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Applications of Polymer Gel Physics: Alcoholic Acute Pancreatitis and Marine Microgel Formation

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APPLICATIONS OF POLYMER GEL PHYSICS: ALCOHOLIC ACUTE PANCREATITIS AND MARINE MICROGEL FORMATION

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To my wife and son.

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ABSTRACT

Gels are a very unique state of matter and play very important roles in nature. As a special intermediate state between solid and liquid, gels have some peculiar characteristics, such as phase transition and ion-exchange. These unique properties allow them to have very broad applications in modern physics and biological sciences. My research has focused on the application of polymer gel physics and engineering to understand a broad scope of biological systems including cells and the ocean. My study on pancreas acinar cells aims to validate a hypothesis to understand the mechanism of initiating of alcoholic acute pancreatitis. Abnormally high $[Ca^{2+}]_C$ in the cytosol side can initiate acute pancreatitis through a Ca^{2+}/K^{+} ion-exchange mechanism with a gel state polyanionic matrix inside the pancreas acinar cell zymogen granules as an ion-exchanger. Ethanol treatment can sensitize the pancreas acinar cells to this ion-exchange process. The application of polymer gel principles to marine microgel system aims to understand the role and mechanism of exopolymers from marine microbes on spontaneous assembly of DOC into highly bioactive polymer gels. The effects of DOC concentration, seawater pH, and seawater temperature on spontaneous assembly kinetics were investigated in this study. A fluorescent-probe assay to measure the fraction of carbon present in self-assembled gels was also developed. The successful applications of polymer gel physics to cell signaling and oceanography demonstrate the great potential of the interdisciplinary approaches in research.

CHAPTER 1

INTRODUCTION

1.1 Motivation and Choice of Alcoholic Acute Pancreatitis and Marine Microgel Formation as Topics

Gels are a form of matter between solid and liquid state. They are usually made of long-chain molecules to form three-dimensional tangled complexes with solvents entrapped inside the networks. The networks hold the liquid from seeping out, and the solvents help to prevent the polymer networks from collapsing, creating a micro-environment that is in thermodynamic equilibrium with surrounding medium. In most cases for natural gels, the solvent is water. Most intriguing characteristics of polymer gels arise from the fact that the polymer chains in such networks are close to each others in the range of Debye field (Tam and Verdugo 1981; Verdugo 1994). Consequently, all weak forces can effectively influence the mutual interactions among polymer chains and between polymer chains and the solvent (including other species in the solvent). Polymer gel matrixes are interconnected by chemical or physical cross-links, such as ionic interactions, hydrophobic interactions, van der waals and hydrogen-bond interactions (Li and Tanaka 1992), keeping the polymer chains in a statistically stable neighborhood. Because of the difference of these connecting forces, the structures of gels range from rigid to easily disperse of viscous fluid. The broad range of structural and ultra-structural features gives polymer gels a unique set of

emergent physical properties, including phase transition (volume transition) between hydrated to condensed states, ion-exchange for preferential selective ions, Donnan potential and swelling kinetics (Tam & Verdugo 1981, Verdugo 1994). These interactions allow polymer gels to play critical roles in various processes happened in nature. At the micron and submicron scale of organization, biopolymer gels make the structural scaffolding of cells and tissues while playing equally important roles in carbon cycling at the global scale of our planet. Although the physical-chemical properties of polymer gels have been studied intensively since the 1940's and the polymer networks have been widely applied from macro to micro scale, new applications of biopolymer gels on some of the most promising and least explored areas of research remain vastly unexplored (Guillard and Hellebust 1971; Benner et al. 1992; Davidson and Marchant 1992; Hedges 1992; Linhardt et al. 1992; Verdugo 1990; Verdugo 1994; Chin et al. 1998).

Previous studies have revealed that the physical properties of biopolymer networks including Donnan effect, dynamics of swelling and phase transitions play a critical role in storage and release of secretory materials in secretory cells (Tam and Verdugo 1981; Verdugo 1994). Abnormally high $[Ca^{2+}]_C$ (free Ca^{2+} concentration in the cytosolic side) has been demonstrated to induce trypsinogen premature activation by releasing of Ca^{2+} and H^+ from a secretory polyanionic gel network inside the zymogen granules (ZGs) through K^+/Ca^{2+} and K^+/H^+ ion-exchange mechanism (Yang et al. 2004; Yang 2004). Since acute pancreatitis is a very serious disease with no treatment available and alcohol abuse is one of the most common triggering factors, I choosed to study the mechanism of alcoholic acute pancreatitis. If ethanol treatment can increase the sensitivity of the polyanionic gel ion exchanger, elevation of $[Ca^{2+}]_C$ could induce acute pancreatitis much more effectively.

The interlinked biological and physical processes by which dissolved organic carbon (DOC) is cycled in the ocean represent one of the most complicated and critical systems on the earth (Chin et al. 1998; Wells 1998). The active exchange between different carbon pools, especially between DOC and POC (particulate organic carbon) in the ocean has been studied from the view of polymer gel assembly and dispersion (Chin et al. 1998). However, a lot of chemical, physical, and biological factors will affect the spontaneous assembly mechanism and most of these processes remain largely unexplored. In my study, I performed a series of extensive research on how these factors affect the marine microgel assembly. These results contribute a rich new framework for many critical questions to the marine carbon cycle.

The selection of alcoholic acute pancreatitis and marine microgel formation as research topics is a continuation and contribution of research done at our lab. Alcoholic acute pancreatitis and marine microgel formation are two totally different processes happened in different biological systems. The research in these two fields is independent. However, gel networks play determing roles in both processes. The extensive investigation of these two systems requires full understanding of various properties of polymer gels and any new findings in these two processes will help us to have better understanding of biophysics of biopolymer networks.

1.2 Arrangement of Dissertation

In my work, I applied polymer gel physics and engineering to understand two totally different but both very challenging biological systems: identification and understanding of the mechanisms to initiate alcoholic acute pancreatitis in pancreatic acinar cells, and understanding the role of polymer assembly in the ocean and its implications in the marine carbon cycle.

In the first section of my dissertation, I applied theories and methods of polymer gel physics to confirm a simple but very important hypothesis. Namely, that ethanol treatment can sensitize the pancreas acinar zymogen granules (ZGs). Following the sensitization, elevated $[Ca^{2+}]_C$ in the cytosol could initiate the experimental acute pancreatitis through the ion-exchanger, a gel state polyanionic matrix inside the ZGs. The detailed experiments and results are explained in Chapter 3.

The second section of the dissertation, which includes chapters 4, 5, 6, and 7, I reported my work on the process of polymer gel spontaneous assembly and its application in the global carbon cycle. Dissolved organic carbon (DOC) is one of the major carbon reservoirs on our planet. Recent evidence suggests that ~ 10% of the DOC pool can enter the microbial loop by forming microgels through spontaneous assembly mechanism (Chin et al. 1998; Wells 1998). However, lots of factors and mechanisms can influence this course and most of these mechanisms remain unexplored. There is still no detection method to estimate the fraction of carbon present in self-assembled marine gels. In chapter 4, I reported my results about the development of a fluorescent-probe assay to estimate the fraction of carbon present in self-assembled gels. This assay is

based on the observation that chlorotetracycline (CTC), a fluorescent probe for bound-Ca^{2+} (Chin et al. 1998), can readily be used to estimate the pool of carbon that exists as microgels. This new assay was applied to measure depth distribution of transects from ALOHA station, kinetics and time variations of self-assembled gels (SAGs) formation, and self-assembled polymer gels formation in alginic solution. This CTC-EDTA fluorescent assay demonstrated comparable effectiveness as flow cytometer and dynamic laser scattering (DLS). This method is also much simpler, less costly, and readily to be used in the field study. In chapter 5, I studied the assembly kinetics of phytoplankton extracellular polymer substances (EPS) and their potential roles in global carbon cycle. Results show that EPS from different phytoplankton species have different assembly mechanisms. Hydrophobic domains are detected in these EPS. In chapter 6, the role of EPS from marine bacteria in the formation of networks including biofilms is studied. My results show that nanomolar concentrations of EPS released by the marine bacteria *Sagitulla stellata* can induce quick assembly of DOC polymers found in seawater. This process is probably driven by hydrophobic rather than electrostatic interactions. Studies using fluorescent resonance energy transfer (FRET) and energy enhancement show that *sargitulla stellata* EPS chains do indeed contain hydrophobic domains that could potentially nucleate the assembly of DOC. Addition of commercial hydrophobic polyesterene beads into seawater DOC pool resulted in similar effects as *sargitulla stella* EPS. These results suggest that by releasing minute quantities of EPS, marine bacteria could eventually capture and concentrate substrates found in the DOC polymer pool. Alternatively, they could build up biofilms by prompting DOC polymers to form networks thereby providing an efficient and intriguing mechanism for bacterial attachment, microbial colonization, and biofilm formation. In chapter 7, I studied the effects of some important environmental

factors, including free DOC polymer concentration, seawater pH, and seawater temperature, on the spontaneous assembly kinetics.

CHAPTER 2

LITERATURE REVIEW

2.1 Acute Pancreatitis

2.1.1 Introduction

Acute pancreatitis is a common, often life-threatening disease with high mortality. An estimated 50,000 to 80,000 cases of acute pancreatitis occur in the United States each year. In 1998, there were 183,000 cases diagnosed in US (Mergener and Baillie 1998). The overall mortality of acute pancreatitis is 10-15%. However, for patients with other severe diseases the overall mortality could increase to 30% (Mergener and Baillie 1998; NIH 2001). Although acute pancreatitis has been recognized for centuries, the initial mechanisms of this disease are still poorly understood (Nam and Murthy 2003). Due to the lack of understanding on the initiating process, currently there is still no specific clinical treatment available (Mergener and Baillie 1998; Nam and Murthy 2003).

The limitation of research efforts that address initiating pathophysiological events in human pancreatitis usually comes from several factors. First, because of the location of pancreas in the retroperitoneal space, it is extremely difficult to obtain pancreas biopsies. Second, patients who admitted to physician and diagnosed as acute pancreatitis patients already passed the initial stages of the disease

(Steer 1999). Currently, much of our knowledge regarding the onset of acute pancreatitis comes from studies on animal models of experimental pancreatitis or from isolated animal pancreas cells. There are five types of experimental animal models of acute pancreatitis: (1) the secretagogue-induced models in which experimental mice were given a supra-dose of cholecystokinin (CCK) or CCK analogue caerulein, which can induce experimental acute pancreatitis (Leach et al. 1991; Rosenzweig et al. 1983; Saluja et al. 1997); (2) the duct obstruction models, which can simulate the gallstone acute pancreatitis; and in these models the outflow of exocrine secretions are mechanically blocked (Steer and Meldolesi 1988); (3) the diet-induced model, in which experimental animals were given a special kind of diet which could induce acute pancreatitis (Saluja and Steer 1999); (4) the duct injection models in which the pancreatic ducts were retrogradely injected with reagents such as bile, bile salts, activated enzymes (Luthen et al. 1998); (5) other models using isolated pancreas cells directly (Bayerdorffer et al. 1985; Halangk and Lerch 2004; Kruger et al. 1998). Although none of these animal models or studies can represent the complex situations in human patients, these results have made the progress to identify some essential early events that lead to pancreas inflammation and acute pancreatitis (Halangk and Lerch 2004).

2.1.2 Onset site of acute pancreatitis

There has been a long-time controversy about where the acute pancreatitis is initiated (Raraty et al. 1999). Early hypothesis suggested that peri-ductal cells were the sites of initial pancreas damage and that pancreatic juice leaked from the pancreatic duct was responsible for the onset of acute pancreatitis (Halangk and Lerch 2004). Later autopsy studies on patients died of acute pancreatitis supported peripancreatic fat necrosis as the onset sites (Kloppel et al. 1986).

Enzymes secreted from acinar cells were supposed to play a central role in the initiating phase. Recent experimental studies have confirmed that the acinar cell is the onset site for damages of pancreas (Raraty et al. 1999; Halangk and Lerch 2004). Genetic studies on patients with hereditary acute pancreatitis also supported the same conclusion (Whitcomb et al. 1996; Lerch et al. 1999). Currently the pancreas acinar cells are widely accepted as the initial sites for acute pancreatitis (Halangk and Lerch 2004). After the establishment of this concept, most cell biology studies of the acute pancreatitis have focused on isolated acinar cells, which also include those have been done in my study.

2.1.3 Premature zymogen activation mechanism in initiation of acute pancreatitis

Pancreas is an important endocrine and exocrine secretion organ in animals. The physiological exocrine function of pancreas includes the synthesis and secretion of digestive enzymes into the small intestine to digest the food constitutes. In the pancreatic tissue, the acinar cells are the protein synthesis factory. More than 90% of the proteins synthesized by acinar cells consist of digestive enzymes, which are transported out of the cell for digestion of food (Steer 1999). Acinar cells also produce proteins that are destined inside the cell to digest intracellular substances. During normal physiological condition, these enzymes remain inactive as zymogens in the process of their synthesis, transport and storage within the acinar cells and after secretion into the pancreatic duct (Lerch and Gorelick 2000). The activation of zymogens requires a proteolytic activation by cleavage of their propeptide. After the pancreatic fluid entering the small intestine, the trypsin zymogen — trypsinogen is activated by the brush border endoprotease enteropeptidase (Gorelick et al. 1992; Gorelick and Otani 1999; Halangk and Lerch 2004). This initial activation of trypsin can further activate

trypsinogen and other zymogens, such as chymotrypsinogen, protelastase, and prophospholipase etc., into their active states (Gorelick et al. 1992).

It is possible that some trypsin activity is generated within acinar cells under normal physiological conditions (Raraty et al. 1999). In animal body there presents some protective mechanisms preventing cell damage from the normal accumulation of trypsin activity. These possible mechanisms include: the existence of pancreatic trypsin inhibitor; the pH environment within zymogen is far away from optimal for enzyme activity; presence of protease capable of degrade active trypsin (Halangk and Lerch 2004). Under some extreme conditions, such as the acute inflammation of pancreas, premature activation of large amounts of trypsinogen could overcome these protective mechanisms and cause the premature release of activated digestive enzymes into the cytosol, which, consequently contribute to cell damage. More than one century ago, this mechanism was suggested as the underling pathogenetic mechanism of acute pancreatitis (Alexander 2002). Ever since then, lots of work has been done to prove the role of premature trypsin zymogen activation as the initial mechanism in the process of acute pancreatitis (Gorelic and Otani 1999; Dufor and Adamson 2003; Oruc and Whitcomb 2004). Modern animal models in acute pancreatitis observed the following phenomena that could support this model in the process of acute pancreatitis: the release of activation peptides of trypsinogen and carboxypeptidase A1 into serum early in acute pancreatitis (Luthen et al. 1998; Mithofer et al. 1998); the increase of pancreatic trypsin activity in the course of experimental pancreatitis (Leach et al. 1991); reduce of injury with serine protease inhibitors (Leytus et al. 1984); and hereditary research evidence (Whitcomb et al. 1996; Lerch et al. 1999).

2.1.4 Ca^{2+} Signaling in the initiation of acute pancreatitis

Many triggering factors of acute pancreatitis have been identified and can be categorized into mechanical, metabolic, infective, vascular, and genetic causes (Raraty et al. 1999). Given the diverse features of all causes and the similar final disease entity, there must be some common pathways linking all the triggering factors together and leading to acute pancreatitis. Many experimental reports indicated similar features in acute pancreatitis (Raraty et al. 1999; Sutton et al. 2003). These observations include the appearance of intracellular vacuoles, premature enzyme activation, loss of acinar cell polarity, and co-localization of zymogens with lysosomal enzymes (Steer 1999; Steer and Meldolesi 1988). All these phenomena are associated with the process of enzyme secretion. These previous results imply that all the triggering factors of acute pancreatitis may be correlated with the digestive enzymes secretion pathway (Ward et al. 1995). Ca^{2+} is a crucial and universal second messenger in the process of stimulus-secretion coupling in acinar cells and might be a common factor in acute pancreatitis (Berridge 1997; Dolman et al. 2003; Niederau et al. 1999).

Animal studies in which the disruption of intracellular Ca^{2+} signaling on premature trypsinogen activation was introduced confirmed the determing role of Ca^{2+} in acute pancreatitis (Dolman et al. 2003; Gerasimenko et al. 1996; Niederau et al. 1999; Raraty et al. 1999). In experimental acute pancreatitis induced by supramaximal CCK or CCK analogue carulein, the premature activation of zymogen was reduced or abolished by depletion of Ca^{2+} by calcium-ATP-ase inhibition, withdraw of extracellular Ca^{2+}, or chelation of intracellular Ca^{2+} with chemical reagents (Halangk and Lerch 2004; Kruger et al. 2000; Saluja and Steer 1999). In animal model experiments that simulated human gallstone-induced acute pancreatitis, the critical role of Ca^{2+} was also observed (Mooren et

al. 2003). Another line of evidence supporting the critical role of Ca^{2+} in initiation of acute pancreatitis came from the observation that some patients suffering hypercaicemia developed acute pancreatitis (Mithofer et al. 1995). Based on these previous reports, the requirement of Ca^{2+} in zymogen premature activation is well accepted (Dolman et al. 2003; Niederau et al. 1999; Raraty et al. 1999; Ward et al. 1995). Many researchers have proposed that elevated intracellular Ca^{2+} is sufficient to induce acute pancreatitis (Raraty et al. 2000; Sutton et al. 2003).

To play the crucial role of intracellular Ca^{2+} in the stimulus-secretion coupling in pancreas acinar cells, it's very important to not only maintain a low intracellular Ca^{2+} level under normal physiological condition, but also subsists a timely Ca^{2+} signaling pathway. Pancreatic acinar cells maintain a Ca^{2+} gradient between cytosol and both extracelluar and internal stores under normal physiological resting conditions (Raraty et al. 1999; Steer 1999). This gradient enables small variation of Ca^{2+} in different localized sites of the cell can be utilized as a signal to control intracellular events, such as enzyme secretion in response to external or internal stimuli. The Ca^{2+} gradient is retained by plasma membrane and endoplasmic reticulum membrane Ca^{2+}-ATPase respectively (Bayerdorffer et al. 1985; Kribben et al. 1983; Steer 1999). The acinar cells develop some mechanisms to sustain a low resting intracellular Ca^{2+}. In the plasma membrane there are store-operated and receptor-operated Ca^{2+} channels (Raraty et al. 1999; Steer 1999). Transient increase of cytosolic Ca^{2+} is pumped out of the cell or into intracellular stores against concentration gradient by ATPases. Influx of extracellular Ca^{2+} into the cell or release from internal stores can be controlled by specific receptors (Raraty et al. 2000).

2.1.5 Ca^{2+}/K^{+} ion-exchange model in the initiation of acute pancreatitis

Although the initiating role of premature activation of trypsinogen and abnormally high Ca^{2+} in acute pancreatitis has been suggested long time ago and widely accepted right now, the exact mechanism of this process is still not clear (Werner et al. 2002). Ion-exchange process has been demonstrated to play a critical role in both mucus secretion in the airway systems and histamine secretion in mast cell (Nguyen et al. 2001; Nguyen et al. 1998; Quesada et al. 2001). A similar ion-exchange mechanism for the premature digestive enzyme activation leading to acute pancreatitis was validated in our lab (Yang et al. 2004; Yang 2004). Our preliminary results indicated that inside pancreas acinar cell ZGs there is a gel state polyanionic matrix that can function as an ion exchanger. On the granules membrane there are Ca^{2+}-activated K^{+} channels (ASK_{Ca}) that import K^{+} into ZGs. When $[Ca^{2+}]_C$ is increased in the cytosol, it causes opening of the ASK_{Ca} channels and K^{+} will move into the lumen of ZGs. The increase in $[K^{+}]_G$ will mobilize bound Ca^{2+} by Ca^{2+}/K^{+} ion-exchange thus increasing the free Ca^{2+} concentration in ZGs ($[Ca^{2+}]_G$), and it will also mobilize bound H^{+} by H^{+}/ K^{+} ion-exchange and decrease the pH ($[pH]_G$) inside ZGs. Both the elevated $[Ca^{2+}]_G$ and decreased $[pH]_G$ not only facilitate the autoactivation of trypsinogen to active trypsin but also stabilize trypsin activity leading to the premature activation of digestive enzymes in pancreatic acinar cells and possible acute pancreatitis (Yang et al. 2004; Yang 2004).

2.1.6 Effects of Ethanol on Acute Pancreatitis

Inflammation of pancreas includes acute events developed from acinar cells with no permanent injury of the organ and chronic pancreatitis with serious destruction, such as fibrosis, scaring, and dysfunction of organs. In human, acute pancreatitis is usually related with mechanical (such as gallstone), metabolic (ethanol abuse, etc.), infective, vascular, and genetic causes (Raraty et al. 1999). Although the causes of this disease are diverse and broad, alcohol consumption is one of the most common factors in both acute and chronic pancreatitis. In US almost one third of all cases of acute pancreatitis are alcohol-related (Mergener and Baillie 1998; Lankisch et al. 1999; Birgisson et al. 2002).

Although the association between alcohol and acute pancreatitis has been established more than one century before, the underlying pathophysiological mechanism of alcoholic acute pancreatitis remains unidentified (Werner et al. 2002; Oruc and Whitcomb 2004). One reason the research dealing alcoholic acute pancreatitis was delayed is because of the lacking of suitable animal models representing human disease. The first animal model study alcoholic acute pancreatitis was developed in 1856 through injection of oliver oil into the pancreatitic duct (Alexander and Schneider 2002). Ever since then, lots of experimental models have been developed (Schneider et al. 2002; Oruc and Whitcomb 2004). Since very few model animals were induced slight or significant acute pancreas inflammation by acute ethanol feeding alone, the severe pathophysiological effect of ethanol is not an independent factor. Current models utilized ethanol feeding in combination with other co-factors to establish the mechanisms that play the role in alcoholic acute pancreatitis. Those include

the effect of acute alcohol administration in combination with CCK or CCK-analogue careulein induced acute pancreatitis (Lu et al. 2002; Pandol et al. 1999; Tachibana et al. 1996), with pancreatitic duct obstruction, with pancreas duct permeability (Mooren et al. 2003; Yamamoto et al. 2003), with virus infection (Clemens and Jerrells 2004; Oruc and Whitcomb 2004), and with cigarette smoking (Hartwig et al. 2000; Rayford 2001; Talamini et al. 2000). These studies provided us with precious though limited knowledge about the initial mechanism of alcoholic acute pancreatitis.

Generally ethanol was proposed to damage pancreas through several mechanisms including: ethanol induced changes in acinar cell protein secretion leading to premature activation of the zymogens; ethanol induced influx of bile, which was more toxic than ethanol; ethanol induced obstruction of the duct system; ethanol induced permeability change of membrane lipids (Singh 1990; Katz et al. 1996). Recent studies found that ethanol can enhance cholecystokin or caerulein induced acinar cell zymogens activation (Katz et al. 1996; Zhao et al. 2002). Thus ethanol is believed to enhance the stimulus-secretion coupling process dependent initiation of acute pancreatitis. In other models, ethanol was also found to sensitize the acinar cells to other co-factors which are causes of acute pancreatitis, such as virus infection, non-oxidative metabolites of ethanol, bile duct obstruction, etc (Hartwig et al. 2000; Morton et al. 2004; Talamini et al. 1996; Schneider et al. 2002).

2.2 Marine Microgel Formation

2.2.1 Introduction

The global climate change has been observed in the past century and will continue to exacerbate in the future. Global warming effect is much more obvious in recent years. Although so far the global mean temperature change is relative modest at around 0.6 ± 0.2 °C, its ecological effects and disruption force on society are evident (Intergovernmental Panel on Climate Change [IPCC], 1990; 2001). In the next 100 years, the predicted change for temperature is faster and bigger than before, exceeding the IPCC 2001 predictions of + 1.4 to 5.8 ^{o}C above the temperatures of the 1990s (Karl and Trenberth 1999; Reilly et al. 2001; World Meteorological Organization [WMO], 2002).

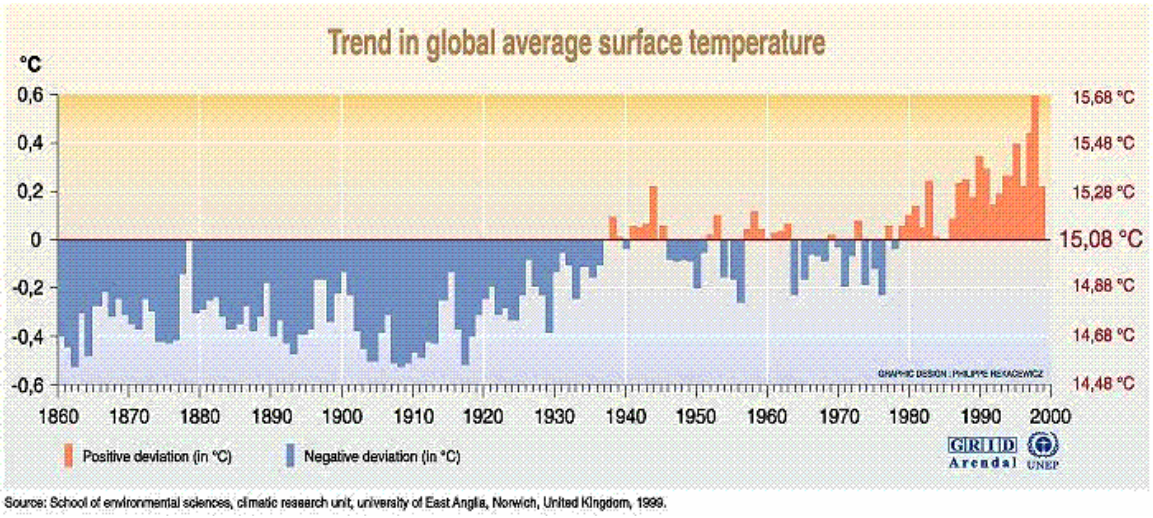


Fig 2-1. Global average temperature trend. Adapted from United Nations Environment Program / GRID-Arendal (http://www.grida.no/climate/vital/17.htm).

Most of the global warming observed before can be attributed to direct or indirect human interventions that have increased green house gases concentrations in the atmosphere (IPCC, 2001). These activities include: addition of new carbon into global carbon cycle through fossil-fuel combustion, deforestation, etc. CO_2 is the most important among the green house gases and it has played an important role in regulating global climate. Prior to the industrial revolution in the nineteenth century, the atmospheric CO_2 concentration varied between ~ 80 and ~ 180 ppmv (Apps and Kurz 1991). The narrow range of CO_2 concentration variation suggests that the global carbon cycle is controlled by biological cycle to maintain balance between net photosynthetic uptake of CO_2 and its total respiration (Falkowski et al. 2000.). Due to human interventions to the global carbon cycle, the atmospheric CO_2 concentration is ~ 370 ppmv now (Falkowski et al. 2000.). Clearly the previous biogeochemical balance was broken by this abrupt increase of CO_2 concentration and hence the global climate has changed.

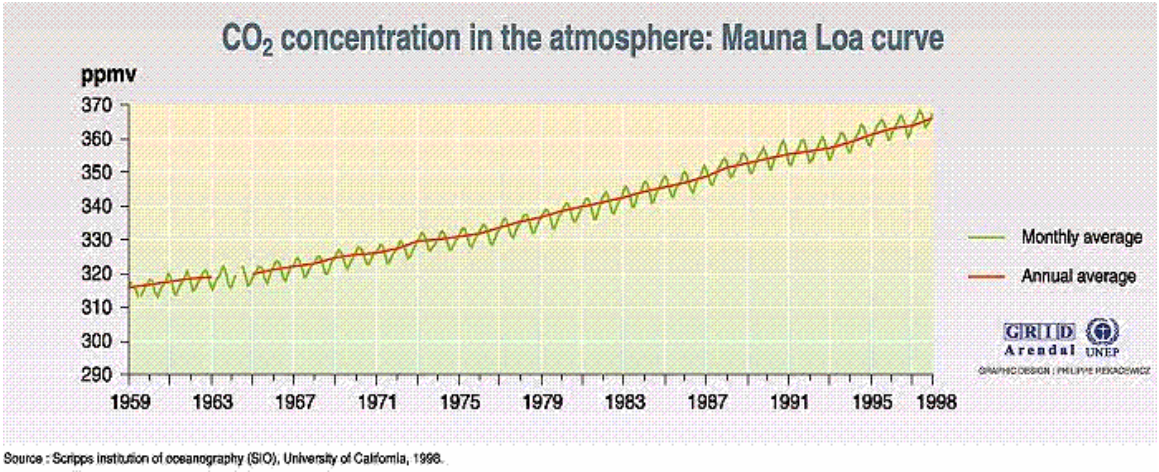


Fig 2-2. CO_2 concentration in the atmosphere. Adapted from United Nations Environment Program / GRID-Arendal (http://www.grida.no/climate/vital/06.htm).

2.2.2 Global carbon cycle and climate change

Over the long revolution of the planet, the accumulation of atmospheric CO_2 to the atmosphere has been resulted in 750 gt C (Houghton 2003; Hedges 1992; Hedges 2000). Each year there is a net addition of 3.4 gt C to the atmosphere. The terrestrial and ocean ecosystem work as active buffers to withdraw CO_2 from the biosphere and maintain the global temperature in a relative level. During 1980 to 1985, 7.6 $\pm$ 0.8 gt C was added to the atmosphere yearly, while only 3.2 $\pm$ 1.0 gt C / yr remains there (Houghton 2000). The rest was processed equally by the ocean and by terrestrial biota and resulted in the accumulation of primary production of carbon in the ocean body finally (Hedges 2000). A large portion of the carbon accumulated in the ocean exists as DOC (Kirchman et al. 1991; Amon and Benner 1996; Benner 2002). DOC and POC (particulate organic carbon) are operational definitions in oceanographic research. For organic carbon can pass through filters of given pore size usually in the range of 0.22~0.8 μm is called DOC, while those pass through the filters are called POC in oceanography science (Verdugo et al. 2004)). DOC is the major reservoir of carbon in the ocean. Although the total concentration of DOC in the ocean is around 700 gt (100 tons / person), it's very dilute. The DOC pool can potentially influence global carbon cycle and climate (Chin et al. 1998; Wells 1998).

Although the DOC and POC pool have similar chemical compositions, both including proteins, polysaccharides, lipids, and oliganucleotides, the DOC pool is refractory and has very low bioactivity. Only the POC pool has higher bioactivity and can be utilized by the ocean ecosystem easily (Kirchman et al. 1991; Kepkay 1994; Amon and Benner 1996). If the DOC remained at its small size, it would be less accessible for microbial utilization and couldn't go into the global carbon

cycle. Any process can convert carbon from the DOC into POC pool and those factors can affect the conversion are very important from the point of view of microbial loop, global carbon cycle, and even for global climate change (Kepkay 1994; Amon and Benner 1996; Benner 2002).

2.2.3 Marine gel phase

It is widely accepted by oceanography scientists that a large part of the marine particles, including DOC and POC, exist in ocean in a gel state (Chin et al. 1998; Wells 1998; Verdugo et al. 2004). Hydrogels are three-dimensional polymer networks imbedded in water (Jensen and Sondergaard 1982; Stordal et al. 1996; Wen et al. 1997; Santschi and Guo 1997; Chin et al. 1998). Gels are a distinctive state of molecular group in which the polymer chains that form their networks are interconnected by chemical or physical cross-links, keeping these chains in a statistically stable background (Tanaka 1992; de Gennes and Léger 1982). The chemical and physical properties of the individual polymer chains and the dielectric characters of the water determine the topology and chemical reactivity of the gel networks and how they interact with the entrapped water, smaller organic or metal ion solutes and living organisms (Tanaka 1992; Verdugo et al. 2004).

In general, chemical complexes composed of covalently cross-linked polymer chains do not disperse, whereas gel networks stabilized by tangles and hydrophobic, or ionic linkages can readily disperse (de Gennes and Léger 1982; Tanaka 1992). The broad range of the structural characters of the gels gives them a unique set of emergent physical properties. Slight alteration in ocean pH, ionic concentration or temperature can trigger extensive and abrupt phase transitions in marine gels that can radically change their size, density, dielectric

properties, chemical reactivities and permeabilities, and hence their potential interactions with enzymes and living organisms (Tanaka 1992).

The marine gel phase exists in the whole size spectrum of organic matter in the ocean from colloids of single macromolecule entangled to water forming network, to macrogels of hundred of μm, such as transparent exopolymer particles (TEP) (Alldredge et al. 1993). The observation that a fraction of the DOC pool remains in reversible equilibrium with self-assembled micron-size networks implies that $\sim 10^{16}$ g C might be found forming a massive microscopic gel phase in seawater (Chin et al. 1998; Verdugo et al. 2004). Recent observations using flow cytometry confirmed that these gels are indeed present in the water column (Verdugo et al. in press) reaching concentrations that are consistent with those expected from previous laboratory observations (Moran and Buesseler 1992; Chin et al. 1998; Guo et al. 2002.). However, a method that can provide a systematic assessment of the budget of carbon found in gel phase in seawater is still not available.

2.2.4 Marine microgel spontaneously assembly theory

The structure properties of marine gel networks make them continuously assemble and disassemble, incorporating (and releasing) a diverse collection of organic matters and ions, including DOC and POC polymers, into a matrix of concomitantly changing volume, porosity, and structure in the ocean environment (Azam 1998; Azam and Long 2001; Orellana and Verdugo 2003). There are many mechanisms that explain assembly and disassembly of marine gels (Twomey 1977; Johnson and Cooke 1980; Alldredge et al. 1993; Chin et al. 1998; Passow 2002). Two major hypotheses have been proposed for the conversion of carbon from DOC to POC: aggregation from multiple collisions and

subsequent adhesion among small particles to form macrogels (Twomey 1977; Jackson 1990), and spontaneous assembly of DOC polymers to form larger submicro gels by gelation and annealing (Chin et al. 1998) (Fig 2-3).

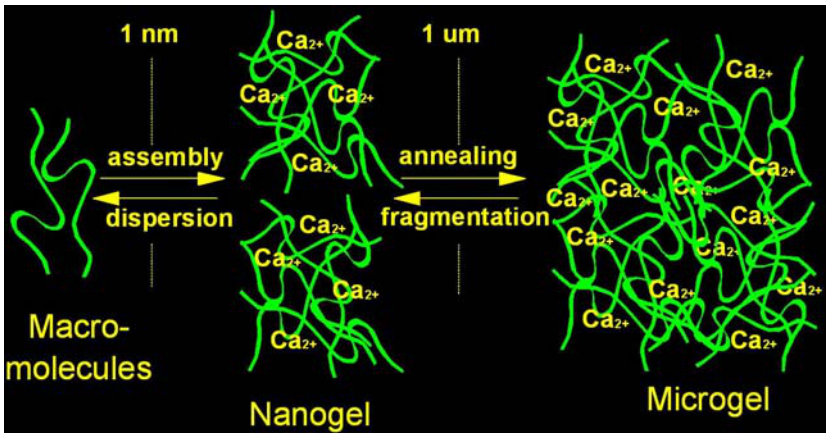


Fig 2-3. Processes transforming from free marine DOC to polymer gels. Wells, 1998; Chin et al. 1998.

Aggregation mechanism was first suggested by aerosol physicists (Twomey 1977) and has been applied to explain particle size spectra in natural waters (McCave 1984; Jackson 1990). Coagulation theory quantitatively describes the rate of change in particle abundances and size distributions generated by the repetitive collision and sticking process (Kepkay 1994; Verdugo et al. 2004). According to this mechanism, aggregation rate is a function of the sizes, concentrations, and stickiness of colliding particles and the intensity of the physical processes colliding them together. For colloidal-sized particles these physical processes include Brownian motion, shear (both laminar and turbulent), differential settlement, surface coagulation, diffusive capture, filtration, and, for

living particles, motility (Kepkay 1994; Verdugo et al. 2004). Brownian motion is the dominant collision mechanism for submicron particles in still seawater such as the deep ocean (McCave 1984; Honeyman and Santschi 1989; Jackson and Burd 1998).

According to polymer gel theory, the polymer complex that forms the matrix of polymer gels stem from spontaneous or induced assembly of polymer chains (Doi and Edwards 1986). Assembly is different from coagulation in that it happens when the inter-chain distance allows polymers to interact with each other via chemical (covalent) or physical bonds (entanglements, electrostatic and hydrogen bonding, or hydrophobic/hydrophilic interactions, van der Waals forces, etc) that result in the formation of cross-linking. Assembly could not be explained by collision or by a stickiness argument process. Whether marine free DOC can self-assemble to form polymer gels has never been demonstrated before the publication of Chin's work in 1998 (Chin et al. 1998). They first demonstrated that free ocean DOC can spontaneously assemble to form marine polymer gels with experimental observations (Fig 2-4). This formation mechanism can be explained with polymer gel theory (Chin et al. 1998).

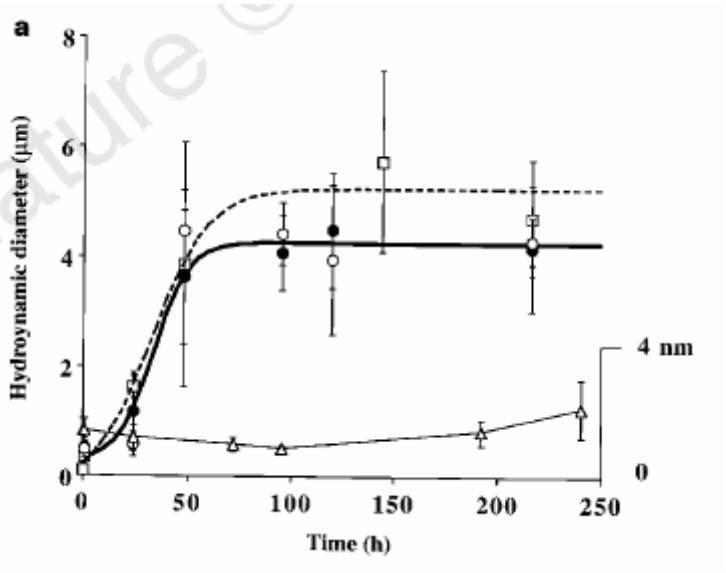


Fig 2-4. Marine gel spontaneous assembly kinetics. Chin et al. 1998

In their work, the kinetics of marine DOC polymer assembly was measured by a dynamic laser scattering (DLS). Results showed that DOC polymers filtered from seawater with filter size 0.22 μm could spontaneously assemble. The assembly process was non-linear and following a second order kinetics. Samples measured with flow cytometry revealed the similar process of assembly. In the process of marine gel assembly, divalent bonds were the essential stabilizing force. Chelation of Ca^{2+}/Mg^{2+} with ethylenediaminetetraacetic acid (EDTA) blocked the self-assembly process completely (Chin et al. 1998).

The spontaneous assembly mechanism of marine gel formation had been well demonstrated and widely accepted (Chin et al. 1998; Wells 1998). However the probability of particle assembly and the stability of the gels formed also depend on chemical and physical characteristics of the individual polymer chains and how they interact with other components of the ocean. Seawater temperature,

turbulence, pressure, and marine microorganism can also affect the assembly. Factors that affect the biopolymers, such as ultraviolet photolysis, enzymatic hydrolysis and presence of organic pollutants, microorganism effects could be potentially very important.

2.2.5 Extracellular polymer substances

Extracellular polymeric substances or exopolymers (EPS) are widely distributed in the ocean water. Microbial EPS produced by marine organisms are released to the seawater forming up to 50% of the DOC polymer pool (Sell et al. 1992). Among all the marine microorganisms, bacterial and phytoplankton are believed to be the major sources of marine EPS (Heissenberger et al. 1996; Singh et al. 1999; Brachvogel et al. 2001). EPS can exist as capsular, slime, or free dissolved matter (Decho 1990). In general, the chemical compositions of EPS are rich in polysaccharides, fair amounts of proteins, small amounts of lipids and nucleic acid (Sutherland 1977; Dcho 1990; Leppard 1995; Davies and Hawkins 1998). The composition of EPS varies considerably between phytoplankton and bacterial. Slight variation of the composition can alter the physico-chemical properties of EPS, thus in turn reflects their fate and role in marine biogeochemical processes (Bhaskar and Bhosle 2005).

EPS have many roles and functions in ocean environment (Wotton 2004; Bhaskar and Bhosle 2005). When associated with organisms, EPS can play roles in attachment, formation of biofilms, aid to locomotion, protection against physico-chemical change in environment, adsorption of nutrients, etc (Decho 1990; Costerton et al. 1978; Hoiczyk 2000; Murray 1995). When EPS are released to the surrounding seawater and become remote from the organisms, the EPS play critical roles in the biogeochemical cycle (Wotton 2004; Bhaskar

and Bhosle 2005). Much of the EPS released in the ocean is in the form of dissolved matter and can make up to 50% of the DOC pool (Sell et al 1992). Particulate size of EPS also exists in the size range of from nm to μm forming colloids. Overall EPS contributes signifitantly to the organic matter pool in the seawater. Dissolved EPS is a major carbon source for marine bacterial and it can be converted to the particulate form through bacterial activity thus become available to higher organisms and enter the microbial loop (Decho 1990; Wotton 1996). Mucus form of EPS can also serve as food sources for animals (Wotton 2004). The unique properties of EPS make them not only a source for organic carbon pool and marine food web, but also play an integral role in the entire biogeochemical process. EPS can provide adhesive bridges to coagulate and scavenge other particles to form larger aggregates like marine snow. EPS are already known for their ability to bind and remove heavy metals and nucleotides from natural water and waste waters (Dhami et al. 1998, Tolley and Macaskie 1993, Wilhelmi and Duncan 1995). Free dissolved polymers of EPS in seawater can potentially intertwine with each other to form a loose hydrated matrix, marine gels (Chin et al. 1998; Wells 1998; Verdugo et al. 2004).

CHAPTER 3

ALCOHOLIC ACUTE PANCREATITIS

3.1 Introduction

Acute pancreatitis is a life-threatening disease with high mortality and multiple causes (Steer 1999; Lerch and Gorelick 2000). Due to our poor understanding of its early stages of development and initial pathological mechanisms involved, currently, there is still no definitive clinical treatment available (Nam and Murthy 2003). A multitude of broad trigger factors of acute pancreatitis has been identified and may be grouped into mechanical, metabolic, infective, vascular, and genetic causes (Raraty et al. 1999). Among them, excessive alcohol abuse alone accounts for around one third of all cases in US (Lankisch et al. 1999; Birgisson et al. 2002). Given the diverse features of all causes and the final similar disease entity, there must be some common pathways linking all the triggering factors together and leading to acute pancreatitis. Almost all experimental models of acute pancreatitis suggest that premature activation of trypsin be the common factor and play an initiating role in the pathogenesis of pancreatitis (Steer 1999; Lerch and Gorelick 2000).

Free calcium is a ubiquitous intracellular second messenger with a variety of physiological effects in cell. It is demonstrated that intracellular Ca^{2+} ($[Ca^{2+}]_C$) acts as important role in the process of stimulus-secretion coupling in acinar cells

and sustained elevation of $[Ca^{2+}]_C$ disrupts the normal zymogen activation process and leads to acute pancreatitis in experimental mice (Frick et al. 1997; Kruger et al. 1998; Gorelick and Otani 1999; Raraty et al. 1999). Our recent results suggest that abnormal elevated $[Ca^{2+}]$ in the cytosol ($[Ca^{2+}]_C$) can initiate acute pancreatitis through K^+ ion exchange mechanism (Yang et al. 2004; Yang 2004). Hence, agents that can sensitize acinar cells to the effects of elevated $[Ca^{2+}]_C$ might exasperate zymogen premature activation and lead to pathology of pancreas inflammation. Although it is well established that alcohol use is one of major risk factors for acute pancreatitis, the mechanisms that ethanol triggers pancreatitis are still not completely understood (Werner et al. 2002). To find out the initiating mechanism for alcoholic acute pancreatitis, we proposed that alcohol treatment might increase the sensitivity of acinar cells to elevated $[Ca^{2+}]_C$ induced zymogen activation. To verify this model, isolated ZGs from mouse pancreas acini were exposed to alcohol directly in the range of 0 to 100 mM (relevant to usual levels of drinking). Changes in pH ($[pH]_G$), Ca^{2+} concentration ($[Ca^{2+}]_G$), and trypsin premature activation inside the pancreatic ZGs after incubation with ethanol were measured as representative markers for the sensitization effects (Frick et al. 1997; Gorelick and Otani 1999; Raraty et al. 1999; Yang et al. 2004; Yang 2004). Results corroborated with the model predictions.

3.2 Experimental Methods

3.2.1 Hypothesis

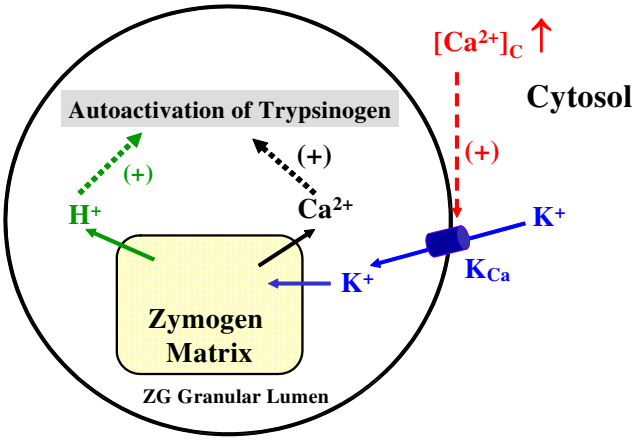


Fig 3-1. Model for ethanol induced acute pancreatitis: treatment of pancreas acinar cell ZGs with ethanol can sensitize the pancreas acinar cells to the effects of elevated-$[Ca^{2+}]_C$, consequently the latter initiate premature activation of trypsinogen via the $[Ca^{2+}]_C$ induced ion-exchange mechanisms (Yang et al. 2004; Yang 2004).

Inside the pancreas acinar cell ZGs, there is a polyanionic matrix. The matrix and intragranular media forms polymer gels. Under normal physiological condition, this gel matrix binds with cation ions such as Ca^{2+} and H^+ to stabilize the gel structure and regulate $[Ca^{2+}]_G$ and $[pH]_G$ level inside the ZGs. A sustained increase of $[Ca^{2+}]_C$ in the cytosolic side of the acinar cells was demonstrated to open the ASK_{Ca} channels on the ZGs membranes and trigger the influx of K^+ into the lumen of the ZGs. The influx of K^+ can free bound Ca^{2+}

and H^+ through an ion exchange mechanism (Yang et al. 2004; Yang 2004). Both the increase of $[Ca^{2+}]_G$ and decrease of $[pH]_G$ can facilitate the trypsinogen premature activation and lead to acute pancreatitis. I plan to test the hypothesis that treatment of pancreas acinar cell ZGs with ethanol can sensitize the pancreas acinar cells to the effects of elevated-$[Ca^{2+}]_C$ (Fig 3-1). Consequently, same level of $[Ca^{2+}]_C$ elevation is expected to induce higher trypsinogen premature activation and exasperate the initiating of acute pancreatitis after incubation with ethanol.

I used isolated ZGs directly instead of pancreas acinar cells. One critical problem associated with monitoring fluorescence intensities emitted from respective indicators in intact cells with thin optical sectioning is that following stimulation secretory granules get mobilized, quickly move out of the focal plane and disappear from the field of observation. Work on isolated secretory granules can not only overcome movement problems, but also reduce the interference from the cytoplasm or other cellular organelles. An intracellular buffer [140 mM potassium glutamate, 100 nM Ca^{2+} (EGTA-buffered), 20 mM Tris, pH 7.4] will be used to retain the physiological functions of the isolated secretory granules. This protocol was successfully applied on secretory granules of goblet cells, mast cells and pancreas acini cells (Nguyen et al. 1998; Quezada et al. 2001; Chin et al. 2002; Yang et al. 2004; Yang 2004).

3.2.2 Isolation of pancreas acinar cells and dye loading

Mice were sacrificed by exposure to CO_2 according to a protocol approved by Florida State University Animal Care Committee. Isolated pancreatic acinar cells were harvested from mouse pancreas with collagenase and cleaned with Hank's solution. Intact pancreatic acinar cells were equilibrated at 37°C for 30 minutes

in Hanks' solution containing 10μM Rhodamine 110, bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide) dihydrochloride (BZiPAR) ($\lambda_{excitation}$ = 498 nm, $\lambda_{emission}$ = 521 nm) (Molecular Probes, Eugene, Oregon) (Raraty et al. 2000; Kruger et al. 2000) to measure trypsin activity within ZGs. The labeled cells were washed with Hanks' solution to remove any excess dyes.

Similarly, 5μM Calcium Orange-5N-AM (K_d = 20 μM, $\lambda_{excitation}$ = 549 nm, $\lambda_{emission}$ = 576 nm) (Molecular Probes, Eugene, Oregon) (Nguyen et al. 1998; Quezada et al. 2003; Chin et al. 2002), 1μM LysoSensor Blue DND-167 (pK_a = 5.1, $\lambda_{excitation}$ = 373 nm, $\lambda_{emission}$ = 425 nm) (Molecular Probes, Eugene, Oregon) or LysoSensor DND-189 (pK_a = 5.2, $\lambda_{excitation}$ = 443 nm, $\lambda_{emission}$ = 505 nm) (Molecular Probes, Eugene, Oregon), were loaded to monitor Ca^{2+} concentration ($[Ca^{2+}]_G$), pH fluctuations ($[pH]_G$) (Nguyen et al. 1998; Quezada et al. 2001; Chin et al. 2002) within the ZGs respectively. These dyes can be loaded separately or in combination with each other.

3.2.3 ZGs isolation and detection of pH ($[pH]_G$), Ca^{2+} ($[Ca^{2+}]_G$), trypsin premature activation inside ZGs

Granules were isolated by following a procedure published elsewhere (Marszalek et al. 1997; Nguyen et al. 1998; Quezada et al. 2001; Chin et al. 2002). Briefly, while labeled cells were suspended in an intracellular solution (140 mM potassium glutamate, 20 mM Tris, 5 mM $MgSO_4$, 10 mM MES, and 2 mM EGTA, at room temperature, pH 7.3) that mimicked the intracellular environment, they were disrupted by brief sonication, and the secretory granules were separated by centrifugation. The granules were then allowed to attach to polylysine-coated chambers filled with intracellular solution containing different $[Ca^{2+}]_C$ as published previously (Nguyen et al. 1998; Quezada et al. 2001; Chin et al. 2002; Yang et al.

2004; Yang 2004). The chambers were mounted and kept at 37°C on the thermo-regulated stage of an inverted Olympus fluorescence microscope. Fluorescence emission of each dye was collected under the excitation of its specific excite wavelength and the intensity was analyzed using commercial digital-imaging softwares (Image-Pro Plus, SharpStack, Media Cybernetics, Silver Spring, MD, USA). The unit for trypsin activity used here was an arbitrary unit (AU) for the fluorescence intensity from BZiPAR. The fluorescence intensity for Calcium Orange-5N-AM and LysoSensor DND-189 or LysoSensor Blue DND-167 was calibrated and converted into Ca^{2+} ($[Ca^{2+}]_G$) and pH ($[pH]_G$) respectively according to procedures published elsewhere (Nguyen et al. 1998; Quezada et al. 2001; Chin et al. 2002; Yang et al. 2004; Yang 2004)

3.3 Results and Discussions

3.3.1 Ethanol-induced trypsin activity, pH ($[pH]_G$) and Ca^{2+} concentration ($[Ca^{2+}]_G$) inside ZGs under various $[Ca^{2+}]_C$ level

To study the effect of ethanol on zymogen activation, isolated mouse pancreas acini granules were treated with increasing concentrations of ethanol under varied free $[Ca^{2+}]_C$ for 120 min. The sensitization effect of ethanol incubation to $[Ca^{2+}]_C$-induced trypsinogen premature activation is dose-dependent (Fig. 3-2A).

Fig 3-2A.

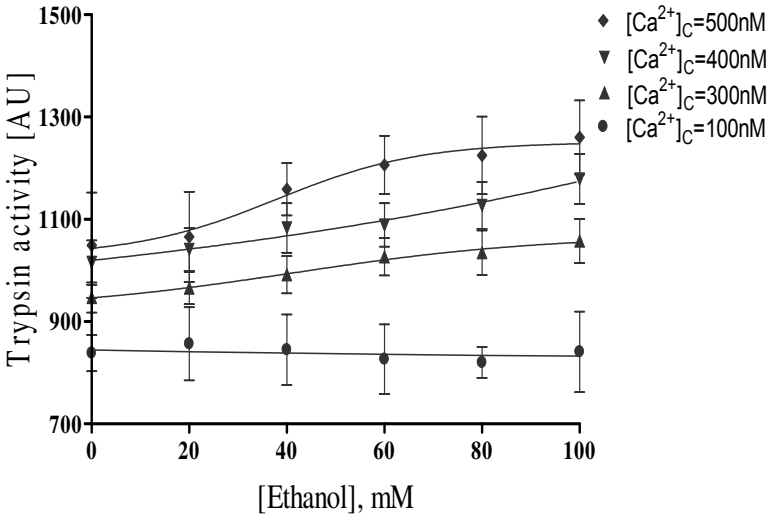


Fig 3-2B.

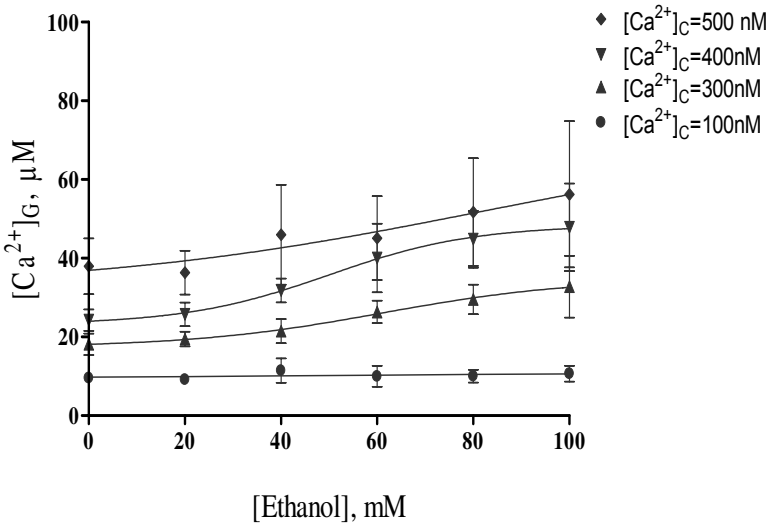


Fig 3-2. Direct effects of ethanol in the intracellular solution on trypsin activity, $[Ca^{2+}]_G$ and $[pH]_G$ within ZGs. Panel A: trypsin activity; B. $[Ca^{2+}]_G$; C. $[pH]_G$. This effect relies on free $[Ca^{2+}]_C$ in cytosol side. Filled circles: $[Ca^{2+}]_C$=100 nM; filled triangles: $[Ca^{2+}]_C$=300 nM; filled inverted triangles: $[Ca^{2+}]_C$=400 nM; filled diamonds: $[Ca^{2+}]_C$=500 nM;

Fig 3-2C

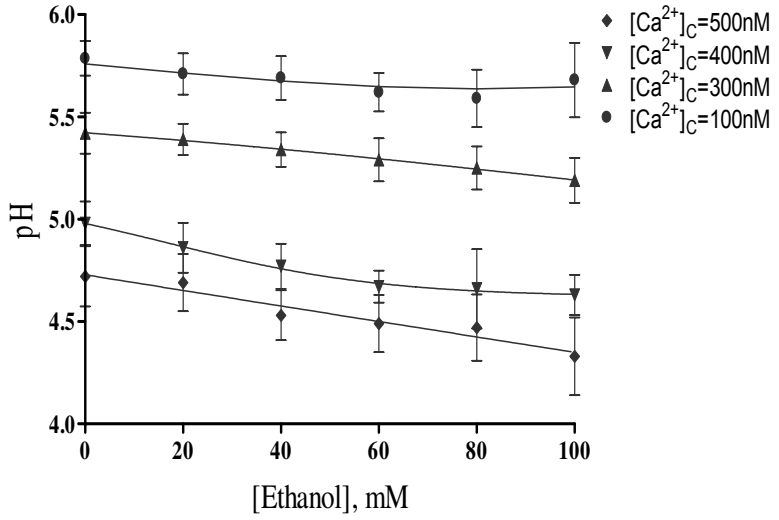


Fig 3-2 Continued

Trypsin activity increase is a substitute marker of zymogen activation within pancreas. Since the intensity of the trypsin activity could not be measured directly, we used the fluorescent intensity from Rhodamin-110 to represent trypsin activity. BZiPAR is a specific substrate for the serine protease trypsin and itself has no fluorescence. After the cleavage of the two oligopeptide side chains from its structure catalized by trypsin, BZiPAR becomes Rhodamin-110 and emits fluorescence. The resting trypsin activity inside ZGs was 838.27 ± 35.65 (AU, mean ± SD, n=35). Treatment of granules with high concentration of $[Ca^{2+}]_C$ (500 nM) alone resulted in a 1.25-fold increase of trypsin activity. Prolonged exposure of $[Ca^{2+}]_C$ (500 nM) treated granules to ethanol (100 mM) resulted in another 1.20-fold enhancement of trypsin activity. This sensitization effect of ethanol was concentration dependent and strongly relied on the free $[Ca^{2+}]_C$ inside intracellular solution. Treatment of ZGs with ethanol alone (0-100 mM) or together with resting $[Ca^{2+}]_C$ (around 100 nM) resulted in no increase of zymogen

activation (Fig 3-2 A). Similar results were obtained for changes in Ca^{2+} concentration ($[Ca^{2+}]_G$) and $[pH]_G$ inside ZGs (Fig. 3-2B, C).

Comparable effects of ethanol on CCK or caerulein-induced zymogen activation in pancreatic acinar cells were found (Zhao et al. 2002; Mark et al. 1996). However, we used isolated pancreas acinar cell ZGs instead of the whole cell according to protocols published previously (Nguyen et al. 1998; Quesada et al. 2001; Chin et al. 2002). Directly working on isolated secretory granules can overcome the movement problem and reduce the interference from the cytoplasm or other cellular organelles.

3.3.2 $[Ca^{2+}]_C$ -induced trypsin activity, pH ($[pH]_G$) and Ca^{2+} concentration ($[Ca^{2+}]_G$) inside ZGs

Either hyper-stimulation of CCK or CCK-analog caerulein can induce acute pancreatitis on experimental mice (Katz et al. 1996; Zhao et al. 2002). This activation appears to be dependent on a rise in $[Ca^{2+}]_C$ (Raraty et al. 1999). The trypsin premature activation can be caused by elevated $[Ca^{2+}]_C$ directly (Michael et al. 2000). We measured the direct effect of $[Ca^{2+}]_C$ on ethanol treated ZGs (Fig 3-3). In the broad range of ethanol concentration used, elevated $[Ca^{2+}]_C$ elicited an increase of trypsin activity to a peak of 1259.98 ± 72.41 (AU, mean ± SD, n=15) from a base line of 838.27 ± 35.65 (AU, mean ± SD, n=35); the calcium concentration inside the ZGs ($[Ca^{2+}]_G$) was elevated 6.10-fold; the pH ($[pH]_G$) decreased from 5.68 to 4.16. When $[Ca^{2+}]_C$ in the intracellular solution remained at normal physiological level (around 100 nM) or lower, there was no measurable zymogen activation. Since the free intra-granular concentration $[Ca^{2+}]_G$ is approximately 10 μM and it is much higher than $[Ca^{2+}]_C$ (~100nM), the

major source for the ethanol-induced $[Ca^{2+}]_G$ increase is from the zymogen granular organelle itself.

Fig 3-3 A

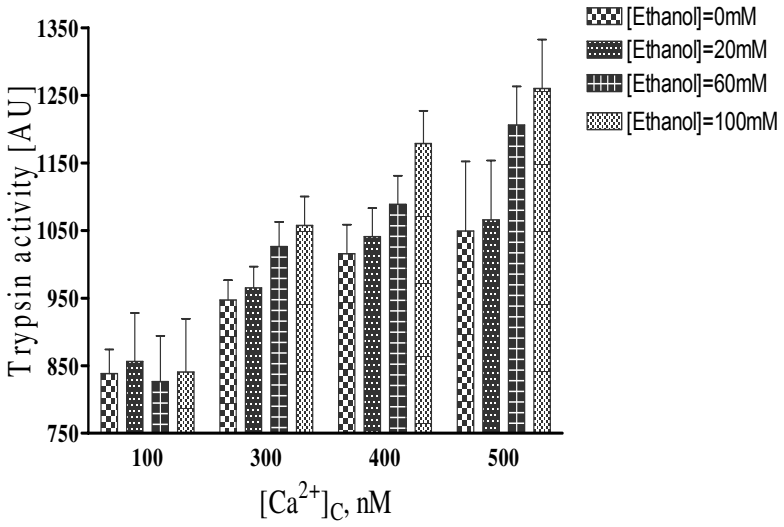


Fig 3-3 B

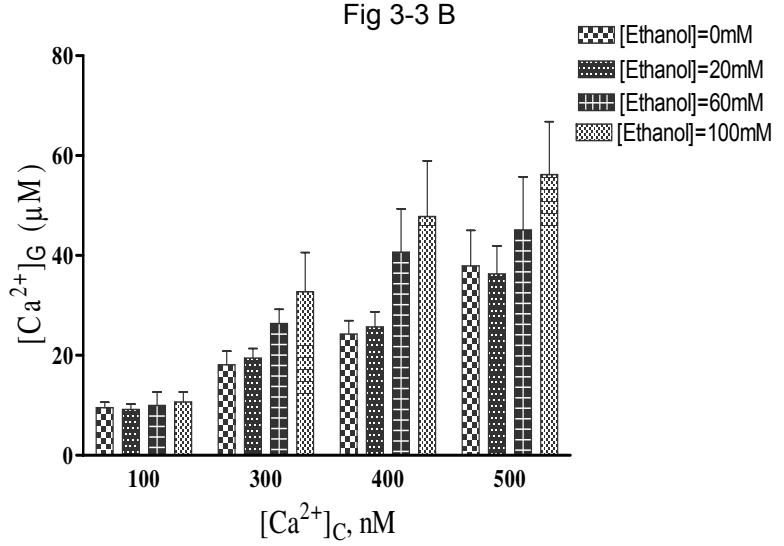


Fig 3-3. Effect of increasing free Ca^{2+} concentration in the intracellular solution ($[Ca^{2+}]_C$) on trypsin activity, $[Ca^{2+}]_G$ and $[pH]_G$ within ZGs, under various ethanol concentration. A. Increasing $[Ca^{2+}]_C$ resulted in the increase of trypsin activity; B. Increasing $[Ca^{2+}]_C$ resulted in the increase of $[Ca^{2+}]_G$; C. Increasing $[Ca^{2+}]_C$ resulted in the increase of $[H^+]_G$, thus the decrease of $[pH]_G$.

Fig 3-3C

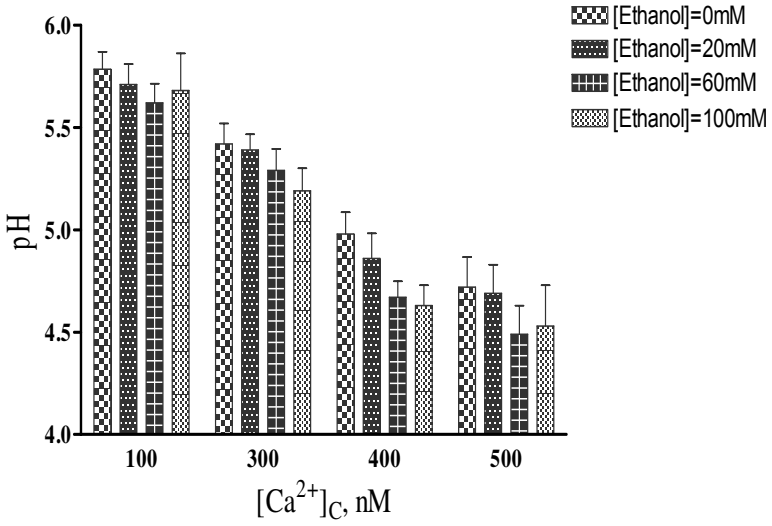


Fig 3-3 Continued

The pre-activation of trypsin is an early and crucial step in acute pancreatitis disease. How could ethanol promote this process? There are probably two mechanisms. First, alcohol consumption may sensitize the sick to the effect of abnormally elevated second messenger $[Ca^{2+}]_C$ induced by multiple triggering factors of acute pancreatitis (Katz et al. 1996; Zhao et al. 2002). Second, ethanol itself may stimulate the increase of $[Ca^{2+}]_C$ directly, the latter then couples trypsin activation and secretion (Hoek et al. 1992; Michael et al. 2000). Ethanol has an effect that may change cell structure and function, including membrane fluidity, protein-protein interaction, and lipid structure (Singh 1990; Katz et al. 1996). Ethanol treatment also has been reported to affect cell signaling (Katz et al. 1996). It was found that ethanol could affect calcium channel conductivity and cGMP production that could facilitate the influx of Ca^{2+} (Hoek et al. 1992).

3.3.3 The effects of ASK_{Ca} channels on the sensitization effects of ethanol

The data above demonstrated that the ethanol treatment can sensitize the pancreas acinar cells to the effects of elevated $[Ca^{2+}]_C$. We further tested whether this process requires the concomitant results from K^+ channels or not. Our previous results demonstrated the existence of ASK_{Ca} channels on the ZGs membranes (Yang et al. 2004; Yang 2004). These channels belong to SK_{Ca} channels and 20 mM or higher tetraethylammonium chloride (TEA) can block this type of channel completely. After the blocking of ASK_{Ca} channels with 20 mM TEA, we measured the direct effect of ethanol treatment under various $[Ca^{2+}]_C$ level. Results show that after the blocking, ethanol treatment could not enhance elevated $[Ca^{2+}]_C$ induced change of trypsin activity, $[Ca^{2+}]_G$ and $[pH]_G$ inside the ZGs. Both $[Ca^{2+}]_G$ and $[pH]_G$ remained the same level as prior to treatment with ethanol (Fig 3-4). These results show that sensitization effects of ethanol rely on the activation (openning) of the ASK_{Ca} channels on the ZGs membranes.

Fig 3-4 A

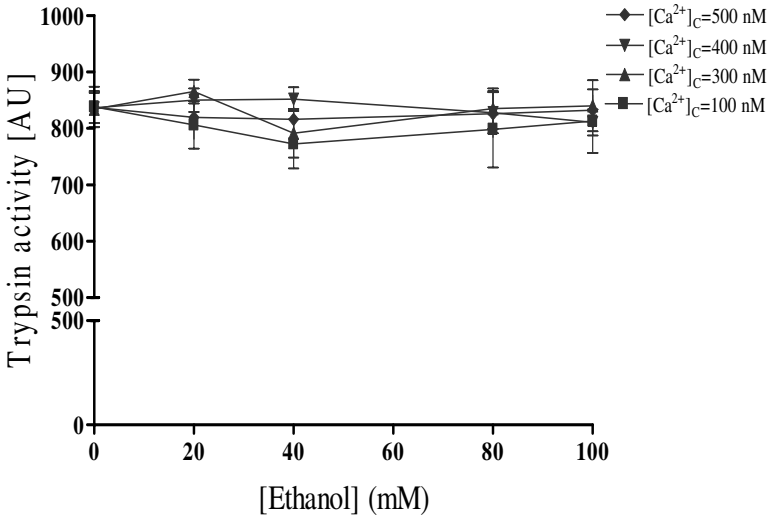


Fig 3-4 B

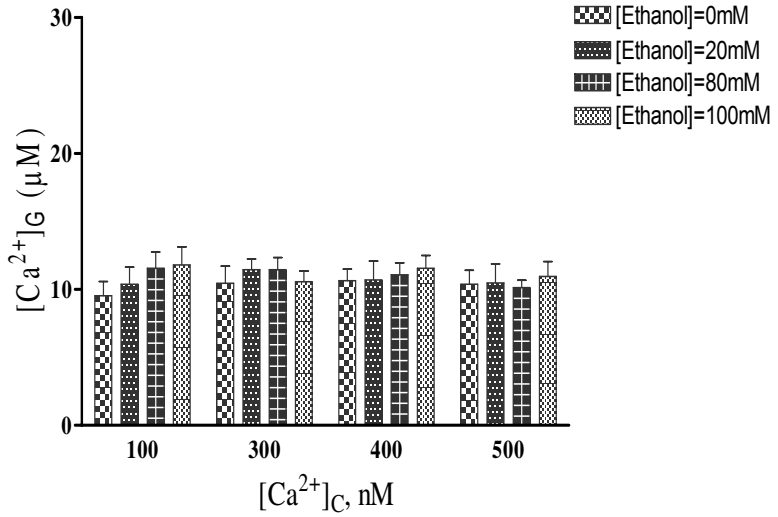


Fig 3-4. Effect of increasing free Ca^{2+} concentration in the intracellular solution ($[Ca^{2+}]_C$) and ethanol treatment on trypsin activity, $[Ca^{2+}]_G$ and $[pH]_G$ within ZGs, after blocking of ASK_{Ca} channels with 20 mM TEA. A. trypsin activity; B. $[Ca^{2+}]_G$; C. $[pH]_G$.

Fig 3-4 C

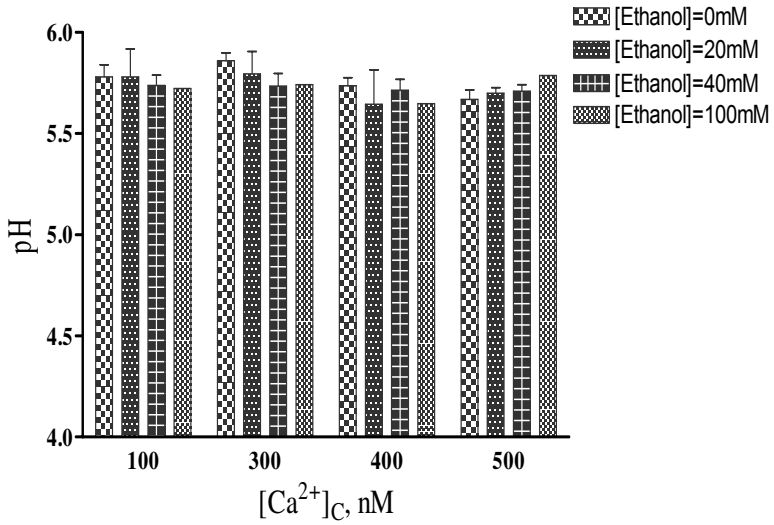


Fig 3-4 Continued

3.4 Conclusion

Ethanol treatment can sensitize the pancreas acinar cell ZGs to the effects of elevated $[Ca^{2+}]_C$-induced experimental acute pancreatitis. After ethanol incubation, same level of elevated $[Ca^{2+}]_C$ was observed to induce higher increase of trypsin activity, $[Ca^{2+}]_G$ and lower $[pH]_G$. The sensitization effects strongly rely on the $[Ca^{2+}]_C$ level in the cytosolic side. Ethanol alone or with resting $[Ca^{2+}]_C$ around 100 nM has no significant stimulus on trypsin premature activation. The sensitization effects require the involvement of the ASK_{Ca} channels on the ZGs membranes. Our present experimental results are quite consistent with the model we previously proposed (Fig 3-1). The results demonstrated that ethanol could potentially induce acute pancreatitis through sensitizing the pancreas acinar cells.

CHAPTER 4

DEVELOPMENT OF A FLUORESCENT-ASSAY TO MEASURE THE FRACTION OF CARBON PRESENT IN SELF-ASSEMBLED GELS

4.1 Introduction

The observation that a fraction of the DOC pool remains in reversible equilibrium with self-assembled micron-size networks implies that ~10^{16} g C might be found forming a massive microscopic gel phase in seawater (Chin et al. 1998; Verdugo et al. 2004). Recent observations using flow cytometry confirmed that these gels are indeed present in the water column (Verdugo et al. in press) reaching concentrations that are consistent with those expected from previous laboratory observations (Moran and Buesseler 1992; Chin et al. 1998; Guo et al. 2002.). However, a systematic assessment of the budget of carbon found in self-assembled gels (SAGs) in seawater, its time variations and geographic and depth distribution require an assay that can be readily implemented on board for the field study without the complications and high cost of flow cytometry. Here we report the development and experimental validation of a simple and reproducible fluorescence method to estimate the fraction of carbon in SAGs in seawater.

This assay is based on the idea that chlortetracycline hydrochloride (CTC), a fluorescent probe for bound Ca^{2+}, can readily be used to estimate the pool of

carbon that binds Ca^{2+} in seawater. This pool consists of Ca^{2+} bound to dissolved polyanionic polymers — including polysaccharides that comprise the vast majority of the dissolved carbon found in seawater (Benner 2002) — as well as Ca^{2+} bound to polymer networks, including both chemical microgels — resulting from cytoskeletal remains or other cellular debris — and self-assembled physical microgels crosslinked by Ca^{2+} (Verdugo et al. in press). CTC fluorescence de-quenching is lower in Ca^{2+} bound to free polymer than in assembled polymer networks resulting in increased CTC fluorescence when SAGs are dispersed by chelating of binding Ca^{2+} with EDTA. The amount of fluorescence increase is proportional to the fraction of SAGs in seawater. This feature can be used to estimate the fraction of carbon contained in SAGs in seawater.

Therefore,

$$C_A \sim C_T \times (F_T - F_A) / F_T$$

C_A =Assemble carbon, C_T = Total carbon, F_T = Total CTC fluorescence,
F_A = Fluorescence CTC bound to SAGs

While standard assays are available to measure C_T, the fraction $(F_A - F_T) / F_T$ can be found by labeling seawater with CTC and measuring fluorescence before and after chelating of Ca^{2+} with EDTA.

4.2 Experimental Methods

4.2.1 Chemicals

CTC was used as bound Ca^{2+} fluorescent indicators. Ethylenediaminetetraacetic acid (EDTA) from Sigma-Aldrich was used to chelate Ca^{2+}.

Sodium chloride, potassium chloride, palcium chloride, magnesium chloride, magnesium sulfate, sodium bicarbonate (Sigma-Aldrich, HPLC reagent), and deionized water from a Milli-Q system (Millipore) were used to prepare the artificial seawater (ASW) and Ca^{2+} free ASW.

4.2.2 Sample collection and pretreatment

Samples were collected from a coastal estuary site in Friday Harbor, Puget Sound (48°32'N, 123°05'W), and the open Pacific Ocean ALOHA station (22°26'N, 158°5'W) respectively.

Vertical profiles were collected during a cruise on March 9, 2004 near ALOHA station (22°26'N, 158°5'W) using CTD/28 Niskin-bottle rosette. Puget Sound samples were taken on May, 2005. Upon collection, samples were filtered at 8 μm (all filters were pre-treated with 0.1 N HCl and rinsed with Milli-Q water to avoid contaminants) and treated with sodium azide (NaN_3) at 0.02% to inhibit microbial activity. All samples were stored in dark at 4 ° C.

4.2.3 Preparation of self-assembled alginic gels

Alginic acid sodium salt (from Macrocystis pyrifera, Sigma-Aldrich) was dissolved in ASW (423.00 mM NaCl, 9.00 mM KCl, 9.27 mM $CaCl_2$, 22.94 mM $MgCl_2$, 25.50 mM $MgSO_4$, 2.14 mM $NaHCO_3$) and Ca^{2+}-free ASW (436.71 mM NaCl, 9.00 mM KCl, 22.94 mM $MgCl_2$, 25.50 mM $MgSO_4$, 2.14 mM $NaHCO_3$, 1mM EGTA) to simulate SAGs in natural seawater. The ASW recipes were adopted from Marine Biological Laboratory, Woods Hole, MA (http://www.mbl.edu/BiologicalBulletin/COMPENDIUM/CompTab3.html).

4.2.4 Fluorescent assay

Each seawater sample was first loaded with 100 μM CTC. The sample was then divided into two portions with equal volume. One portion of the sample was mixed with EDTA (final concentration 20 mM) to chelate the Ca^{2+} in the sample. A Corning 530 pH meter was used to measure pH. The pH decrease after chelating with EDTA was titrated back to original state with 10 N NaOH.

Static fluorescence was measured with a Shimadzu RF-5000U fluorescence spectrometer controlled by a DR-15 controller/printer. The exciting wavelength used here was 380 nm and emission wavelength was 522 nm (Oliver et al. 2000).

4.3 Results and Discussions

4.3.1 EDTA and CTC fluorescence

Fluorescence quenching refers to many factors that reduce the quantum yield of the fluorophore without shifting the wavelength of the fluorescence emission spectrum. Quenching may involve resonance energy transfer, interactions of fluorophore with quenchers to form non-fluorescent complexes, energy transfer, or collisional quenching (The Handbook — A Guide to Fluorescent Probes and Labeling Technologies. Molecular Probes, Eugene, OR). The local concentration or distance between fluoropores control the amount of fluorescent quenching, in this case, usually referred as self-quenching (Wu et al. 1995; Kwon and Carson 1998). During seawater self-assembly forming SAGs, the DOC-polymers annealed and aggregated to form submicron-meter gels. CTC is a fluorescence dye that associates with bound Ca^{2+} on DOC polymers (Verdugo et al. in press, Chin et al. 1998). With the formation of these three-dimensional physical networks, the distance between DOC-polymers is reduced and so is the distance among CTC fluorophores. This proximity of CTC molecules causes CTC fluorescence self-quenching and the decrease of total CTC fluorescence. The extent of self-quenching was determined by the decrease of the distance and the concentration of proximate of CTC molecules. Chelating of Ca^{2+} with EDTA resulted in dispersion of the SAGs and segregation of CTC labeled DOC polymers. This separation resulted in the decrease of CTC self-quenching in SAGs, or CTC dequenching. This increase of CTC fluorescence due to CTC dequenching provides the basis for the CTC-EDTA assay in this work. Based on the increase of CTC fluorescence induced by EDTA, we can estimate the percentage of organic carbon as SAGs in the seawater samples (Fig 4-1).

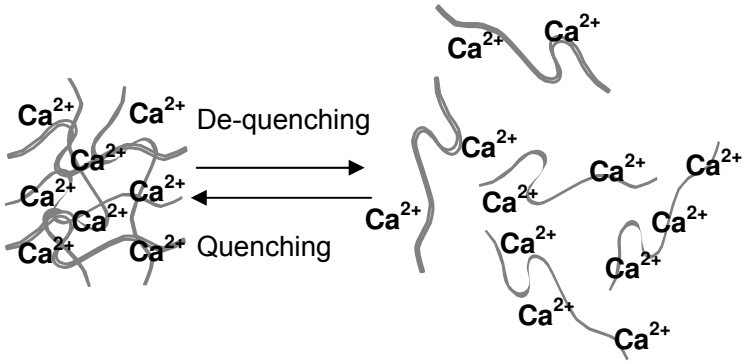


Fig 4-1. Fluorescent quenching and de-quenching: during marine microgel spontaneous assembly process, there is CTC fluorescence quenching, while in the reverse process, micogels dispersion into free DOC polymers, de-quenching happened.

4.3.2 Application of the assay on self assembled polymer gels

We dispersed a series of alginic salt into ASW to simulate SAGs in seawater. As a control, we also prepared alginic salt into Ca^{2+} free ASW. For alginic salt polymer gel in normal ASW, the fluorescence difference increased from 7.41% to 11.87% with the increase of alginic salt concentration from 2.5×10^{-5} to 5.0×10^{-5} (w/w). While the alginic salt concentration was further increased to 1.0×10^{-4} (w/w), the fluorescence difference remained at 11.75% (Fig 4-2).

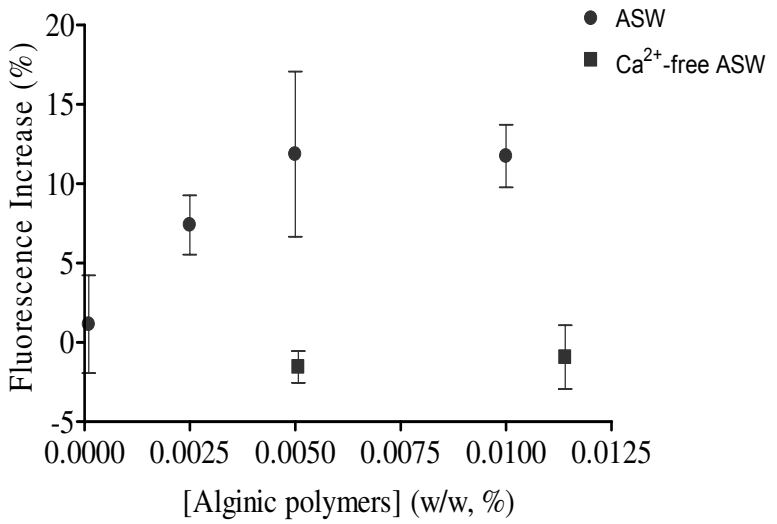


Fig 4-2. Formation of self-assembled polymer gels in alginic salt solution measured with fluorescent probe assay. Filled circles refer to ASW with Ca^{2+}, while filed squares are ASW with no Ca^{2+}.

Alginic acid sodium salt is a straight-chain, hydrophilic, colloidal, polyuronic acid composed primarily of anhydro-β-D-mannuronic acid residues with 1→4 linkage. It is very difficult to dissolve into solution. But once dispersed in solute, alginic molecules will assemble to polymer gels in artificial seawater with Ca^{2+} momentarily. Sample with 5.0×10^{-5} (w/w) alginic salt already reached saturation. Even if we increased alginic salt to 1.0×10^{-4} (w/w), the concentration of self-assembled gels couldn't increase. So after chelating of Ca^{2+} with EDTA, the fluorescence difference remained at the same level as alginic salt concentration at 5.0×10^{-5} (w/w). For control experiments, since there was no stabilizing force from Ca^{2+} and no self-assembled alginic polymer gels assembled, fluorescent probe assay couldn't detect any fluorescence increase.

4.3.3 Depth transects distribution of SAGs in seawater

The assay was performed in triplicate on various depth samples from ALOHA station. The distribution of SAGs varies widely depending on depth at which samples were collected (Fig 4-3A).

Fig 4-3 A.

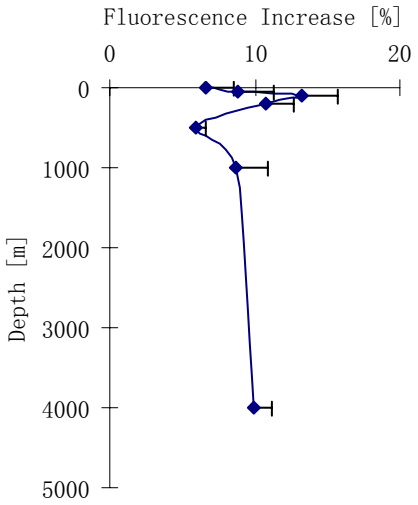


Fig 4-3. Depth distribution of transects of samples from ALOHA station. Panel A) is measured with fluorescent probe assay and panel B) is distribution of SAGs from the same samples measured with flow cytometer (Verdugo et al. in press).

Fig 4-3 B.

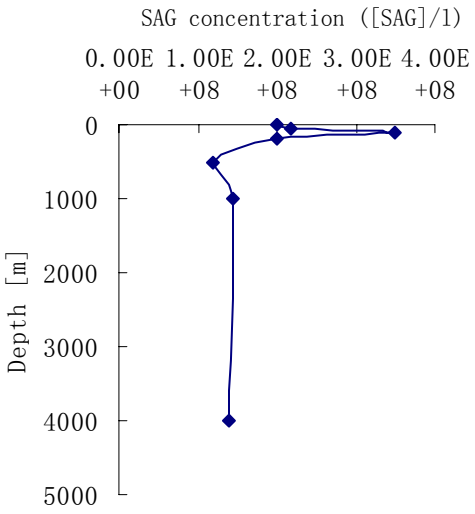


Fig 4-3 Continued

The percentage of fluorescence difference from samples at depth 0 and 50 m were 6.62% and 8.89% respectively. The highest signal was detected at depth 100 m (13.26%). Samples from deeper depth demonstrated slightly lower fluorescence difference, going from 8.71% at depth 1000 m to 9.94% at 4000 m. These results from fluorescent probe assay are quite consistent with a recent report about SAGs count with flow cytometry assay on the same transects (Fig 4-3B, Verdugo et al. in press). ALOHA surface seawater [SAGs] decreased from 3.5×10^8 to 2×10^8 SAG$\times$L^{-1} at depth 100 and 0 m respectively. The highest [SAGs] was detected at depth 100 m (3.5×10^8 SAG$\times$L^{-1}). Samples collected between depth 1000 and 4000 m showed relative slow variation in SAGs, between 1.4×10^8 to 1.45×10^8 SAG$\times$L^{-1}.

According to polymer gel theory, the probability of formation of SAGs increases with the second power of polymer length (de Gennes et al. 1982; Edwards 1974). So SAGs assemble more readily from larger and higher molecular weight DOC. The maximum concentration of high molecular weight DOC near ALOHA station was found between depth 80 and 110 m (Hernes and Benner 2002). Therefore, it is not surprising that the maximum abundance of SAGs near ALOHA occur at 100 m. Closer to the sea surface area, SAGs and large polymers are more exposed to UV photo-cleavage and microorganisms scavenging (Orellana et al. 2000; Orellana and Verdugo 2003). The resulting short polymers fail to self-assemble into SAGs. These previous reports are consistent with the finding that the fluorescence difference decreased near seawater surface.

4.3.4 Kinetics and time variation of DOC self assembly

According to the recently reported first direct experimental evidence for self assembly theory using dynamic laser scattering, DOC polymers in 0.22 μm-filtered seawater can spontaneously assemble, forming gels of 200 nm to 1 μm in about 30 minutes and equilibrating to 5~6 μm in ~60 hours (Chin et al. 1998; Wells 1998). We applied the fluorescent probe assay on the kinetics of SAGs formation from samples taken from Friday Harbor Bay at Puget Sound. The results from fluorescent probe assay and dynamic laser scattering are quite similar (Fig 4-4).

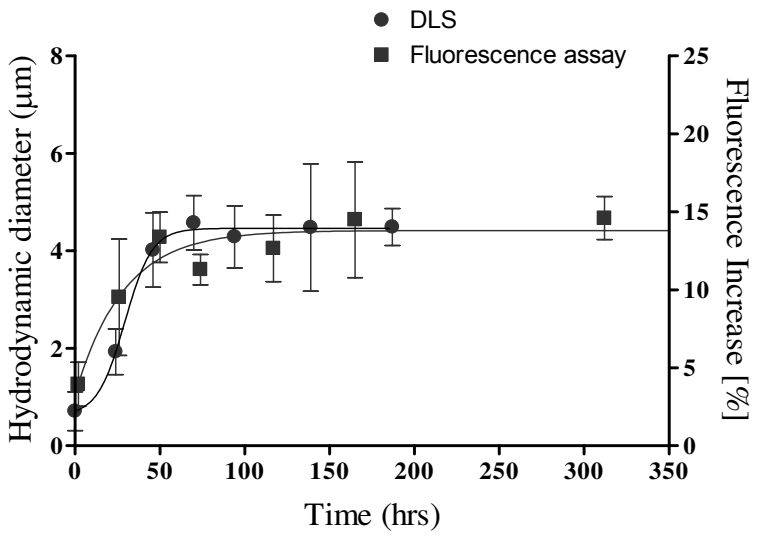


Fig 4-4. Kinetics and time variation of self-assembled gels. The assembly was monitored by measuring particle size and fluorescence difference after cheating with EDTA using dynamic laser scattering (filled circles) and fluorescent probe assay method (filled squares) respectively.

To measure the DOC gels self-assembly kinetics, we further filtered the sample with 0.22 μm clean filter. Immediately after the filtration, we applied the fluorescent probe assay on the sample and this process took around 2 hours. The fluorescence difference decreased to around 3.95%. With the formation of SAGs, fluorescent probe assay showed the increase of fluorescence difference. It reached steady state of 13.29% after 50 hours. This result demonstrated the presence and formation process of SAGs in seawater. It further showed that the fluorescent probe assay could be used as a simple and reliable method to estimate the fraction of SAGs in ocean environment.

4.4 Conclusion

A simple and straightforward fluorescent assay for estimation of SAGs in natural seawater was developed and experimentally validated. This assay has been applied to measure the fraction of SAGs for the depth distribution of transects from Hawaii ALOHA station, kinetics and time variations, and in alginic polymer solution. For the same samples, the CTC-EDTA fluorescent assay method detected similar results as we measured with flow cytometer or dynamic laser scattering (DLS). This demonstrated that the fluorescent probe assay has the same precision and accuracy as flow cytometry and dynamic laser scattering. But compared with both techniques, this CTC-EDTA assay was much less complicated, and cost-effective. Although this method has been used mainly for laboratory-based work, the equipment set-up is compact, portable and robust enough for ship-borne measurements.

CHAPTER 5

SPONTANEOUS ASSEMBLY OF EXOPOLYMERS FROM PHYTOPLANKTON

5.1 Introduction

The climate of our planet has changed over the past century. The global mean temperature has increased 0.6 ± 0.2 ^{o}C so far (Intergovernmental Panel on Climate Change [IPCC], 1990; 2001). The IPCC has predicted that the change in the next 100 years will be faster and larger. The earth's mean surface temperature will exceed 1.4 ~ 5.8 ^{o}C above the temperature of the 1990s (Karl and Trenberth 1999; Reilly et al. 2001; World Meteorological Organization [WMO], 2002). Most of the global warming observed is caused by human interventions that have increased the concentration of green house gases. CO_2 is the major green house gas and its atmospheric concentration has increase from around 80-180 ppmv (parts per million by volume) to ~ 370 ppmv because of the human kind's disruption of the global carbon cycle (Apps and Kurz 1991; Falkowski et al. 2000). The ecological disaster effects of this disruption are becoming more and more evident.

The terrestrial land and oceans are the buffers to withdraw CO_2 from the atmosphere. The almost equal processing of CO_2 by land and ocean biota resulted in the final accumulation of 750 gt C of DOC in the ocean, which was almost in equilibrium with the carbon at atmosphere prior to the twentieth century

(Houghton 2003; Hedges 1992; Hedges 2000). DOC is the primary carbon reservoir on the earth. Because of its high refractory and extremely low bioactivity, the bulk of DOC was believed to be useless for the microbial loop and they couldn't take part in the global carbon cycle. Recently, it was suggested that ~ 10% of the DOC pool can enter the microbial loop and global carbon cycle through spontaneous assembly forming microgels that can eventually utilized and scavenged by marine bacteria (Chin et al. 1998; Wells 1998). Since DOC comes directly or indirectly from phytoplankton as a result of photosynthetic activity (Wotton 2004; Bhaskar and Bhosle 2005), it is critical to find out the mechanism of conversion of free EPS polymers from phytoplankton to microgels.

Phytoplanktons are the oceanic equivalents of terrestrial plants, forming the basis of virtually all marine food webs. The total phytoplankton biomass outweighs that of all the marine animals (zooplankton, fish, whales) put together, and phytoplankton productivity is one of the primary forces in regulating our planetary climate - for instance via impacts on atmospheric carbon dioxide levels which are tightly linked to the oceanic concentrations.

In this chapter, I studied the assembly mechanisms of EPS from three typical marine phytoplanktons, *Emiliania huxleyi*, *Skeletonema costatum,* and *Synechococcus* (Hung and Santschi 2004; Hung et al. 2005). *Emiliania huxleyi* is by far the most abundant of the coccolithophores on our planet, and is extremely widespread, occurring in all except the polar oceans. It has the capacity to occur in massive blooms when water conditions are favourable. During these blooms the numbers of *Emiliania huxleyi* cells usually outnumber those of all other species combined, frequently accounting for 80 or 90% or more of the total number of phytoplankton cells in the water (Delille, 2003). *Skeletonema costatum* cells are in column shape. They are connected by

numerous tiny spines, in long-chained form, normally 2 pigment bodies, with nucleus at the centre of cells. The diatom is commonly found in almost all sorts of seawater and it is extremely abundant in many temperate areas. *Skeletonema costatum* is a characteristic phytoplankton of coastal eutrophication. Marine unicellular *Synechococcus* group occupy an important position at the base of the marine food chain (Drebes 1974; Medlin et al. 1991). *Synechococcus* have the ability to acquire major nutrients and trace metals from the submicromolar concentrations found in the oligotrophic open seas and their light-harvesting apparatus is uniquely adapted to the spectral quality of light in the ocean. *Synechococcus* are the main source of primary production in oligotrophic, pelagic marine waters. They can cause destructive blooms, producing neurotoxins. Their growth is generally limited by the concentration of nutrients and trace metals such as iron and phosphorus (Campell et al. 1998; Zouni et al. 2001).

The assembly kinetics of EPS extracted from these phytoplanktons were investigated using a traditional way, DLS (Hung and Santschi 2005; Chin et al. 1998). The possible mechanism driving their spontaneous assembly were studied. Results show that different phytoplankton EPS have uniquely different assembly mechanisms.

5.2 Experimental Methods

5.2.1 Hypothesis

In the spontaneous assembly process of DOC polymers, both electrostatic force from Ca^{2+} and hydrophobic interactions play important roles (Chin et al. 1998). Our hypothesis is that EPS from phytoplankton can assemble via Ca^{2+} cross-linking and via hydrophobic interaction.

5.2.2 Separation and purification of exopolymers from phytoplankton

Extracellular polymeric substances from phytoplankton *Emiliania huxleyi*, *Skeletonema costatum,* and *Synechococcus* were extracted and purified using repeated alcohol precipitation, centrifugation, and enzymatic digestions procedures by researchers at Texas A&M University at Galveston according to procedures published previously (Hung and Santschi 2005). In brief, samples from these three phytoplanktons were inoculated and cultured in soy broth (30 g of soy broth into 1000 ml of distilled water) for overnight. An aliquot from each phytoplankton culture was taken and centrifuged at 3500 rpm for 30 min. After centrifugation, the culture was divided into a pellet and a supernatant layer. EPS were then isolated from the pellet according to a procedure published previously (Kushner et al.1992). The supernatant was added with a proteinase solution to hydrolyze proteins of the soy broth and the resulted solution was incubated at 37 °C for 12 h at 70 rpm. After alcohol precipitation with four volumes of 95% ethanol and 5% methanol in the refrigerator for 12 h, a precipitate was formed and removed by membrane separation. The precipitate was disolved and

washed with the same alcohol precipitation procedures for three times. The final clear solution was dialyzed for 5 days using a 6–8 kDa membrane, and resulted in the fraction of EPS used in our research (Hung and Santschi 2005)

5.2.3 Particle sizing

100 $\mu g.L^{-1}$ EPS was dissolved with ASW (423.00 mM NaCl, 9.00 mM KCl, 9.27 mM $CaCl_2$, 22.94 mM $MgCl_2$, 25.50 mM $MgSO_4$, 2.14 mM $NaHCO_3$). The assembly of microgels was monitored through measuring particle size by dynamics laser scattering (DLS) following protocols published elsewhere (Chin et al. 1998). Briefly, samples were shaken, and refiltered through a 0.22-μm Millipore membrane (pre-washed with 0.1N HCl). Aliquots were then poured directly into scattering cells. The scattering cells were positioned in the goniometer of a Brookhaven laser spectrometer (Brookhaven Instruments, NY, USA). Polymer assembly was monitored for 8~10 days, by analyzing the scattering fluctuations detected at a 45 scattering angle. The autocorrelation function of the scattering intensity fluctuations was averaged over a 10-min sampling time, using a Brookhaven BI 9000AT autocorrelator. Particle size distribution was calculated by the CONTIN method (Provencher 1982; Chin et al. 1998). Each measurement was taken in triplicate in 10-ml at room temperature. Calibration of the DLS method was conducted using standard suspensions of latex microspheres (Polysciences, PA, USA).

5.2.4 Fluorescence measurement

Nile-red was used without further treatment. ASW sample was labeled with 13 μM Nile-red. Static fluorescence before and after addition of EPS were

measured with a Shimadzu RF-5000U fluorescence. The exciting wavelength used here was 568 nm and emission wavelength was 633 nm (Yablon and Schilowitz 2004).

5.3 Results and Discussions

5.3.1 Assembly of exopolymers from phytoplankton in ASW

The spontaneous assembly of exopolymers from *Emiliania huxleyi* was measured by dispersing the EPS into ASW with Ca^{2+} and monitoring the formation of microgels in the following 6-8 days with DLS. Results show that EPS from *Emiliania huxleyi* can self-assemble following a first order kinetics. The assembly process can reach steady in ~ 42 hrs yielding microgels of 3.5 μm. To test the possible role from electrostatic interactions of Ca^{2+}, we dissolved the EPS into ASW with no Ca^{2+} and repeated the same measurements. The data show that the EPS can also assemble in Ca^{2+}-free ASW. However, the equilibrium size of the microgels in Ca^{2+}- free ASW is much smaller, which is only ~ 1.8 μm after 140 hrs. This phenomenon is quite different from the self-assembly of seawater DOC polymers. After the blocking of Ca^{2+} with EDTA, the latter could not assemble at all. This result suggest that Ca^{2+} cross-linking is not the only stabilizing force for the formation of microgels from *Emiliania huxleyi* EPS (Fig 5-1 A).

Fig 5-1 A

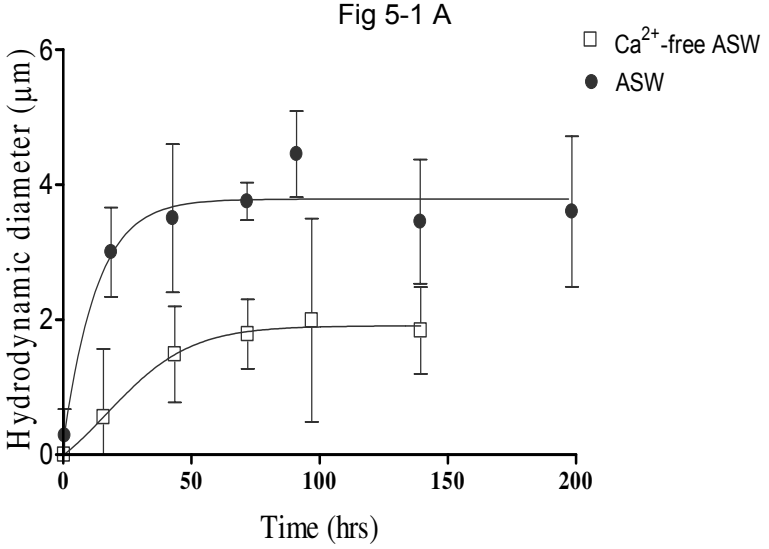


Fig 5-1 B

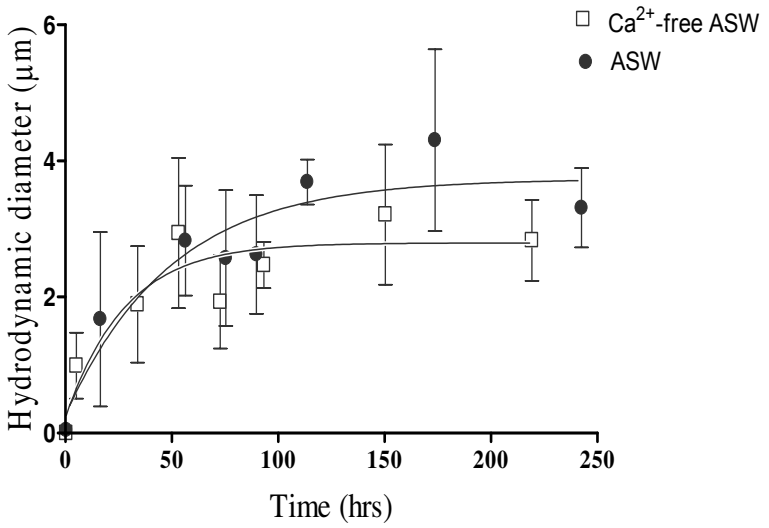


Fig 5-1. Spontaneous assembly of exopolymers from phytoplankton in ASW. A: EPS from *Emiliania huxleyi*; B: EPS from *Skeletonema costatum*; C: EPS from *Synechococcus*. Filled circles: ASW with Ca^{2+}; Squares: Ca^{2+}-free ASW.

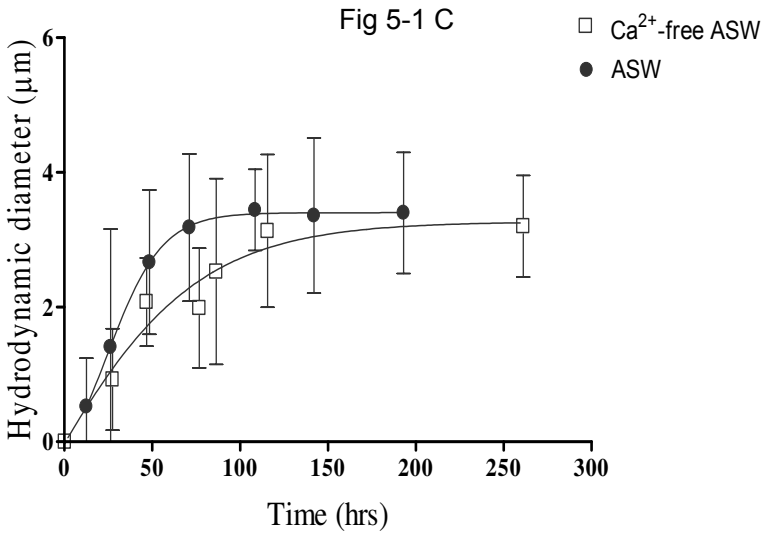


Fig 5-1 Continued

Similarly, we measured the spontaneous assembly of EPS from *Skeletonema costatum* (Fig 5-1 B), and *Synechococcus* (Fig 5-1 C). The results show that both EPS polymers demonstrated different assembly kinetics compared with the EPS secreted by *Emiliania huxleyi* and polymers from DOC pool in the ocean (Chin et al. 1998). Both EPS polymers can assemble in ASW either with or without Ca^{2+}. The data suggest that the assembly of these two EPS is not via Ca^{2+} stabilization.

In natural seawater and ASW, except for the electrostatic force from Ca^{2+}, hydrophobic and hydrophilic interactions could also stabilize the formation of polymer gel networks spontaneously (Verdugo et al. 2004). For the assembly of EPS from phytoplankton *Emiliania huxleyi*, *Skeletonema costatum,* and *Synechococcus*, the hydrophobic linking could provide part or major nucleating driving forces. Due to the highly complexity of the chemical structures of these EPS polymers and the non-specific characteristics of the hydrophobic interaction,

we couldn't provide direct evidence to show that stabilization force for the spontaneous assembly of the EPS comes from hydrophobic force. However, our studies introduced later demonstrated the existence of hydrophobic regions within the structure of these EPS. The hydrophobic interactions from these hydrophobic domains could nucleate the formation of microgels.

5.3.2 Energy enhancement of Nile-red fluorescence by exopolymers from phytoplankton

EPS polymers from marine organisms have very complex chemical compositions and they vary considerably between phytoplankton and bacterial. Even different species of phytoplankton can secrete different exopolymers. Slight change of the composition can affect their physico-chemical properties, hence their role and fate in the biogeochemical cycle (Wotton 2004; Bhaskar and Bhosle 2005). The major components of these EPS from phytoplankton are carbohydrates, up to 50% of the total carbon (Table 5-1). The acidic groups in these EPS are mainly carboxylate, sulphate, and phosphate. These EPS also contain around 2-8% proteins, rendering them with hydrophobic groups (Hung and Santschi 2005).

Table 5-1 Chemical compositions of EPS from phytoplankton. Adapted from Hung and Santschi, 2005.

I.D.	TCHO/OC (%)	Protein-C/OC (%)	URA/OC (%)	C/PO4 (mole ratio)	C/SO4 (mole ratio)
Synechococcus	13.18	3.13	1.38	2567	316
Emiliania huxleyi	58.32	2.07	7.37	39648	137
Skeletonema costatum	35.59	2.35	9.62	9513	248

Due to the highly complexity of their chemical composition, it is almost impossible to detect the precise hydrophobic groups or regions inside these EPS polymers. With the help of Nile-red (The Handbook — A Guide to Fluorescent Probes and Labeling Technologies. Molecular Probes, Eugene, OR), a widely used fluorescent probe specific for hydrophobic domains, we detected the existence of hydrophobic regions inside the EPS from phytoplankton *Emiliania huxleyi*, *Skeletonema costatum,* and *Synechococcus*.

Fig 5-2 A

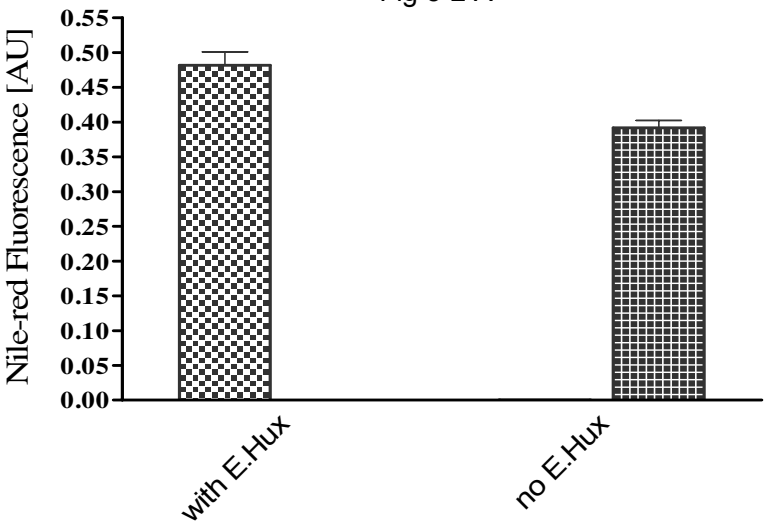


Fig 5-2 B

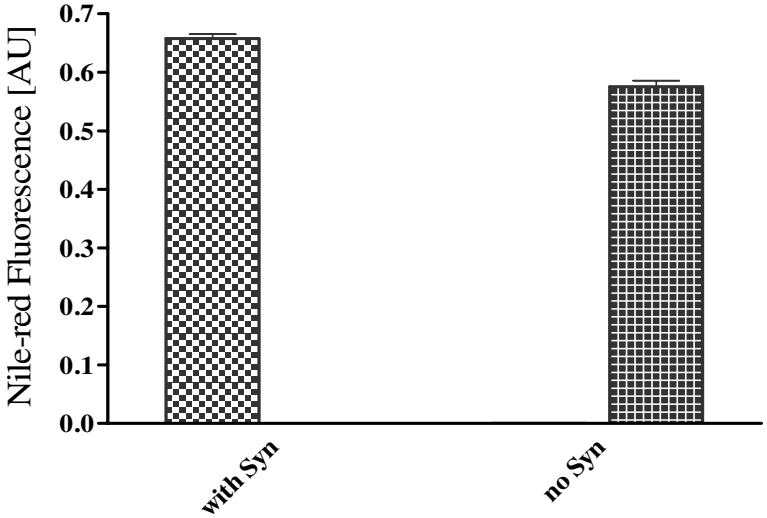


Fig 5-2. The detection of hydrophobic domains with Nile-red. Nile-red is a fluorescence dye specific for hydrophobic regions (EX_m / EM_m= 568/633 nm). In the ASW, Nile-red emitted very weak fluorescence signal. With addition of 100 $\mu g.L^{-1}$ EPS, the fluorescent intensity was enhanced ~15%. A, *Emiliania huxleyi*; B, *Synechococcus*; C, *Skeletonema costatum*.

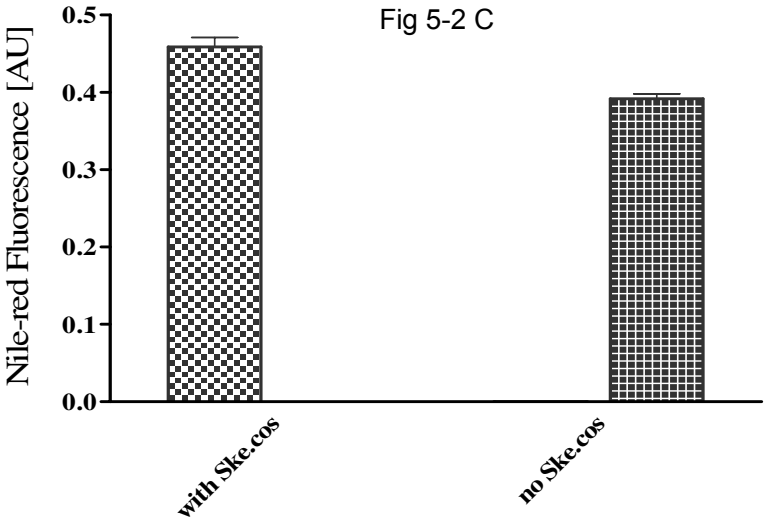


Fig 5-2 Continued

Nile-red is a particularly effective solvatochromic dye consisting of a rigid aromatic group and an exocyclic diethylamine group (Yablon and Schilowitz 2004). Its absorption and fluorescence depends on the physical properties of surrounding solvent environment, such as polarity. Fluorescence spectra of Nile-red recorded at a 568 nm excitation wavelength showed very weak signal at maximum emission wavelength 633 nm in polar ASW solvent. When we added 100 $\mu g.L^{-1}$ EPS from these phytoplanktons into the seawater sample labeled with Nile-red, the fluorescence signal was enhanced ~15%. These results indicateded that hydrophobic domains indeed exist in the EPS polymers that could provide the stabilizing force for the spontaneous assembly forming microgels.

5.4 Conclusions

While electrostatic force from Ca^{2+} cross-linking is one of the integral factors for the assembly of extracellular polymer substances from phytoplankton *Emiliania huxleyi*, hydrophobic interactions provides another important role in its spontaneous assembly kinetics. For EPS from *Synechococcus* and *Skeletonema costatum*, hydrophobic nucleating is the major stabilizing force for their formation of networks. Results from Nile-red fluorescence enhancement substantiate the existence of these hydrophobic domains in the EPS. These results show that different phytoplankton EPS possess uniquely different mechanisms for the formation of microgels.

CHAPTER 6

EFFECTS OF EXOPOLYMERS ON MARINE MICOGEL ASSEMBLY AND BIOFILM FORMATION

6.1 Introduction

The finding that polymers found in the seawater DOC pool can self-assemble forming microscopic gels has broad implications for the understanding of how carbon is cycled in the ocean. Marine self-assembled microgels represent a potentially massive shunt between the pool of largely refractory DOC moieties and a stock of POC made of concentrated highly biosusceptible porous bacterial substrate (Wells 1998; Chin et al. 1998; Verdugo et al. 2004; Verdugo et al. in press). EPS produced by marine organisms including bacterial and phytoplankton (Hoagland et al. 1993; Wingender et al. 1999) are released to the seawater forming up to 50% of the ocean DOC polymer pool (Sell and Overbeck 1992; Kirchman et al. 2001). EPS have important function in regulating various processes including particle formation, sedimentation, cycling of dissolved metals, and marine biogeochemical cycling, etc (Bhaskar and Bhosle 2005; Decho 2000; Passow 2002). However, the role of EPS in the formation of DOC-polymer gels remains unknown. The experiments presented here were designed to investigate interaction of EPS polymers and polymes found in the DOC pool that results in the formation of hydrated polymer networks of marine microgels. Using EPS released by *Sargitulla stella* as a model, our results show that

nanomolar concentrations of *Sargitulla stella* EPS can drastically accelerate the self-assembly of DOC polymers. *Sargitulla stella* EPS changes DOC polymer self-assembly from a second to a first order kinetics, also turning self-assembly independent of Ca^{2+} bonds. An alternative source of low energy cross-linking of DOC is via the formation of hydrophobic bonds. Experiments conducted in Ca^{2+}-free ASW indicate that *Sargitulla stella* EPS can self-assemble in a Ca^{2+}-independent, temperature-dependent fashion, strongly suggesting that *Sargitulla stella* EPS -induced cross-linking of DOC-polymers might result not from electrostatic but from hydrophobic bonding. Studies using FRET show that *Sargitulla stella* expolymer chains do indeed contain hydrophobic domains that could potentially drive their own assembly as well as nucleate the assembly of DOC. To further test the hypothesis that hydrophobic agents can nucleate DOC polymer self-assembly, we used polystyrene nanospheres. Addition of these hydrophobic nanospheres to freshly 0.2 μm-filtered seawater result in changes in DOC assembly that virtually mimic those produced by *Sargitulla stella* EPS, supporting the idea that polymers released by marine bacteria might be operating as a nucleating agent for DOC assembly and microgel formation.

These observations suggest that *Sargitulla stella* EPS can form self-assembling networks that could serve to bind bacteria to specific substrates. In seawater however, bacteria need not to release a large amount of EPS to create polymer networks since trace amounts of *Sargitulla stella* EPS can efficiently recruit polymers found in the DOC pool to nucleate the formation of DOC polymer microgels. These findings open a new frontier of exploration to understand the microgel/bacteria interaction in the ocean. They further provide intriguing new leads to investigate the mechanisms of biofilm formation, not only by marine bacteria, but perhaps also by bacterial pathogens affecting humans and other species.

6.2 Materials and Methods

6.2.1 Chemicals

Nile red was used as hydrophobic domain indicators (Molecular Probes, Inc, Eugene, OR , USA). Ethylenediaminetetraacetic acid (EDTA) from Sigma-Aldrich was used to chelate Ca^{2+}. Sodium chloride, potassium chloride, calcium chloride, magnesium chloride, magnesium sulfate, sodium bicarbonate (Sigma-Aldrich, HPLC reagent), and deionized water from a Milli-Q system (Millipore) were used to prepare the ASW (423.00 mM NaCl, 9.00 mM KCl, 9.27 mM $CaCl_2$, 22.94 mM $MgCl_2$, 25.50 mM $MgSO_4$, 2.14 mM $NaHCO_3$), Ca^{2+} free ASW (436.71 mM NaCl, 9.00 mM KCl, 22.94 mM $MgCl_2$, 25.50 mM $MgSO_4$, 2.14 mM $NaHCO_3$, 1mM EGTA), and Ca^{2+}/Mg^{2+} free ASW (461.85 mM NaCl, 10.73 mM KCl, 2.14 mM $NaHCO_3$, 7.04 mM Na_2SO_4, 1mM EGTA). The ASW recipes were adopted from Marine Biological Laboratory, Woods Hole, MA (http://www.mbl.edu/BiologicalBulletin/COMPENDIUM/CompTab3.html).

6.2.2 Sample collection and pretreatment

Seawater samples were collected from a coastal estuary site in Friday Harbor, Washington in May, 2004. Upon collection, samples were gravity filtered through a sterile fiberglass membrane and 0.22 μm Millipore filter (all filters were pre-treated with 0.1N HCl and rinsed with Milli-Q water to avoid contaminants) and treated with sodium azide (NaN_3) at 0.02% to inhibit microbial activity. All samples were stored in clean sterile bottles in the dark at 4° C.

6.2.3 Isolation and purification of *Sargitulla stella* EPS

Polysaccharide-rich *Sargitulla stella* EPS were extracted and purified from marine bacteria *Sargitulla stella* using repeated alcohol precipitation, centrifugation, and enzymatic digestions procedures by researchers at Texas A&M University at Galveston according to methods introduced in previous chapters and published elsewhere (Hung and Santschi 2005).

6.2.4 Particle sizing

The assembly of microgels was monitored through measuring particle size by DLS following protocols published elsewhere (Chin et al. 1998). Briefly, seawater samples were shaken, and refiltered through a 0.22-μm Millipore membrane (pre-washed with 0.1N HCl). Aliquots were then poured directly into scattering cells. The scattering cells were positioned in the goniometer of a Brookhaven laser spectrometer (Brookhaven Instruments, NY, USA). Polymer assembly was monitored for 8~10 days, by analyzing the scattering fluctuations detected at a 45 scattering angle. The autocorrelation function of the scattering intensity fluctuations was averaged over a 10-min sampling time, using a Brookhaven BI 9000AT autocorrelator. Particle size distribution was calculated by the CONTIN method (Provencher 1982; Chin et al. 1998). Each measurement was taken in triplicate in 10-ml at room temperature. Calibration of the DLS method was conducted using standard suspensions of latex microspheres (Polysciences, PA, USA).

6.2.5 *Sargitulla stella* EPS self-assembly

Assembly of free *Sargitulla stella* EPS in ASW, ASW with 10mM EDTA, Ca^{2+}-free ASW, and Ca^{2+}/Mg^{2+}-free ASW was initiated by dispersing the *Sargitulla stella* EPS into the solutions and filtering with 0.22 μm Millipore filter (prewashed with 0.1 N HCl). The final concentration of *Sargitulla stella* EPS was set at 20 or 100 $\mu g.L^{-1}$. The hydrodynamic diameter of the assembled microgels was monitored by DLS during the next 8-10 days.

6.2.6 Exposure of free DOC polymer assembly to *Sargitulla stella* EPS

Seawater were shaken and mixed with 20 or 100 $\mu g.L^{-1}$ *Sargitulla stella* EPS and refiltered through 0.22 μm Millipore filter (prewashed with 0.1 N HCl). As in the previous report, DOC polymer assembly and microgel formation kinetics were monitored with DLS at room temperature.

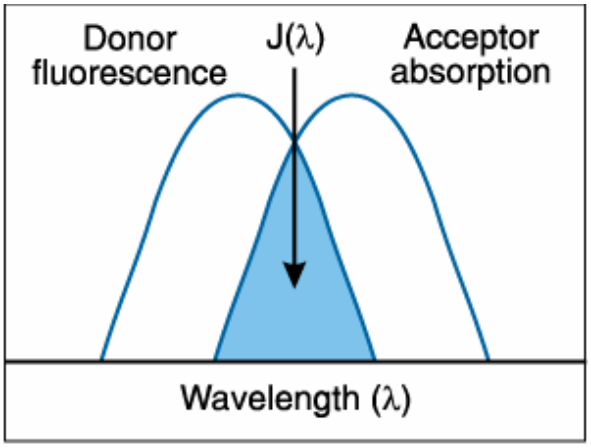
6.2.7 Exposure of free DOC polymer assembly to polyesterene microspheres

Polystyrene hydrophobic Microspheres were obtained from Bangs Laboratories, Inc. (Fishers, IN, USA). The mean diameter of the beads is 24 nm. Prior to use, the beads were pre-washed with MilliQ-water to remove detergents and other contaminants and subsequently suspended in seawater to reach a concentration of 1.322×10^{13} microspheres per ml. The suspension was then filtered through 0.22 μm Millipore filter (prewashed with 0.1 N HCl) and poured directly into the scattering cells. The DOC polymer assembly and microgel formation kinetics were monitored with the same protocol.

6.2.8 Fluorescent enhancement and FRET assays

Nile-red fluorescence (λ_{ex} = 580 nm; λ_{em} = 633 nm) increases the emission intensity when bound to hydrophobic recidues (Yablon and Schilowitz, 2004). This feature can be used to detect the presence of hydrobobic domains in organic moieties like *Sargitulla stella* EPS. In this case ASW sample was labeled with 13 μM Nile-red, and the increase of fluorescence before and after addition of *Sargitulla stella* EPS was measured with a Shimadzu RF-5000U fluorescence spectrometer.

The FRET assay (Fig 6-1) takes advantage of the fact that *Sargitulla stella* EPS are fluorescent. When excited at λ_{ex} = 285 nm *Sargitulla stella* EPS emit at a narrow band centered at λ_{em} = 580 nm (see Fig 6-9 A), that is precisely within the range of exitation of Nile-red. Therefore, if the emission site in *Sargitulla stella* EPS is very near to the domains of its hydrophobic recidues where Nile-red is bound, exiting *Sargitulla stella* EPS at λ_{ex} = 285 nm can couple its λ_{em} = 633 nm emission to excite Nile-red lighting up the emission of Nile-red at λ_{em} = 633 nm. In this case, after addition of *Sargitulla stella* EPS into Nile-red labeled ASW, the fluorescence emission spectra of Nile-red and *Sargitulla stella* EPS were taken at λ_{em} = 633 nm and λ_{em} = 580 nm respectively, while exciting *Sargitulla stella* EPS at λ_{ex} = 285 nm.



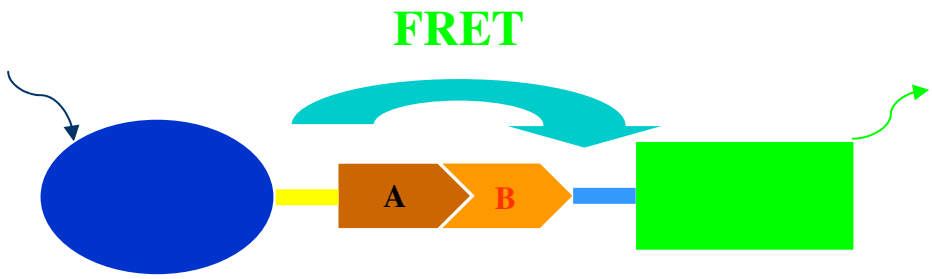


Fig 6-1. Introduction to Fluorescent Resonance Energy Transfer (FRET) technique. Adapted from The Handbook — A Guide to Fluorescent Probes and Labeling Technologies. Molecular Probes, Eugene, OR.

6.3 Results

6.3.1 Spontaneous assembly of *Sargitulla stella* EPS

Polymers in seawater DOC pool can self-assembly into microscopic gels stabilized by tangles and electrostatic cross-linking from Ca^{2+} (Chin et al. 1998). The spontaneous assembly of EPS was monitored by dispersing *Sargitulla stella* EPS into ASW and measuring polymer assembly and microgel formation with DLS. Like seawater DOC polymers, both 20 and 100 $\mu g.L^{-1}$ *Sargitulla stella* EPS can spontaneously assemble in normal ASW (Fig. 6-2.). However, lower concentration of *Sargitulla stella* EPS (20 $\mu g.L^{-1}$) yielded smaller microgels and required longer time to reach steady state. At higher concentration (100 $\mu g.L^{-1}$) *Sargitulla stella* EPS could yield microgels of ~4-6 μm and reach equilibrium in ~ 80h, while the microgel size of 20 $\mu g.L^{-1}$ *Sargitulla stella* EPS sample was still less than 4 μm and tended to grow after 8 days. Unlike DOC polymers, the *Sargitulla stella* EPS polymers exhibited characteristic first-order kinetics.

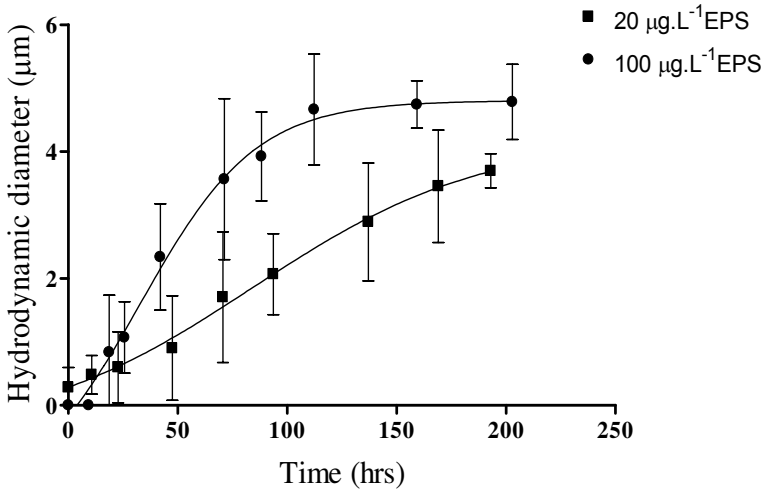


Fig.6-2. Both 20 (filled squares) and 100 (filled circles) μg.L^{-1} *Sargitulla stella* EPS can self-assemble in ASW following a characteristics first order kinetics. 100 μg.L^{-1} EPS polymers could yield microgels of ~4-6 μm and reach equilibrium in ~ 80h, while the microgel size of 20 μg.L^{-1} EPS sample was still less than 4 μm and tended to grow after 8 days.

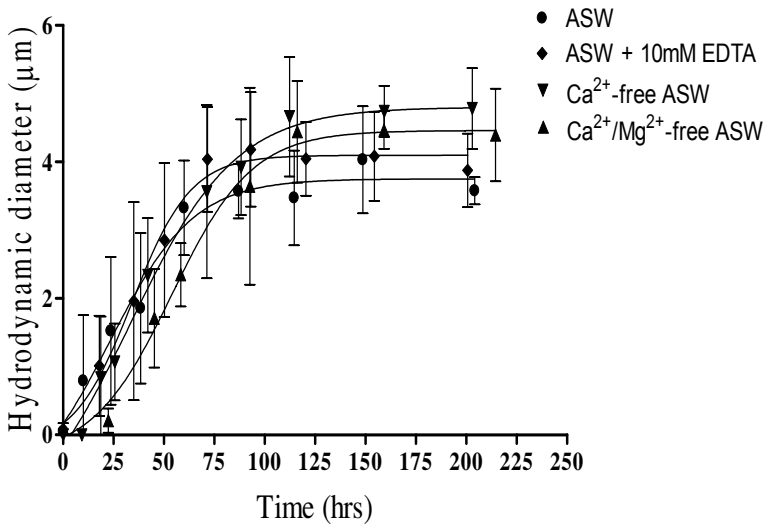


Fig. 6-3. Spontaneous assembly of 100 μg.L^{-1} *Sargitulla stella* EPS in ASW (filled inverted triangles), ASW with 10mM EDTA (filled triangles), Ca^{2+}-free ASW (filled circles), Ca^{2+}/Mg^{2+}-free ASW (filled diamonds). Note there is no statistical difference among the assembly kinetics.

After chelating of Ca^{2+} in ASW with 10 mM EDTA, the microgel assembly kinetics couldn't be disrupted and the *Sargitulla stella* EPS demonstrated the same assembly kinetics as in ASW without chelating of Ca^{2+} (Fig. 6-3.). Usually divalent bonds are required to stabilize the formation of various types of polymer gels (Verdugo 1994). For seawater DOC microgels, chelation of Ca^{2+} and Mg^{2+} by addition of EDTA could restrain polymer assembly and microgel formation (Chin et al. 1998). To further verify the result, we repeated the assembly of *Sargitulla stella* EPS in Ca^{2+}-free ASW and Ca^{2+}/Mg^{2+}-free ASW (Fig. 6-3.). The results showed no significant difference from ASW and from Ca^{2+}-free, Ca^{2+}/Mg^{2+}-free ASW. All these data strongly suggested that the stabilization of SE microgel does not rely on the electrostatic force from divalent Ca^{2+} or Mg^{2+} in ASW.

6.3.2 Effects of *Sargitulla stella* EPS on free DOC polymers assembly

Microbial EPS are widely distributed in the ocean waters (Decho 1990; Wingender 1999). Much of the EPS is released in the dissolved form and they can contribute up to 50% of the semi-labile DOC pool. They played crucial roles in marine environments and biogeochemical processes, including microbial loop, cycling of dissolved metals, sedimentation, and organic carbon mineralization (Wingender 1999; Passow 2002). They could also play critical roles in the processes that direct the exchange between the DOC and POC pools in the ocean and cycling of marine organic matter. Recently it was observed and experimentally validated that the marine polymer gels, which make up of most part of the POC pool including colloids, transparent exopolymer particles, and marine snows, can assemble from DOC polymers (Chin et al. 1998; Verdugo et al. 2004). The effects of *Sargitulla stella* EPS on the spontaneous assembly of this process were investigated. 20 or 100 $\mu g.L^{-1}$ *Sargitulla stella* EPS was added to seawater samples and the DOC polymer self-assembly kinetics was monitored following standard protocols (Chin et al. 1998).

With addition of 20 $\mu g.L^{-1}$ *Sargitulla stella* EPS, the time for DOC polymers to reach equilibrium with self-assembled microscopic gels decreased from typically ~50-83h (Chin et al. 1998) to ~36h (Fig. 6-4.). When the *Sargitulla stella* EPS concentration increased from 20 to 100 $\mu g.L^{-1}$, the equilibrium time was further cut down to ~12-24h (Fig. 6-5.). Under both cases, the characteristic DOC polymer assembly kinetics was accelerated from sigmoid course resembling second order kinetics to first order process. The equilibrium size of the microgels was not significantly different with or without addition of *Sargitulla stella* EPS.

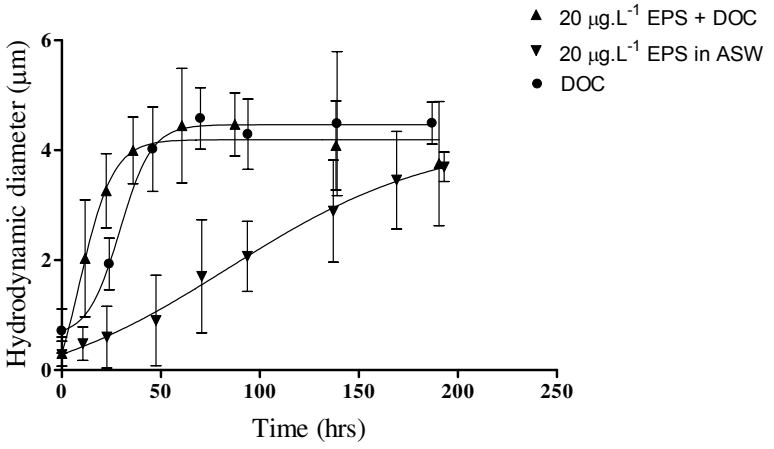


Fig. 6-4. Effects of *Sargitulla stella* EPS on the assembly of DOC-polymers from Friday Harbor seawater. Data points in filled triangles represent the DOM spontaneous assembly kinetics with addition of 20 $\mu g.L^{-1}$ EPS. Filled circles and inverted triangles are control kinetics of DOC polymers from natural seawater and 20 $\mu g.L^{-1}$ EPS in ASW. With addition of 20 $\mu g.L^{-1}$ EPS, the time for DOC polymers to reach equilibrium with self-assembled microscopic gels decreased from typically ~50-83h to ~36h.

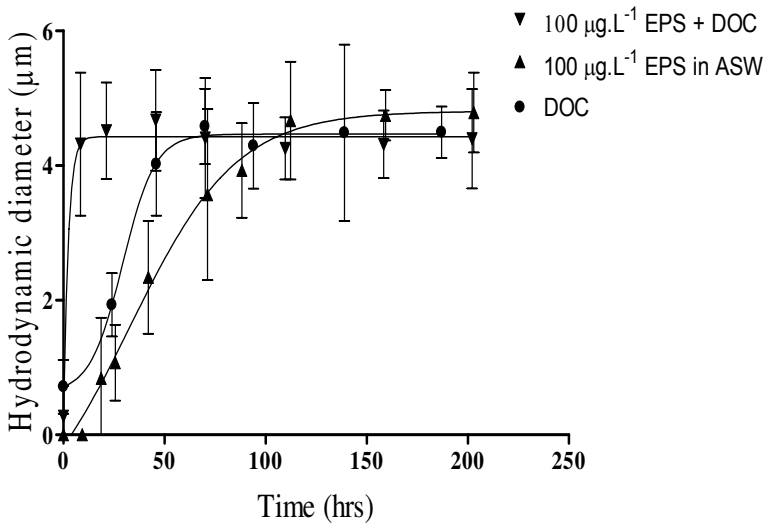


Fig. 6-5. Effetcs of 100 μg.L^{-1} *Sargitulla stella* EPS on the assembly kinetics of DOC polymers. The assembly kinetics was drastically accelerated from sigmoid course resembling second order kinetics (filled circles) to first order process (filled inverted triangles). Filled triangles data is the control result for assembly of 100 μg.L^{-1} *Sargitulla stella* EPS in ASW.

To further investigate whether the speeding effects from *Sargitulla stella* EPS rely on the electrostatic force from Ca^{2+}/Mg^{2+} in the seawater, we added 10 mM EDTA at the same time when we put 100 μg.L^{-1} *Sargitulla stella* EPS into the seawater sample (Fig. 6-6.). The assembly kinetics showed no statistical difference. Unlike the self-assembly of DOC polymers in natural seawater, chelating of Ca^{2+} could no longer inhibit marine gels formation with addition of 100 μg.L^{-1} *Sargitulla stella* EPS. Without Ca^{2+}, *Sargitulla stella* EPS demonstrated the same effects and extent on acceleration of DOC spontaneous assembly.

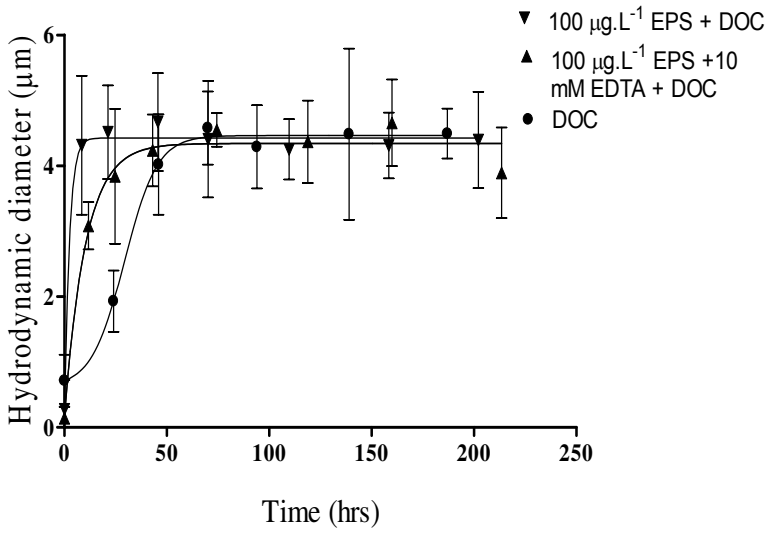


Fig. 6-6. Effects of 100 μg.L^{-1} *Sargitulla stella* EPS on the spontaneous assemble of DOC polymers after chelating of Ca^{2+} in seawater sample with 10 mM EDTA. Notice that with addition of 100 μg.L^{-1} *Sargitulla stella* EPS, Chelationg of Ca^{2+} in seawater samples could neither inhibit the DOC polymers assembly, nor restrain the acceleration effects from EPS. There is no significant difference from with (filled triangles) or with no (filled inverted triangles) chelating of Ca^{2+} for DOC polymer assembly kinectis after addition of 100 μg.L^{-1} *Sargitulla stella* EPS. Filled circles data is the control curve for natural seawater DOC polymer assembly kinetics.

6.3.3 Temperature dependent assemble characteristics of *sargitulla stella* EPS

The spontaneous assembly of *Sargitulla stella* EPS at different temperatures was measured. The results demonstrated that the assembly of *Sargitulla stella* EPS is strongly dependent upon temperature (Fig 6-7).

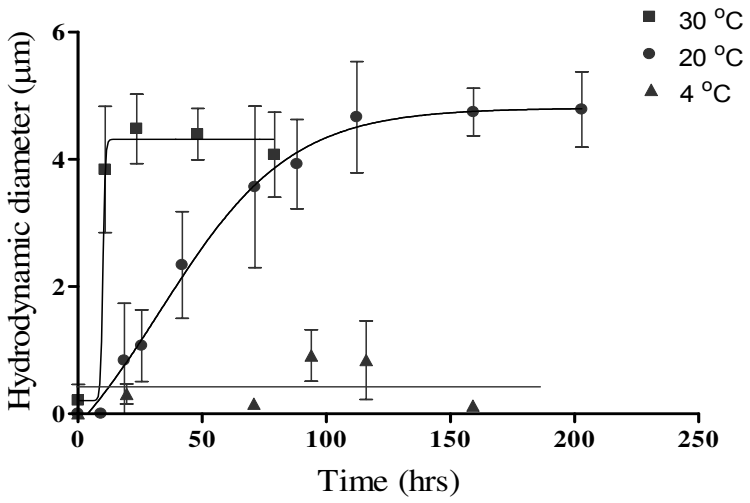


Fig 6-7. Effects of temperature on the spontaneous assembly of *sargitulla stella* EPS in ASW. (filled triangles, 4 °C; filled circles, 20 °C; filled squares, 30 °C.)

When we increase the temperature from room temperature to 30 °C, the time for 100 μg.L^{-1} *Sargitulla stella* EPS to reach assembly equilibrium state at ASW was shortened from normally ~ 80 hrs to 8~10 hrs. While at low temperature (4 °C), we still could not detect bigger size particles (> 0.8 μm) after 150 hrs. These results are quite consistent with the spontaneous assembly theory and our previous results about *Sargitulla stella* EPS. Our previous results show that the driving force for the assembly of *Sargitulla stella* EPS is not from electrostatic interactions of Ca^{2+}/Mg^{2+}, but most probably from hydrophobic stabilizations. Hydrophobic force is largely decided by the entropy term, but not by enthalpy of interaction (Smith and Wood, Biological Molecules (Molecules and Cell Biochemistry), Springer). So the hydrophobic effect is strongly relied on the environment temperature. According to polymer spontaneously assembly theory, the first stage of the marine polymer gel assembly is relied on the hydrophobic forces (Chin et al. 1998). Since the assembly of *Sargitulla stella* EPS is driven by

hydrophobic nucleating and this force is strongly dependent on temperature, the change of environment temperatures affects *Sargitulla stella* EPS assembly.

6.3.4 Detection of hydrophobic domains with FRET technique

Sargitulla stella EPS could not only self-assemble without electrostatic stabilizing from Ca^{2+}/Mg^{2+}, but also can accelerate DOC polymer spontaneous aggregation (Fig. 6-4, 6-5, 6-6.). These results imply that *Sargitulla stella* EPS could function as nucleating reagent for DOC polymer assembly, and this facilitation is most probably driven by hydrophobic interactions rather than electrostatic interactions as in spontaneous DOC polymer assembly kinetics. The composition of EPS varies noticeably, depending on species, age, and growth conditions, etc. Generally, EPS are rich in high molecular weight polysaccharides, fair amounts of proteins, non-sugar moieties, small amounts of lipids and nucleic acid (Hoagland et al. 1993; Wingender 1999). Hydrophobic groups could be widely distributed among those EPS components.

Fig 6-8 A

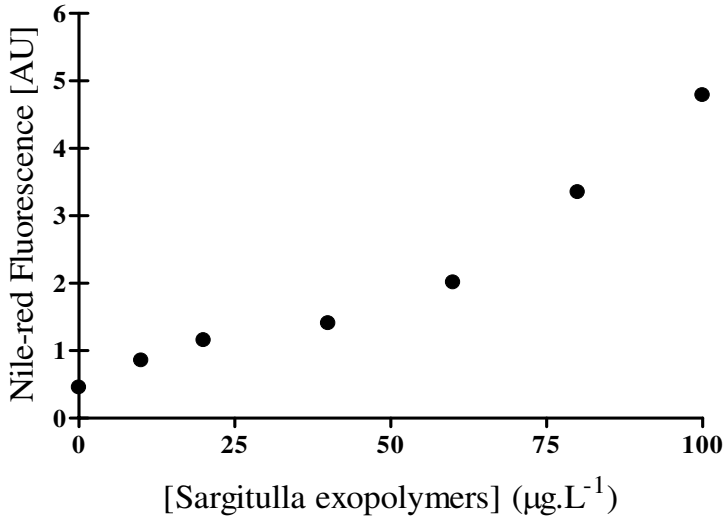


Fig 6-8 B

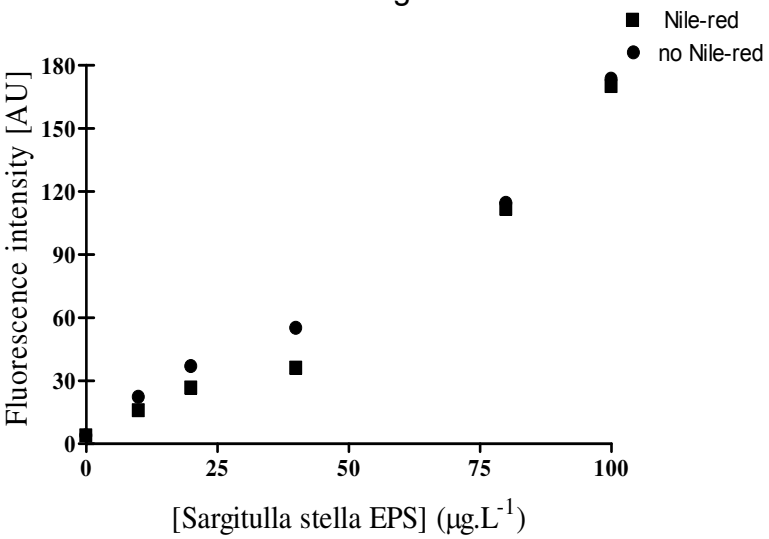


Fig 6-8. Energy transfer between *sargitulla stella* EPS and Nile-red. Panel A: Nile-red fluorescence intensity (AU); Panel B: filled circles refer to fluorescence emission from *sargitulla stella* EPS alone, while filled squares refer to fluorescence emission from *sargitulla stella* EPS after addition of Nile-red. Note the decrease of *sargitulla stella* EPS fluorescence with energy transfer to Nile-red.

Due to the highly complexity characteristics of the EPS chemistry, it is difficult to identify the specific hydrophobic groups in the *Sargitulla stella* EPS polymers. However, our investigation demonstrated the existence of hydrophobic domains within *Sargitulla stella* EPS polymers. Nile-red is a particularly effective solvatochromic fluorescent dye consisting of a rigid aromatic group and an exocyclic diethylamine group (Yablon and Schilowitz 2004). Its absorption and fluorescence depends on the physical properties of surrounding solvent environment, such as polarity.

Fig 6-9 A

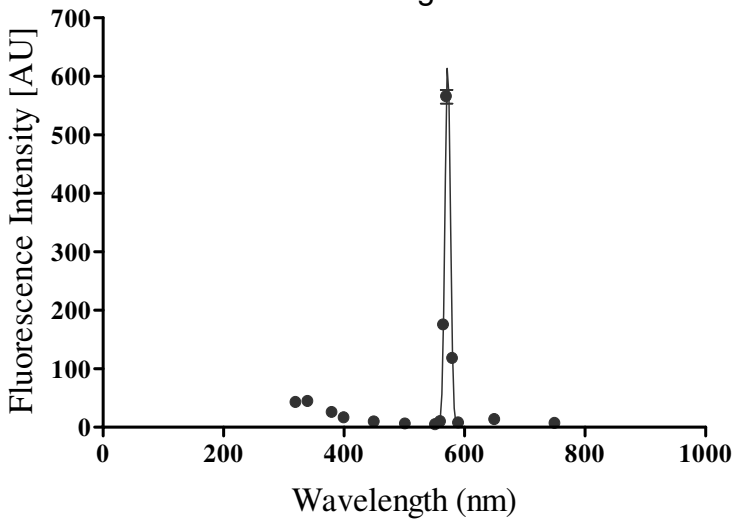


Fig 6-9. Fluorescence spectrum of *Sargitulla stella* EPS and Nile-red. Panel A: *Sargitulla stella* EPS; B: Nile-red, from MSDS.

Fig 6-9 B

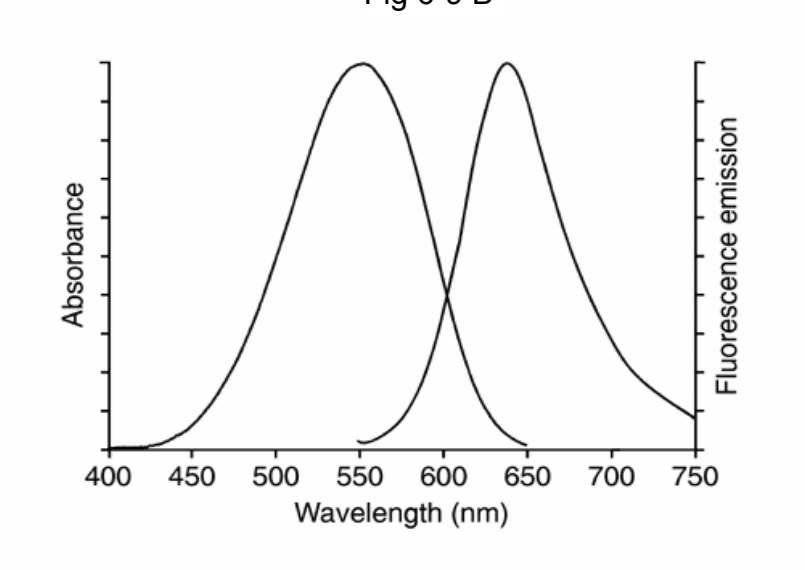


Fig 6-9 Continued

Fluorescence spectra of Nile-red recorded at a 568 nm excitation wavelength showed very weak signal at maximum emission wavelength 633 nm in polar ASW solvent (Fig 6-9 B). When we added 100 $\mu g.L^{-1}$ *Sargitulla stella* EPS into the ASW sample labeled with Nile-red, the fluorescence signal was enhanced ~4-5 times (Fig. 6-10.).

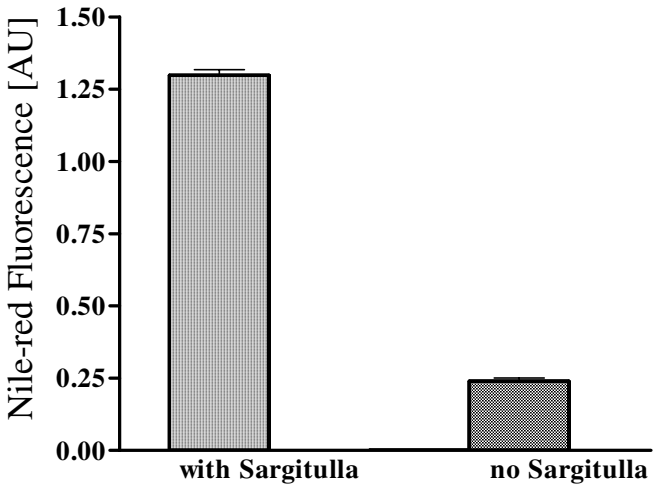


Fig 6-10. With a specific fluorescent dye Nile-red the existence of hydrophobic region was detected in *Sargitulla stella* EPS. Nile-red is a fluorescence dye specific for hydrophobic groups (EX_m / EM_m= 568/633 nm). In the ASW, Nile-red emitted very weak fluorescence signal. With addition of 100 $\mu g.L^{-1}$ *Sargitulla stella* EPS, the fluorescent intensity was enhanced ~4-5 fold. *Sargitulla stella* EPS has no fluorescence under EX_m / EM_m=568/633

The outcome means that addition of *Sargitulla stella* EPS decreased Nile-red proximate solvent polarity. This result implies the existence of hydrophobic regions in *Sargitulla stella* EPS polymeric structures. Absorption and fluorescence spectroscopy scan indicated that *Sargitulla stella* EPS has absorbance and fluorescence emission spectra in ultra-violet (285 nm) and green (570 nm) wavelength respectively (Fig. 6-9A.). Under EX/EM=568/633 nm, *Sargitulla stella* EPS itself could not emit fluorescence signal. Since the absorption spectra of the Nile-red (568 nm) overlaps the fluorescence emission spectra of *Sargitulla stella* EPS polymer, we could also use the FRET technique to test the hydrophobic groups in the *Sargitulla stella* EPS (The Handbook — A Guide to Fluorescent Probes and Labeling Technologies. Molecular Probes, Eugene, OR). With addition of *Sargitulla stella* EPS into Nile-red labeled ASW, a

new fluorescence signal from Nile-red appeared at emission wavelength 633 nm under excitation from 285 nm, but not from 568nm. Meanwhile, we could also detect the decrease of *Sargitulla stella* EPS fluorescence at 570 nm (Fig. 6-8A, B.).

6.3.5 Mimic of the effects of *Sargitulla stella* EPS with hydrophobic polyesterene beads

Further investigation demonstrated that hydrophobic force could indeed accelerate the DOC polymer assembly. The seawater samples were mixed with 0.01% (1.322×10^{13} microspheres per ml) plain polystyrene hydrophobic beads and the DOC polymer spontaneous assembly kinetics was measured with the same method. Addition of hydrophobic beads into seawater DOC sample resulted in similar results on marine microgels formation as *Sargitulla stella* EPS addition (Fig. 6-11.).

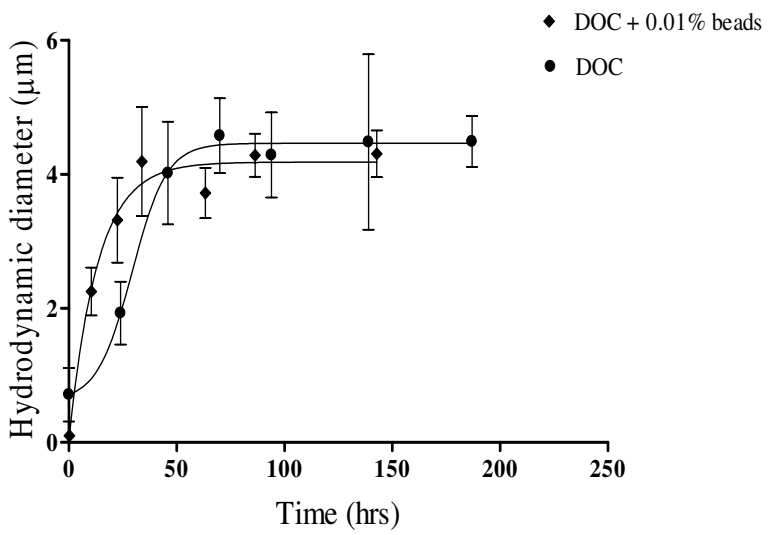


Fig. 6-11. Addition of 0.01% polyesterne particles into seawater samples could shorten the time for DOC polymers to reach equilibrium with self-assembled microscopic gels from typically ~50-83h (filled circles) to ~40h (filled diamonds), which has similar effects as *Sargitulla stella* EPS. The average polyesterne particle size was 20 nm.

6.4 Discussion

According to polymer gel theory the polymer networks that form the matrix of polymer gels result from spontaneous or induced assembly of polymer chains (Doi and Edwards 1986). Assembly that result in the formation of physical crosslinks occurs when the inter-chain distance allows polymers to interact with each other via low energy physical interactions, such as entanglements, electrostatic and hydrogen bonding, or hydrophobic interactions, van der Waals forces, etc. In the case of DOC polymer assembly forming marine hydrogels, divalent bonds from Ca^{2+}/Mg^{2+} in the seawater are demonstrated to be the main stabilizing force (Chin et al. 1998). The present results show that hydrophobic

interactions can drive bacterial EPS spontaneous assembly and EPS can function as nucleating agent for DOC polymer annealing and aggregation. Although hydrophobic force is the driving force for the role of *Sargitulla stella* EPS in these processes, to some extent other factors, such as chain size and dynamic topology of the EPS polymers, polyelectrolyte properties and charge intensities, etc, should also contribute the assembly process.

EPS produced by marine microorganisms in natural environment are complex and difficult to identify. Generally EPS contain large quantities of high molecular weight polysaccharides, fair amounts of proteins, non-sugar moieties, small amounts of lipids and nucleic acid. The acidic groups in the EPS were mainly composed of carboxylate, sulphate, and phosphate. Besides the hydrophilic characteristics, these EPS also contained 2-8% amphiphilic proteins, rendering them hydrophobic regions (Hung and Santschi 2005).

Another important role of bacterial EPS involves microbial attachment and biofilm formation. Before the formation of biofilm, one of the initial steps is that the microorganism should come closer to a surface where the low energy physical attractions hold them from removed by moderate shear forces. Both biotic and abiotic material can provide the initiating surface in natural and engineered systems. Recent discoveries derived around 7×10^{15} g of organic carbon present in microgels in ocean environment (Verdugo et al. in press). Gels may serve as surface for bacterial attachment and following by biofilm formation. Alledredge et al (1998) found the most direct evidence for bacterial attach to marine gel through differentially stained seawater samples for TEP and bacterial. This study found a large portion of bacterial would attach to the TEP gels (Alledredge et al. 1998). Due to the very native characteristics of these TEP gels, it is impossible to examine further how these bacterial respond to the gels and much of our

knowledge come from hypothesis and expectations. At the early stage of bacterial binding with gels and before biofilm formation, the attachment is very weak and physical factors of the surface, such as electrostatic forces, hydrophobic interaction, surface charges, etc, can reverse the bacterial from the surface. Our present data provide strong evidence how bacterial can bind with the surface tightly and accelerate the biofilm formation. The *Sargitulla stella* bacterial can secrete EPS polymers to the ocean and the *Sargitulla stella* EPS can self-assembly forming microgels to provide building blocks for the bacterial and other microorganisms to colonize. These EPS polymers can also bind with DOC polymers and other marine gels through hydrophobic interactions and accelerate the development of biofilm formation by speeding the micogels spontaneous assembly kinetics.

CHAPTER 7

EFFECTS OF ENVIRONMENTAL FACTORS ON KINETICS OF MARINE MICROGEL ASSEMBLY

7.1 Introduction

Global carbon cycling and greenhouse effects are two topics not only arrested attention from scientists, but also from political leaders and general ordinance from each corner of the world (Karl and Trenberth 1999). One of the most important determinants of global climate is the cycling of atmospheric gases, particularly CO_2. The equilibrium between CO_2 released and CO_2 recaptured from the atmosphere is drastically unbalanced. Burning of fossil fuels and other human interventions are releasing roughly 6.5×10^9 metric tons of CO_2/year to the air (Hedges 1992; Hedges 2000). The increase of atmospheric CO_2 and other gases is responsible for the greenhouse effect that blocks heat releasing, disturb global thermal equilibrium, and results in progressive climate disruption.

The processing of green house gas by land and ocean biota resulted in primary production of organic carbon. A large pool of the organic carbon is stored in ocean in the form of DOC (Houghton 2003; Hedges 1992; Hedges 2000). DOC is an operational definition of those pass through filters of given pore size usually in the range of 0.22~1 μm, while those pass through the filters are called POC (Verdugo et al. 2004). Although DOC has very similar chemistry composition as

POC, DOC is much more refractory and has very low bioactivity. So the POC pool has much determinant role on the microbial loop, global carbon cycling and finally climate change. Recently it is experimentally validated that there exists a spontaneous assembly mechanism converting the carbon from DOC to POC pool (Chin et al. 1998). It has been estimated and experimentally calculated that at least 10% of the total DOC in the ocean is transformed into a special form of POC, marine micogels composed of three-dimensional networks of biopolymers (Chin et al. 1998; Verdugo et al. 2004). This result provides profound new insight into the cycling of ocean organic carbon. However, the chemical and physical characteristics of the ocean environment affect the assembly kinetics strongly and these environmental factors remain largely unexplored.

7.2 Methods

7.2.1 Sample collection and pretreatment

Samples were collected from a coastal estuary site in Friday Harbor, Puget Sound, WA using CTD/28 Niskin-bottle rosette. Upon collection, samples were filtered at 0.22 μm (all filters were pre-treated with 0.1N HCl and rinsed with Milli-Q water to avoid contaminants) and treated with sodium azide (NaN_3) at 0.02% to inhibit microbial activity. All samples were stored in dark at 4° C.

7.2.2 Preparation of samples with different concentration of DOC polymers

To mimic seawater with different concentration of polymeric material, regular seawater samples were diluted with artificial seawater (423.00 mM NaCl, 9.00 mM KCl, 9.27 mM $CaCl_2$, 22.94 mM $MgCl_2$, 25.50 mM $MgSO_4$, 2.14 mM $NaHCO_3$) at different ratios.

7.2.3 Adjustment of seawater sample pH

The pH of seawater samples was adjusted with 3 M HCl and 10 N NaOH respectively.

7.2.4 Control of the temperature

Immediately after the start of monitoring of the assembly kinetics, the samples were put in incubators at pre-set temperatures. The DLS setup was connected with a temperature control unit. Before each measurement of the sample, the DLS temperature was adjusted to respective level.

7.2.5 The spontaneous assembly kinetics measurement

The assembly of DOC polymer gels was monitored by measuring particle size with a DLS protocol published elsewhere and mentioned in above chapters (Chin et al. 1998). Briefly, Aliquots (10-ml) were then poured directly into scattering cells. The scattering cells were positioned in the goniometer of a Brookhaven laser spectrometer (Brookhaven Instruments, NY, USA). Polymer assembly was monitored for 8~10 days, by analyzing the scattering fluctuations detected at a 45 scattering angle. The autocorrelation function of the scattering intensity

fluctuations was averaged over a 10-min sampling time, using a Brookhaven BI 9000AT autocorrelator. Particle size distribution was calculated by the CONTIN method (Provencher 1982; Chin et al. 1998).

7.3 Results and Discussions

7.3.1 Effects of pH on Marine Microgel Assembly

The pH of the ocean is decided by the equilibrium between atmospheric CO_2 concentration and dissolved calcium carbonate. The calcium carbonate provides a buffer against the pH change either natural or man-made. The ocean calcium carbonate works just like buffers used in chemical laboratory through altering the proportions of the abundant weak acids bicarbonate (HCO_3^{2-}) and carbonate (CO_3^{2-}) (Apps and Kurz 1991). Currently the pH in the bulk ocean body is remained to a slightly alkaline pH of 7.6 to 8.2. The buffer capacity of the calcium carbonate can only work in a certain pH range. With the build-up of CO_2 in the atmosphere and additional adsorption of CO_2 into the ocean, this capacity is broken down and the ocean is turning more acidic (Houghton 2000).

At normal pH level, the DOC polymer matrix has the polyanionic nature. Divalent bonds from Ca^{2+} can stabilize these polyanionic polymers to form an ionic-stabilized three dimensional gel networks but not covalently crosslinked networks. Since the alteration of the ocean pH will finally change the electrical distribution of the DOC polymers, pH should also affect the self-assembly of DOC

polymers. Our results show that the DOC polymers have similar assembly kinetics in alkaline or near alkaline seawater (pH 6.0-9.0). With the decrease of pH from 6.0 to 5.5, the assembly kinetics was greatly delayed. The typical time to reach equilibrium size was decreased from ~ 50 hrs to ~140 hrs. When the pH was further decreased from 5.5 to 3.0, the assembly kinetics didn't change that much. These results demonstrated that pH level affected the DOC polymers assembly seriously (Fig 7-1.). Although for the whole ocean body, the pH may not change from 9.0 to 3.0, large pH changes could happen in regional or highly localized area for natural or biological reasons.

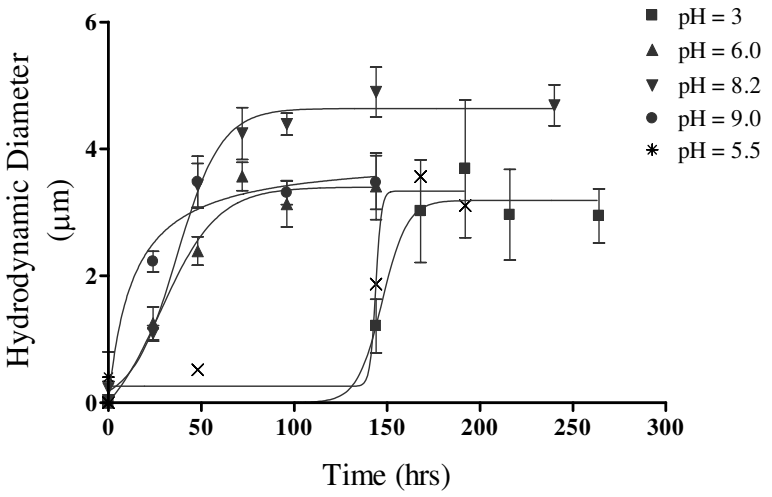


Fig 7-1. The effects of pH on DOC polymer spontaneous assembly kinetics. At filled circles, pH= 9.0; filled inverted triangles, pH= 8.2; filled triangles, pH=6.0; x refers to pH at 5.5; filleded squares, pH=3.0.

7.3.2 Effects of free DOC polymer concentration on marine microgel assembly

The organic carbon in the DOC pool can be converted into POC through the assembly of dissolved polymeric matrix into nano-gels and subsequently secondary aggregation and annealing into microgels, a theory called spontaneous assembly mechanism (Chin et al. 1998). According to this mechanism, the rate of the self-assembly is primarily determined by the concentration of the DOC polymers. The distribution of DOC in the ocean varies not only with location and depth, but its production also changes seasonally, primarily with the onset and decline of algal blooms (Wotton 2004; Bhaskar and Bhosle 2005). To mimic the variation of DOC in the ocean and its effects on the spontaneous assembly of DOC polymers, we diluted seawater samples taken from Friday Harbor, WA, with standard artificial and monitored their assembly kinetics with the standard DLS protocol (Fig 7-2.).

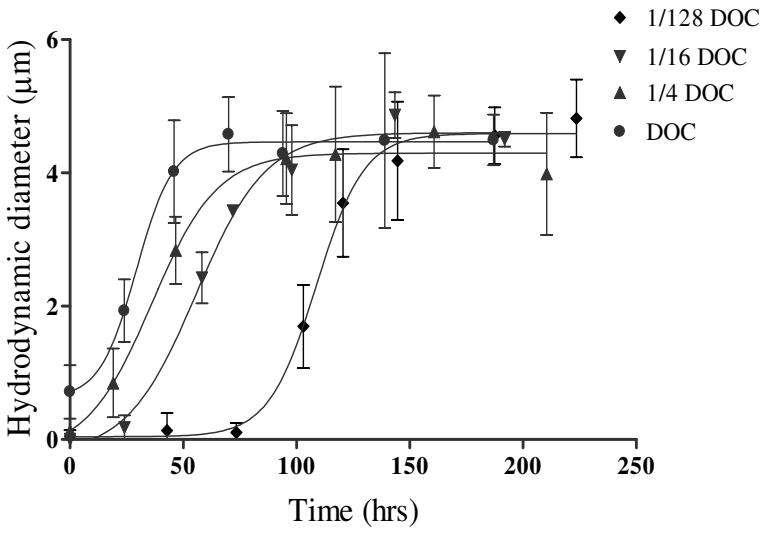


Fig 7-2. Effects of DOC polymer concentration on marine microgel formation.

The results show that the dilution of seawater with ASW will delay the assembly of DOC polymers obviously. With the dilution ratio from ¼ to 1/128, the typical time to reach equilibrium size extended from ~ 50 hrs to ~ 130 hrs. However the dilution operation won't change the final size of the microgel particles. These results demonstrated that the first stage of the marine polymer assembly is decided by a critical concentration of free DOC polymers with limiting sizes. With the dilution of the seawater, it will take longer time for smaller size free polymers to aggregate to form bigger particles and to accumulate and reach the critical concentrations.

7.3.3 Effects of temperature on marine microgel assembly

According to polymer gel theory, the assembly of polymers happened when the inter-chain distances between polymers allow them to interact with each other. For the self-assembly of marine polymer gels, the networks was resulted through physical interactions including entanglements, electrostatic and hydrogen bonding, or hydrophobic/hydrophilic interactions, van der Waals forces, etc (Verdugo et al. 2004). These physical forces, especially the hydrophobic interactions, are strongly dependent on the temperature. According to Chin et al's spontaneously theory, during the first stage of marine nanogel formation, the hydrophobic force and air-seawater interface play crucial roles (Chin et al. 1998). Any factor can change the surface microlayer or hydrophobic interactions should change the microgel assembly.

Since hydrophobic force is largely decided by the entropy term, but not by enthalpy of interaction, the alteration of temperature should change the hydrophobic force obviously. Our results about the effects of temperature on marine microgel spontaneous assembly kinetics confirmed this prediction and

Chin's model (Fig 7-3). When we increased temperature from 20 to 30 °C, the characteristics assembly kinetics of DOC polymers was accelerated from second order resembling a sigmoidal course to first order kinetics, and the typical time to reach equilibrium was shorted from ~ 50 hrs to ~ 15 hrs. If we further increased the temperature from 30 to 37 °C, there is no obvious acceleration of polymer assembly as we increased the temperature from 20 to 30 °C. This results show that the effect of temperature on hydrophobic force is non-linear. At 4 °C, there is no marine microgel formation. After waiting for 5-6 days, we still could not detect the formation of bigger size particles.

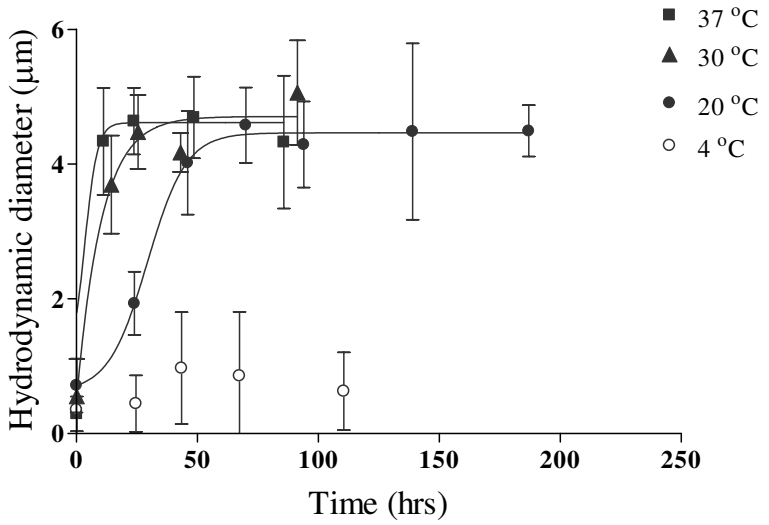


Fig 7-3. Effects of temperature on marine DOC polymers self-assembly (circles, 4 °C; filled circles, 20 °C; filled triangles, 30 °C; filled squares, 37 °C.)

7.4 Conclusion

Around 10% of the low bioactivity biopolymeric material in the ocean DOC pool can enter the microbial loop by forming microscopic gels through a spontaneous assembly mechanism (Chin et al. 1998; Well 1998). The rate of this process is strongly influenced by these DOC polymers and their interactions with the ocean environment. Our studies here show that free DOC polymer concentration, seawater pH, and seawater temperature have drastic impact on the conversion process. The assembly kinetics is greatly affected by concentration of free DOC polymers. Below the critical assembly concentration, the assembly process is largely delayed until the accumulation of enough particles. Our results are consistent with the observation that DOC production varies seasonally and TEP production follows DOC production (Wotton 2004; Bhaskar and Bhosle 2005). Temperature has strong impacts on the assembly kinetics possibly due to its effect on hydrophobic interactions. Seawater pH can also affect the assembly kinetics of marine microgels.

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTION

The goal of this work is to apply the principles of polymer gel physics and engineering to study alcoholic acute pancreatitis and marine microgel formation.

In alcoholic acute pancreatitis, our major finding is that ethanol treatment can sensitize the pancreas acinar cells to the effects of abnomaly high $[Ca^{2+}]_C$-induced premature zymogen activation leading to acute pancreatitis. After ethanol incubation, same level of $[Ca^{2+}]_C$-increase was observed to lead to higher trypsin activity, $[Ca^{2+}]_G$, and lower $[pH^+]_G$ inside the ZGs membrane. The sensitization effects relied on the opening of the ASK_{Ca} channels on the ZGs. These results not only provided us mechanism understanding of the initiating of alcoholic acute pancreatitis, but also gave us insights for the development of possible potential treatments. Our results demonstrated that blocking of the ASK_{Ca} channels could inhibit the effects of ethanol completely. So any possible pharmacological blockers and inhibitors of this ASK_{Ca} channel could serve potential drugs for this disease.

In the marine microgel formation field, my results demonstrated the effects of exopolymers from marine microbes on conversion of DOC into highly bioactive microscopic gels. The results show that these EPS could not only self-assembly into marine microgels, they could also drastically accelerate the assembly of DOC polymers through hydrophobic nucleating. These results provide a new

frontier to understand the microgel/bacteria interaction in the ocean. The observations further offer intriguing new leads to investigate the mechanisms of biofilm formation, not only by marine bacteria, but perhaps also by microbial pathogens affecting humans and other species. Our development of a fluorescent probe assay can also provide marine researchers a reliable, but simple and potable method to measure the fraction of organic carbon presented in SAGs.

There are still lot of work to do and new direction to follow up. For alcoholic acute pancreatitis, recognizing additional factors that increase the susceptibility of human pancreas to the sensitization effect of alcohol is a growing interest of research. In the field of the effects of exopolymers from marine organisms on marine microgel formation, our results provided the first evidence that marine bacterial can accelerate marine microgel assembly and biofilm formation. However, our results mainly come from the EPS extractant from *sargitulla stella*. It will be beneficial to expand the study to include more marine microorganisms.

APPENDIX

A.1 Acronyms and Special Characters

ALOHA	Open Pacific Ocean ALOHA station (22°26'N, 158°5'W)
[AU]	Arbitrary unit
ASK_{Ca}	Ca^{2+}-activated K^+ channels
ASW	Artificial seawater
BZiPAR	Rhodamine 110, bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide) dihydrochloride
Ca^{2+}	Free calcium ions
$[Ca^{2+}]_C$	Free calcium ions concentration in cytosol side
$[Ca^{2+}]_G$	Free calcium ions concentration inside zymogen granules
C_A	Total carbon concentration in self-assembled gels
CCK	Cholecystokinine
CTC	Chlortetracycline hydrochloride
C_T	Total carbon concentration
DLS	Dynamic laser scattering
DOC	Dissolved organic carbon
EDTA	Ethylenediaminetetraacetic acid
EGTA	Acetic acid, (ethylenebis(oxyethylenenitrilo)) tetra-; ethylene glycolb bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
E.Hux	*Emiliania huxleyi*
EPS	Extra-cellular polymeric substances or exopolymeric substances
FHL	Friday Harbor Laboratories, University of Washington at Seattle
FRET	Fluorescence resonance energy transfer
F_T	Total CTC fluorescence intensity
F_A	CTC fluorescence intensity with self-assembled gels
gt C	10^9 tons of carbon
$[H^+]_G$	Hydrogen ions concentration inside zymogen granules
hrs	Hours
IPCC	Intergovernmental Panel on Climate Change
K^+	Free potassium ions
$[K^+]_G$	Free potassium ions concentration inside zymogen granules
m	Meter
Mg^{2+}	Free meganisiums
Nile-red	A lipid probe for hydrophobic groups

OC	Organic carbon
$[pH]_G$	pH inside zymogen granules
POC	Particulate organic carbon
ppmv	Parts per million by volume
Protein-C	Carbon present in proteins
SAGs	Self-assembled gels
[SAG]/l	Number of self-assembled gels per liter
Ske	*Skeletonema costatu*, a type of phytoplankton
Syn	*Synechococcus*, a type of phytoplankton
TCHO	Total carbonhydrates
TEA	Tetraethylammonium chloride, blocker for K^+ channels
TEP	Transparent exopolymer particles
WMO	World Meteorological Organization
ZGs	Zymogen granules, an organelle inside pancreas acinar cell

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(4) Yong-Xue Ding, K.Yang, Nikki. Sweeney, Wei-chun Chin. Ethanol augments premature zymogen activation in acute pancreatitis. BMES 2004 Fall Annual Meeting.

(5) Yong-Xue Ding, Anothony Rodriguez, Wei-Chun Chin, Chin-Chang Hung, Peter.H Santschi, Pedro. Verdugo. Effects of microbial extracellular substances on the assembly of dissolved organic matter into microgels. ASLO 2005 Meeting.

(6) Anthony Rodriguez, Yong-Xue Ding, Wei-Chun Chin**,** Laura Gamble, David Castner, Pedro Verdugo. Iron ion accelerates the kinetics of spontaneous assembly of DOM polymer for the formation of marine microgels. ASLO 2005 Meeting.

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(8) Yong-Xue Ding, Anothony Rodriguez, Wei-Chun Chin, Chin-Chang Hung, Peter.H Santschi, Pedro. Verdugo. Exopolymers from Marine Bacteria Can Accelerate Biopolymer Self-assembly and Biofilm Formation. UWEB 2005 Summer Symposium.

(9) Yong-Xue Ding, Wei-Chun Chin, Anothony Rodriguez, Chin-Chang Hung, Peter.H Santschi, Pedro. Verdugo. Marine Bacteria Exopolymers Induce Microgel Assembly Through Hydrophobic Interactions: Implications for Biofilm Formation and Substrate Scavenging. ASLO 2006 Conference.