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Encapsulation of selenium in chitosan nanoparticles improves selenium availability and protects cells from selenium-induced DNA damage response

Shu Zhang[a], Yangchao Luo[a], Huawei Zeng[b], Qin Wang[a], Fei Tian[c], Jiuzhou Song[c], Wen-Hsing Cheng[a,*]

[a]Department of Nutrition and Food Science, University of Maryland, College Park, MD 20742, USA
[b]USDA, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202, USA
[c]Department of Animal and Avian Sciences, University of Maryland, College Park, MD 20742, USA

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Abstract

Selenium, an essential mineral, plays important roles in optimizing human health. Chitosan (CS) is an effective, naturally oriented material for synthesizing nanoparticles with preferable properties such as biocompatibility, biodegradation and resistance to certain enzymes. We have recently shown that cellular exposure to selenium compounds activates ataxia-telangiectasia mutated (ATM)-dependent DNA damage responses, a tumorigenesis barrier. To test whether nanoencapsulation of selenium modulates the cellular response to selenium compounds, the HCT 116 cancerous and the MRC-5 normal cells were treated with Na_2SeO_3 and methylseleninic acid (MSeA) encapsulated in CS/polyphosphate nanoparticles. Analyses of cellular selenium levels demonstrate that (1) the nanoencapsulation enhances selenium levels in cells after exposure to Na_2SeO_3 and MSeA (1–10 μM); (2) cells retained more selenium when treated with Na_2SeO_3 than with MSeA; (3) selenium levels are greater in HCT 116 than in MRC-5 cells after Na_2SeO_3, but not MSeA, exposure. Survival analysis shows that CS encapsulation desensitizes HCT 116 and MRC-5 cells to Na_2SeO_3 or MSeA exposure. Immunofluorescent analysis demonstrates that CS encapsulation attenuates the selenium-induced ATM phosphorylation on Ser-1981, and the extent is greater in HCT 116 than in MRC-5 cells. Our results reveal features of selenium nanoencapsulation in CS, including increased selenium retention in cells and decreased cellular sensitivity and DNA damage response to selenium exposure.

Keywords: Selenium; Chitosan; Nanoparticles; DNA damage; Cancer

1. Introduction

Selenium is an essential mineral that maintains optimal health in life. Although the recent Selenium and Vitamin E Cancer Prevention Trial (SELECT) fails to demonstrate selenomethionine as an effective chemoprevention agent against prostate cancer [1], the Nutritional Prevention of Cancer (NPC) and other epidemiological trials, together with ample animal studies, have established the anticarcinogenic potential of selenium in decreasing risks of prostate and colorectal cancer, as well as an inverse association between selenium status and cancer risk [2–4]. The above observations appear to be unexpected. However, a further analysis has suggested that daily selenium supplements do not benefit all people, and cancer risk reduction by selenium is seen only in men with suboptimal selenium levels (<1.53 μmol/L plasma) prior to entering the trial (a subgroup evaluated by NPC but not by SELECT) [5].

The biological function of selenium is exerted through selenoproteins and selenium metabolites, both of which are implicated in the regulation of tumorigenesis [6,7]. At least one-third of the 25 selenoproteins in humans exhibit antioxidative activities, but selenium metabolites are also known to induce reactive oxygen species (ROS) [8]. Selenium may mitigate tumorigenesis at the initiation, promotion and progression stages through different mechanisms. It is well studied that selenium at lethal doses kill cancerous cells via induction of apoptosis and cell-cycle arrest [9-12]. Recently, it has been unveiled that selenium at low doses can activate an early barrier of tumorigenesis, namely, the ataxia-telangiectasia mutated (ATM)-dependent senescence [13], specifically in the non-cancerous MRC-5 cells but not in the cancerous HCT 116 cells [14]. Upon various types of stress, the ATM kinase is rapidly phosphorylated on Ser-1981 (pATM Ser-1981) and mediates multiple downstream pathways of DNA damage checkpoint and repair responses [15].

Chitosan (CS) is a linear, biodegradable polysaccharide rich in the exoskeleton of crustaceans. Chitosan nanoparticles are ideal carriers of bioactive and therapeutic compounds such as quercetin, venlafaxine hydrochloride and oligonucleotides [16]. Of note, the nature of positive charges in acidic solutions allows CS to target cells with negatively charged microenvironment, a feature intrinsic to some cancers. Conjugation of selenite or selenic group ($-SeO_3$) to CS has been shown to promote cell death in human sarcoma and leukemia

Abbreviations: ATM, ataxia-telangiectasia mutated; MSeA, methylseleninic acid; MTT, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NPC, Nutritional Prevention of Cancer Trial; pATM Ser-1981, phosphorylation of ATM on Ser-1981; ROS, reactive oxygen species; SELECT, Selenium and Vitamin E Cancer Prevention Trial.

* Corresponding author. Tel.: +1 301 405 2940; fax: +1 301 314 3313.
E-mail address: whcheng@umd.edu (W.-H. Cheng).

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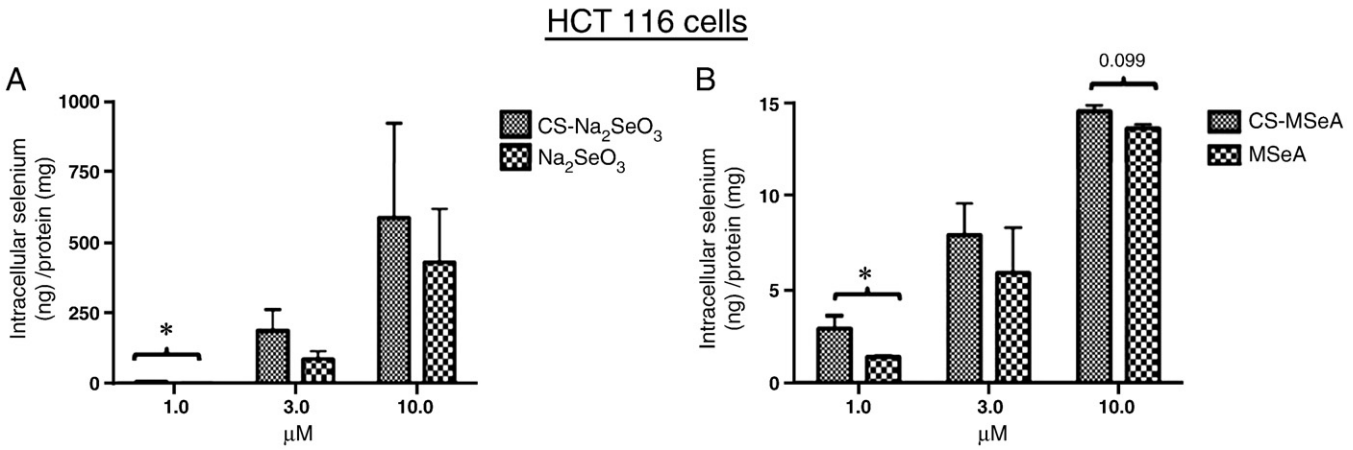

Fig. 1. Effect of CS encapsulation on cellular selenium levels in the HCT 116 human colorectal adenocarcinoma cells treated with selenium compounds. Exponentially growing HCT 116 cells were treated with Na_2SeO_3 (A), MSeA (B) and the CS-encapsulated forms (1–10 μM) for 24 h, followed by analyses of selenium levels using hydride-generation atomic absorption spectrometry and protein concentration using a bicinchoninic acid protein assay. Na_2SeO_3, sodium selenite; MSeA, methylseleninic acid; *P<.05.

K562 cells [17]. Recently, selenite was successfully encapsulated into CS/TPP nanoparticles, which exhibited enhanced antioxidant activities and controlled release *in vitro* [18]. However, little is known about the effect of CS encapsulation on cellular selenium levels and the cellular DNA damage response to selenium compounds. In particular, MSeA is one of the most efficacious selenium compounds that suppress tumors in animal models [19], and our previous results show that MRC-5 non-cancerous fibroblasts are more sensitive than HCT 116 cancer cells to selenium-induced DNA damage responses [14]. To investigate whether nanoencapsulation in CS affects the efficacy of selenium compounds in targeted delivery and DNA damage response, we treated HCT 116 and MRC-5 cells with MSeA, selenite and the selenium-loaded CS nanoparticles to assess cellular selenium levels, cell survival and DNA damage response after the selenium exposure.

2. Materials and methods

2.1. Cell cultures and chemicals

The HCT 116 human colorectal adenocarcinoma cells and MRC-5 normal lung fibroblasts were obtained and cultured as described previously [14]. Sodium selenite (Na_2SeO_3), MSeA, CS (low molecular weight, 92% deacytelation degree), sodium tripolyphosphate and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Selenium-loaded CS nanoparticles

The selenium-loaded CS/TPP nanoparticles (CS-Na_2SeO_3, or CS-MSeA) were prepared by a previously reported method [18]. In brief, serial Na_3SeO_3 or MSeA dilutions (dissolved in deionized water) were dropwise added to CS solution (32.6 μM, dissolved in 1% acetic acid) with mild stirring for 30 min, and then TPP solution (dissolved in deionized water) was dropwise added into the above system with mild stirring for 30 min. On the basis of the reported optimization method [18], CS concentration was set as 32.6 μM, and weight ratio of CS to TPP was set as 3:1 for Na_3SeO_3 and 5:1 for MSeA.

2.3. Cell survival assays

Twenty-four hours after being seeded into 24-well plates, the exponentially growing cells were treated with Na_2SeO_3, MSeA, CS-Na_2SeO_3 and CS-MSeA (0.1–10 μM) for 24 h. Cell survival was estimated by a MTT assay [20]. Briefly, PBS-washed cells were incubated with 500 μl MTT (1 mg/ml) for 3 h at 37°C. DMSO was then added and the solubilized formazan was quantified spectrophotometrically at 595 nm (FLUOstar OPTIMA, BMG LABTECH, Cary, NC, USA). The MTT value for the cells treated with CS alone at 0.1 μM was set as 1.

2.4. Selenium analysis

Cellular selenium levels were measured using hydride-generation atomic absorption spectrometry, normalized by total protein levels and expressed as

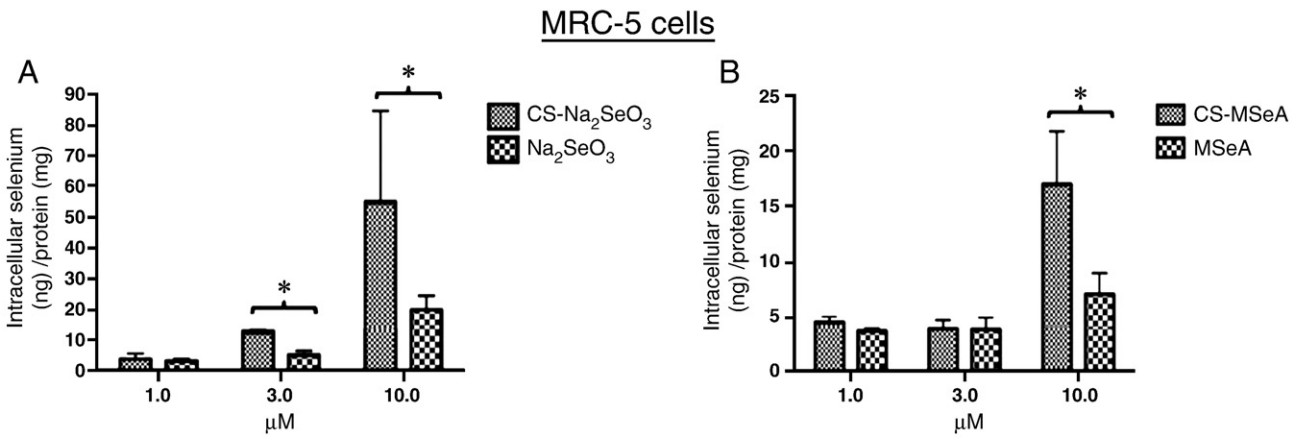

Fig. 2. Effect of CS encapsulation on cellular selenium levels in the MRC-5 normal human lung fibroblasts treated with selenium compounds. Exponentially growing MRC-5 cells were treated with Na_2SeO_3 (A), MSeA (B) and the CS-encapsulated forms (1–10 μM) for 24 h. The biochemical analyses and keys are as described in Fig. 1 legend.

nanograms of intracellular selenium per milligram of protein as described previously [21]. Protein concentrations were measured by a bicinchoninic acid protein assay (Thermo, Rockford, IL, USA).

2.5. Immunofluorescence

Immunofluorescent analysis of ATM and pATM Ser-1981, including primary antibodies and fluorescence microscope conditions, was performed as described previously [14]. Cells containing at least five pATM Ser-1981 foci are defined as focus-positive cells. Five pictures were randomly taken from each slide (n=3).

2.6. Statistical analysis

The data were analyzed by using the SAS version 9.0 software (SAS Institute, Inc., Cary, NC, USA). A two-tailed Student's t test was applied to determine the statistical significance between the treatments and the control. Linear regression was applied to determine the cellular selenium content (Figs. 1 and 2) after cellular response to gradient concentrations of selenium.

3. Results

3.1. Effect of CS encapsulation on intracellular selenium levels in HCT 116 and MRC-5 cells

We began this study by asking whether encapsulation of selenium in CS nanoparticles impacted selenium levels in cells treated with selenium compounds. Cellular selenium levels were increased in a dose-dependent manner (1–10 μM, P<.05) within 24 h after treatment of HCT 116 (Fig. 1) and MRC-5 (Fig. 2) cells with Na_2SeO_3, MSeA, CS-Na_2SeO_3 and CS-MSeA. In HCT 116 cells, nanoencapsulation of Na_2SeO_3 (Fig. 1A) and MSeA (Fig. 1B) in CS at 1 μM resulted in significantly (P<.05) elevated cellular selenium levels; however, such effect was not statistically significant when the cells were treated with higher doses of selenium. In contrast, selenium levels in MRC-5 cells was significantly (P<.05) enhanced by treatment of the selenium compounds at 10 μM (CS-Na_2SeO_3 vs. Na_2SeO_3, 1.7-fold, Fig. 2A; CS-MSeA vs. MSeA, 1.4-fold, Fig. 2B) and 3 μM (CS-Na_2SeO_3 vs. Na_2SeO_3, 1.5-fold, Fig. 2A), but not at 1 μM. Considering selenium specification, selenium levels were greater (P<.05) after cellular exposure to CS-Na_2SeO_3 than to CS-MSeA in HCT 116 cells (25-fold, 3 μM; 30-fold, 10 μM, Fig. 1) and in MCR-5 cells (2.2-fold, 10 μM, Fig. 2). Similarly, cellular selenium levels were greater after cellular exposure to Na_2SeO_3 than to MSeA in HCT 116 and MRC-5 cells. Considering cell types, selenium levels were greater (P<.05) in HCT 116 cells than in MRC-5 cells after treatment with CS-Na_2SeO_3 (13-fold, 3 μM; 10-fold, 10 μM), Na_2SeO_3 (16-fold, 3 μM; 21-fold, 10 μM), CS-MSeA (1.1-fold, 3 μM) and MSeA (1.2-fold, 10 μM). Taken together, after cellular exposure to the selenium compounds, intracellular selenium levels are enhanced by nanoencapsulation in CS, greater when treated with Na_2SeO_3 than with MSeA, and higher in HCT 116 than in MRC-5 cells.

3.2. Effect of CS encapsulation on selenium-induced toxicity in HCT 116 and MRC-5 cells

We next performed survival assays to determine the effect of nanoencapsulation in CS on the cellular sensitivity to Na_2SeO_3 and MSeA. Results from the MTT analysis showed that HCT 116 and MRC-5 cells were sensitive to Na_2SeO_3 (Figs. 3A and 4A) and MSeA (Figs. 3B and 4B) at doses ranging from 0.1 to 10 μM; however, encapsulation in CS significantly (P<.05) desensitized the cells to the Na_2SeO_3 and the MSeA exposure. Consistent with our previous report [14], MRC-5 cells were more sensitive than HCT 116 cells to the selenium compounds at low doses (0.1 and 1 μM). Treatment of HCT 116 and MRC-5 cells with CS alone (0.1–10 μM) did not impact on cellular sensitivity. Therefore, encapsulation in CS could protect both HCT 116 and MRC-5 cells from the killing effect of selenium compounds.

3.3. Effect of CS encapsulation on selenium-induced pATM Ser-1981 focus formation in HCT 116 and MRC-5 cells

We have previously shown that pATM Ser-1981 foci in the nucleus, a marker of the ATM DNA damage pathway activation, were induced by selenium compounds in HCT 116 and MRC-5 cells [14,22]. Therefore, we employed immunofluorescence analyses to test whether nanoencapsulation in CS can affect pATM Ser-1981 focus formation induced by the selenium compounds (10 μM). There was no detectable pATM Ser-1981 focus in HCT 116 and MRC-5 cells 6–24 h after treatment with CS only (data not shown). In HCT-116 cells, treatment of Na_2SeO_3 (Fig. 5A) or MSeA (Fig. 5B) induced pATM Ser-1981 focus formation at 6 h. Interestingly, encapsulation of Na_2SeO_3 and MSeA in CS nanoparticles suppressed pATM Ser-1981 focus formation almost completely and by four-fold, respectively. At 24 h, the selenium-induced pATM Ser-1981 foci subsided, and the extents were comparable in the presence or absence of CS. In MRC-5 cells, in contrast, the encapsulation did not greatly alleviate pATM Ser-1981 focus formation induced by Na_2SeO_3 (Fig. 6A) or MSeA (Fig. 6B) at 6 h. At 24 h, the selenium-induced pATM Ser-1981 foci subsided, but

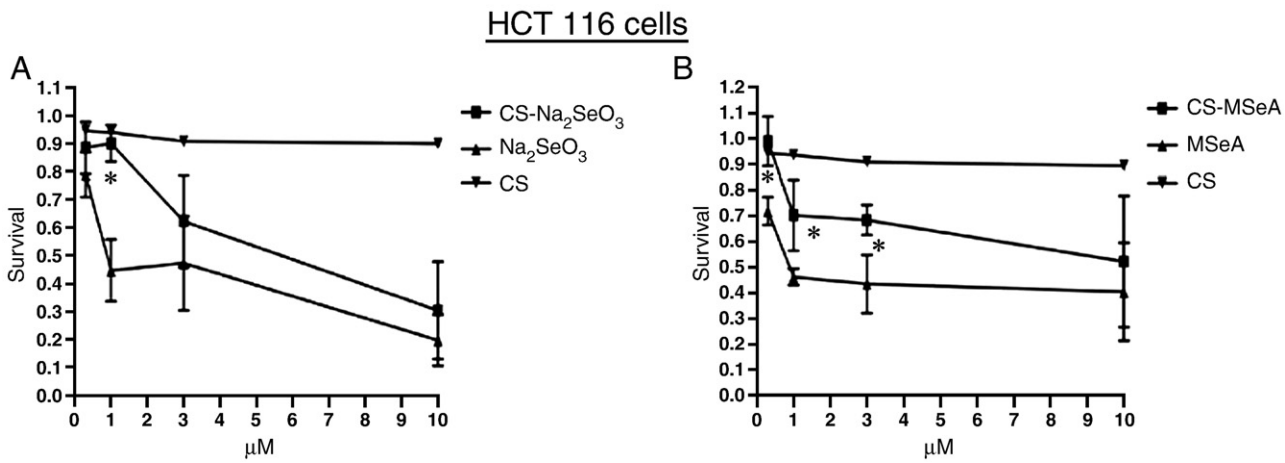

Fig. 3. Effect of CS encapsulation on the sensitivity of HCT 116 cells to selenium compounds. Exponentially growing HCT 116 cells were treated with Na_2SeO_3 (A), MSeA (B) and the CS-encapsulated forms (0.1–10 μM) for 24 h, followed by a MTT assay. Na_2SeO_3, Sodium selenite; MSeA, methylseleninic acid; *P<.05, Na_2SeO_3 vs. CS-Na_2SeO_3 or MSeA vs. CS-MSeA at the indicated concentrations.

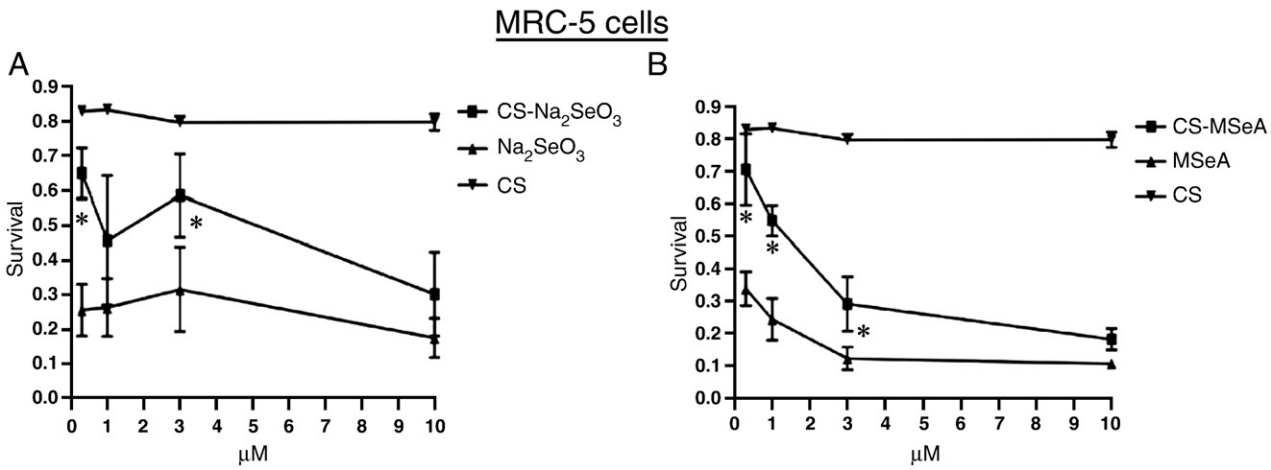

Fig. 4. Effect of CS encapsulation on the sensitivity of MRC-5 cells to selenium compounds. Exponentially growing MRC-5 cells were treated with Na_2SeO_3 (A), MSeA (B) and the CS-encapsulated forms (0.1–10 μM) for 24 h, followed by a MTT assay. The keys are as described in Fig. 3 legend.

encapsulation in CS further suppressed ($P<.05$) the focus formation. Taken together, encapsulation of selenium in CS nanoparticles suppressed the selenium-induced pATM Ser-1981 focus formation differentially between HCT 116 and MRC-5 cells.

4. Discussion

Our current results demonstrate that, in cultured cells treated with Na_2SeO_3 and MSeA, nanoencapsulation of selenium in CS increases cellular selenium levels, desensitizes the cells to selenium compounds and decreases pATM Ser-1981 DNA damage foci. Our previous publications showed that exposure of selenium compounds to cells induces pATM Ser-1981 focus formation in a ROS-dependent manner [14] and that CS and CS-Na_2SeO_3 exhibit antioxidative properties *in vitro* [18]. Therefore, the anti-oxidative CS may dominate the pro-oxidative Na_2SeO_3 and MSeA when treated with the CS-encapsulated selenium compounds. This may explain why increasing cellular selenium levels by nanoencapsulation of selenium in CS does not confer a sensitized cellular response to the selenium compounds. Therefore, although the results implicate the feasibility of increased selenium target delivery to cells via CS encapsulation, this does not seem to be an ideal approach for selenium chemoprevention. Nonetheless, we suggest here the potential of employing CS encapsulation to improve selenium delivery or retention in cells with decreased toxicity.

Why do the cancerous HCT 116 cells contain more selenium than the non-cancerous MRC-5 cells after treatment with Na_2SeO_3, but not with MSeA? Why does CS enhance cellular selenium levels at high doses in MCR-5 cells, but at low doses in HCT 116 cells? One feature of solid tumor is the extracellular acidosis. The microenvironment of pH gradient favors uptake and retention of weak acidic molecules [23]. The weak acid nature of selenite ($pK_{a1}=2.46$ and $pK_{a2}=7.31$), as opposed to MSeA ($pK_a=8.5$), may help Na_2SeO_3 to be more efficiently taken into cancerous cells than into noncancerous cells. The positively charged CS may enhance selenium delivery at low doses in the negatively charged HCT 116 cancerous cells. At 10 μM, selenium delivery and retention may be saturated in HCT 116 cells but not in MRC-5 cells. Whatever the mechanism, the results show for the first time that selenium encapsulation in CS nanoparticles improves selenium delivery and/or retention in cells, and the effective doses differ between cancerous and non-cancerous cells. Apparently, supplementing selenium that is encapsulated in CS shows enhanced cellular selenium levels in a manner dependent on the types of cells and the forms of selenium compounds.

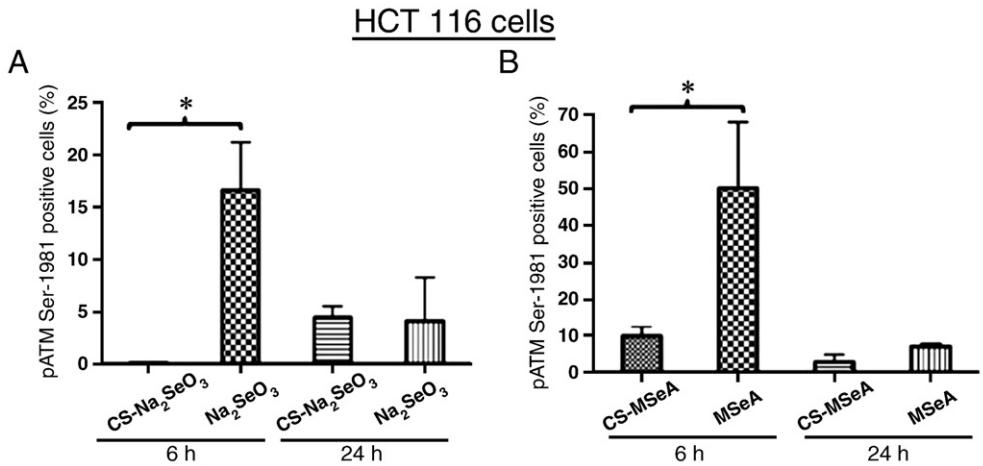

Fig. 5. Effect of CS encapsulation on selenium-induced pATM Ser-1981 in HCT 116 cells. Exponentially growing HCT 116 cells were treated with Na_2SeO_3 (A), MSeA (B) and the CS-encapsulated forms (10 μM) for 6 and 24 h, followed by immunofluorescent analysis of pATM Ser-1981 and ATM expression. Cells containing >5 pATM Ser-1981 foci are defined as pATM Ser-1981-positive cells. Na_2SeO_3, Sodium selenite; MSeA, methylseleninic acid; *$P<.05$.

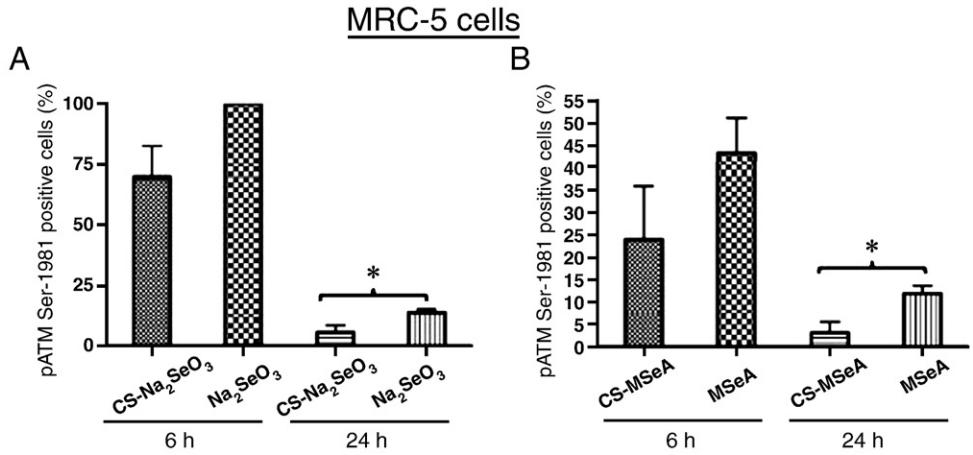

Fig. 6. Effect of CS encapsulation on selenium-induced phosphorylation of ATM at Ser-1981 (pATM Ser-1981) in MRC-5 cells. Exponentially growing MRC-5 cells were treated with Na_2SeO_3 (A), MSeA (B) and the CS-encapsulated forms (10 μM) for 6 and 24 h, followed by immunofluorescent analysis of pATM Ser-1981 and ATM expression. The keys are as described in Fig. 5 legend.

Why does selenium encapsulation in CS increase cellular selenium levels yet decrease selenium-induced cell death and DNA damage response? First, CS *per se*, as an antioxidant [18], may suppress the selenium-induced ROS formation and the associated DNA damage response and cell death. Second, selenium compounds may be sequestered in CS polymers and thus are not metabolically available for the induction of DNA damage response. Our selenium analysis method employs acid digestion to measure all available selenium in cells. Thus, the results may not represent all the bioavailable selenium speciation. To this end, selenium-induced ROS formation is likely to be buffered by the antioxidative properties of CS, and the sequestered selenium does not efficiently act on the activation of DNA damage response. Moreover, cell culture media usually do not supplement with additional selenium, and the source of selenium in cultured cells is typically from serum only. Indeed, this condition seems not likely to support the full expression of selenoprotein. Supplementation of additional selenium to cell culture media is likely to increase selenoprotein expression, and CS encapsulation, in principle, may increase selenium retention or delivery that can facilitate selenoprotein expression. It is of future interest to study the bioavailability of selenium compounds encapsulated by nanoparticles and to assess selenoprotein expression in cultured cells supplemented with selenium and CS-encapsulated selenium.

Our approaches employing cell models and CS encapsulation are of physiological relevance that can equate the expected physiologic levels of cellular selenium exposure to that through the diet or other clinical delivery mechanisms. It has been documented that the selenium concentration at 10 μM in cultured media represents a physiologically achievable plasma level [24]. Moreover, blood selenium concentrations of human populations (free living people) worldwide and Dakota population are 0.29–40.5 and 3.24 μM, respectively [25]. With high selenium intake, the additional selenium can enrich the methylselenol pool by the methylation pathway [26,27]. Interestingly, although 70–80% of the selenium compounds are released from CS nanoparticles within 1 h in a condition reminiscent of stomach pH, the selenium release can be greatly attenuated when co-coated with the zein protein [18]. Furthermore, the nature of high surface charge (+37 to 50 mV) in CS-encapsulated selenium compounds is likely to facilitate their absorption through the gastrointestinal tract [18], due to the molecular attractive forces formed by an electrostatic interaction between positively charged CS and negatively charged mucosal surface [28]. Thus, the selenium concentrations tested in the present study are likely to be within the physiologic level.

In summary, this study provides evidence that selenium compounds can be efficiently delivered to cells and exhibit lowered DNA damage response by encapsulation in CS nanoparticles. Development of the nanodelivery system of selenium compounds may improve selenium bioavailability and facilitate selenoprotein expression when selenium level is low. Thus, the novel selenium delivery system with increased specificity and decreased toxicity may have significant dietary and therapeutic intervention potential.

Acknowledgments

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