ABSTRACT

Title of Document: EFFECT OF CHITOSAN ON THE INDUCTION OF DNA DAMAGE RESPONSE BY SELENIUM COMPOUNDS.

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Selenium (Se)， a nutrient trace mineral, plays important roles in optimizing human health. Chitosan is an effective, natural-oriented material for synthesizing nanopolymers, with preferable properties such as biocompatibility, biodegradation and resistance to certain enzymes. In this study, encapsulated Na_2SeO_3 and methylseleninic acid (MSeA) with low and medium molecular weight chitosan were used to determine the efficacy of Se in mitigating tumorigenesis. We applied Se compounds, which is from sub-lethal to lethal dose, to colon cancer cell line HCT-116 and normal fibroblasts cell line MRC-5. Analysis of cellular selenium content demonstrated that: 1) Na_2SeO_3, but not MSeA, treatment resulted in a greater Se retention in HCT-116 than in MRC-5 cells, 2) chitosan

encapsulation enhanced Se contents in cells treated with the various Se preparations. Cell survival analysis showed that chitosan encapsulation protected HCT-116 and MRC-5 cells from Na_2SeO_3 or MSeA induced toxicity. Moreover, this beneficial effect was greater in MRC-5 cells. MSeA encapsulated with chitosan induced phosphorylated ATM Ser-1981 formation in MRC-5 and HCT-116 cells to a less extent as compared to MSeA alone treatment. Taken together, the results suggest that, when encapsulated with chitosan, cells are less susceptible to Se treatment, possibly through a mechanism by which the presence of chitosan attenuates Se-induced activation of ATM and corresponding DNA damage response pathway.

EFFECT OF CHITOSAN ON THE INDUCTION OF DNA DAMAGE RESPONSE BY SELENIUM COMPOUNDS.

By

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Dedication

To my parents: Ms. Shi Chen and Mr. Yiqun Zhang.

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Chapter 1: Literature review

1.1 Early studies on Selenium (Se)

Se was first identified as a by-product of sulfuric acid production by a Swedish chemist Jons Jacob Berzelius in 1817 (Brown and Arthur, 2001). It is a non-metal trace element with the atomic number 34, and is in the same chemical family with oxygen, sulfur, tellurium, and polonium. Se exists in nature as a combination of inorganic (such as selenide, selenate, and selenite) and organic (such as selenomethionine and selenocysteine) forms.

Se was known only as a toxicant (Fels and Cheldelin, 1949) until 1952 when a biological role of Se was discovered in microorganisms (Postgate, 1952). The nutritional essentiality of Se was identified by Schwarz and Foltz in a seminal report indicating that dietary Se supplementation is required to prevent liver necrosis in rats (Schwarz and Foltz, 1958). Later on, it was demonstrated that Se is an integral component required for glutathione peroxidase (GPX) activity (Rotruck et al., 1973; Flohe et al., 1973). Evidence supporting selenocysteine as the 21st amino acid was first shown in eubacteria and archaebacteria systems. Stadtman and colleagues metabolically labeled *Clostridium sticklandii* with ^{75}Se and found that Se exists in proteins in the form of selenocysteine (Cone et al., 1976; Stadtman, 1989). Then, the *Gpx1* gene was cloned from mice and UGA was identified as the codon for selenocysteine incorporation (Chambers et al., 1986).

1.2 Selenoprotein

The biological function of Se is primarily implemented through its incorporation into three forms of Se-containing proteins (Behne and Kyriakopoulos, 2001). First, Se can be non-specifically incorporated into methionine-rich proteins, such as those found in the Brazil nut (Gander et al., 1991). Second, Se can be specifically incorporated into selenoproteins. The key features of selenoproteins include the decoding of UGA codon as selenocysteine (Sec) residues and the requirement of a stem-loop secondary structure in the 3'-UTR region of selenoprotein mRNA, also known as Sec insertion sequence (SECIS). There are specific tRNA ($Cys^{[Sec]}$-tRNA) for Sec and a RNA-binding protein complex that recognizes the stem-loop secondary structure and facilitates decoding the codon UGA, which would otherwise act as a stop codon (Bock et al., 1991). Third, there are specifically Se-containing proteins that sequester Se through an unknown mechanism, examples of which include the uncharacterized liver protein 56K (SLP-56) and the liver fatty acid binding protein (SLP-14) (Behne et al., 1994).

By scanning the entire genome for SECIS, it was shown that there are a total of 25 selenopoteins in humans (Kryukov et al., 2003). They include five glutathione peroxidases (GPX1-GPX4, GPX6), three iodothyronine deiodinases (DI1-DI3), three thioredoxin reductases (TR1-TR3), the 15-kDa selenoprotein (Sep 15), selenoprotein H, I, K, M, N, O, P, R, S, T, U, V and selenophosphate synthetase-2 (SPS2). Except for selenoprotein P, which has ten Sec residues, selenoproteins only contain a single Sec residue (Burk and Hill, 1999). The Sec residue is critical for the enzymatic activity of

selenoproteins; for instance, substitution of Sec in TR-1 with a cysteine results in decreased enzymatic activity (Hatfield and Gladyshev, 2002; Lee et al., 2000).

Of note, more than one-third of selenoproteins are known to exhibit antioxidant properties. The GPX family uses glutathione as a reducing equivalent to eliminate hydrogen peroxide, organic hydroperoxides and phospholipid hydroperoxides (Brigelius-Flohe, 1999). The TR family controls the redox state of thioredoxin (Bjornstedtet al., 1995). Although selenoprotein P, a major extracellular selenoprotein, is generally considered a Se carrier, it has been shown to eliminate phospholipid hydroperoxides and thus is considered as a selenoperoxidase (Saito et al., 1999; Takebe et al., 2002).

1.3 Se as a dietary component promoting human health

Se is essential for certain metabolic pathways that support optimal human health. For example, dietary Se deficiency leads to cardiomyopathy seen in patients with Keshan disease, a childhood endemic disease occurring in part of China where the soil is deficient in Se (Guanqing, 1979). Recently, hypomorphic mutation in *SECISBP2*, a gene whose product influences selenoprotein synthesis, has been studied, the result of which suggest this gene a potential target for treating human hereditary diseases, such as thyroid hormone disorders (Dumitrescu et al., 2005). At nutritional or supranutritional levels, Se is known as a chemopreventive agent that counteracts tumorigenesis through cell cycle control, apoptosis and other yet-to-be-verified mechanisms (Jackson and Combs, 2008).

Se is one of the most extensively studied trace elements with cancer chemopreventive properties. *In vivo,* Se is capable of inhibiting cancer cell growth and inducing apoptotic cell death by inducing ROS (Reactive Oxygen Species) formation via interaction with GSH. Excessive formation of ROS leads to cell cycle arrest and accumulated DNA damage in tumor cells (Lu et al., 1995); (Spallholz et al., 2001); (Stewart et al., 1999b). Several underlying mechanisms of such effect have been elucidated. Se-induced apoptosis in prostate cancer cells is dependent on p53 (Zhao et al., 2006); Se also alters cancer cells from apoptosis-resistant to apoptosis-prone (Hu et al., 2006); (Li et al., 2007).

In addition, antioxidants can counteract selenite-induced apoptosis by eliminating ROS (Shen et al., 2001). Se compounds negatively affect carcinogen activation and metabolism through inhibition of phase I enzymes and induction of phase II enzymes, which subsequently induce cell cycle arrest (Ganther, 1999); (Harrison et al., 1997). In animal models, Se suppresses carcinogenesis, which is initiated by carcinogen treatment. Se is also capable of inhibiting colonial expansion of a carcinogen-transformed population (Combs and Gray, 1998).

1.4 Se compounds as prevention for colon cancer

Colon cancer is regarded as a severe threat leading to cancer-related death in Untied States. Accumulating evidence indicates that Se is an effective chemopreventive agent against colon cancer in human. The proposed mechanisms of Se on colon cancer include 1) inhibition of cytosine methyltransferases (Fiala et al., 1998) and cyclooxygenase (Baines et al., 2002).

An organic form of Se, selenomethionine, inhibits growth of human colon cancer cell lines HCT116 through p53 mediated G2/M cell cycle arrest as well as apoptosis (Goel et al., 2006). In addition, activation of ERK (Extracellular Signal-Regulated Kinase) and the subsequent phosphorylation of RSK (Ribosomal S6 Kinase) and histone H3 have also been suggested as target of Selenomethionine (Goulet et al., 2007). cDNA microarrays reveal statistically significant changes of 50 genes after Se challenge, for example, CX26 (connexin26) or DAP-1 (Death associated protein 1) etc., exists in Se treated HCT116 cells.

1.5 Effect of Se in counteracting ROS

Anti-carcinogenic activity by selenium and its metabolites are correlated with the production of ROS. Although the ROS theory of aging was proposed 50 years ago, there have been no definitive studies to prove or disprove the theory. ROS, such as superoxides, hydroxy free radicals, and hydrogen peroxides are widely dispersed *in vivo* (Harman, 1956); they are by-products of redox cycling during mitochondrial respiration, modification of protein-thiols and methionine mimicry (Jackson and Combs, 2008).

ROS can also be generated from exogenous sources, for instance UV and ionizing irradiation (Finkel and Holbrook, 2000). Because GPX1 accounts for much of the H_2O_2-eliminating activity of Se (Cheng et al., 1997), it is logical to address the ROS theory of aging by investigating Se and GPX1 activity. By comparing aged and juvenile rats, it has been suggested that GPX activity can be up-regulated during the aging process in an attempt to suppress lipid oxidation accumulation in mitochondria (Nohl et al., 1979).

Nonetheless, available evidence has not conclusively identified a role of GPX1 in lifespan extension in mouse models (Ladiges et al., 2009). Interestingly, *Gpx4*$^{-/-}$mice are embryonic lethal while *Gpx4*$^{+/-}$mice exhibit extended lifespan, possibly because the spontaneous tumors in *Gpx4*$^{+/-}$mice are more susceptible than wild-type mice to apoptosis induced by the intrinsic oxidative stress as a result of the loss of one *Gpx4* allele (Ran et al., 2007).

1.6 The ATM pathway in DNA damage response

Ataxia-telangiectasia (A-T) was identified as an autosomal recessive disorder that is characterized by early onset progressive cerebellar ataxia, oculocutaneous telangiectasia, lymphoid tumors and susceptibility to bronchopulmonary disease (Boder, 1985). Several abnormalities such as cellular radio sensitivity, checkpoint defects and chromosomal instability are characteristics of A-T. In clinical study, A-T patients show immunodeficiency and insulin-resistant diabetes (Lavin and Shiloh, 1997). Genetically, A-T is attributed to ATM mutation. ATM is a Ser/Thr protein kinase and a member of phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family (Savitsky et al., 1995).

ATM functions by forming a complex with MRE11–RAD50–NBS1 (MRN). Nijmegen breakage syndrome (*NBS1* mutantions) and A-T-like disorder (*MRE11* mutations) have certain symptoms in common with A-T (Carney et al., 1998); (Matsuura et al., 1998); (Varon et al., 1998); (Stewart et al., 1999a). As described by Lavin's research group, expression of ATM is activated in response to DNA damage (Double Strand Breaks

DSBs) and signals to the DNA damage checkpoint to repress cell proliferation and facilitate DNA repair. In A-T cells, loss of γH2AX (Phosphorylated Histone H2AX) foci leads to failure of DNA fraction repair, whereas non-homologous-end-joining (NHEJ) repairs DNA DSBs in an ATM-independent manner.

Mechanisms of DNA damage repair with rapid recruiting of repair proteins to accurate foci have been elucidated gradually. As for DSBs repair, the MRN complex recognizes damaged foci and its accumulation regulated by the mediator of DNA-damage checkpoint protein-1 (MDC1) adaptor protein. Thereafter, ATM localizes to the DSBs foci and associates with MRN complex at this initial repair stage, which guarantees complete activation of ATM and γH2AX is phosphorylated as downstream substrate of ATM. However, P53 was proved firstly to be substrate of incompletely activated ATM both *in vitro* and *in vivo* (Banin et al., 1998); (Canman et al., 1998); (Khanna et al., 1998). To date, up to 700 phosphoproteins have been identified as targets of ATM (Matsuoka et al., 2007); (Linding et al., 2007).

The order in which those DNA repair proteins assemble in ATM signaling pathway is postulated as: ATM phosphorylate histone variant of γH2AX to produce γH2AX initially. A second DSBs response protein MDC1 binds to γH2AX via breast cancer susceptibility protein-1 (BRCA1) C-terminal (BRCT) domain and this is key step for recognition and repair DNA damage. NBS1, as a component of MRN complex, localizes to breaks in the same manner as MDC1. The RING-finger ubiquitin ligase RNF8 ubiquitylates γH2AX and facilitates the accumulation of p53-binding protein-1 and BRCA1 by interacting with phosphorylated MDC1. Of interest, ATM plays a key role to phosphorylate a series of proteins, which in turn, produce signal cascade during DNA damage repair machinery.

As an essential role in mediating DSBs, ATM undergoes autophosphorylation on at least three sites (Ser367, Ser1893, Ser1981) during activation process. In addition, DNA DSBs induce the acetylation of ATM in parallel to Ser1981 is another post-translational modification of ATM as well (Sun et al., 2005). Exceptions are examples from human cells, in which ATM can be activated without phosphorylation on Ser1981 (Hamer et al., 2004); (Powers et al., 2004). Thus the phosphorylation of ATM could be relevant to the nature of stimulus.

Treated with ATM small interfering RNA (siRNA), selenite induced apoptosis has been alleviated in mouse embryonic fibroblast NIH 3T3 cells, implying an involvement of DNA damage regulator ATM. In the same study, selenite also stimulates Ser-139 phosphorylation of the ATM/ATR substrate H2AX (Zhou et al., 2003). It is likely that the selenite-induced apoptosis is attributed to ATM mediated cell damage.

1.7 Chitosan as a favorable material to incorporate selenium as nanopolymer

Chitosan is found in the exoskeleton of crustaceans of marine arthropods and insects, which is available in abundant supply. Several properties make it as a favorable material for synthesizing nanopolymer: it is non-toxic, biocompatible and biodegradable, yet resistant to enzymatic degradation in normal biological environment (Chandy and Sharma, 1990).

In biochemical aspect, it is polysaccharide β (1–4) 2-amino-2-deoxy-D-glucosemodified from chitin, which is converted to chitosan by alkaline deacetylation Co-polymer of N-acetyl D-glucosamine and D-glucosamine (Onishi and Machida, 1999). Chitosan varies

in the molecular weight (5–2000 kDa) and degree of N-acetylation (40–98%), depending on the processing method [Cancer, chitosan nanoparticles and catalytic nucleic acids (Hejazi and Amiji, 2003). Common techniques established to produce chitosan include chemical cross-linking (Banerjee et al., 2002), emulsification solvent diffusion (Nafee et al., 2007), complex coacervation (Leong et al., 1998) and ionotropic gelation (Ma et al., 2005). Due to the D-glucosamine residue with a pKa value of 6.2–7.0, chitosan can only be soluble in acid pH solutions (Luangtana-anan et al., 2005). Also, its encapsulation efficiency and release profiles largely depend on the molecular weight. Previous study postulated that high molecular weight chitosan with a high deacetylation degree improves plasmid DNA encapsulation efficiency (Bozkir and Saka, 2004).

The surface of chitosan is positive-charged, thus it is able to deliver drugs/genes specifically to negative-charged tumor cells and enter the nucleus directly (Chitosan microparticles encapsulating PEDF plasmid demonstrate efficacy in an orthotopic metastatic model of osteosarcoma). Moreover, it releases drugs/gene in a suspending manner (Zhang and Neau, 2001).

In vitro degradation of chitosan by a commercial enzyme preparation: effect of molecular weight and degree of deacetylation]. Mucoadhesive property of chitosan enhances drug absorption, simply by re-arranging the junction proteins (Illum et al., 1994); (Pan et al., 2002). In another word, it can overcome the permeability barrier posed by the epithelia, attributing to taking up of endosomes (Sakuma et al., 2001). Chitosan is able to induce apoptosis of bladder tumor cells by activating caspase-3 or other yet unclear mechanisms. It own demonstrates growth inhibition on cancer cells (Hasegawa et

al., 2001). In addition, it suppresses growth of hepatocellular carcinoma to a certain extent (Qi et al., 2007).

Seleno-short-chain chitosan (SSCC, molecular weight between 5,000 and 10,000 Da) inhibit proliferation of human leukemia K562 cells in a dose-dependent and time-dependent manner. K562 treated by SSCC was primarily accumulated in G (2) /M phase, which is evidenced by flow cytometry analysis. Subsequently, this arrested growth is associated with K562 cell apoptosis (Liu et al., 2008).

Packed siRNA by sodium tripolyphosphate (TPP)–chitosan nanoparticles demonstrated comparable gene silencing effects compared with Lipofectamine 2000 transfections. However, based on MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay for cytotoxicity, less than 50% cell death was observed, which was not expected result from gene knockdown. The conflicting data calls for further investigation to siRNA-chitosan nanoparticles as a potential therapeutic, especially in terms of tumor growth inhibition and normal tissue protection (Katas and Alpar, 2006).

Besides delivery of siRNA as gene therapy, the docetaxel-loaded hydrophobically modified glycol chitosan (DTX-HGC) nanoparticles has been reported to facilitate antitumor efficacy of free DTX, inducing less tumor volume and increased survival rate in A549 lung cancer cells-bearing mice and reduced the anticancer drug toxicity compared to that of free DTX (Hwang et al., 2008).

1.8 Hypothesis of this thesis

Based on findings in 2003 (Zhou et al., 2003), selenium compound sodium selenite triggers ATM mediated apoptosis in NIH3T3 mouse embryonic fibroblast cells. Moreover, sodium selenite phosphorylated Ser-139 of ATM substrate H2AX in HL60 cells. Therefore, it is suggested that selenium induces fibroblast apoptosis, which can attribute to DNA damage and subsequently activation of ATM pathway. In addition, specific phosphorylation site can be altered by treatment of selenium.

Since chitosan-nanopolymers have been proved to be an efficient delivery of gene therapy drugs and chemical compounds such as siRNA and selenium. Of note, the seleno-short-chain chitosan (SSCC) exert anticancer effect on K562 cells by arresting cell growth. In our lab, we have recently identified novel barriers of tumorigenesis induced by selenium compounds, namely senescence and DNA damage response.

Collectively, the activation of tumorigenesis barriers is thought to attribute to the toxic aspects of suspernutritional level of selenium. To address this issue, we aim at providing a targeted delivery of selenium to colon cancer cells. Since cancer cell surface is negative charged; in contrast, chitosan particles own positive surface charges. Hence, we raise a question that whether Chitisan-Se has influence on the efficacy of Se delivery and on the activation of tumorigenesis barriers, for example, the phosphorylation of DNA damage repair factor ATM.

In combination of chitosan-coated selenium and DNA damage repair, this study mainly focuses on effect of chitosan selenium nanopolymer on cell viability, oxidative stress generation and DNA damage associated ATM pathway. Here we compare effect of chitosan conjugated sodium selenite, methylseleninic acid and original selenium compounds in normal human fibroblast cell and human colon cancer cell.

To unveil the difference between normal and cancer cell in response to Chitosan-selenium and original selenium, this research includes assessment on cell survival, ROS production and ATM Ser 1981 phosphorylation.

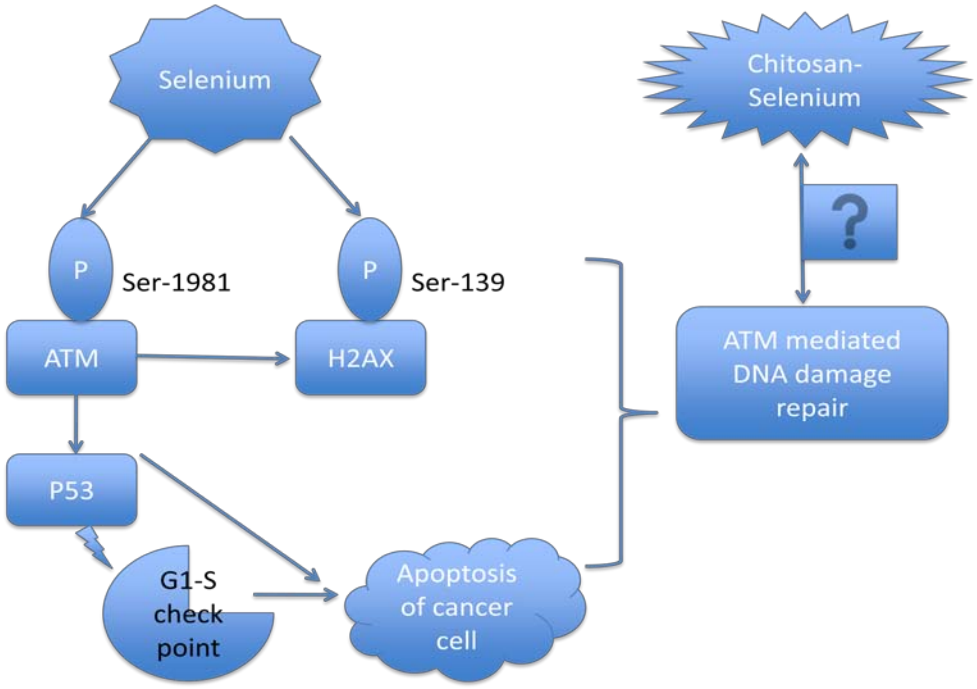


Figure 1.1 Eluciated mechanisms underlying Se induced cancer cell apoptosis and unknown mechanisms we investigate.

P: Ser phosphorylation sites.

Chapter 2: Review: Selenoproteins and the aging brain (Review paper accepted in 2009)

2.1 Selenium in the brain

In addition to the well-documented functions of Se in the antioxidant system and in the regulation of the thyroid and immune function (St Germain et al., 2009), recent advances have indicated a role of Se in the maintenance of brain function. Se is widely distributed throughout the body, but is particularly well maintained in the brain, even upon prolonged dietary Se deficiency (Schweizer et al., 2004). In the brain, the highest concentration of Se was found in the gray matter, an area responsible for chemical synaptic communication (Hock et al., 1975). It has been shown that rats on a Se-deficient diet for thirteen weeks retained Se in their brain, while their plasma Se concentrations were depleted (Prohaska and Ganther 1976). After intraperitoneal injection of $^{75}SeO_3^{2-}$ into Se-deficient rats, the brain rapidly sequesters a large portion of the available Se (Trapp and Millam 1975). In the brain, it was found that the cerebellum accumulated the highest concentration of Se, followed by the cortex, medulla oblongata, cerebral hemisphere, and the spinal cord. Interestingly, Se retention in the brain depends on selenoprotein P (Nakayama et al., 2007).

Because the body preferentially allocates available Se to the brain during Se deficiency, Se may play an essential role in the brain. More evidence for the brain being at the apex of Se retention is provided by a study showing that a six generation Se deficiency in rats

caused a more than 99% reduction of Se concentration in the liver, blood, skeletal tissue, and muscle, while the brain retained only 60% of the Se, even under generational Se deficiency (Kyriakopoulos et al., 2000). Likewise, the mRNA level of twelve selenoproteins in young pigs fed a Se-deficient diet remains highly expressed in the pituitary gland, but is significantly reduced in other organs such as the liver (Zhou et al., 2009). Moreover, Se concentration in Alzheimer's brains was found to be 60% of the age-matched control individuals (Hawkes and Hornbostel, 1996). These results suggest that Se is critical for the maintenance of brain function, and is appropriated to the brain from the remaining part of the body even when the body Se availability is scarce.

2.2 Selenoproteins and neurodegeneration

Evidence suggests a link between Se and neuronal disorders via redox regulation. Low levels of Se are associated with cognitive impairment, depression, anxiety and hostility humans (Rayman and Rayman 2002), and Se deficiency was found to be associated with decreased expression of brain-derived neurotrophic factor mRNA in the developing brain of rat pups (Mitchell et al., 1998). Increased oxidative stress has been linked to neuronal disorders (Rosen, 1993), including Alzheimer's disease (Ishrat et al., 2009), multiple sclerosis, Batten's disease, Parkinson's disease and brain tumors (Chen and Berry, 2003; Schweizer et al., 2004). In particular, Alzheimer's disease is characterized by memory loss with age, a symptom that is linked to ROS-induced neuron damage. Levels of Se are elevated in amygdala (Cornett et al., 1998) and in microsomesin the temporal lobe of patients with Alzheimer's (Wenstrup et al., 1990). Moreover, investigation of the

dementia caused by Alzheimer's type revealed that total GPX activity and Se content in the plasma decreased with age in the patients (Ceballos-Picot et al., 1996; Basun et al., 1991). Likewise, hematogenous cells derived from patients suffering from multiple sclerosis and Batten's disease show lowered Se concentration and decreased GPX activity (Clausen et al., 1988). Furthermore, the level of Se in the plasma is decreased in patients with malignant tumors of central nervous origins (Philipov and Tzatchev, 1988). Intriguingly, long-term culture of neuronal cells requires medium conditions of basal elements supplemented with either serum or Se (Tian et al. 2002).

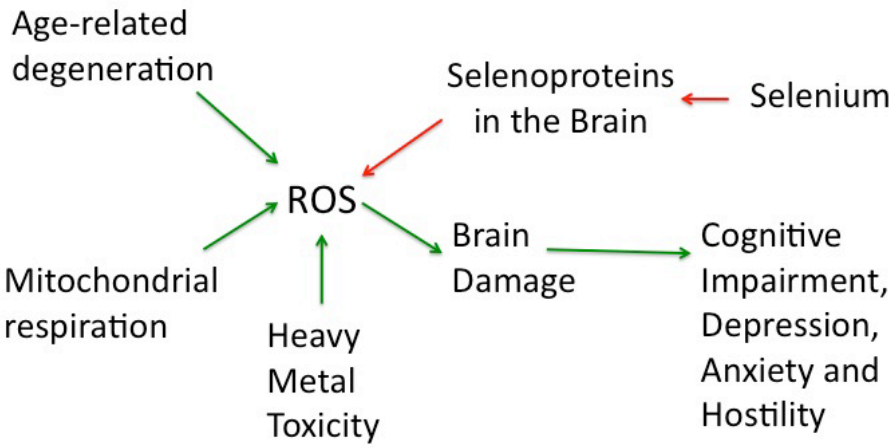


Figure 2.1 Function of ROS in the aging brain as a consequence of selenium status. Most brain related neurodegenerative diseases have increased free radical production, and decreased radical scavenging.

Considering the location of antioxidant activity in the brain, some functionally characterized selenoproteins have been postulated to protect against neurodegeneration via redox regulation. The selenoproteins that are implicated in brain maintenance include GPX1, GPX4, selenoprotein P, thioredoxin reductases, selenoprotein W, and

selenoprotein M (Korotkov et al., 2002; Steinbrenner and Sies, 2009; Drukarch et al., 1998; Dringen et al., 1999; Whanger, 2001; Jeong et al., 2002). Compared with age-controlled normal individuals, Alzheimer's patients showed a significant age-dependent decrease in the Se-dependent glutathione peroxidase activity in the plasma and red blood cells (Ceballos-Picot et al., 1996). Moreover, an increase in thioredoxin reductase activity has been observed in the brain of Alzheimer's patients (Lovell et al., 2000). In contrast, a recent report showed that the level of Se in plasma and cerebrospinal fluid from Alzheimer's patients are comparable to normal individuals (Gerhardsson et al., 2008). Thus, changes of the selenoenzyme activities appear to be confined to the brain.

Parkinson's disease is another age-related degeneration characterized by loss of the dopaminergic cells caused by the formation of ROS through dopamine's own metabolism (Kupsch and Oertel, 1994). The link between selenoproteins and Parkinson's disease is an emerging field of study. Using the 6-OHDA-treated rats as a model of Parkinson's disease, it was found that administration of Se increased antioxidant activity, thus protecting the dopaminergic cells from oxidative damage. Consequently, more dopamine cells were able to survive after Se treatment (Zafar et al., 2003). Neuronal systems experiencing acute stress, such as epilepsy and stroke, exhibit lowered GPX activity in plasma and decreased GPX4 activity in neuronal cells (Ramaekers et al., 1994; Weber et al., 1991). In animal models, selenoprotein P knockout mice exhibit reduced growth and develop ataxia, as well as decreased GPX and TR activities in the brain (Schomburg et al., 2003). Transgenic mice that overexpress human GPX1 showed decreased susceptibility to neuronal injury as a result of cerebral ischemia reperfusion (Ishibashi et

al., 2002).

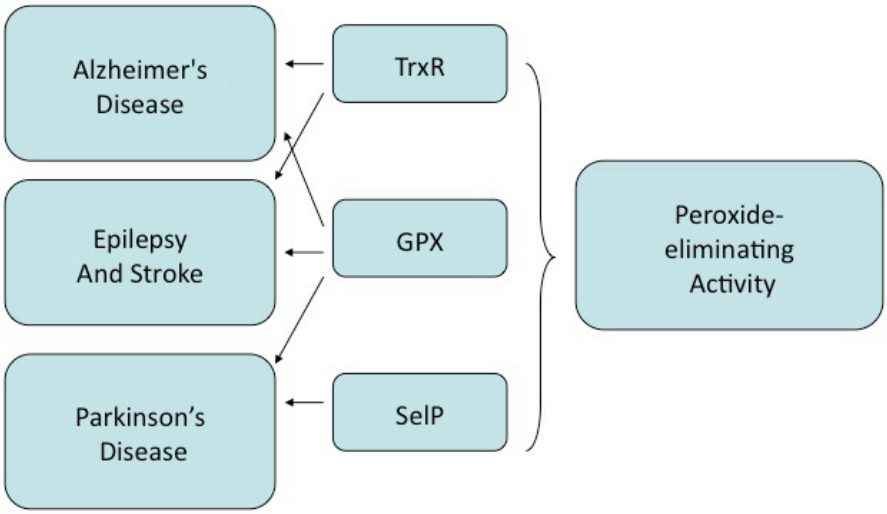


Figure 2.2 Peroxide-eliminating selenoproteins involved in various neurodegenerative diseases. Selenium is thought to confer free radical scavenging abilities in the brain by its incorporation into selenoproteins. Some selenoproteins are tissue specific, while others are more general.

2.3 The Se-dependent glutathione peroxidase

The mammalian GPX was originally identified as an antioxidant enzyme that protects membrane lipids and macromolecules from oxidative damage in 1957 (Mills, 1957). To date, there are five Se-dependent GPXs known to exist in mammals: the cytosolic GPX1, GPX2 (also known as gastrointestinal GPX), GPX3 (or plasma GPX), GPX4 (or phospholipid hydroperoxide GPX), and the newly identified GPX6.

Systematic investigations revealed that in human and rodent brains, GPX1 is ubiquitously expressed in both neurons and glial cells, and functions as a ROS scavenger. It is known that GPX1 localizes at damaged brain regions surrounding substantia nigra and infarcted

regions in patients suffering from Parkinson's disease (Damier et al., 1993; Takizawa et al., 1994). Furthermore, by employing the Alzheimer's disease related (APP) transgenic mice, a recent study demonstrated that the GPX activity can protect against oxidative stress induced by neurotoxic agents in the hippocampus (Garcia et al., 2009). From a nutraceutical perspective, extracts of panax ginseng, a Korean red ginseng, has been shown to activate GPX activity and protect against transient cerebral ischemia-induced brain damage in the rats (Kim et al., 2009). In contrast, another study showed that overexpression of GPX1 in mice does not improve neurogenesis in juvenile mice that have sustained brain injury (Potts et al., 2009).

GPX1, the most abundant Se-dependent GPX, is expressed in various types of neuronal cells including neurons, astrocytes, and glial cells (Lindenau et al., 1998; Takizawa et al., 1994). Using primary cultured neurons isolated from *Gpx1*$^{-/-}$ mice, it was shown that GPX1 protects against H_2O_2 induced oxidative stress and the toxicity of amyloid β (Aβ) peptides, the major component of senile plagues and cause of Alzheimer's disease (Crack et al., 2006). In contrast, it was reported that *Gpx1*$^{-/-}$ mice are more resistant to kainic acid induced seizure and neurodegeneration (Jiang et al., 2000). As such, further studies are needed to elucidate the role of GPX1 in response to various types of brain damage in different regions.

In rats, cortical neurons and astrocytes are prone to oxidative stress induced by paraquat (Schmuck et al., 2002). The first identified phenotype of *Gpx1*$^{-/-}$ mice was an increased susceptibility to acute paraquat-induced lethality (Cheng et al., 1998). This suggests a role for GPX1in the protection against brain damage. The GPX1 protein is highly expressed in microglia and the Lewy bodies with abnormal aggregates of proteins, two

hallmark features of Parkinson's disease and dementia (Power and Blumbergs, 2009), suggesting that GPX1 is involved in the degradation of the Lewy bodies.

GPX4 is a multifunctional selenoprotein expressed as cytosolic, mitochondrial, and nuclear isoforms. Compared to the tetrameric GPX1, GPX4 is a monomer and eliminates phospholipid hydroperoxides (Ursini et al., 1985). GPX4 and vitamin E may work together to suppress lipid peroxidation on biological membranes and lipoproteins (Ursini and Bindoli, 1987). Although *Gpx4*$^{-/-}$ mice are embryonic lethal, embryonic fibroblasts derived from *Gpx4*$^{-/-}$ mice are hypersensitive to inducers of oxidative stress, including γ-irradiation, paraquat, *tert*-butyl hydroperoxide, and hydrogen peroxide (Yant et al., 2003). In adult brains, mitochondrial and cytosolic GPX4 isoforms are mainly expressed in neuronal cells, but are not expressed in glial cells, as evidenced by immunohistochemistry and mRNA analyses (Savaskan et al., 2007). GPX4 is up-regulatedin response to brain injury only in regions of the brain surrounding astrocytes, not in other regions of the brain such as those with a high density of neurons. Savaskan and colleagues postulated that up-regulation of GPX4 in the astrocytes protected brain cells from apoptosis. Mutations in DJ-1 cause autosomal recessive, early-onset Parkinson's disease. Because DJ-1 binds GPX4 mRNA and suppresses the protein expression at the post-transcriptional level, GPX4 protein level is up-regulated in the DJ-1 deficient Parkinson's disease (Blackinton et al., 2009). Moreover, GPX4 has been proposed as a sensor of oxidative stress in neuronal cells, triggering the 12/15-lipoxygenase derived lipid peroxidation and apoptosis-inducing factor-mediated cell death pathways (Seiler et al., 2008). Consistent with this, *Gpx4*$^{+/-}$ mice accumulate beta-amyloid precursor protein cleavage enzyme 1 (BACE1) and oxidized lipids in the brain,

suggesting an increased amyloid plaque burden, a hallmark of Alzheimer's disease (Chen et al., 2008). Overexpression of the mitochondrial form of GPX4 reverses the retardation phenotype in the developmental brain of the guanine-rich sequence-binding factor 1 (Grsf1) knockout mice, suggesting that GPX4 plays an important role in the embryonic brain development (Ufer et al., 2008). Similarly, GPX4 has been shown to eliminate excessive oxidants and amyloid plaques in primary cortical neurons isolated from *Gpx4* overexpression mice (Ran et al., 2006). Moreover, GPX4 is highly expressed in neurons located at olfactory bulb, hippocampus, cerebral cortex, and cerebellar cortex in mice (Zhang et al., 2008). Knockdown of mitochondrial GPX4 in mice resulted in impaired development due to apoptosis in the cerebral cells in the hindbrain (Borchert et al., 2006). In conclusion, GPX1 and GPX4 may collectively eliminate various types of oxidants in the brain.

2.4 Thioredoxin reductases

Thioredoxin reductases (TR) belong to a family of selenoproteins that regulate the redox status of thioredoxin (Txn) (Arner and Holmgren, 2000). The Txn-dependent system has been implicated in the age-related degeneration such as Alzheimer's and Parkinson's disease (Andersen, 2004). There are three members of TR in mammals: the cytosolic thioredoxin reductase (TR-1) (Gladyshev et al., 1996), the mitochondrial thioredoxin reductase (TR-2) (Gasdaska et al., 1999), and the thioredoxin-glutaredoxin reductase (TR-3) (Sun et al., 2001a). While TR-1 and TR-2 are expressed ubiquitously, TR-3 is primarily expressed in testis. Thioredoxin reductases are flavoproteins, possessing two

catalytically interacting reactive centers. The redox active site, located in theC-terminal,contains a selenocysteine residue; therefore thioredoxin reductase activity can be modified by dietary Se (Soerensen et al, 2008). Not surprisingly, the expression and activity of TR are maintained in the brain under Se deficiency (Schomburg et al., 2003). Homozygous mutants of TR can cause embryonic death at the blastocysts stage (Matsui et al., 1996); however, embryos taken from *Txnrd2*$^{-/-}$ mice 10.5 days postcoitus show severe neuronal pathologies, like anterior neural tube defects (Nonn et al., 2003). Using neuron system specific *Txnrd1*$^{-/-}$ or *Txnrd2*$^{-/-}$ mice, it was found that the *Txnrd2*$^{-/-}$ mice appear normal, while *Txnrd1*$^{-/-}$ mice have ataxia, tremor, and a smaller body size (Soerensen et al., 2008). At the cellular level, the *Txnrd1*$^{-/-}$ mice with TR-1 knockout only in the neuron cells develop impaired Bergmann glia and neuronal precursor cells, as well as hypoplasia in various regions in the brain. Moreover, the following reports suggest that TR-1 and TR-2 are required for brain development and the maintenance of redox status in the brain. First, TR-1 in embryos has highest expression in neuronal tissues such as the developing forebrain, rhombomeres and the neural tube (Kanemasa et al., 1992). Second, mice with a brain-specific knockout of *Txnrd1,* the gene that encodes TR-1, are viable, but exhibit growth retardation and a movement disorder attributed to cerebellar dysfunction (Tronche et al., 1999).

Incubation of neuronal PC-12 cells with the TR inhibitor 1-chloro-2,4-dinitrobenzene (CDNB) resulted in elevated ROS levels and in the activation of the c-Jun N-terminal kinase and mitogen activated protein kinase 4 stress response signaling pathway (Seyfried and Wullner, 2007). Thus, TR is considered as a therapeutic target for ROS-induced neurodegenerative diseases.Exposure of albino mice to toxic doses of fluorescent

light resulted in an increase in the TR protein expression, increased nuclear translocation of the protein, and the binding of Nrf2 to the antioxidant response element of TR mRNA (Tanito et al., 2007). Furthermore, treatment of the mouse photoreceptor-derived 661W cells with 4-hydroxynonenal, a lipid peroxide, alleviated H_2O_2-induced cell damage and increased TR expression in an Nrf2-dependent manner. Hence, the interaction between TR and the Nrf2-ARE pathway could be the target of 4-HNE‘s therapeutic ability.

2.5 Selenoprotein P

Selenoprotein P is central to Se homeostasis in mammals and is considered a reliable biomarker for total body Se status (Hill et al., 1996; Burk and Hill, 2005). Because selenoprotein P contains ten Sec residues and is estimated to store 60% of the Se in plasma; it is considered as a Se transporter that can sequester a large portion of extracellular Se (Hill et al., 1996; Brown and Arthur, 2001; Burket al., 1991). Selenoprotein P issecreted primarily from the liver as a glycoprotein and is associated with the ApoER2 liporeceptor in humans and mice (Read et al., 1990; Takebe et al., 2002). Interestingly, results from mouse studies have suggested an epistatic effect of selenoprotein P and ApoER2 on the maintenance of brain function. The *SelP*$^{-/-}$ mice or the *ApoER2*$^{-/-}$ mice fed a Se-deficient diet manifest features of neuronal disorders, and the *ApoER2*$^{-/-}$mice have more severe symptoms than the*SelP*$^{-/-}$ mice (Nakayama et al., 2007). The N-terminal domain of selenoprotein P contains one Sec residue in the UxxC redox motif (U, Sec; C, Cys), while the C-terminal domain contains nine Sec residues. In rats, there are four isoforms of selenoprotein P whichcontain different amounts of Sec residues

resultingfrom differential splicing in the C-terminal region (Himeno et al., 1996). Selenoprotein P also differs from others selenoproteins because it contains two SECIS elements in the 3'-UTR of the mRNA (Read et al., 1990).

Selenoprotein P plays a central role in Se homeostasis and is necessary for optimal health in the brain (Nakayama et al., 2007). In humans, selenoprotein P mRNA expresses in cerebrospinal fluid, neurons, fiber tracts, and ependymal cells (Scharpf et al., 2007). Interestingly, selenoprotein P was found to be the neuronal survival-promoting factor in fractionated bovine serum (Yan and Barrett, 1998). Moreover, genomic analysis suggests that selenoprotein P single nucleotide polymorphisms are associated with the risk of glioma pathogenesis (Rajaraman et al., 2009). The Se rich, C-terminal selenoprotein P is required to prioritize body Se to the brain (Nakayama et al., 2007). This is consistent with the view that optimal selenoprotein expression and activity can be maintained in the brain even though the body Se status is depleted.Thus, Se in the brain may function independently from Se residing in other regions of the body.Selenoprotein P can be synthesized and secreted from cultured rat and human astrocytes (Yang et al., 2000; Steinbrenner et al., 2006a). Interestingly, mice with targeted disruption of the selenocysteine tRNA gene transcription activation factor (STAF) or selenoprotein P display overlapping neurological phenotypes to a great extent (Carlson et al., 2009). Moreover, there is an 80% reduction in selenocysteine tRNA level in the brain of the *Staf*$^{-/-}$ mice.

Selenoprotein P has also been linked to the protection against Alzheimer's disease. Analysis of the postmortem Alzheimer's brain by immunohistochemistry demonstrated that selenoprotein P localizes at the pathological Aβ plaques and neurofibrillary tangles,

where levels of oxidative stress are high (Bellinger et al., 2008). Thus, selenoprotein P may act as an antioxidant to protect against the damage in the Alzheimer's brain. Likewise, it has been shown that selenoprotein P protects astrocytes against oxidative stress induced by *tert*-butyl hydroperoxide (Steinbrenner et al., 2006b).

Sepp1$^{-/-}$ mice fed with a Se-deficient diet show increased severity in motor impairment, rotarod test and pole climb compared to the control group (Hill et al., 2004). *Sepp1*$^{-/-}$ mice have dystrophic and degenerated axons in their brainstems and cervical spinal cords regardless of the Se level in the diet. *Sepp1*$^{\Delta 240–361}$ mice fed a Se-deficient diet exhibited less severe neurodisorders than *Sepp1*$^{-/-}$ mice (Hill et al. 2007). Moreover, brain Se concentrations are significantly decreased in the *Sepp1*$^{\Delta 240–361}$ mice, whereas Se contents in the liver and kidney remain comparable to wild-type mice. Since the region between amino acid residues 240-361 in selenoprotein P includes several Sec residues, the results from using the *Sepp1*$^{\Delta 240–361}$ mice indicate critical roles of the Sec residues, in selenoprotein P, in Se retention of brain and optimal health.

2.6 Selenoprotein W

Selenoprotein W was originally purified in cytosol from the liver of lambs, and since has been known to prevent white muscle disease in domestic animals (Vendeland et al., 1993; Whanger, 2000). The glutathione molecule shows intrinsic affinity to selenoprotein W (Dringen et al., 1999), which may explain why this selenoprotein is capable of reducing protein disulfide bonds in the *Escherichia coli* cytoplasm (Prinz et al., 1997), as well as protecting against H_2O_2-induced oxidative stress *in vivo* (Jeong et al., 2002). Although

selenoprotein W exists in many tissues, its expression is preserved in the brain under Se deficiency in rats (Yeh et al., 1997). In particular, the expression of selenoprotein W in cortex, cerebellum, and thalamus remained constant under Se deficiency (Sun et al., 2001b). Thus, selenoprotein W expression is maintained in certainregions of the brain in response to different stages of Se deficiency, suggesting a role of selenoprotein W in redox regulation in the brain.

Experiments employing cultured neuronal cells have indicated a role of selenoprotein W in the brain.Increased expression of selenoprotein W protects the rat glial cells against oxidative stress induced by heavy metal or the 2, 2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) (Sun et al., 2001c; Amantana et al., 2002). Treatment of SH-SY5Y neuronal blastoma cells with methylmercury,a neurotoxicant, results in decreased selenoprotein W mRNA expression in a glutathione dependent manner (Kim et al., 2005). Recently, high levels of selenoprotein W mRNA and protein expression have been found in the developing central nerve system of rodents (Chung et al., 2009). Collectively, these results suggest selenoprotein W cansafeguard developing neuronal cells from oxidative attack of endogenous and exogenous origins.

2.7 Selenoprotein H

Selenoprotein H is a newly discovered 14-kDa nucleolar protein expressed primarily in the thymus and brain, but also during embryogenesis and tumorigenesis (Novoselov et al., 2007). Similar to other selenoenzymes, selenoprotein H shares the conserved CXXU (C: Cys; U, Sec) motif. Selenoprotein H has been shown to limit lipid peroxidation

of*Drosophila melanogaster* embryos (Morozova et al., 2003), and can protect H22 neuronal cells against UV-induced DNA damage and superoxide production (Ben et al., 2007). Although the physiological function of mammalian selenoprotein H is largely unknown, these results suggest a role of this selenoprotein in the brain as an antioxidant.

2.8 Selenoprotein M

Selenoprotein M is a newly identified selenoprotein containing a SECIS distinct from other selenoproteins; it lacks the invariant adenosines typically found in SECIS (Korotkov et al., 2002). Based on the study, the secondary structure of selenoprotein M is similar to Sep15, selenoprotein W and selenoprotein T. Selenoprotein M mRNA is expressed in various tissues in mice, butbrain has the highest levels. Recent results suggest that selenoprotein M may regulate the pathogenesis of Alzheimer's disease. Overexpression of the human selenoprotein M in rats causes a redox shift resulting in a reduced redox status in the brain. The induction of the ERK signaling, which is associated with the inhibition of alpha/gamma secretase-mediated proteolysis and the phosphorylation of the Tau protein at several residues, also resulted from selenoproteins M overexpression in rats (Yim et al., 2009). These responses improve brain function, and may prevent the Alzheimer's brain from sustained degeneration; thus suggesting a promising role of selenoprotein M in the treatment of diseases associated with neurodegeneration.

2.9 Perspectives on selenoproteins in neurological aging

Se and selenoproteins are indispensable in the maintenance of the physiological functions of the brain, especially in attenuating neuron damage induced by oxidative stress. Based on previous studies in animal models and clinical trials, it is clear that selenoproteins, such as GPX, TrxR and SelP, have a critical role in protecting mammalian neurodegeneration either by eliminating ROS or by stimulating related antioxidant pathways. The body of evidence accumulated emphasizes the importance of selenoproteins in maintaining proper brain function; however, more data should be collected in the form of *in vivo* experiments.

A fascinating perspective in the role of selenoproteins in neurological aging is their potential interaction with telomeres, a tandem TTAGGG repeat at chromosome ends. The length of the telomeres in cells derived from the hippocampus is longer In Alzheimer's patients than in control individuals; however, in buccal and white blood cells from the same Alzheimer's patients, the telomere lengths are significantly shorter than the control groups (Thomas, 2008). Thus, Alzheimer's patients have evolved unique mechanism for telomere maintenance. Recently, it was shown that a form of vitamin E could provide protection to telomeres in the aging brain (Tanaka, 2007). Tanaka and colleagues showed that age-dependent telomere shortening in human brain microvascular endotheliocytes is negated by phosphorylated alpha-tocopherol, possibly through alpha-tocopherol's ability to decreaseintracellular oxidative-stress. Given the antioxidant similarities between Se and vitamin E, it would be interesting to determine if Se had a similar effect in the brain in the context of telomeres. In agreement with this view, Se, in the form of selenite,

prevents telomere length shortening during cellular aging in the normal human L-02 hepatocyte (Liu et al., 2004). There is, however, evidence that telomere shortening in the brain does increase the chances of developing atypical and malignant meningiomas and gliomas, pathologies more commonly associated with aged populations (Sanson and Cornu 2000). Altogether, future research is needed to elucidate the mechanism by which Se mediates telomere functions, in the brain.

Another prospective avenue of a role of Se in the protection against aging brain could be independent from its role in decreasing oxidative stress; but as of yet, the collective body of evidence in the field has not produced sufficient results supporting this hypothesis. There has been evidence that suggests that Se associates with other minerals (Cakatay et al. 2008; Maret 2008; Liu et al. 2009). Therefore, Se's relationship with other minerals and vitamins could elucidate a novel role of Se in the aging brain. This will provide a more thorough understanding in the biological function of Se in the brain.

Chapter 3: Materials and Methods

3.1 Materials

3.1.1 Cell lines

Table 3.1 Cell lines

Cell	Description	References
MRC-5	Normal Human Fetal Lung Fibroblast	(Jacobs et al., 1970)
HCT116	Human Colon Cancer	(Schroy et al., 1995)

3.1.2 Chitosan and selenium compounds

Table 3.2 Chitosan capsulated selenium compounds. Na_2SeO_3: Sodium Selenite MSeA: Methylseleninic Acid

Chitosan (CS) concentration	Sample	Se compound encapsulated	Weight ratio (CS : Se compound)	Particle size (nm)
1.5 mg/mL	L1	Na_2SeO_3	3:1	350-450
	L2	MSeA		90-100
	M1	Na_2SeO_3	3:1	650-750
	M2	MSeA		650-750

3.1.3 Chemicals and reagents

The major chemicals and reagents used in this research are listed in Appendix I

3.1.4 Solutions and their ingredients

Buffer, solution and gel were prepared according to the formulation listed in Appendix II.

3.1.5 Commercial kits

The main commercial kits used in this research are listed in Appendix III.

3.1.6 Equipments and facilities

All equipments and facilities have been provided by Department of Nutrition and Food Science, University of Maryland, College Park. The inventory is shown Appendix IV.

3.2 Methods

3.2.1 Preparation of Chitosan-Se compounds

A) Low and medium molecular weight chitosan (Sigma), sodium tripolyphosphate (TPP) (Pfaltz & Bauer) were of bio-analytical grade.

Chitosan-TPP nanoparticles were prepared according to the ionotropic gelation process. In brief, chitosan was dissolved in 1% (weight /volume) equal volume of acetic acid (Fisher) under sonication until the solution was transparent. Different amount of sodium selenite (Sigma) or methylseleninic acid (Sigma) was dissolved together with chitosan in order to obtain the different mass ratio of chitosan/ selenium compounds (sodium selenite, methylseleninic acid). The ratio of chitosan to selenium compound was 3:1. Then, equal volume TPP aqueous solution was added dropwise to chitosan solution, which was stirred for 30 minutes at room temperature to obtain nanoparticles.

B) Preparation of original selenium compounds

Sodium selenite (SSe, Na_2SeO_3) and methylseleninic acid (MSeA, CH_3SeO_2H) were suspended in 1X phosphate-buffered saline (PBS) and dilute to work concentration as needed.

3.2.2 Cell culture

MRC-5 (Coriel) and HCT-116 (ATCC) were cell lines used in this study. MRC-5 were cultured in MEM (Minimum Essential Medium, Mediatech) with 15% fetal bovine serum (Sigma), 1ng/mL essential amino acid (Mediatech), 1ng/mL nonessential amino acid (Mediatech), 1ng/mL vitamins (Mediatech), 100U/mL penicillin/ strepcillin (Mediatech), 100U/mL amphotericin B (Mediatech) and 5μg/mL plasimocin (InvivoGen) at 37°C in a

5% CO2 incubator. HCT-116 cells were cultured in D-MEM (Dulbecco's Modified Eagle Medium medium, Mediatech) with 10% fetal bovine serum (Sigma), 100u/mL penicillin/ strepcillin (Mediatech), 100U/mL amphotericin B (Mediatech) and 5µg/mL plasimocin (InvivoGen).

MRC-5 and HCT-116 were seeded in corresponding medium and cultured to 90% confluence. At second day, nano-selenium, original Na_2SeO_3, MSeA (Sigma) were applied to both cells lines in concentration of 0.3µM, 1µM, 3µM, and 10µM respectively, according to preliminary survival test.

3.2.3 Selenium concentration assessment

First, prepared samples were washed by 1XPBS for four times. Second, these cells were then dissolved in 3.0 mL-4mL of 0.2 mol/L NaOH (Mallinckrodf) and 0.2% SDS (Teknova), and protein was measured using the BCA (Brown et al., 1989) (bicinchoninic acid) kit (Thermo) following the instsruction. After that, cellular Se concentration was determined by hydride-generation atomic absorption spectrometry. Samples were treated for analysis by predigestion in nitric acid and hydrogen peroxide, followed by high-temperature ashing in the presence of $Mg(NO_3)_2$ as an aid to prevent Se volatilization.

3.2.4 Cell survival assay

A) MTT

MRC-5 and HCT-116 cells were seeded in 24-well plates (Greiner Bio-One) and treated by four selenium compound described in *2.1.2* and two chitosan control at above four dosages. Cells were incubated with reagents for 48 hours. At the end time point, cells were washed twice with 1XPBS and then incubated with 500 µL MTT (Calbiochem) at work concentration of 1mM for 3 hours. After incubation, MTT was dumped out and cells were washed twice with 1XPBS. DMSO (Mallinckrodt) was applied to each well at room temperature. Plates should be tapped lightly to mix DMSO and absorbed MTT well. Once MTT was dissolved in DMSO, plates were read directly by FLUOstar OPTIMA plate reader (BMG Labtech) at absorption wavelength of 595nm.

3.2.5 Immunofluorescence assay

HCT-116 and MRC-5 cells were cultured on coverslips in 6-well plates (Greiner Bio-One) and treated the next day. The cells then were washed with PBS once and fixed with 4% paraformaldehyde (Fisher) for 15 min at room temperature and washed with 1XPBS once. The fixed cells were then permeabilized in 90% methanol (Pharmco AAPER) for 10 min at -20°C and washed with 1XPBS once. Then cells were incubated with 0.3% Triton-100 (Calbiochem) for at room temperature for 10mins, then followed by three times' washing with 1XPBS (5 min each time). After that, cells were blocked in 3% BSA (Calbiochem) in PBS for 1 hour. Wash samples with 1XPBS three times, for 5mins each time. The immunoblotting step was performed using primary antibodies of total ATM (Epitomics) and ATM-Ser1981 (Rockland) overnight at 4°C. After incubation, cells were washed with 1XPBS three times, 5 mins each time. Incubate samples with secondary

immunofluorescence antibodies (1:200 for anti-mouse and anti-rabbit) (Invitrogen) in BSA for 1 hour at room temperature. Wash samples with 1XPBS three times. Antifade DAPI dye (Invitrogen) applied to the slide. A coverglass was mounted on top of the slide and sealed with nail polish. Prepared slides were observed under a Zeiss AxioObserver 100 fluorescence microscope (Carl Zeiss) for image acquisition. Softwarae Axiovision 4.7 (Carl Zeiss) was used to obtain the images. Fluorescence indicating nucleus was observed by using the filter cube set 49 for DAPI (exitation 365nm, filter 395nm, and emission 445nm), fluorescence indicating expression of total ATM protein by filter cube set 38 for GFP (exitation 470nm, filter 495nm, and emission 525nm) and fluorescence indicating expression of phosphorylated ATM Ser 1981 by filter cube set 43 for DsRed (exitation 550nm, filter 570 nm, and emission 605nm). Each individual treatment was taken 3 images, which under same magnification scale (630X) and exposure time (87.6ms for DAPI, 332.8ms for GFP and 450.2ms for DsRed).

3.2.6 ROS assay

MRC-5 and HCT-116 cells were seeded in black 96-well plate (NalgeNunc) and cultured for one day. Desired concentration of nano-selenium and original selenium were pipetted in wells and incubate for 9 and 24 h. Culture media was poured out and 1XPBS was added to wash cells once. Dump PBS and add 50 μl ROS dye 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Invitrogen), which is a cell-permeate indicator for ROS. Final concentration of ROS dye should be 10 μM. Bottom optic was selected to measure fluorescence and samples were read at time 0.

Thereafter, ROS was incubated with samples for 40 min. After incubation, 5mL of lysis buffer was mixed with cocktail I (Roche) and Phenylmethylsulphonyl fluoride (PMSF) (Calbiochem) at ratio of 50:1 and 200:1 respectively. Fluorescence values have been corrected using standard curve obtained from BCA assay.

Chapter 4: Results

4.1 Cellular Selenium Content Assessment

4.1.1 Delivery efficacy of Chitosan-Na_2SeO_3

Chitosan-Se and original Se were applied to HCT-116 at doses of 0.3, 1, 3, 10 μM. In accordance with Figure 4.1.1, at doses of 0.3 and 1 μM, no significant difference of cellular Se concentration was observed among three types of selenium compounds. However, cellular concentration of L-Na_2SeO_3 was jumped to 400 at dose 3 μM and 1200 μg/L at 10 μM, which meant higher delivery efficacy of L-Na_2SeO_3 compared to other two. The overall absorption of three Na_2SeO_3 types at dose 10 μM was above 500 μg/L and followed a dose-dependent manner.

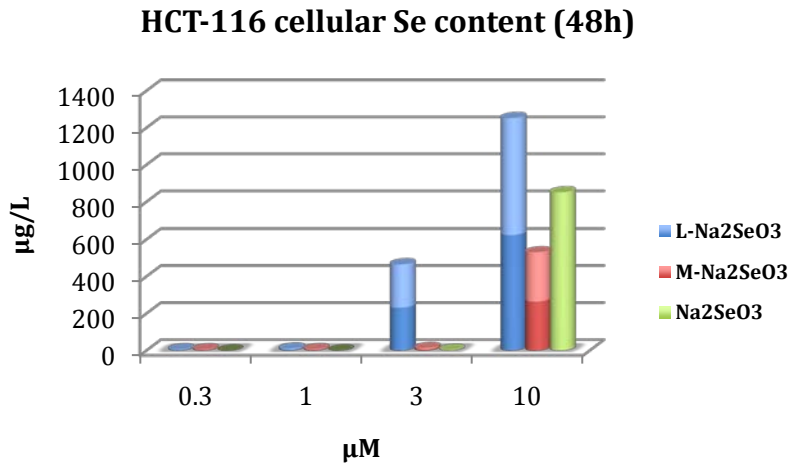


Figure 4.1.1 Cellular Se concentration in human colon cancer cells HCT-116. Absorption of three types of Na_2SeO_3 increased in a dose dependent manner. Among them, L-Na_2SeO_3 exhibited most high delivery efficacy, which reaches up to 1200 μg/L. Na_2SeO_3 is inorganic selenium compound; L-Na_2SeO_3 is encapsulated by low molecular weight chitosan; M-Na_2SeO_3 is encapsulated by medium molecular weight chitosan.

In MRC-5, which is normal lung fibroblast cell, cellular Se content increased with dosage as shown in Figure 4.1.2. However, the overall absorption of three Na_2SeO_3 types dropped dramatically, which was no more than 40 μg/L, almost 1/30 compared that of HCT-116. Of note, Chitosan-Na_2SeO_3 exhibited higher delivery efficacy than uncoated one.

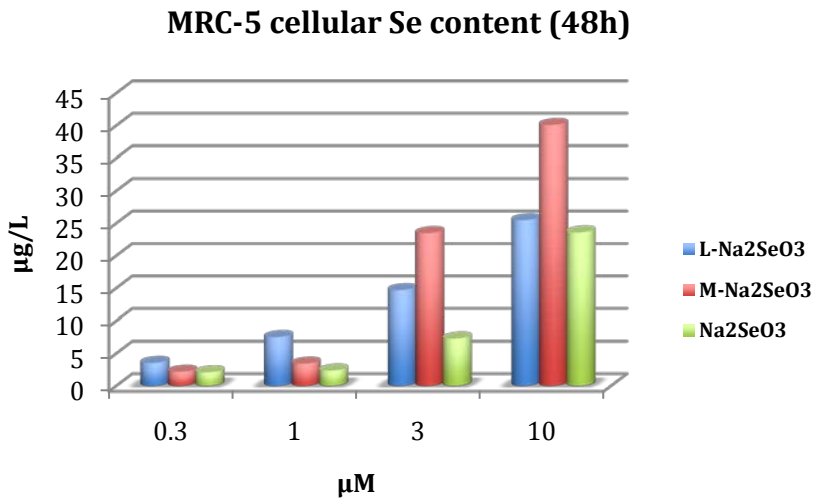


Figure 4.1.2 Cellular Se concentration in human normal lung fibroblast cells MRC-5. Absorption of three types of Na_2SeO_3 in MRC-5 had similar trend as that of HCT-116. However, the absorption scale is much lower in comparison with HCT-116, the maximum concentration was no more than 40 μg/L. Among three types of Na_2SeO_3, M-Na_2SeO_3 showed higher efficacy in delivery.

4.1.2 Delivery efficacy of Chitosan-MSeA

As illustrated in Figure 4.1.3 and Figure 4.1.4, in both cell lines, cellular content of MSeA compounds fell into similar range (2-40μg/L). To HCT-116, the cellular concentration of chitosan-MSeA was much less than that of chitosan-Na_2SeO_3, which meant deliver efficacy was dependent on selenium type. To MRC-5, delivery efficacy of chitosan-MSeA was same as chitosan-Na_2SeO_3, with slight decline. Interestingly, the overall uptaking of chitosan coated ones was less, whereas uptaking of original form was more in HCT-116. This phenomenon can attribute to specific targeting of Chitosan-MSeA to cancer cells and, therefore, promoted toxic effect and induced decreased cell viability.

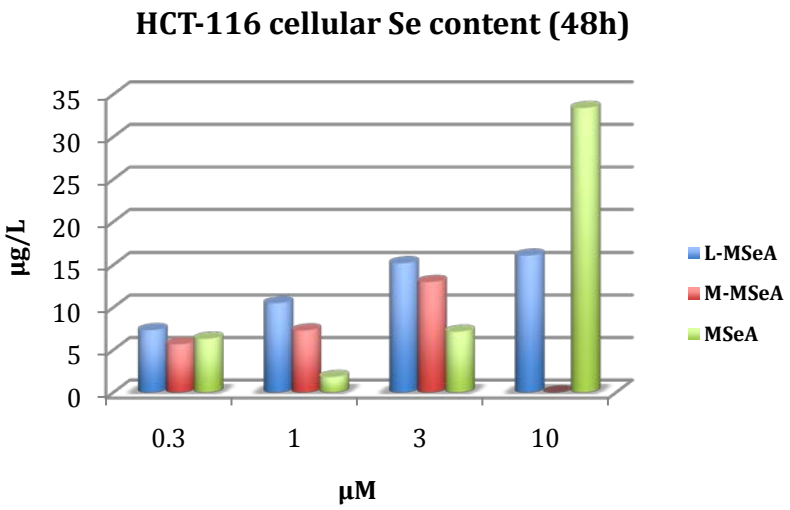


Figure 4.1.3 Cellular Se concentration in human colon cancer cells HCT-116. Absorption of three MSeA followed doses except no M-MSeA at 10 μM. Compared to Na_2SeO_3, deliver efficacy of MSeA decreased to 30 μg/L approximately. MSeA (Methylseleninic Acid) is organic selenium compound; L-MSeA is encapsulated by low molecular weight chitosan; M-MSeA is encapsulated by medium molecular weight chitosan.

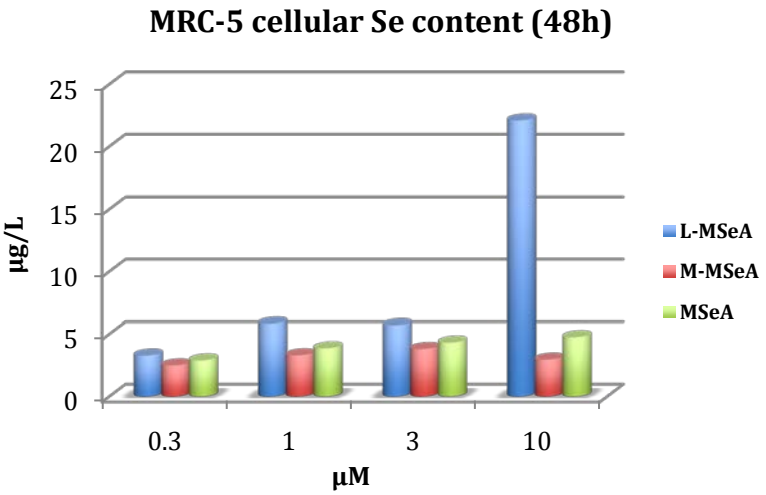


Figure 4.1.4 Cellular Se concentration in human normal lung fibroblast cells MRC-5. Overall uptake of three types of MSeA was a little bit less than that of HCT-116 cells. Cellular concentration of L-MSeA reached 20%, which was highest compared to other two selenium compounds. However, unlike Na_2SeO_3, MRC-5 demonstrated similar uptaking scale of three MSeA compounds as HCT-116.

4.2 Cell survival assay by MTT

4.2.1 Chitosan-Na_2SeO_3 nanoparticles inhibit cell viability in colon cancer HCT-116 cells

Cell survival rate was calculated as:

(MTT absorption w/ treatment) X100%/ (MTT abosorption w/o treatment)

According to Figure 4.2.1, 10 µM L-Na_2SeO_3 and M-Na_2SeO_3 reduced survival rate of HCT-116 to 30% and 40% respectively, which was higher than 20% induced by Na_2SeO_3 only. Student two-tailed T-test has been used to compare Chitosan-Na_2SeO_3 and Na_2SeO_3. $P<0.01$ between L-Na_2SeO_3 and Na_2SeO_3, as indicated by ** in figure 4.2.1. This resistance to chitosan-Na_2SeO_3 could be due to sustained releasing profile of

chitosan-encapsulated particles. In other words, partial release of chitosan-Se may attenuate expected toxic effect even with a high abosorption by cancer cells. Chitosan was applied to culture medium as background control, which exerted little toxic effect on viability of HCT-116.

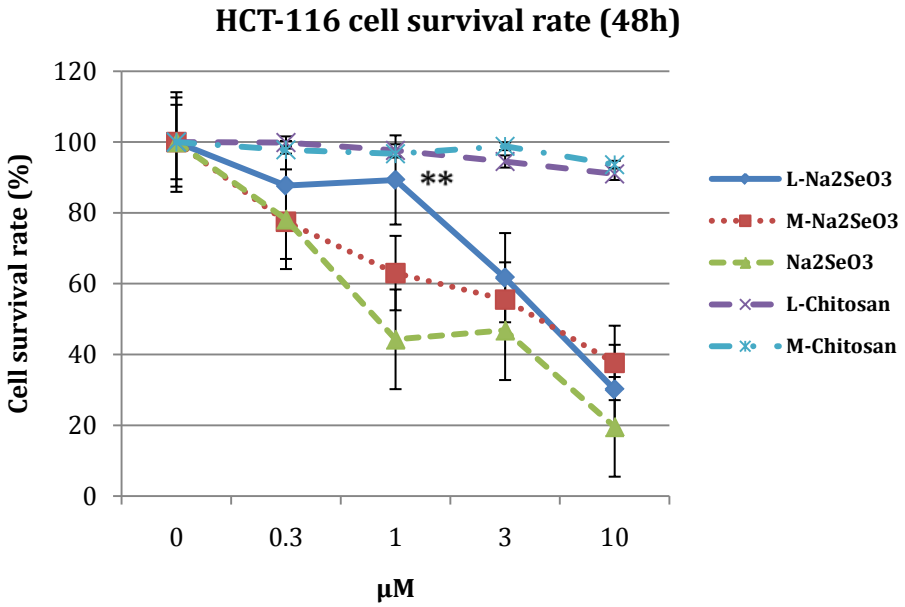


Figure 4.2.1 Cell survival rate interpreted from MTT assay in human colon cancer cells HCT-116. Cell survival rate of L-Na_2SeO_3, M-Na_2SeO_3 and Na_2SeO_3 decreased to 40%, 30% and 20% approximately. L-Chitosan and M-Chitosan, showing >90% survival rate, were added as background control. L-Chitosan is low molecular chitosan w/o selenium; M-Chitosan is medium molecular chitosan w/o selenium.

** Represents $P<0.01$, when compare chitosan-Na_2SeO_3 and Na_2SeO_3.

MRC-5 had a dose-dependent decline in survival rate. The slope in Figure 4.2.2 was more flat in contrast to rapidly declined slope in Figure 4.2.1. The overall higher survival rate in chitosan-Na_2SeO_3 applied normal cells, which was approximately 50%, can be explained by lower absorption of Na_2SeO_3 compounds. Despite the 10% increase

induced by chitosan-Na_2SeO_3, there was little difference produced by original Na_2SeO_3 between cancer and normal cells. Chitosan applied to MRC-5 had reverse effect evidenced by approximately 85% viability under 10 μM.

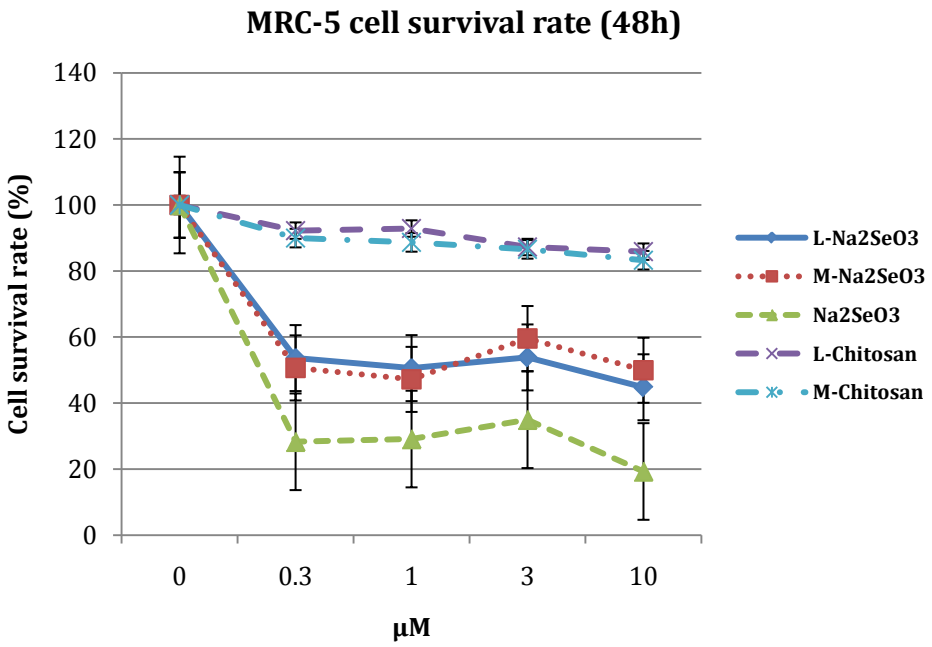


Figure 4.2.2 Cell survival rate obtained from MTT assay in human normal lung fibroblast cells MRC-5. Cell survival rate of L-Na_2SeO_3, M-Na_2SeO_3 and Na_2SeO_3 decreased to 50%, 45% and 20% approximately. L-Chitosan and M-Chitosan, showing >80% survival rate, were added as background control.

4.2.2 Chitosan-MSeA nanoparticles inhibit cell viability in colon cancer HCT-116 cells

Based on Figure 4.2.3 and Figure 4.2.4, both cell lines incubated with three MSeA compounds exhibited survival rate ranges from 40-50% at dose 10 μM. It can be explained by similar ability in MSeAs absorption. Again, compared to original MSeA,

Chitosan-MSeA was more protective for normal cells, having 15% elevated survival rate at average than original MSeA.

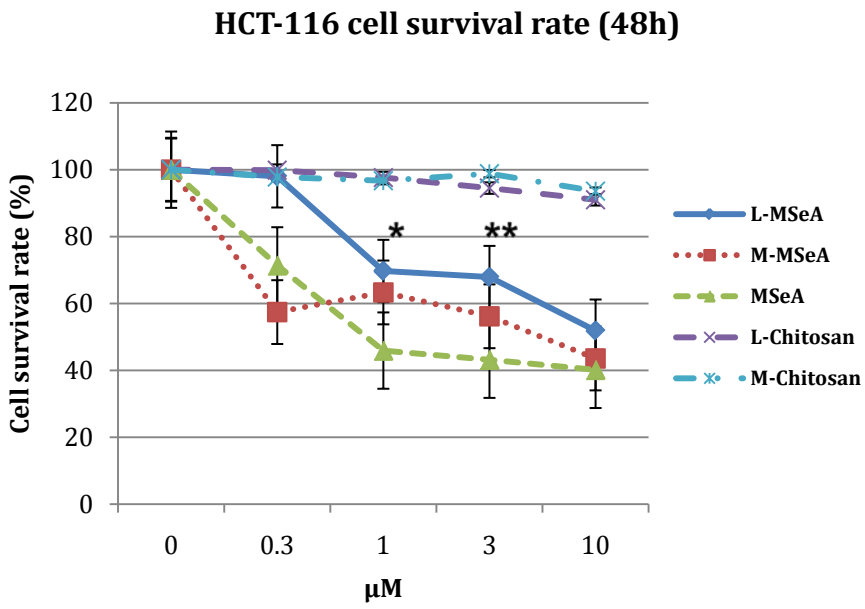


Figure 4.2.3 Cell survival rate obtained from MTT assay in human colon cancer cells HCT-116. Cell survival rate of L-MSeA, M-MSeA and MSeA decreased to 55%, 45% and 40% approximately. L-Chitosan and M-Chitosan, showing >90% survival rate, were added as background control.

** Represents $P<0.01$; * represents $P<0.05$, when compare chitosan-Na_2SeO_3 and Na_2SeO_3.

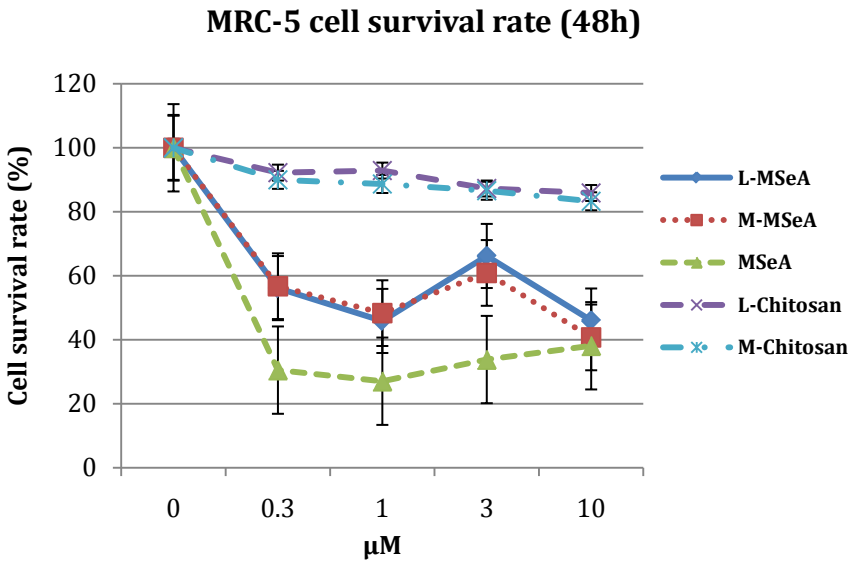


Figure 4.2.4 Cell survival rate obtained from MTT assay in human normal lung fibroblast cells MRC-5. Cell survival rate of L-MSeA, M-MSeA and MSeA decreased to 65%, 45% and 40% approximately. L-Chitosan and M-Chitosan, showing >80% survival rate, were added as background control.

4.3 ROS production of Chitosan-Se

4.3.1 ROS production was stimulated by Chitosan-Se in cancer cell HCT-116 after 9-hour treatment

ROS production was calculated as:

(ROS emission w/ treatment)/ (ROS emission w/o treatment)

According to Figure 4.3.1, 10 μM L-Na_2SeO_3 and M-Na_2SeO_3 stimulated ROS production to 15 and 16 folds respectively in HCT-116, higher than 7 folds by Na_2SeO_3 only. For Chitosan-Na_2SeO_3 applied cells, the curve climbed rapidly at higher doses 3 μM and 10 μM. This elevated ROS production triggered by chitosan-Na_2SeO_3 is probably

due to relatively higher releasing profile of chitosan-encapsulated particles at early stage of treatment (9 hours). L-Chitosan and M-Chitosan, showing <2 folds' production of ROS, had little reverse effect as oxidative stress to HCT-116.

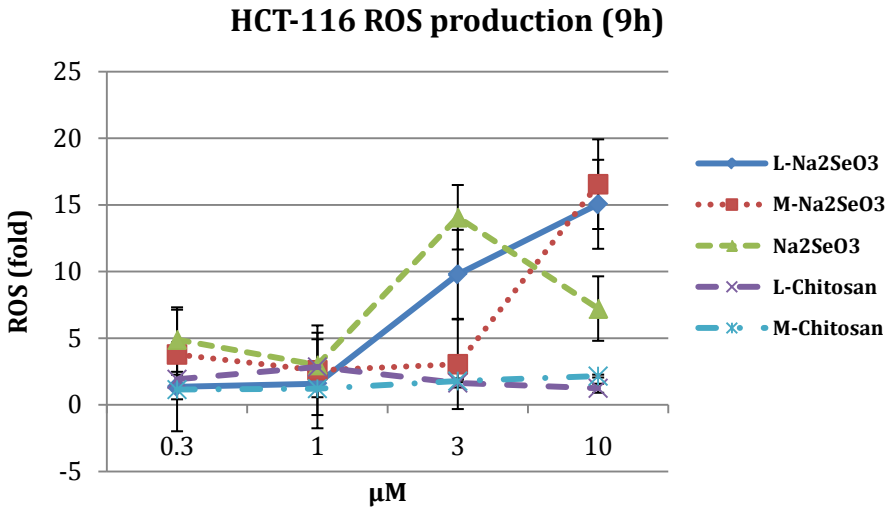


Figure 4.3.1 ROS (Reactive Oxygen Species) production in human colon cancer cells HCT-116. ROS production of 9-hour treatment by L-Na_2SeO_3, M-Na_2SeO_3 and Na_2SeO_3 increased to 15, 16 and 7 folds approximately. L-Chitosan and M-Chitosan, showing <2 folds' production of ROS, were added as background control.

In contrast to the ascending trend of ROS production in HCT-116, ROS moderately decreased along with doses in MRC-5. Under 9-hour challenging, ROS production induced by L-Na_2SeO_3, M-Na_2SeO_3 and Na_2SeO_3 decreased to 2.75, 2.25 and 2 folds approximately, as illustrated in Figure 4.3.2. No obvious difference between Chitosan-Na_2SeO_3 and Na_2SeO_3 was observed, yet there was distinguished Chitosan-Na_2SeO_3 suppressed ROS production in MRC-5 in contrast to HCT-116. L-Chitosan and M-Chitosan, showing <2 folds' production of ROS, indicating that Chitosan did not behavior as oxidative stress to MRC-5 as well as HCT-116 at early stage incubation.

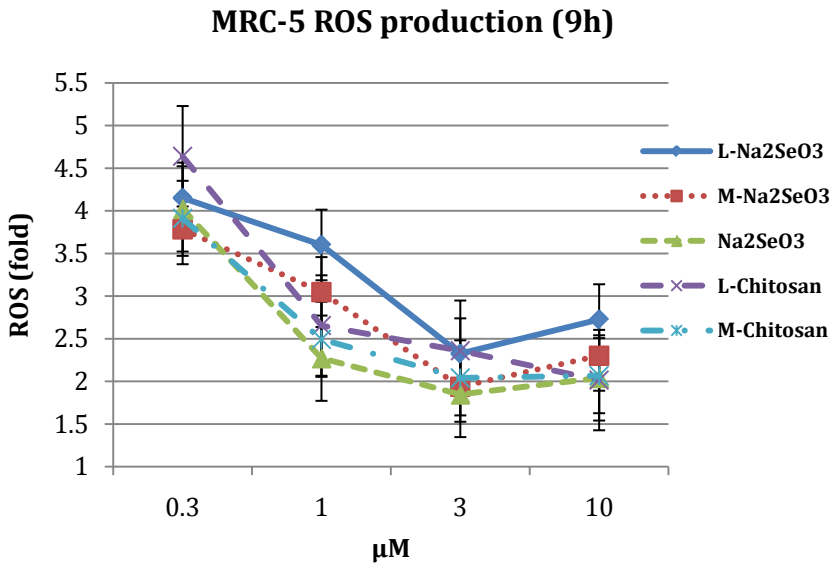


Figure 4.3.2 ROS production in human normal lung fibroblast cells MRC-5. ROS production of 9-hour treatment by L-Na_2SeO_3, M-Na_2SeO_3 and Na_2SeO_3 decreased to 2.75, 2.25 and 2 folds approximately. L-Chitosan and M-Chitosan, showing <2 folds' production of ROS, were added as background control.

Applied with three MSeA compounds, HCT-116 and MRC-5 demonstrated same ROS production pattern as that of three Na_2SeO_3 compounds, in accordance with Figure 4.3.3 and Figure 4.3.4. In HCT-116, ROS productions were 10, 17 folds produced with L-MSeA and M-MSeA, in comparison to 4 folds by MSeA only. At this early stage, Chitosan-MSeA exerted elevated oxidative stress than MSeA in HCT-116. In MRC-5, ROS production was 2, 1, 2 folds approximately, indicating Chitosan-MSeA did generate excessive ROS in MRC-5.

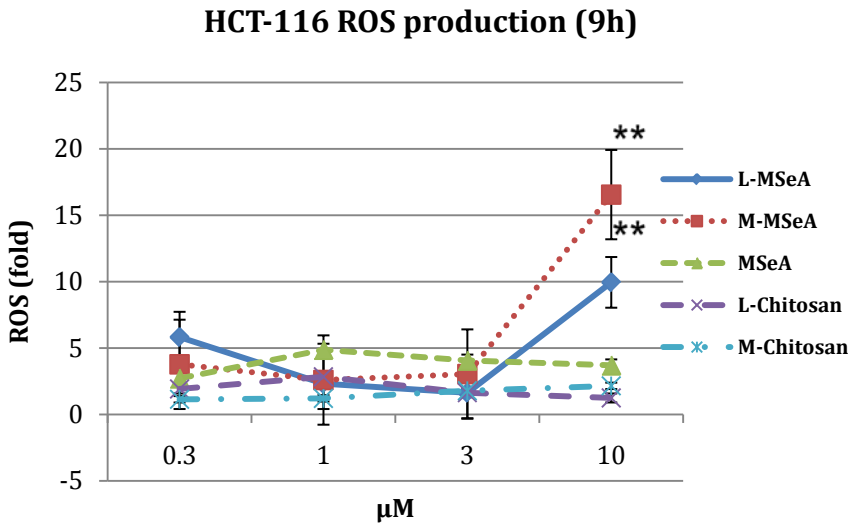


Figure 4.3.3 ROS (Reactive Oxygen Species) production in human colon cancer cells HCT-116. ROS production of 9-hour treatment by L-MSeA, M-MSeA and MSeA increased to 10, 17 and 4 folds approximately. L-Chitosan and M-Chitosan, showing <2.5 folds' production of ROS, were added as background control.

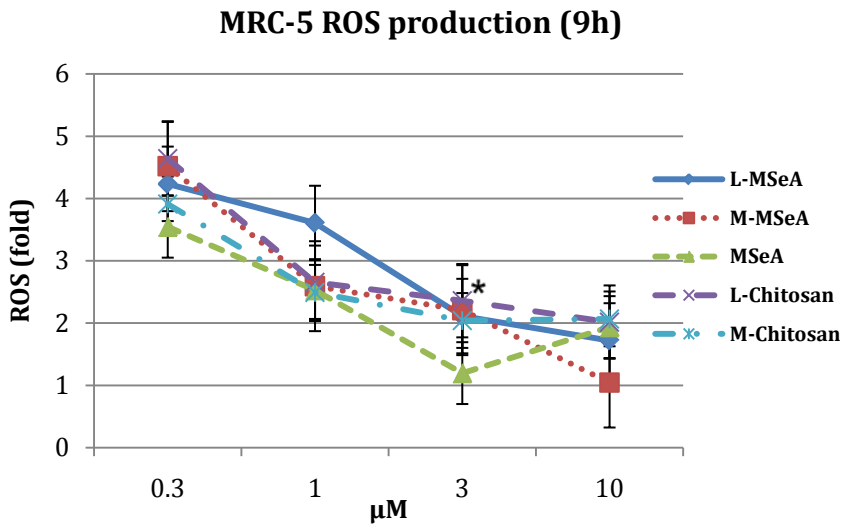


Figure 4.3.4 ROS (Reactive Oxygen Species) production in human normal lung fibroblast cells MRC-5. ROS production of 9-hour treatment by L-MSeA, M-MSeA and MSeA decreased to 2, 1, 2 folds approximately. L-Chitosan and M-Chitosan, showing <2 folds' production of ROS, were added as background control.

4.3.2 ROS production was inhibited by Chitosan-Se in cancer cell HCT-116 after 24-hour treatment

Based on Figure 4.3.5, striking decreased of ROS production in HCT 116 was observed at later stage (24h). ROS production of 24-hour treatment by L-Na_2SeO_3, M-Na_2SeO_3 and Na_2SeO_3 decreased to 1, 1 and 3 folds approximately. Chitosan-Na_2SeO_3 exhibited relative stronger influence on elimination of ROS, evidenced by 1 fold compared to 3 fold induced by Na_2SeO_3. As background control, Chitosan particles were not able to attenuate ROS in a time-dependent manner.

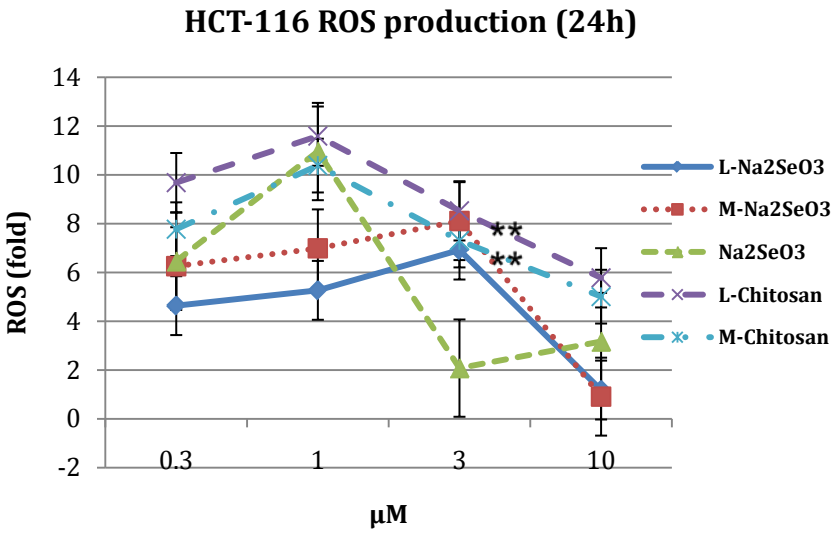


Figure 4.3.5 ROS production in human colon cancer cells HCT-116. ROS production of 24-hour treatment by L-Na_2SeO_3, M-Na_2SeO_3 and Na_2SeO_3 decreased to 1, 1 and 3 folds approximately. L-Chitosan and M-Chitosan, showing >5 folds' production of ROS, were added as background control.

On contrary, ROS produced by L-Na_2SeO_3 and M-Na_2SeO_3 in MRC-5, increased up to 4, 5.5 folds, but Na_2SeO_3 kept at 1 folds as at early stage. It seemed that 24h's treatment

under Chitosan-Na_2SeO_3 could be more toxic to MRC-5, attributing to excess oxidative stress. Chitosan particles as control did not reverse the increased ROS.

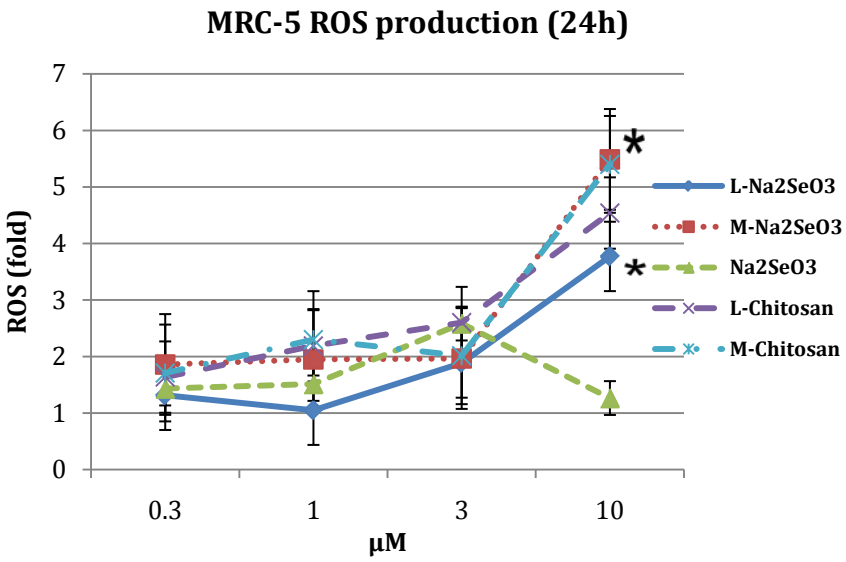


Figure 4.3.6 ROS production in human normal lung fibroblast cells MRC-5. ROS production of 24-hour treatment by L-Na_2SeO_3 and M-Na_2SeO_3 increased to 4, 5.5, but Na_2SeO_3 kept at 1 fold approximately. L-Chitosan and M-Chitosan, showing >4.5 folds' production of ROS, were added as background control.

In HCT-116, we can notice a similar pattern existing in MSAs compounds (Figure 4.3.7). ROS production of 24-hour treatment by L-MSeA, M-MSeA and MSeA decreased to 1 fold correspondingly in a dose dependent manner. Nevertheless, Chitosan-MSeA attenuated ROS (1.25 folds) compared to MSeA (5 folds) in MRC-5 (Figure 4.3.8), showing an advange of chitosan-MSeA in protecting MRC-5 from oxidative stress damage.

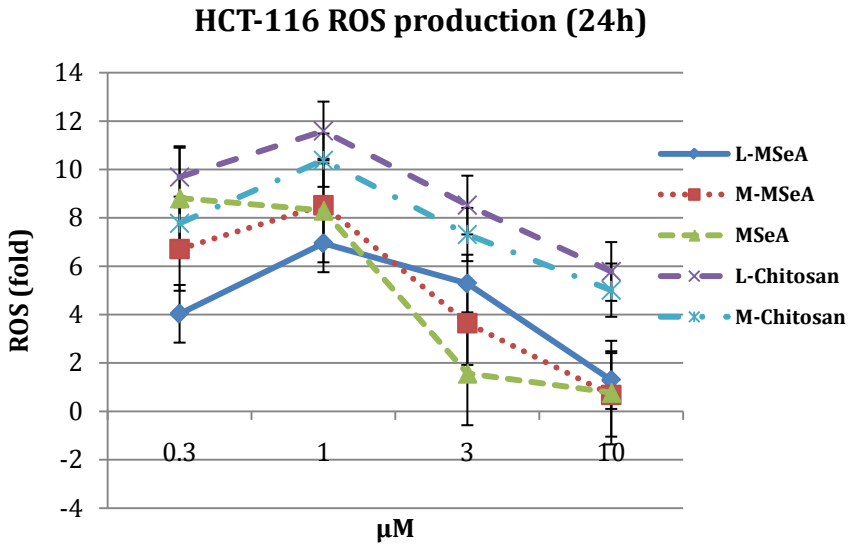


Figure 4.3.7 ROS (Reactive Oxygen Species) production in human colon cancer cells HCT-116. ROS production of 24-hour treatment by L-MSeA, M-MSeA and MSeA decreased to 1, 1 and 1 folds approximately. L-Chitosan and M-Chitosan, showing >5 folds' production of ROS, were added as background control.

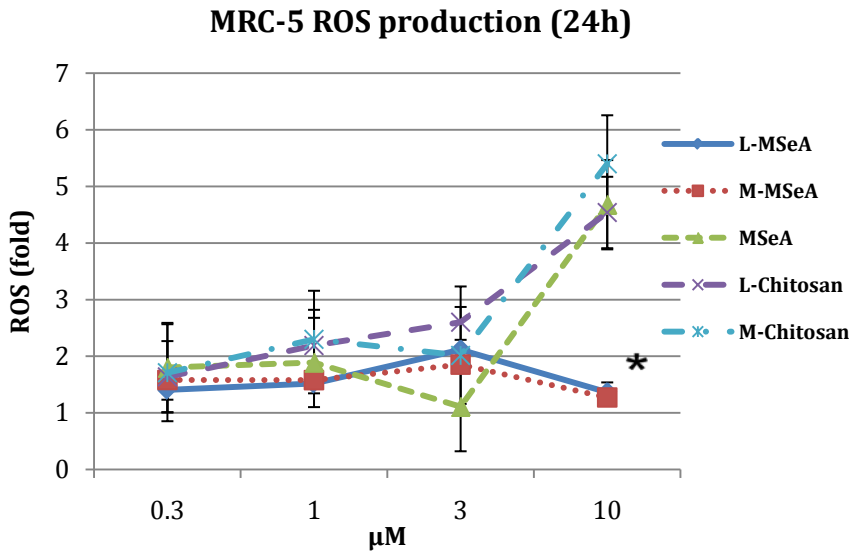


Figure 4.3.8 ROS production in human normal lung fibroblast cells MRC-5. ROS production of 24-hour treatment by MSeA increased to 5 folds, whereas L-MSeA and M-MSeA produced 1.25 fold ROS. L-Chitosan and M-Chitosan, showing >4.5 folds' production of ROS, were added as background control.

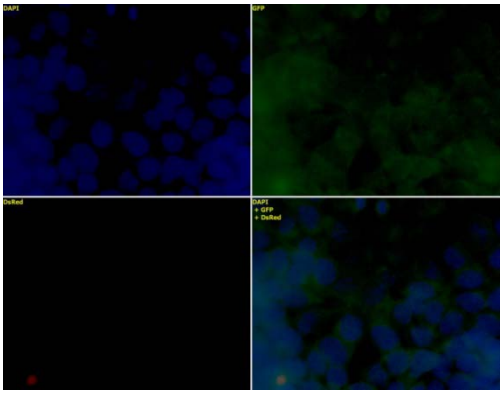
4.4 Expression of pATM Ser-1981

4.4.1 Chitosan-Se up-regulated pATM Ser-1981 expression in normal cell MRC-5 at 6h

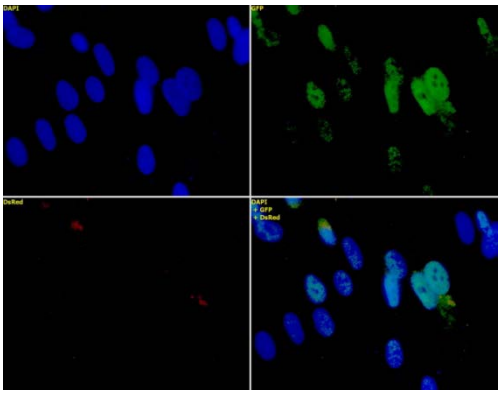
Theoretically, expression of pATM Ser-1981 is elevated in response to DNA double-strand-break damage. In context of this study, oxidative stress and supernutritional level of selenium are potential stimulus for pATM Ser-1981 and associated ATM DNA damage repair pathway. Here we utilized immunofluorescence to examine celluar expression and distribution of total ATM and phoporylated ATM at Ser-1981 activated by selenium compounds.

Photographs of MSeAs 6-hour treatment were taken as examples. As background control, chitosan only had no significant influence on activation of pATM ser-1981 neither in HCT-116 (Figure 4.4.1 A) nor MRC-5 (Figure 4.4.1 B). Percentage of pATM ser-1981 positive cells was 10% in HCT-116 cell (Figure 4.4.1 C) and 65% in MRC-5 (Figure 4.4.1 D) respectively. Percentage of pATM ser-1981 positive cells was 20% in HCT-116 cell (Figure 4.4.1 E) and 30% in MRC-5 (Figure 4.4.1 F) respectively. Chitosan-MSA treated MRC-5 (Figure 4.4.1 D) demonstrated more intensive expression of pATM ser-1981 than HCT-116 (Figure 4.4.1 C).

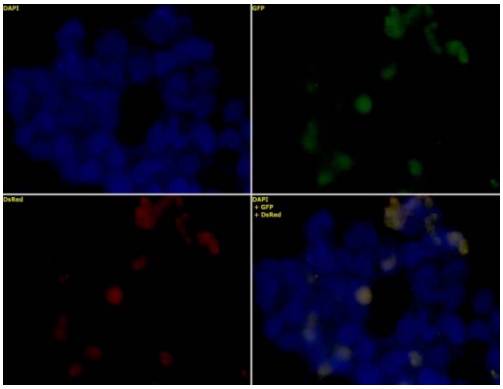
In consistence with results got from ROS production, inhibited phosphorylation of Ser-1981 by chiotsan-MSeA could not counteract with ROS production, and subsequently, ROS level of 9-hour treatment was elevated in HCT-116. Of note, chiotsan-MSeA exhibited 35% more positive cells in MRC-5 than MSeA only, in contrast to 10% less in HCT-116. Anti-ROS function of Chitosan-MSeA can be obtained through increased ATM activation in MRC-5.



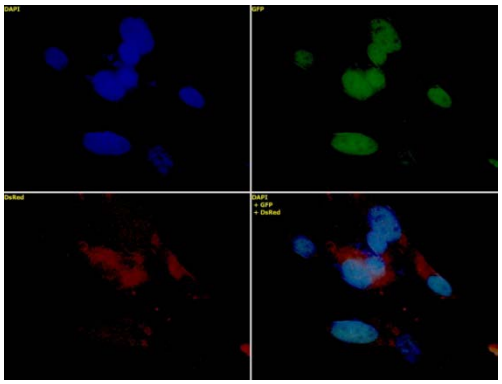
A HCT-116-Chitosan-10µM-6h



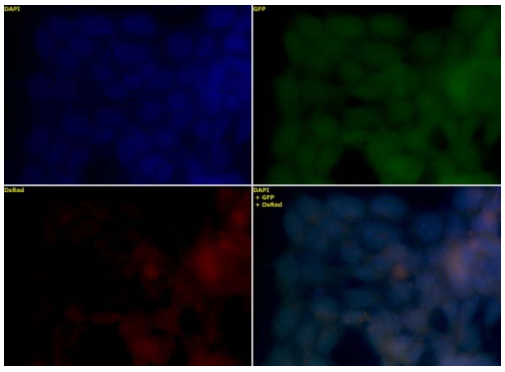
B MRC-5-Chitosan-10µM-6h



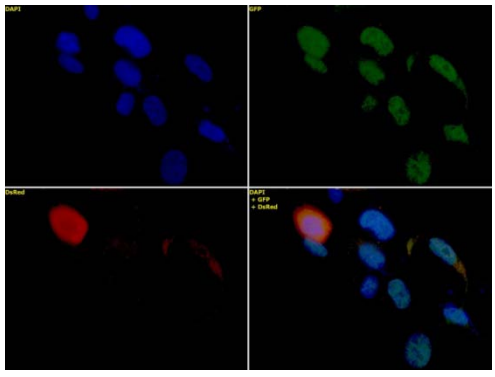
C HCT-116-L-MSeA-10µM-6h



D MRC-5-L-MSeA-10µM-6h



E HCT-116-MSeA-10µM-6h



F MRC-5-MSeA-10µM-6h

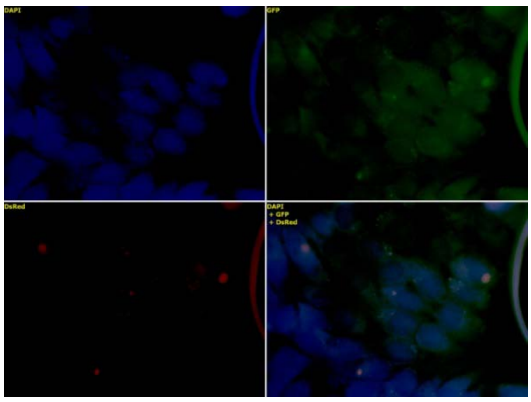
Figure 4.4.1 Expression of total ATM and pATM ser-1981 after 6-hour treatment of Chitosan-MSeA. Blue signal represents nuclear; Green signal represents total ATM; Red signal represents pATM ser-1981; Three colours represent merged image A, B: Challenged by Chitosan only No significant expression of pATM ser-1981 was observed neither in HCT-116 or MRC-5. C, D: Percentage of pATM ser-1981 positive was 10% in HCT-116 cell and 65% in MRC-5 respectively; E, F: Percentage of pATM ser-1981 positive was 20% in HCT-116 cell and 30% in MRC-5 respectively. D taken from Chitosan-MSA treated MRC-5 demonstrated more intensive expression of pATM ser-1981 than E from HCT-116.

Photographs of MSeAs 24-hour treatment were taken as examples. As background control, chitosan only had no significant influence on activation of pATM ser-1981 in HCT-116 (Figure 4.4.2 A), yet moderate stimulation pATM ser-1981 of in MRC-5 (Figure 4.4.2 B). Percentage of pATM ser-1981 positive cells was 95% in HCT-116 cell (Figure 4.4.2 C) and 35% in MRC-5 (Figure 4.4.2 D) respectively. Percentage of pATM ser-1981 positive cells was 20% in HCT-116 cell (Figure 4.4.2 E) and 90% in MRC-5 (Figure 4.4.2 F) respectively. Chitosan-MSA treated MRC-5 (Figure 4.4.2 D) demonstrated more intensive expression of pATM ser-1981 than HCT-116 (Figure 4.4.2 C).

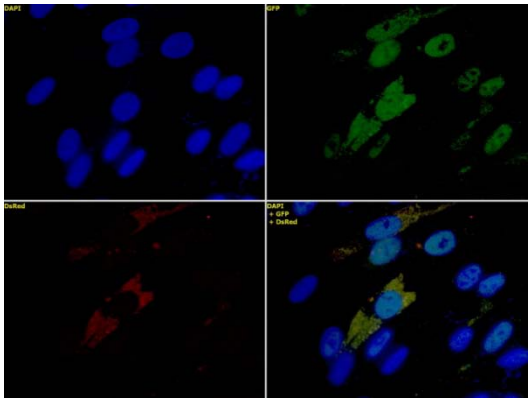
4.4.2 Chitosan-Se down-regulated pATM Ser-1981 expression in normal cell MRC-5 at 24h

In consistence with results got from ROS production, enhanced expression of phosphorylation of Ser-1981 by chitosan-MSeA eliminated ROS production, and subsequently, ROS level of 24-hour treatment was reduced in HCT-116. However, no such protective role of chitosan-MSeA existed in MRC-5. Of note, chiotsan-MSeA exhibited 70% more positive cells than MSeA only in HCT-116, in contrast to 65% less

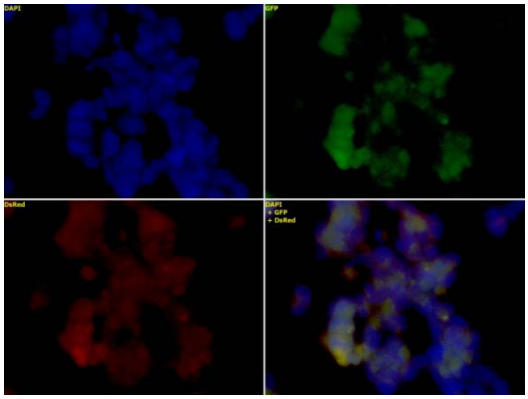
in MRC-5. Anti-ROS function of Chitosan-MSeA can be observed through increased ATM activation in HCT-116 after 24-hour treatment.



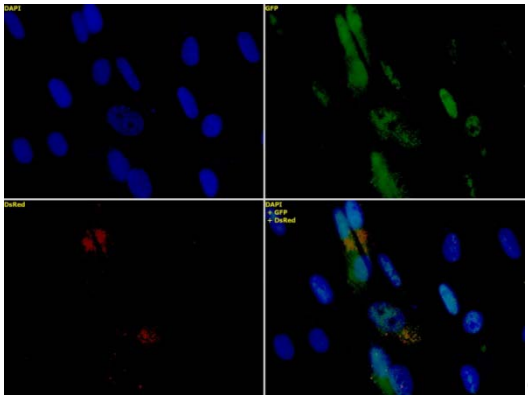
A HCT-116-Chitosan-10μM-24h



B MRC-5-Chitosan-10μM-24h

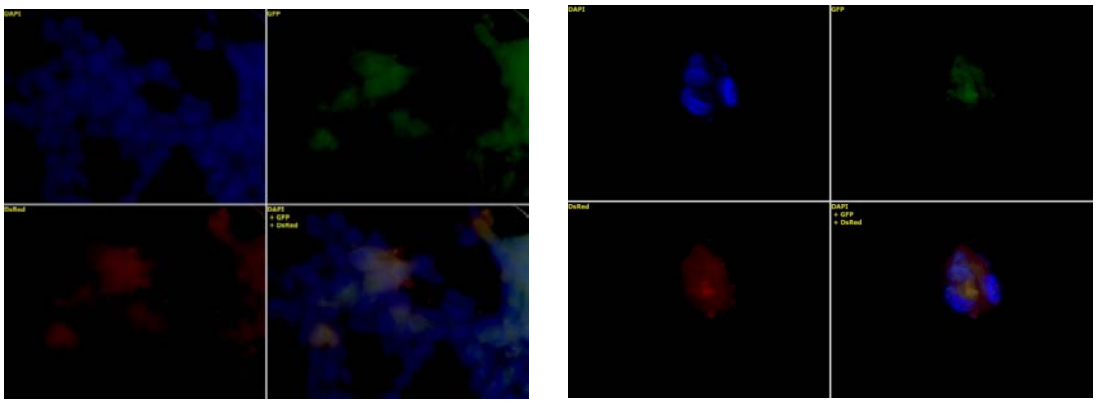


HCT-116-L-MSeA-10μM-24h



C

D MRC-5-L-MSeA-10μM-24h

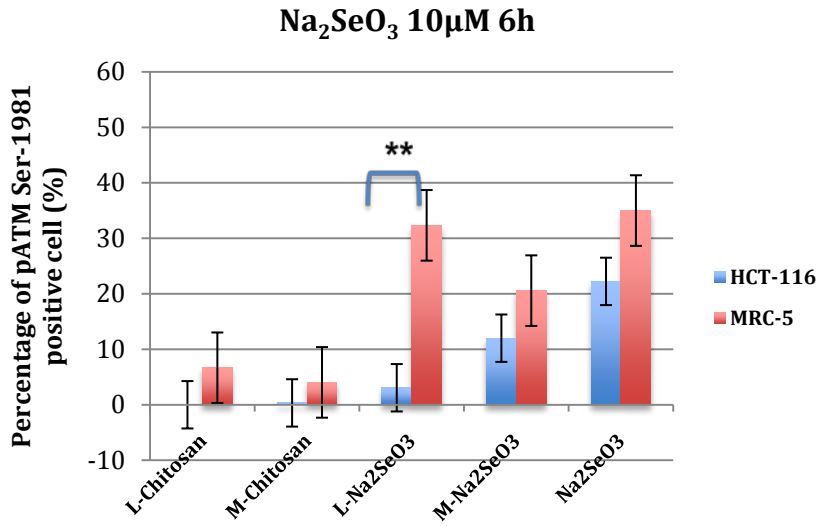


E HCT-116-MSeA-10μM-24h F MRC-5-MSeA-10μM-24h

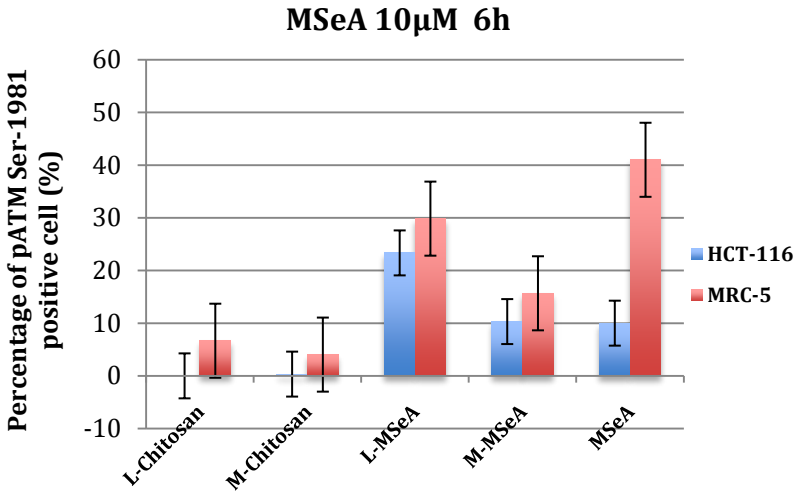
Figure 4.4.2 Expression of total ATM and pATM ser-1981 after 24-hour treatment of Chitosan-MSeA. A, B: HCT-116 and MRC-5 were challenged by Chitosan only. No expression of pATM ser-1981 was observed in HCT-116, but 13% showed red signal in MRC-5. C, D: Percentage of pATM ser-1981 positive was 95% in HCT-116 cell and 35% in MRC-5 respectively; E, F: Percentage of pATM ser-1981 positive was 25% in HCT-116 cell and 90% in MRC-5 respectively. C taken from Chitosan-MSA treated HCT-116 demonstrated more intensive expression of pATM ser-1981 than D from MRC-5.

4.4.3 Quantitative results based on immunofluorescence images

To further interpret phosphorylaton degree of ATM under each treatment, percentage of pATM Ser-1981 positive cell was calculated as follow. Among them, L-Na_2SeO_3 induces highly significant increase of pATM Ser-1981 in MRC-5 cells after 6-hour incubation. Since ATM is a DNA damage repair protein, we infer that L- Na_2SeO_3 may function with higher DNA damage repair efficacy than other groupmates.



A

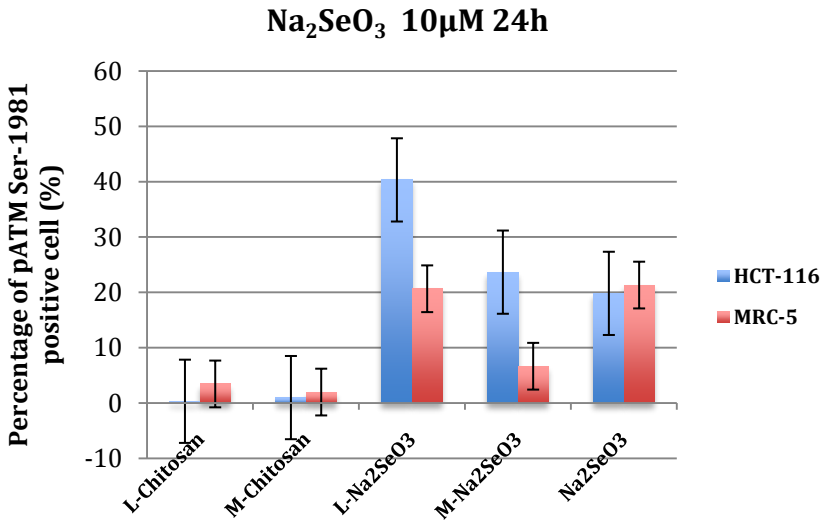


B

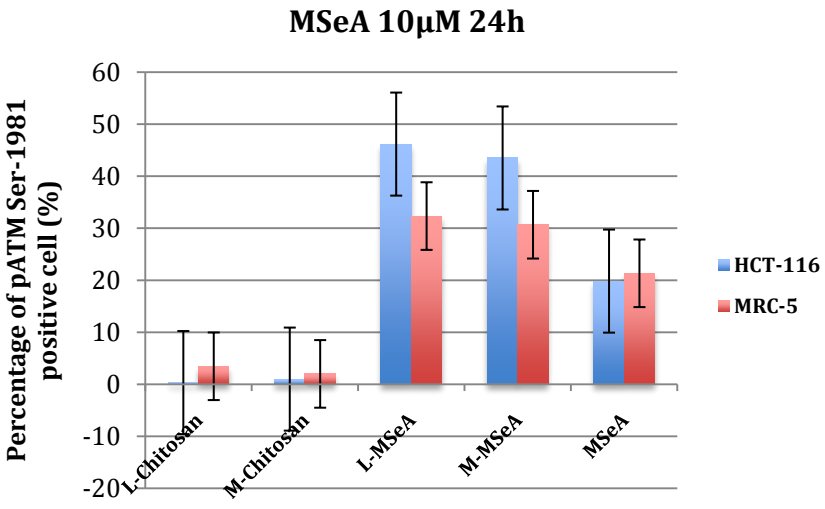
Figure 4.4.3 Percentage of pATM Ser-1981 for 6-hour treatment. H represents HCT-116 cells; M represents MRC-5 cells. P represents P value derived from t-test; * represents $P<0.05$; ** represents $P<0.01$. Percentage difference between two cell lines was analyzed by Student T test, two tails, Type 3.

A are samples treated with Na_2SeO_3; B are samples treated with MSeA.

However, when incubation extended to 24 hours, MRC-5 exhibited autophosphorylation of ATM without interference of Se compounds, which was evidenced by control groups. MRC-5 had overall less positive cells, which was due to less DNA damaged. In addition, the chitosan-Se led to higher positive cells than original compounds in HCT-116 than MRC-5, implying chitosan-conjugated Se caused more DNA damage in cancer cells after 24-hour incubation.



A



B

Figure 4.4.4 Percentage of pATM Ser-1981 for 24-hour treatment. H represents HCT-116 cells; M represents MRC-5 cells.

A are samples treated with Na_2SeO_3; B are samples treated with MSeA.

Chapter 5 Discussion and Perspective

5.1 Roles of selenium in cell absorption and cell viability

For cellular Se content, Na_2SeO_3 compounds demonstrated overall 30-40 times higher efficacy to cancer cells than normal cells. However, MSeA compounds were delivered to both cells with slightly higher concentration scale in HCT-116. Among selenium compounds, low molecular chitosan-Na_2SeO_3 owned the highest delivery efficacy. For chitosan-MSeA, interestingly, the original MSeA was higher in HCT-116 at dose 10μM. It is possible that increased absorption of chitosan-MSeA causes more cell death, and in turn, less cell available for Se assessment.

The MTT assay reveals a retained viability induced by chitosan-Se of both cells. In addition, the chitosan-Se is more protective than orginal Se, especially to normal cells. It may be explained by a suspended releasing profile of nanopartiles causes attenuated toxicity to cells. Besides, cell culture medium for HCT-116 with chitosan-Se was approximately 8.0 and that for MRC-5 was about 7.3. The alkaline solution may mitigate surface charge of chitosan and hence no improved deliver efficacy.

5.2 Roles of selenium in activation of pATM Ser-1981

In combination of ROS production and pATM Ser-1981 activation, we figure out ROS production is correlated with phosphorylation of ATM. Super-nutritional dosage of

selenium (10μM), especially chitosan-Se, is able to trigger activation of pATM Ser-1981 in normal cells at early stage (6h), without significant influence in cancer cells. This activation of ATM probably trigger ATM-mediated DNA DSBs repair pathway and fight against further production of ROS. That is why ROS production at 9 hour was decreased in normal cells rather than in cancer cells. Moreover, chitosan-Se stimulated increased ROS in HCT-116, indicating it's more toxic to cancer cells after 6-hour incubation.

Again, effects of selenium have been reversed in both cells after 24-hour incubation. Chitosan-Se can activate ATM in cancer cell. Meanwhile, Chitosan-MSeA protected normal cells more than original one by generating less ROS. Therefore, Chitosan-Se could facilitate cancer cells in DNA damage repair and elimination of ROS in 24-hour period, but still demonstrate advantages over original selenium in ROS suppression for normal cells.

5.3 Perspective

Selenium has been postulated as an antioxidant as well as an efficient chemopreventive trace element for cancer therapy. By incorporating into chitosan nanoparticles, its releasing profile and chemoproperties can be altered. This study investigated systemically in chemopreventive function of chitosan-Se and original Se in terms of cell viability, oxidative stress stimulation and activation of ATM, which is known as initiator of DNA DSBs repair.

By analyzing these parameters comprehensively, we found out that chitosan-facilitated delivery of Se to cancer cells, but still constrained to certain Se type and incubation time. Accurate releasing profile of chitosan-Se should be measured, in that absorption of chitosan-Se does not necessarily equals to complete cellular release of Se. Furthermore, time point set to MTT, ROS and immunofluorescence assessment should be optimized. To address potential function of chitosan-Se in ATM-mediated DNA damage pathway, we may also need to examine more factors, such as rH2AX and MRN complex, which can describe our hypothesis in a more appropriate manner.

Appendices

Appendix I: Chemicals and reagents

10XTBS	Bio-Rad 170-6435
10XTG	Bio-Rad 161-0771
10XTBS	Bio-Rad 170-6435
10% SDS	Teknova S0184
Amphotericin B	Mediatech 30003-CF
Acetic acid	Fisher 086636
ATM antibody	Epitomics YF-10-17-02C
ATM Ser-1981 antibody	Rockland 20772
BSA	Calbiochem D00069588
Chitosan-Low molecular weight	Sigma 448869
Chitosan-Medium molecular weight	Sigma 448877
DAPI fluorescence staining dye	Invitrogen 447892
DMEM	Cellgro 10-017-CV
DMSO	Mallinckrodt H31J18
Essential amino acid	Mediatech 25030-CI
Ethanol (absolute)	Pharmco AAPER C0910191
EDTA	Quality Biological 351-027-10
Fetal Bovine Serum	Sigma 028K0381
Glycerol	Sigma G-5516
KCl	Baker 4001-01
KH_2PO_4	Baker 4008-01

MEM	Mediatech 10010-CI
MTT	Calbiochem 475989
Methanol	Pharmco AAPER 0704046
Methylseleninic acid	Sigma 541281
NaCl	Baker 4058-05
Na_2HPO_4	Sigma S-7907
NaOH	Mallinckrodf
Non essential amino acid	Mediatech 25025-CI
Non-Fat Dry Milk	Bio-Rad 170-6404
NP40	Calbiochem 492016
Paraformaldehyde	Fisher 066298
Penicillin-Streptomycin	Cellgro 30-002-CI
Phenylmethylsulphonyl fluoride	Calbiochem D00006306
Plasimocin	InvivoGen MPT-31-005
Protease inhibitor cocktail tablets	Roche 12553900
Puromycin	Calbiochem 540222
ROS dye (CM-H_2DCFDA)	Invitrogen 537553
Secondary antibody-anti-mouse	Introgen Alexa Fluor® 594
Secondary antibody-anti-rabbit	Introgen Alexa Fluor® 488
Sodium tripolyphosphate (TPP)	Pfalfz & Bauer S07555
Sodium selenite	Sigma S5261
Tris-HCl	Quality Biological 351-007-101
Triton-100	Calbiochem D00014550
Trypsin EDTA, 1X	Cellgro 25-052-C
Tween20	Calbiochem 655204
Vitamins	Mediatech 25020-CI

Appendix II: Solutions and their ingredients

10X PBS	80 g NaCl, 10 g KCl, 72 g Na_2HPO_4, 12 g KH_2PO_4, 1 L dd H_20
MEM Cell culture medium	500 mL MEM medium, 15% Fetal Bovine Serum, 1% Penicillin-Streptomycin
D-MEM Cell culture medium	500 mL DMEM medium, 10% Fetal Bovine Serum, 100U/mL Penicillin-Streptomycin, 100U/mL amphotericin B, 5µg/mL plasimocin
Blocking buffer	3 g BSA, 100 mL PBS
Lysis buffer	50 mM Tris-HCl (7.4), 250 mM NaCl, 5 mM EDTA, 0.1% NP40
MEM cell culture medium	500 mL DMEM medium, 10% Fetal Bovine Serum, 100U/mL Penicillin-Streptomycin, 100U/mL amphotericin B, 5µg/mL plasimocin
MTT	(0.083g MTT is dissolved in 10mL 100% ethanol as a stock concentration of 20mM) (serum free medium dilute 20mM MTT to 1mM final)
TBS-T	100 mL 10XTBS, 900 mL ddH_20, 10 mL 10% Tween20

Appendix III: Commercial kits

BCA™ Protein Assay Kit	Thermo JI124811

Appendix IV: Equipments and facilities

Balance	Denver Instrument S-403
Biological Safety Cabinet	Thermo 109578

Cell culture incubator	Thermo 3595
FLUOstar OPTIMA	BMG 413-3128
Isotemp Air Bath	Fisher 11-715-1250
Isotemp Water Bath	Fisher 15-462-01
Legen RT centrifuge	Thermo 75004377
Optic Microscope	Motic AE21
Rocker	VWR 12620-906
Roto-Shake Genie	Scientific Industries S1-1100
Zeiss fluorescence microscope	Carl Zeiss Axio Observer 100
Vortex-Genie	Scientific Industries 2-401968

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