bleeding obviously. All this also supported our ulcerative colitis model assumption. PS II just have same effect on inducing IBD compared to DSS.
Figure 14: Pullulan sulfate II (PS II) induced colitis. Its 4 centimeter long. Bleeding, swelling, and shorter. The whole colon is with erosion.

Figure 15: the specificity for colon is confirmed by the small intestine length. Its 30 centimeter long. No bleeding, no swelling is found. No erosion is found either. The whole small intestine is healthy, not inflammation feature is found.

On the other hand, unlike the colon, small intestine is free of the PS II affection. It keeps just as seem as the healthy small intestine. In this case, it is 30 centimeter long.
No bleeding, swelling and erosion is found.

Figure 16: Normal length of small intestine of pullulan sulfate II group confirming the specificity of PSII for colon.

Its 30 centimeter long. No bleeding, no swelling is found. No erosion is found either. The whole small intestine is healthy, not inflammation feature is found.

Figure 17: PS II, CS and DSS show shorter colon, which is a solid evidence of colitis. DSS—dextran sulfate sodium; PS II—pullulan sulfate II (DS=2.1); PS I—pullulan sulfate I (DS=0.7); P—pullulan; C—cyclodextrin; CS—cyclodextrin sulfate.
Another typical feature of inflammation bowel disease is shorter colon. Due to the inflammation, mice immune system is clearing those sick cells, mice have a shorter colon.

Figure 18: Length of small intestine for polysaccharides tested. Inflammation is not present in the small intestine. DSS—dextran sulfate sodium; PS II—pullulan sulfate II (DS=2.1); PS I—pullulan sulfate I (DS=0.7); P—pullulan; C—cyclodextrin; CS—cyclodextrin sulfate.

3.4 Histology

Figure 19: colitis is confirmed by histology (H&E staining.). DSS PSI and pullulan is used as control. DSS—dextran sulfate sodium, PS I—low sulfate rate pullulan sulfate. P—pullulan.
DSS, PS II, PS I and Pullulan were dissolved with water at 3 percent concentration. And then feed to mice over a span of 7 days. Then mice was sacrificed, the colon was buried with paraffin and H&E staining. A: DSS colon, the base of crypt is totally destroyed by the inflammation. B: PS II colon, the base of crypt is totally destroyed by the inflammation. C: Pullulan colon, tissue remain integrated. No inflammation was found. D: PS I colon, tissue remain integrated. No inflammation was found.

Histological appearance of the mice colonic mucosa after DSS administration. Mucosal injury was produced and characterized by necrosis of epithelium, focal ulcerative of the mucosa and diffused infiltration of neutrophil and lymphocyte (20x).

Figure 20: the specificity for colon is confirmed by histology (H&E staining). DSS PSI and pullulan is used as control. DSS—dextran sulfate sodium, PS I—low sulfate rate pullulan sulfate. P—pullulan.

DSS, PS II, PS I and Pullulan were dissolved with water at 3 percent concentration. And then feed to mice over a span of 7 days. Then mice was sacrificed, the small intestine was buried with paraffin and H&E staining. A: DSS colon, the base of crypt remains integrated, villus structure is clear and no inflammation was found. B: PS
II small intestine, the base of crypt remains integrated, villus structure is clear and no inflammation was found. C: Pullulan small intestine, tissue remain integrated. No inflammation was found. D: PS I small intestine, tissue remain integrated. No inflammation was found.

3.5 PCR

![Colon TNF α](image)

**Figure 21**: Colonic cytokine measurement (TNF alpha) using PS II induced colitis (fold expression change of mRNA). P—pullulan, PS 1—low sulfate rate pullulan. PS II—high sulfate rate pullulan. DSS—dextran sulfate sodium

TNF α is chosen as primer. PS II and DSS have an incredible increment compared to Pullulan and PS I, thus inflammation is detected in PS II and DSS treated tissue.

Tumor necrosis factor (TNF alpha) is a well-known adipokine, which is used as a cell signaling protein. It is related to systemic inflammation and is able to make up an acute reaction. TNF alpha can be generated by activated macrophages, CD4+ lymphocytes, NK cells and eosinophils.
The basic role of TNF alpha is to regulate body immune system. It can induce fever, cachexia, inflammation even has potential to inhibit tumorigenesis. It also respond to sepsis via IL 1 and IL 6 producing cells.

It is made use of a variety of human diseases, especially inflammatory bowel disease (29). Fig 5 shows different expression of TNF alpha in colon and small intestine tissue. In colon tissue, it has a significant expression on PS group and DSS group. And no expression on P and PSI group. On the other hand, it has no obvious distinction between PS, PS I, DSS and P groups.

In this case, PS II and DSS colon shows an absolutely higher TNF alpha level compare to healthy tissue, thus pullulan and PS I group, which indicates the inflammation.

**Figure 22: Colonic cytokine measurement (IL-10) using PS II induced colitis (fold expression change of mRNA).**  
P—pullulan, PS 1—low sulfate rate pullulan .PS II—high sulfate rate pullulan. DSS—dextran sulfate sodium.
Interleukin-10, in short, IL-10 is a well-known anti-inflammation cytokine. It is encoded IL-10 gene.

IL-10 protein is a homodimer, which is consisted with 178 amino acid.

In normal cells, IL-10 protein is inhibited, only very few amount IL-10 protein can be detected. While in inflammation cells, as an anti-inflammation cytokine, it is highly expressed to fix body inflammation. In other words, it will be obviously increased in inflammation cells (31).

In this case, PS II is just as effective as DSS inducing IBD. Even stronger—PS II has a 41 relatively fold change compare to DSS 22 fold change.

In this case, PS II and DSS colon shows an absolutely higher IL-10 level compare to healthy tissue, thus pullulan and PS I group, which indicates the late severe inflammation.

**Figure 23:** PS II induced colitis is confirmed by the fold change of cox 2 of colon. P—pullulan, PS 1—low sulfate rate pullulan sulfate, PS II—high sulfate rate pullulan sulfate. DSS—dextran sulfate sodium
Cyclooxygenase-2, in short Cox-2, is a primarily responsible for inflammation. In 1991, it is discovered that COX exists in 2 form, Cox-1 and Cox-2. Cox-2 is relative to inflammation, but on the other hand, it is not for gastrointestinal integrity or platelet aggregation, which means Cox-2 inhibiter will work as an anti-inflammation, but minimize the risk of bleeding and gastrointestinal toxicity. Now it is developed and people are already make benefits from it.

In our research, Cox-2 is chosen as another inflammation bio marker. In PS II and DSS group, it shows a dramatic increment compare to P and PS I group, which also proved our hypothesis that PS II works just same as DSS. PS II is able to induce severe inflammation on colon tissue.

3.6 IHC

Immunohistochemistry is a very important and useful method to detect inflammation on the digestive track.

IL-6 and TNF alpha are chosen as the detection bio- maker protein.
Figure 24: PS II induced colitis is confirmed by IHC, IL-6 is used as primary antibody. A: pullulan sulfate I (PS II) I colon. B: dextran sulfate sodium (DSS) colon. C: pullulan sulfate I (PS I) colon. D: pullulan (P) colon.

As shown in Fig 25, A and B is PS II and DSS tissue. The whole tissue is anabrotic, base and base crypt is totally melt, cells is everywhere, and not organized. Especially PS II, brown is DAB color of IL-6, while purple is hematoxylin counterstain. The expression of the IL6 protein is significantly increasing in the colonic tissue attesting of severe colitis. On the other hand. Hematoxylin is used as counterstain, there is no IL-6 expression on C and D, but the purple color of hematoxylin. Which is a clear evidence that PS II is able to induce severe inflammation on colon. And cooperation A and B. we can tell that the whole tissue is broken in A, on picture B, there is some part is still remain organized.
Figure 25: IHC picture of IL-6 on small intestine tissue. A: pullulan sulfate II (PS II) small intestine, B: dextran sulfate sodium (DSS) small intestine, C: Pulluan (P) small intestine, D: pullulan sulfate I (PS I) small intestine.

As shown in Fig 26. there is no broken tissue is found. All the cells are organized by its tissue, and no IL-6 expression is found on this picture. Only the healthy tissue is shown by hematoxylin.
As they are shown in Fig 27 and 28, there is a huge increment of TNF alpha expression on PS II and DSS colon tissue, while the expression is lower on Pullulan and
PS I group. Which means that healthy tissue does not have a large TNF alpha expression, while the inflammation tissue has a lot.

The A and B of Fig 28 are less organized, which means the tissue is histologically inflammation.

![Figure 28: CS induced colitis is confirmed by IHC. CS colon (left) and normal colon (right).](image)

In Fig. 28, IL-6 was used as 1° Ab to detect the inflammation on CS colon. There is obviously IL-6 expression (brown color) on CS colon tissue, which indicates a strong inflammation on the CS treated mice colon.

### 3.7 Histologic score

Histological scoring was decided by three factors.
Table 6 inflammation scoring is based on severity of inflammation, crypt damage and ulceration.

<table>
<thead>
<tr>
<th>scores</th>
<th>severity of inflammation</th>
<th>crypt damage</th>
<th>ulceration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>no inflammation was found</td>
<td>intact crypt</td>
<td>no ulcer</td>
</tr>
<tr>
<td>1</td>
<td>granulocyte numbers was increasing in lamina</td>
<td>1/3 base lost</td>
<td>small spot of ulcer</td>
</tr>
<tr>
<td>2</td>
<td>inflammatory cells were found in the submucosa</td>
<td>2/3 base lost</td>
<td>3 or 4 foci of ulcer</td>
</tr>
<tr>
<td>3</td>
<td>none</td>
<td>whole base lost</td>
<td>extensive ulcer</td>
</tr>
<tr>
<td>4</td>
<td>none</td>
<td>erosion on epithelial surface</td>
<td>none</td>
</tr>
</tbody>
</table>

As it is shown above, Pullulan and Cyclodextrin do not induce inflammation. Low degree of substitution pullulan barely induce colitis. PS II induced a stronger inflammation on mice compare to DSS and CS.
3.8 Immunofluorescence

Based on the paraffin sections, immunofluorescence is finished with IL-6, TNF alpha and P53 used as primary antibody.

Recently, a high relative link between chronic inflammatory and cancer is found. Which is supported by the negative regulation of NF-κB, a major inflammation regulator. And P 53 is what it is relative to. P 53 is a major tumor suppressor.

However, for reasons, once the P53 is active, NF-κB activity, inflammation and immune responses will be decreased. Therefore, P53 has anti-cancer function.

Figure 30: PS II induced colitis is confirmed by IF colon data, P53 is used as 1° Ab. P 53 is used as 1° Ab. A: P 53 on PS II colon. B: DAPI on same tissue of A. C: P53 on DSS colon. D: DAPI on same tissue of C. E: P53 on PS I colon. F: DAPI on same tissue of E. G: P53 on Pullulan colon. H: DAPI on same tissue of G.
Figure 31: the specificity for colon is confirmed by IF small intestine data. **P53 is used as 1° Ab.**

As shown in Fig 33, P 53 is used as primary antibody. To observe the P 53 (green) of Pullulan and PS I group, a longer exposure time is used compared to DAPI. On the other hand, DSS and PS II group even take a shorter exposure time to observe the P 53 (green). Which means that DSS and PS II have a strong fluorescence signal compared to PS I and Pullulan group. Thus DSS and PS II have a high P53 level. Which indicates that there is strong inflammation in PS II and DSS treated mice colon, while no inflammation is found in Pullulan and PS I treated mice.
Figure 32: PS II induced colitis is confirmed by IF. IL-6 is used as 1° Ab. A: IL-6 on PS II colon. B: DAPI on same tissue of A. C: IL-6 on DSS colon. D: DAPI on same tissue of C. E: IL-6 on PS I colon. F: DAPI on same tissue of E. G: IL-6 on Pullulan colon. H: DAPI on same tissue of G.

As shown in Fig34, all P53 group (green) used 400ms to expose, while DAPI (blue) just use 50µs. which still indicates that no difference of P53 expression of PS II, DSS, PS I and Pullulan. This proved the inflammation selectivity on colon.

As shown in Fig34, Il-6 is used as primary antibody. To observe the IL-6 (green) of Pullulan and PS I group, a longer exposure time is used compared to DAPI. On the
other hand, DSS and PS II group even take a shorter exposure time to observe the IL-6 (green). But still, we didn’t observe any green (IL-6). This indicates that DSS and PS II have a strong fluorescence signal compare to PS I and Pullulan group. Thus DSS and PS II have a high IL-6 level.

![Image](image_url)

**Figure 33: IF small intestine data**, IL-6 is used as 1° Ab. A: IL-6 on PS II small intestine. B: DAPI on same section slide of A. C: IL-6 on DSS small intestine. D: DAPI on same section slide of C. E: IL-6 on PS I small intestine. F: DAPI on same section slide of E. G: IL-6 on Pullulan small intestine. H: DAPI on same section slide of G.

However, as shown in Fig 35. For all IL-6 group (green), a much longer exposure time is used, 400ms. And all the DAPI group, which is blue, a shorter exposure time (50 µs) is applied. However, we are not able to confirm IL-6 in PS II small intestine. This
result means, in small intestine, IL-6 is not expressed. Thus PS II has an inflammation selectivity on colon.
We also tried IL-6 on liver tissue. All IL-6 picture share a long exposure time of 400ms, while the DAPI picture share a short exposure time of 50µs. Thus the IL-6 stays inhibited in liver, due to the positive relation between IL-6 and inflammation, no inflammation is found in liver, which proved the specificity of PS II induced colitis for colon.

To detect the inflammation on CS colon tissue, IL-6 is used as 1° Ab, which shows IL-6 (green) (left). DAPI is used as counterstain, which shows DAPI (blue, on the right). The IL-6 is obviously increasing.

Figure 35: CS induced colitis is confirmed by IF, IL-6 is used as 1° Ab.

Figure 36: the specificity of CS induced colitis for colon is confirmed by IF pictures of CS small intestine, IL-6 is used as 1° Ab.
However, no IL-6 signal (green) is found at same exposure time: 40μs. only DAPI (blue) can be seen. Which indicates that no IL-6 expressed in small intestine after 7 days CS treatment on mice.

4 CONCLUSIONS

A series of experiments were finished including primary organic compound synthesis, animal experiments and advanced biochemistry analysis methods. A high degree of substitution Pullulan (PS II), a low degree of substitution Pullulan (PS I) and cyclodextrin sulfate were successfully synthesized with pyridine sulfate complex, then preliminarily purified. The sulfate group was verified and the sulfate rate of substitution was detected by gravimetric system. Polysaccharide sulfates were fed to mice in 3% drinking water over a period of 7 days, the body weight change is recorded every day. PS II and CS showed a better time effect on inducing IBD over a span of 6 days compared to DSS model which need a week. Furthermore, the anatomy also proved the inflammation by the appearance of shorter colon, bleeding in colon, swelling colon and bloody stool. CS and PS II kept mice losing weight just as DSS performed. H&E staining showed relatively similar level of inflammation to the DSS for the colon cross section. On the other hand, no inflammation was found in small intestine and liver cross section. Pullulan sulfate successfully induced inflammatory markers as shown by the qPCR data: IL10, Cox-2, TNF alpha.
Immunofluorescence indicated that PS II and CS both were able to increase the P 53 and IL-6 in mice colon. The specificity on colon other than small intestine also proved the inflammation is UC not CD.

The selective location—colon, also indicated that the inflammation is UC not CD. On the other hand, no inflammation occurred in small intestine and liver, which is also proved by histology, immunofluorescence and immunohistochemistry. The hypothesis that pullulan sulfate and cyclodextrin sulfate show at least same ability on inducing inflammation on colon was supported by all these evidence.
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APPENDICES

(A) $^1$H NMR

Figure 37: $^1$H NMR spectrum of pullulan

Figure 38: $^1$H NMR spectrum of pullulan sulfate
### Table 6, qPCR primer sequence

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (Sense, Antisense)</th>
<th>Product Size, bp</th>
<th>Cycle Number</th>
<th>Annealing T°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>5’- CCGGAGAGGAGACTTCACAG -3’</td>
<td>421</td>
<td>30</td>
<td>62</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’- GGAAATTGGGGTAGGAAGGA -3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>5’- TACTGAACTTCCGGGGTGATTGGTCC -3’</td>
<td>290</td>
<td>24</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>5’- CAGCCTTGTCCTTGAAGAAGAACC -3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>5’- ACCACAGTCCATGCCATCAC -3’</td>
<td>450</td>
<td>15</td>
<td>65</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’- CACCACCCCTGTTGCTGTAGCC -3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>5’- TTCAACTCTGTCTCCTCTCCT-3’</td>
<td>554</td>
<td>32</td>
<td>56</td>
</tr>
<tr>
<td>P53</td>
<td>5’- CAGCCCTGTCTGTCTCCAG-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>