Functionalization of selenium nanoparticles with olive polyphenols – impact on toxicity and antioxidative activity

ABSTRACT

Selenium nanoparticles (SeNPs) represent novel selenium (Se) formulation characterized by improved biocompatibility and a wider therapeutic range in comparison to inorganic Se. The aim of this work was to investigate the possibilities of functionalization of SeNPs with olive pomace extract (OPE), rich in health-promoting polyphenols, and to obtain innovative forms of nutraceuticals. Cytotoxic and antioxidative activities of four types of SeNPs (polyvinylpyrrolidone stabilized (PVP SeNPs), polysorbate stabilized (PS SeNPs), polyvinylpyrrolidone stabilized and functionalized using OPE (f PVP SeNPs) and polysorbate stabilized and functionalized using OPE (f PS SeNPs)) were investigated. SeNPs showed lower toxicity on human hepatocellular carcinoma (HepG2) and human colorectal adenocarcinoma (Caco2) cells compared to selenite. Functionalization with polyphenols significantly improved their direct antiradical (f PVP SeNPs: 24.4 ± 1.84 and f PS SeNPs: 30.9 ± 2.47 mg TE/mmol Se) and reducing properties (f PVP SeNPs: 50 ± 3.16 and f PS SeNPs: 53.6 ± 3.22 mg GAE/mmol) compared to non-functionalized SeNPs. The significant impact of tested SeNPs on intracellular antioxidative mechanisms has been observed and it was dependent on both cell type and physicochemical properties of SeNPs, indicating the complexity of involved mechanisms.

Keywords: selenium nanoparticles, polyphenols, cytotoxicity, antioxidative activity, reactive oxygen species, reduced glutathione

Selenium (Se) is an essential nutrient and its biological role is primarily related to redox metabolism, thyroid function, and the immune system (1–3). The absorption of Se takes place in the small intestine; organic forms and selenate utilize active transport while selenite appears to cross the intestinal barrier by passive diffusion (4). Organic Se forms have higher bioavailability compared to inorganic Se (5), which are additionally characterized by a narrow therapeutic range (6). It has been reported that supplementation with Se...
has beneficial effects on antioxidative status (7) and has been additionally investigated in cancer prevention, however with unclear conclusions (8, 9).

Selenium nanoparticles (SeNPs) are a relatively new type of Se formulation that gained much attention due to their more favorable properties compared to inorganic and organic Se forms. In the nano form, Se is present as elemental Se (0), which is formed by the reduction of inorganic Se salts. SeNPs are characterized by a high surface-to-volume ratio, giving them unique physico-chemical properties. It has been shown that SeNPs have enhanced biological activity and improved biocompatibility and bioavailability compared to other forms (10–12).

The ability to functionalize the surface of SeNPs led to the investigation of their application as nutritional supplements, therapeutic agents, or drug delivery vehicles. Nowadays, greener synthesis approaches are being intensively investigated, and they often include plant-mediated nanoparticle generation, where plant extracts rich in bioactive compounds are used as reducing compounds or stabilizing agents. This approach can result in higher yields, lower ecological footprint of the process, and improved properties of NPs including size, stability, and the possibility of surface modification.

Olive pomace is a food waste remaining in large amounts after olive oil production and it is a source of valuable health-promoting components, such as tyrosol, hydroxytyrosol, and oleuropein (13, 14). As shown in our previous studies, polyphenol-rich olive pomace extract (OPE) with emphasized antioxidative efficiency can be obtained from olive pomace by utilizing efficient green extraction techniques (15–17). It has been shown that OPE has a strong antioxidative effect in vitro, primarily through radical scavenging activity and GSH preservation. Polyphenols found in olives have further been recognized as antimicrobial and anti-inflammatory agents (18, 19).

Since previously obtained results indicated successful functionalization of SeNPs with OPE-derived bioactive compounds (20), the aim of this study was to investigate the impact of functionalization on the biological properties of SeNPs focusing primarily on their antioxidative- and cytotoxic activity. Namely, available in vitro research shows that SeNPs can exert cytotoxic effects through induction of oxidative stress which in turn leads to DNA damage, apoptosis, and necrosis (21–24). On the other hand, they also show chemopreventive, anti-inflammatory, and antioxidative effects both in vitro and in vivo (25–28). The biological response to SeNPs is therefore highly dependent on applied concentrations and before mentioned physicochemical characteristics (that can be modified through functionalization). Considering the role of oxidative stress in the cytotoxicity of SeNPs and a high antioxidative potential of major OPE active compounds used for SeNP functionalization, we hypothesized that SeNPs functionalized with OPE would have reduced toxicity along with improved antioxidative properties and effects on cell models, compared to control SeNPs obtained through regular chemical synthesis.

EXPERIMENTAL

Chemicals and materials

Trolox® was purchased from the Tokyo chemical industry (Japan). ABTS reagents were purchased from Thermo Fischer Scientific (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-
-diphenyltetrazolium bromide (MTT) reagent was acquired from Carbosynth Limited (UK). Fetal bovine serum (FBS) and non-essential amino acid (NEAA) were purchased from Capricorn Scientific (Germany). DMSO was obtained from Kemika (Croatia). EDTA was purchased from Lonza (Switzerland). The rest of the chemicals and materials were purchased from Sigma-Aldrich (USA).

**Green synthesis and characterization of SeNPs**

The detailed SeNPs synthesis and characterization process has been described in our previous work (20) and the composition of reaction mixtures is presented in Table I. In brief, the synthesis consisted of the reduction of sodium selenite using ascorbic acid. Surface stabilization was achieved by using polyvinylpyrrolidone (PVP) or polysorbate 20 (PS), while OPE was used for additional surface modification of SeNPs. Four different types of SeNPs were synthesised – two types of functionalized SeNPs (f PS SeNPs and f PVP SeNPs) and their respective non-functionalised parallels obtained through standard chemical synthesis (PS SeNPs and PVP SeNPs. Obtained nano-systems were purified using dialysis membranes (dialysis tubing cellulose; MWC 14,000 Da).

<table>
<thead>
<tr>
<th>SeNPs</th>
<th>ASC (0.1 mol L(^{-1}))</th>
<th>PVP (1 %)</th>
<th>PS</th>
<th>OPE (1 %)</th>
<th>Na(_2)SeO(_3) (0.1 mol L(^{-1}))</th>
<th>UPW</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVP SeNPs</td>
<td>3.3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0.33</td>
<td>23.37</td>
<td>30</td>
</tr>
<tr>
<td>PS SeNPs</td>
<td>1.7</td>
<td>0</td>
<td>0.08</td>
<td>0</td>
<td>0.35</td>
<td>27.87</td>
<td>30</td>
</tr>
<tr>
<td>f PVP SeNPs</td>
<td>3.3</td>
<td>3</td>
<td>0</td>
<td>5</td>
<td>0.33</td>
<td>18.37</td>
<td>30</td>
</tr>
<tr>
<td>f PS SeNPs</td>
<td>1.7</td>
<td>0</td>
<td>0.08</td>
<td>5</td>
<td>0.35</td>
<td>22.87</td>
<td>30</td>
</tr>
</tbody>
</table>

SeNPs – selenium nanoparticles; ASC – L-ascorbic acid; PVP – polyvinylpyrrolidone; PS – polysorbate Tween\(^{®}\) 20; OPE – olive pomace extract; UPW – ultrapure water

**Determination of Se content**

Sample preparation included digestion of SeNPs in ultrapure water. The total content of Se was determined using an Inductively Coupled Plasma Optic Emission Spectrometer (ICP-OES; Perkin Elmer Optima 5300 DV, USA).

**Determination of Trolox equivalent antioxidative capacity (TEAC) and total reducing potential of SeNPs**

Antiradical activity of SeNPs was investigated using TEAC assay which is based on the decrease in absorbance of 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS\(^{+}\)) radical in the presence of the radical-scavenging compound (29). The ABTS\(^{+}\) radical was prepared by mixing 7 mmol L\(^{-1}\) solution of ABTS with 1.63 mmol L\(^{-1}\) potassium persulfate (1:1). The reaction took place for 12 to 16 h, at room temperature, protected from
light. Prior to experiments, the ABTS+ radical solution was diluted to the absorbance value of approximately 0.7. The assay was performed in a 96-well plate by mixing the ABTS+ radical with SeNPs or ultra-pure water for blank control. The microplate was shaken for 60 s, and incubated for 90 s at 30 °C, after which the absorbance was measured at 750 nm using Victor X3 (Perkin Elmer). Trolox® concentrations in the range from 3–100 mg L⁻¹ were prepared to obtain a standard curve. The results were expressed as the amount of Trolox® equivalents per mmol of Se (mg TE/mmol Se).

The total reducing potential of the SeNPs was analyzed by Folin-Ciocalteu (FC) assay (30). Briefly, 50 µL of 0.2 mol L⁻¹ water solution of FC reagent was mixed with 20 µL of SeNPs or ultra-pure water as blank in a 96-well plate. The plate was shaken for 60 s and incubated for 5 min at 37 °C. Then, 160 µL of 0.7 mol L⁻¹ sodium carbonate was added to each well and the plate was shaken for an additional 60 s and incubated for 30 min at 37 °C. The absorbance was measured at 750 nm using Victor X3. A range of concentrations of gallic acid (3–100 mg L⁻¹) were prepared to obtain a standard curve. The results were expressed as the amount of gallic acid equivalents in mmol of Se (mg GAE/mmol Se).

**Cell culture**

Cell lines used in this study were human hepatocellular carcinoma (HepG2) and human colorectal adenocarcinoma (Caco2). The cells were grown in controlled conditions in a cell culture incubator at 37 °C and 5 % CO₂. HepG2 were cultured in Eagle’s Minimum Essential Medium (EMEM) supplemented with 10 % FBS, 1 % Antibiotic/Antimycotic 1 % NEAA, and 4 mmol L⁻¹ L-glutamine. Caco2 were cultured in Dulbecco’s Modified Eagle’s Medium (supplemented with 20 % FBS, 1 % A/A, 1 % NEAA, and 4 mmol L⁻¹ L-glutamine. The media was changed every few days. After the cells reached about 80 % confluency, they were detached from the flask surface using 1× trypsin-ethylenediaminetetraacetic acid (EDTA; 2.5 % in Hanks’ Balanced Salt Solution (HBSS)w/o Ca, Mg) solution and seeded in 96 well plates 20 000 cells per well (hemocytometer, Germany). The cells were then allowed to rest for 48 h.

**Determination of SeNPs cytotoxicity**

Potential cytotoxicity of SeNPs was investigated by measuring their effect on intracellular ROS formation and intracellular content of GSH.

To measure the relative amount of reactive oxygen species (ROS) in cell cultures, 2',7'-dichlorofluorescin diacetate (DCFH-DA) assay was performed. The non-fluorescent form of DCFH-DA dye can enter the cell, after which the acetate group is cleaved (DFCH) which renders it unable to exit across the membrane. In the next step, the DCFH reacts with ROS which results in the fluorescent form of the dye (DCF), and its fluorescence can easily be measured (31). In the experiment, the media was removed, and the cells were incubated with 25 µmol L⁻¹ DCFH-DA for 45 min. The excess of dye was then removed and the cells were treated for 3 h with SeNPs or sodium selenite dissolved in PBS. Negative controls were incubated with PBS and positive controls were treated with 100 µmol L⁻¹ of tert-butyl hydroperoxide (tBOOH) in PBS. The fluorescence was then measured at 485/535 nm using Victor X3. For relative quantification of GSH, monochlorobimane (mBCl) assay was carried out. The assay is based on reduction of non-fluorescent form of dye to a fluorescent form,
in a reaction with GSH (32). The culture medium was removed followed by the addition of the fresh medium. The cells were treated with SeNPs or sodium selenite for 3 h. Negative controls were treated with the same volume of ultra-pure water and positive controls were treated with 100 µM of tBOOH. The treatment-solutions were removed and the cells were washed with PBS, followed by the addition of 40 µmol L⁻¹ mBCl reagent in PBS. After 30 min of incubation, the fluorescence intensity was measured at 355/460 nm, using Victor X3.

**Determination of SeNPs antioxidative activity in cell culture model**

Efficiency of SeNPs against chemically induced oxidative stress was investigated by measuring the viability of the cells previously incubated with SeNPs and after the exposure to the prooxidant.

For this purpose, two methodological approaches were used. In the first, the cells were treated with non-toxic concentrations of SeNPs or sodium selenite for 24 h. The negative and positive controls were treated with the same volume of ultra-pure water. On the next day, the test cells and positive controls were treated with 300 µmol L⁻¹ tBOOH prepared in PBS, for 5 h. To the wells with negative controls, the same volumes of PBS were added. The media was then removed and the cells were washed once with PBS. The MTT reagent was added and the plates were incubated for 3 h. PBS was used to dissolve the formazan crystals and the plate was put on a shaker for 45 min at 350 rpm. The absorbance was measured at 520 nm using Victor X3.

In the second protocol, fluorescent DCFH-DA assay was used to investigate ROS content in the cells pre-incubated with SeNPs and followed by the addition of prooxidant. The cells were treated with non-toxic concentrations of SeNPs or sodium selenite for 24 h. The negative and positive controls were treated with the same volume of ultra-pure water. On the next day, the media was removed and cells were incubated with 25 µmol L⁻¹ DCFH-DA for 45 min. The excess of dye was removed and the test cells and positive controls were treated with 100 µmol L⁻¹ tBOOH for 3 h. Negative controls were incubated with the same volume of PBS. The fluorescence was measured at 485/535 nm using Victor X3.

**Data analysis**

All experiments were performed in quadruplicates. Results were expressed as average values of measurements and standard deviation. Data was processed using Microsoft Office Excel (USA) and GraphPad® Prism 6 Software (USA). The analysis included a one-way analysis of variance (ANOVA) with a post hoc Tukey test of multiple comparisons. The differences between groups were considered statistically significant if $p < 0.05$.

**RESULTS AND DISCUSSION**

As explained previously, the SeNPs analyzed within this work were obtained by ascorbic acid-mediated reduction of sodium selenite in the presence of PVP or PS as stabilization agents. Further modification of SeNPs’ surface was achieved by bioactive compounds from OPE used in the synthesis process as described in our previous work (20). We hypothesized that SeNPs would show lower toxicity and improved antioxidative activity
in relation to inorganic Se forms (selenium) and furthermore that the surface modification with OPE-derived antioxidants should result in improved reducing and radical-scavenging properties of functionalized SeNPs in comparison to their respective non-functionalised parallels.

**Cytotoxicity of SeNPs**

The first step in testing the biological activity of novel compounds is their toxicity assessment. In this work, we compared the toxicity of novel SeNPs to inorganic Se forms and investigated the significance of SeNPs’ surface functionalization. Since it is known that SeNP's toxicity is in great part related to oxidative stress induction, we investigated their impact on the cellular content of ROS and GSH. The aim was to assess the changes in short-term redox equilibrium since GSH is one of the first non-enzymatic lines of cellular defense against oxidative injury (33). The three cysteine residues that constitute GSH make it a very potent antioxidant that directly scavenges free radicals (34).

As presented in Fig. 1, the increase in ROS content as a response to treatment with either selenite or SeNPs (15 mg mL\(^{-1}\)), was not observed in any of the cell models evaluated in this work, indicating low short-term cytotoxicity of tested Se formulations. The average values of ROS content in HepG2 cells treated with SeNPs were slightly increased in comparison with control cells, however, observed changes were not statistically significant. Similar observations were made in the Caco2 cell model. The described results are in accordance with our previous work on Caco2 cells, where we also did not detect elevations in ROS as a result of SeNPs treatment (35).

Data presented in Fig. 2 shows significant differences in the average values of GSH content in the cells treated with sodium selenite compared to corresponding controls. Depletion of GSH is evidenced in both cell types, but is more pronounced in HepG2 cells, where GSH values, after the treatment with sodium selenite, were reduced to 15.43 ± 5.84 % of normal GSH levels observed in negative controls. This is consistent with one of the previous studies on *in vitro* toxicity of sodium selenite, where GSH impairment was also observed in the HepG2 cells treated with sodium selenite (36). Caco2 cells were proved to be less sensitive to GSH depletion, with GSH levels being reduced to 62.65 ± 3.99 %

![Fig. 1. Relative content of cellular ROS after the treatment with SeNPs in: a) HepG2 and b) Caco2 cells assessed by DCFH-DA assay.](image_url)
compared to those observed in non-treated control cells. It can be assumed that GSH was depleted in reaction with sodium selenite in the process of protecting the cells from elevation of ROS and oxidative stress induction. Interestingly, the treatment with SeNPs did not reduce GSH content in the HepG2 cells. Conversely, Zheng et al. (37) reported elevated levels of superoxide radical in HepG2 cells treated with SeNPs, while in vivo study on SeNPs performed by Guo et al. (38) showed a reduction in antioxidant levels including GSH, SOD, GPx, and catalase. These findings suggest that the prooxidative effect of SeNPs is variable and dependent on the identity and characteristics of the type of SeNPs evaluated and the type of cells or tissues that interact with nanoparticles. In the case of Caco2 cells, a significant reduction of GSH was observed only in the case of treatment with PS SeNPs. Surface stabilization of this type of nanoparticles is achieved by using surfactant polysorbate 20. It can be assumed that in complex environments such as cell systems and culture media, a certain amount of polysorbate molecules is being released from NPs’ surface. Polysorbate 20, as well as some other surfactants, can influence the solubility of membrane components, and in turn enhance their permeability (39, 40). Furthermore, disruptions in membrane integrity and the small diameter of PS SeNPs make them more potent for cellular internalization and consequently lead to cytotoxic effects (41).

Since it was noticed that in the case of treatment with f PS SeNPs, the average value of GSH content was not significantly different in comparison with control, it can be speculated that polyphenol modification of NPs’ surface modulates the response of Caco2 cells to SeNPs, by increasing their biocompatibility.

**Antioxidative activity of SeNPs**

The antioxidative activity of SeNPs was analysed in terms of their direct reducing- and antiradical activity in chemical models. Additionally, their ability to improve viability after exposure to prooxidant and decrease intracellular ROS in cell lines exposed to prooxidant was also investigated.

Results presented in Fig. 3 show that functionalized SeNPs f PVP and f PS had significantly higher antiradical properties (24.4 ± 1.84 and 30.9 ± 2.47 mg TE/mmol Se) in comparison with non-functionalized SeNPs (0.6 ± 0.48 and 7.30 ± 1.15 mg TE/mmol Se), respectively. The same trend was observed in results obtained by FC assay providing
insight into the total reducing capacity of SeNPs. Obtained results ranged from $14.9 \pm 0.48$ and $21.7 \pm 0.68$ mg GAE/mmol Se for PVP SeNPs and PS SeNPs to $50 \pm 3.16$ and $53.6 \pm 3.22$ mg GAE/mmol Se for $\ell$ PVP SeNPs and $\ell$ PS SeNPs, respectively. This can be explained by the high reducing and antiradical activity of OPE that has been reported previously (16), and also provides evidence of the successful binding of polyphenols to SeNPs’ surface. Other bioactive components derived from plant extracts have already been applied in SeNPs synthesis with the aim of enhancing their antioxidative properties. For example, glucan and rosmarinic acid have been utilized to stabilize SeNPs and improve their antiradical activity (42). Other studies have investigated the antioxidative properties of SeNPs generated using Emblica officinalis or Withania somnifera-derived extracts (43, 44). It was shown that these types of SeNPs are potent at scavenging free radicals. Another promising strategy to improve the antioxidative activity of SeNPs is to use plant or microbe-derived polysaccharides. Qui et al. (45) have synthesised SeNPs using pectin and reported strong radical scavenging activity, while Liu et al. (46) utilized Oudemansiella radicata derived polysaccharide and showed enhanced activity of SeNPs against free radicals DPPH and ABTS*, compared to control SeNPs prepared without the polysaccharide.

The experimental setup for antioxidative assays conducted in HepG2 and Caco2 cells included pretreatment of cells with non-toxic concentrations of SeNPs for 24 h (at two concentration levels), followed by the addition of prooxidant tBOOH, as described previously by Radić et al. (17). Non-toxic SeNPs concentrations were determined in our previous study, where we tested the impact of SeNPs on cell viability in HepG2 and Caco2 cells after 24 h-exposure (20). Analyzed SeNPs showed cytoprotective effects that could be attributed to, both direct scavenging of tBOOH or cellular response to pretreatment with Se formulations, which consequently protects the cells from prooxidant effects of tBOOH. As shown in Fig. 4, the cell viability of both cell lines was significantly higher in the case of pretreatment with SeNPs compared to positive controls that were only incubated with tBOOH. Interestingly, all tested nanoparticles exhibited protective effects, independent of their direct antiradical and reductive potential (presented in Fig. 3) indicating that the observed effect was due to the cellular response and not only the direct scavenging of tBOOH by SeNPs. Furthermore, as evident in the case of HepG2 cells, observed effects were concentration-dependent, where lower SeNP concentrations had a more pronounced protective
effect. This suggests that lowering the burden of SeNPs makes the cells more resilient to the prooxidative effects of tBOOH. This is in accordance with the results obtained by Cheng et al. (47), who designed and tested the antioxidative properties of SeNPs in IPEC-J2 cells. Evaluated SeNPs have also shown protective effects in Caco2 cells, independent of
concentration applied, except in the case of PSSeNPs. This is consistent with prooxidative effects that were shown to deplete GSH levels in Caco2 cells (Fig. 2). Protective effects in colon epithelial cells against oxidative damage by biogenically synthesized SeNPs have also been shown by Xu et al. (48). Results also show that the viability of cells pretreated with sodium selenite is comparable to non-treated control cells, indicating a strong protec-

Fig. 5. Relative content of ROS in: a) HepG2 and b) Caco2 cells pretreated with SeNPs or selenite followed by treatment with tBOOH.
tive effect. Having in mind the prooxidative effect of selenite (Fig. 2), this protective effect could be the result of a redox reaction that might occur between tBOOH and selenite. Namely, as described in the Experimental section, the prooxidant is added directly to the media containing sodium selenite, enabling such reaction. Results obtained by Thiry et al. (49), who investigated the absorption of selenite in Caco2 cells, showed that the majority of selenite remained in culture media and was not absorbed by the cells, further supporting our assumption.

Another indicator of SeNPs’ antioxidative activity was the relative content of intracellular ROS measured in SeNP-pretreated cells, after exposure to prooxidant tBOOH. The methodology in this case includes the removal of SeNPs from the cells prior to the addition of tBOOH as described in the Experimental section. Therefore, the observed effect of SeNPs or selenite is solely due to the potential induction of cellular response to prooxidant treatment. As presented in Fig. 5, in HepG2 cells, the protective effects of SeNPs were observed in the cases of pretreatment with higher concentrations of SeNPs. It can be hypothesized that higher concentrations of SeNPs led to higher cellular internalization (50), which consequently resulted in the observed protective effects. Different responses when observing the applied concentration could be seen in Caco2 cells. Since observed effects are dependent on the absorbed SeNP fraction, it can be hypothesized that Caco2 cells, that are able to fully polarize into differentiated monolayers displaying brush border (microvilli), internalize SeNPs more efficiently, leading to noticeable protective efficiency at lower SeNP concentrations.

Our results are consistent with other available (even though scarce) data about the antioxidative efficiency of SeNPs. Zhai et al. (51) have demonstrated results similar to those obtained in our study, where the ROS formation induced by incubation with prooxidant was significantly decreased after the pretreatment with SeNPs. Song et al. (52) have further contributed to understanding the mechanisms involved in observed effects by identifying the changes in expression of Nrf2 and its downstream genes after exposure of cells to SeNPs.

CONCLUSIONS

The Se nanoformulations analysed in this work did not induce oxidative stress in terms of elevation in ROS content and didn’t negatively impact cellular levels of GSH (with the exception of polysorbate-stabilized, non-functionalized SeNP). SeNPs have been shown to have a significantly reduced prooxidative effect compared to sodium selenite. The functionalization of SeNPs using OPE resulted in improved direct antiradical and reductive capacity. Cell-based tests showed that SeNPs show protective effects in the form of protecting the viability of cells after exposure to prooxidants, independently of their direct antiradical activity. The significant impact of SeNPs on cell resistance to prooxidant exposure, measured by impact on intracellular ROS, was more pronounced in Caco2 cells, probably due to more efficient internalization of SeNPs in comparison to HepG2 cells.

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project administration TV; funding acquisition TV.

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