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Original Contribution

FREE RADICAL SCAVENGING EFFICIENCY OF NANO-SE IN VITRO

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Abstract—In this study, we showed that smaller size particles of Nano-Se have better scavenging effects on the following free radicals: carbon-centered free radicals ($R^{\bullet}$) generated from 2,2'-azo-bis-(2-amidinopropane) hydrochloride (AAPH), the relatively stable free radical 1,1-diphenyl-2-picryhydrazyl (DPPH), the superoxide anion ($O_2^{\bullet -}$) generated from the xanthine/xanthine oxidase (X/XO) system, singlet oxygen ($^1O_2$) generated by irradiated hemoporphyrin. Furthermore, the three sizes of Nano-Se studied also show protective effects against the oxidation of DNA. The three samples all have potential size-dependent characteristics on scavenging the free radicals. Although in this study we regarded Nano-Se as a whole without considering interactions between BSA and the red selenium nano-particles, there is the possibility that the apparent free radical scavenging effects may be partially contributed by such interactions.

Keywords—Nano red elemental selenium (Nano-Se), Free radicals, Bovine serum albumin (BSA), Size-dependent, Nanoparticles-protein interactions

INTRODUCTION

Selenium, as one of the essential elements for the health of mammalian animals, has key functions in the balancing of the redox system, the proper functions of the immune system, and anticarcinogenetic effects [1–6]. The supplementation of food with selenium is usually limited to selenides, such as sodium selenite (Na_2Se0_3), ebselen, and other selenium-containing organic compounds. These seleno-compounds comprise redox states of +6, +4, and −2. However, a few studies have been focused on Se in zero redox state (Se^0).

It is known that gray and black elemental Se are biologically inert, which may be due to their insolubility. However, there is one kind of red elemental particulate selenium, observed in several kinds of bacteria, that has promising uses in the environmental protection from the pollution of the excessive selenium [7–9]. These elemental selenium particles are formed in the bacteria with the detoxification of excess selenium and have only about 2% bioavailability compared with selenite.

Nano red elemental selenium (Nano-Se) with the size range of 5–100 nm, can be synthesized by reducing selenite in an environment containing bovine serum albumin (BSA), which is able to adhere to Se atoms and control the size of their aggregation [10]: the sizes of the nanoparticles are dependent on BSA concentration in the preparation system. BSA at higher concentration produces smaller Nano-Se particles, hence a series of Nano-Se particles of different sizes were prepared by varying the concentration of BSA. It is well known that some materials have many new properties when particle sizes are reduced to the nanometer scale, such as large surface area, quantum effects, and high reactivity. In the work of Zhang and his colleagues, it was shown that Nano-Se has a similar bioavailability in rat, and much less acute toxicity in mice compared with selenite [10]. The results suggest that the biological activities of Nano-Se may come from the special properties of the nanoparticles.

Here we were interested in investigating the different free radical savaging efficiencies of Nano-Se with different sizes: small size (5∼15 nm), medium size (20∼60 nm), and large size (80∼200 nm). Efficiencies were

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measured with the aid of an electron spin resonance (ESR) spectrometer and an ultra-weak luminescence recorder.

MATERIALS AND METHODS

Chemicals

Nano red elemental selenium particles (Nano-Se, the three sizes are abbreviated to S, M, and L, respectively) were synthesized by Dr. Jin-Song Zhang (University of Science and Technology of China). α-(4-pyridyl-1-oxide) N-t-butylnitrone (4-POBN), 2,2,6,6-tetramethyl-4-piperidone (TEMPONE), and 1,1-diphenyl-2-picryhydrazyl (DPPH) were purchased from Sigma (St. Louis, MO, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich (Milwaukee, WI, USA). 2,2'-azo-bis-(2-amidinopropane) hydrochloride (AAPH) was purchased from Wako Pure Chemical . Bovine serum albumin (BSA) was purchased from Boehringer Mannheim (Mannheim, Germany). Hemoporphyrin (HP), xanthine (X), diethyldithiocarbamate (DETC), 1,10-phenothroline (OP), ascorbic acid, 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol), and all other chemicals made in China were analytical grade.

Preparation of small, medium and large sizes of Nano-Se

One ml 25 mM sodium selenite was mixed with 4 ml 25 mM GSH containing 200, 20, and 2 mg BSA for the preparations of the small, medium, and large sizes Nano-Se, respectively. The pH of the mixture was adjusted to 7.2 with 1.0 M sodium hydroxide, during which the red elemental Se and oxidized glutathione (GSSG) formed. The red solution was dialyzed against doubly distilled water for 96 h with the water changing every 24 h to separate GSSG from Nano-Se. The final solution containing Nano-Se and BSA was lyophilized and stored at room temperature. Transmission electron microscopy (TEM) showed the sizes of red elemental Se were 5~15 nm (small size, designed as S), 20~60 nm (medium size, designed as M) and 80~200 nm (large size, designed as L). The concentrations of Se were tested using the fluorescent method developed by Watkinson [11].

Assay for the carbon-centered free radicals generated from AAPH

This assay had the same protocol as described in [12], with some modification. The reaction mixtures containing 20 mM AAPH, 0.1 M 4-POBN, and various concentrations of Nano-Se were incubated at 45°C in water bath for 30 min, and then the ESR spectra of the carbon-centered free radicals were recorded.

DPPH radical assay

This assay had the same protocol as described in [12], with some modification. DPPH was dissolved in ethanol to give a 500 μM solution and mixed with the same volume of Nano-Se for 120 s the ESR spectra were recorded. In the time scan experiment the ESR spectra were recorded every 2 min from 2 min to 10 min.

Superoxide anion assay

This assay had the same protocol as described in [12], with some modification. Superoxide anion was generated by the X/XO system. The reaction mixtures containing 0.133 mM xanthine, 6.67 mU/ml xanthine oxidase, 0.143 mM luminol (dissolved by 100 mM Na_2CO_3/$NaHCO_3$ buffer, pH 10), and Nano-Se. The chemiluminescence intensities were recorded with a WDD-1 luminescence recorder just after 20 s the reagents were mixed. The scavenging effects of Nano-Se were calculated by

$$\textit{Scavenging Percent} = \frac{I_0 - I_x}{I_0} \times 100\%$$

Here I_x and I_0 were the chemiluminescence intensities of samples with and without Nano-Se, respectively.

Singlet oxygen assay

This assay had the same protocol as described in [12], with some modification. Singlet oxygen was generated by the irradiated hemoporphyrin/EDTA system. The reaction mixtures containing 1.60 mM hemoporphyrin, 80 mM TEMPONE, and Nano-Se were transferred to a quartz capillary and fitted into the cavity of the Varian-109 ESR spectrometer. The mixtures were irradiated by a Xe lamp (power 1 kW and at a distance of 70 cm) at room temperature and the ESR spectra were recorded every 30 s.

Nitric oxide assay

The investigation of nitric oxide (NO) scavenging effects of Nano-Se had the same protocol as described in [13], with some modification. The reaction mixture containing 100 μl NO-saturated water and 500 μl Nano-Se of various concentrations was put in the dark for 30 min for reaction to take place. Then were added 250 μl $Na_2S_2O_4$ (20 mM), 250 μl Fe^{2+} (6 mM) and 250 μl DETC (12 mM), respectively. After reacting for 10 min, 1 ml ethyl acetate (saturated by nitrogen before use) was added for extraction. The samples were centrifuged at 15,000 $\times$ *g* for 15 min; the ESR spectra of the organic layers were recorded at room temperature.

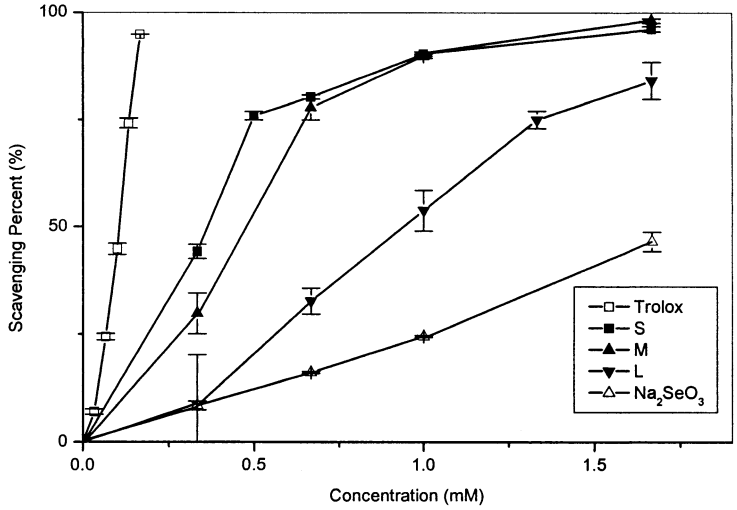

Fig. 1. The dose-dependent and size-dependent scavenging effects of three sizes of Nano-Se on the free radicals generated from AAPH. Trolox, Na_2SeO_3 were control groups and the effects of Nano-Se are size-dependent: S > M > L. The data are shown as mean ± SD (n = 3).

ESR experiments

The samples were transferred to a quartz capillary and placed in the cavity of a Varian-109 ESR spectrometer.

The conditions used to obtain ESR spectra were as follows: center field 0.3327 T, microwave frequency 100 kHz, microwave power 20 mW, modulation amplitude 2 gauss, scan width 200 gauss, time constant 0.128 s, temperature 298 K.

The scavenging effects of Nano-Se on the free radicals were calculated by

$$\textit{Scavenging Percent} = \frac{H_0 - H_x}{H_0} \times 100\%$$

Here H_0 and H_x were the average ESR signal intensities (n = 3) of samples with and without Nano-Se, respectively. And the data were expressed as mean ± SD.

The three sizes of Nano-Se had no detectable ESR signals near the center field.

Protection of DNA against oxidative damage by Nano-Se

The investigation of protection of DNA against damage by Nano-Se had the same protocol as described in [14]. One milliliter (525 μM/75 μM) OP/Cu^{2+} and 150 μl (1 μg/ml) DNA were premixed, then 150 μl (3 mM) ascorbic acid and 200 μl (3%) H_2O_2 were added to initiate the luminescence reaction. Emission intensities were recorded with a BPCL-4 ultra-weak chemiluminescence analyzer with a computerized high-sensitivity single-photon counter (SPC) at 20°C. The voltage in the photo-multiplier was kept 1000 V and the spectral range of chemiluminescence recorded was 340–800 nm. Before measuring the effects of Nano-Se on the chemiluminescence from DNA oxidation, different concentrations of Nano-Se were premixed with $Cu^{2+}(OP)_2$/DNA solution, respectively. Then the chemiluminescent measurement was recorded as described above.

Roles of pure BSA in free radical scavenging effects

The content of BSA in the sample S, M, and L of 5 mM was 40 mg/ml, 4 mg/ml, and 0.4 mg/ml, respectively.

The roles of pure BSA were evaluated by the scavenging effects on DPPH and free radicals generated from AAPH. The conditions of ESR experiments were all the same as above.

Statistical analysis

Data were presented as the mean ± SD. The differences between the groups were examined using Student's t-test. A p value of less than .05 was considered statistically significant.

RESULTS

Scavenging activities of Nano-Se on the free radicals generated from AAPH

The dose-dependent scavenging effects on the free radicals generated from AAPH are shown in Fig. 1. The IC_{50} values of trolox, S, M, L, and Na_2SeO_3 were 0.10 mM, 0.36 mM, 0.48 mM, 0.94 mM, and 1.70 mM, respectively.

There was one potential size-dependent sequence of Nano-Se on scavenging free radicals generated from AAPH: S and M had similar effects and were both better

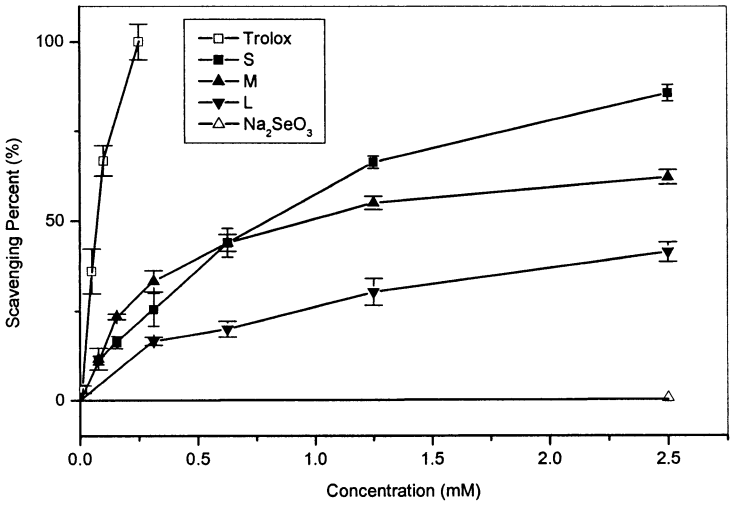

Fig. 2. The dose-dependent and size-dependent scavenging effects of three sizes of Nano-Se on DPPH. Trolox, Na_2SeO_3 were control groups and the effects of Nano-Se are size-dependent: S > M > L. The data are shown as mean ± SD (n = 3).

than L. And the relative difference between M and L was more significant at low concentrations than that at high concentrations.

Scavenging effects of Nano-Se on DPPH

The dose-dependent scavenging effects of Nano-Se on DPPH were evaluated. As shown in Fig. 2, trolox was very strong and the Nano-Se only had medium effects and Na_2SeO_3 had no effect. The IC_{50} values of trolox, S, and M were 0.070 mM, 0.80 mM, and 0.95 mM, respectively. At the concentration of 2.5 mM, L had 41.5 ± 2.7% scavenging effect.

There was also one potential size-dependent sequence of Nano-Se on scavenging DPPH: S and M had similar effects at low concentrations and were both better than L; while the differences between S and M became significant at higher concentrations.

The time-dependent scavenging effects of the three sizes of Nano-Se were studied as shown in Fig. 3. With increased time, more DPPH was scavenged by Nano-Se. The interactions of Nano-Se with DPPH were also size-dependent: S was the fastest, and L was the slowest.

Scavenging activities of Nano-Se on superoxide radical

Superoxide radicals were generated by the X/XO system, which is a biological model in vitro, and the scavenging effect of Nano-Se on superoxide radical was evaluated in this system. The apparent scavenging effects

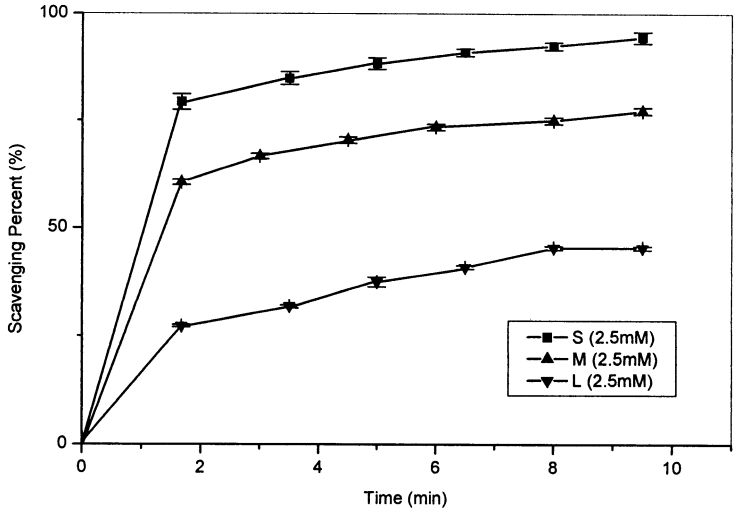

Fig. 3. Comparison of the time-dependent scavenging effects of Nano-Se on DPPH. The data are shown as the mean ± SD (n = 3).

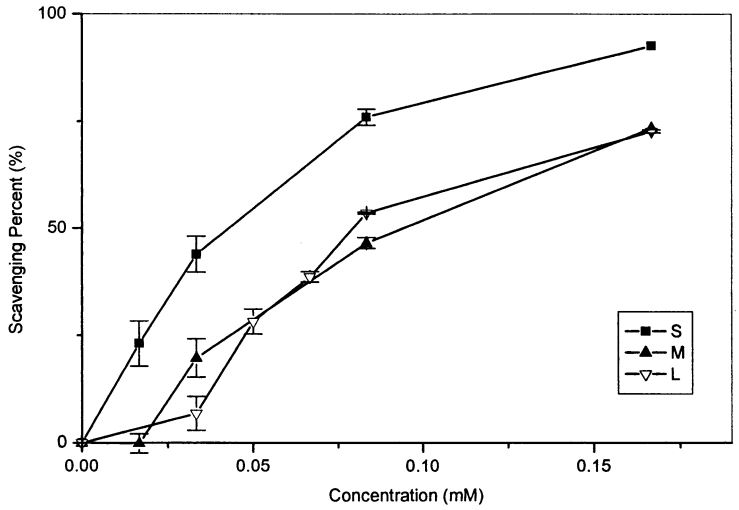

Fig. 4. The scavenging effect of Nano-Se on superoxide radical. The data are shown as the mean ± SD (n = 3).

of Nano-Se were dose-dependent. The results in Fig. 4 showed that S was the most efficacious one and both M and L are not significantly different in their capacities for scavenging superoxide radical. The IC_{50} values of the S, M, and L are 40, 90, and 80 μM, respectively.

Scavenging effects of Nano-Se on singlet oxygen

The singlet oxygen was generated by the irradiated hemoporphyrin/EDTA system. The scavenging capacities were evaluated by the decreased area of the scavenging curves compared with the control group: different patterns of the scavenging curves indicate different mechanisms for scavenging singlet oxygen.

In Fig. 5, M and L are compared at two indicated concentrations: M shows a better scavenging effect on singlet oxygen. The heights and the widths of the curves for M are both less than those of L. The ratio of the efficiencies of M and L was 2:1.

In order to compare with the other singlet oxygen scavenging reagents, BSA, Na_2SeO_3 and trolox were also tested (see Table 1). The results are shown in Fig. 6. Trolox is a strong antioxidant and its effect in the concentration of 0.20 mM, could match the 0.50 mM of L. Na_2SeO_3 has nearly an equal effect with L in the concentration of 1.0 mM. BSA in 1.0 mg/ml was even stronger than 0.50 mM of M.

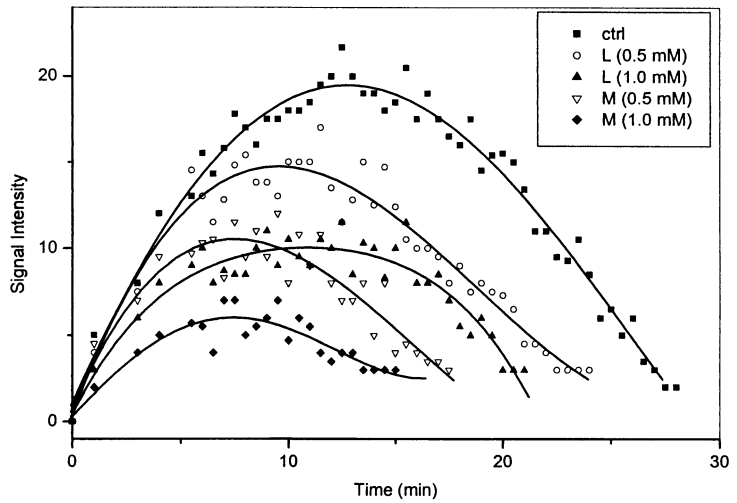

Fig. 5. Dose-dependent and size-dependent effects of Nano-Se on scavenging singlet oxygen. The curves on the graph are polynomials fitted to the original data. The analyses of the curves are shown in Table 1. For a given concentration of Se, there is one potential sequence of Nano-Se on scavenging singlet oxygen: M > L.

Table 1. Analyses of the Scavenging Curves in Figures 5 and 6

Sample	Area	Peak at (min)	Width (min)	Height
Control	363.5	13.0	19.2	19.5
L-0.50 mM	235.3	9.5	16.5	14.7
Trolox-0.20 mM	183.7	8.2	14.4	13.7
Na_2SeO_3-1.0 mM	173.0	8.2	19.2	10.0
L-1.0 mM	159.1	10.9	17.1	10.0
M-0.50 mM	131.2	7.2	12.9	10.5
BSA-1.0 mg/ml	91.9	4.7	11.0	9.0
M-1.0 mM	68.7	7.5	11.7	6.0

Areas under the curves indicate the total amounts of singlet oxygen generated in the reaction system. The peaks indicate how fast the maximum chemiluminescence was reached and the heights of the peaks indicate the maximum concentrations of singlet oxygen. The widths indicate the lifetime of singlet oxygen. There are size-dependent effects of Nano-Se on scavenging singlet oxygen: M > L.

Scavenging activities of Nano-Se on nitric oxide

The scavenging effect of S was studied as shown in Fig. 7. It shows that S has a strong dose-dependent effect on NO.

Protection of DNA against oxidative damage by Nano-Se

The protection effects of antioxidants on DNA were evaluated by the decreased height and the delayed time of the chemiluminescence peaks. The results in Fig. 8 show that there is a potential sequence of the protection roles of the three Nano-Se against the oxidative damage: S > M > L.

Comparation with pure BSA in free radical scavenging effects

The content of BSA in the sample S, M, and L of 5 mM is 40, 4, and 0.4 mg/ml, respectively. In order to evaluate the effects of BSA in Nano-Se, we tested the effects of pure BSA and found it did have significant effects especially at high concentrations.

As shown in Fig. 9 using the concentrations of BSA as the X axis, the pure BSA has about 50% scavenging effects at the concentration of 13 mg/ml on scavenging free radicals generated from AAPH. With the concentrations getting lower, the effects dropped quickly to about 15% at 1 mg/ml and 2% at 0.1 mg/ml.

The similar effects of pure BSA on scavenging DPPH are shown in Fig. 10. The effects of BSA on DPPH dropped from nearly 50% at 20 mg/ml to 10% at 2 mg/ml and 1% at 0.2 mg/ml.

These two figures indicate that the influences of BSA on the scavenging effects decrease with increased size of the Nano-Se. In other words, S is influenced the most by BSA and L was almost not influenced.

DISCUSSION

In this study we regarded Nano-Se as a whole. Because BSA could not be isolated simply, BSA and the elemental Se were inseparable partners in Nano-Se. If BSA was separated out of the whole system of Nano-Se, the red elemental Se nano-particles would aggregate into bigger nano-particles, so we could only study the characteristics of Nano-Se as a whole.

As shown in Figs. 1 to 8, Nano-Se really has good free radical scavenging effects on different free radicals, such as DPPH, free radicals generated by APPH, superoxide anion, singlet oxygen, and NO. And as shown in Figs. 1 to 6 and 8, Nano-Se had one potential size-dependent sequence in both the free radical scavenging effects and the antioxidant effects; namely, the smaller, the better.

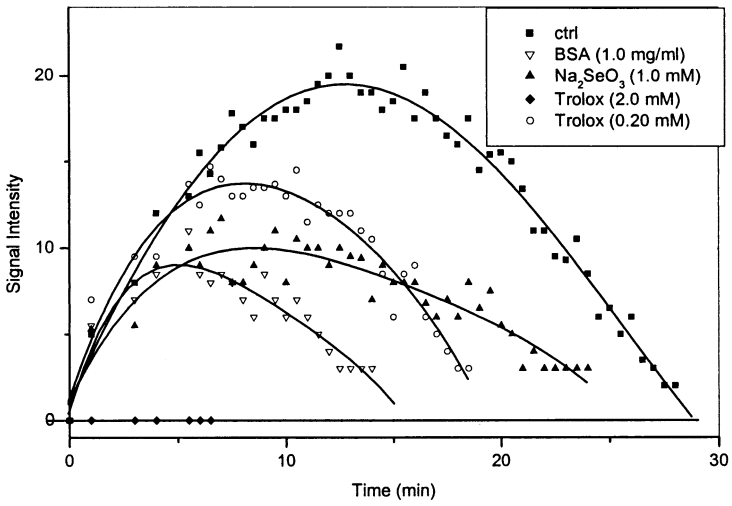

Fig. 6. Comparison of the scavenging effects of BSA (1.0 mg/ml), Na_2SeO_3 (1.0 mM) and trolox on singlet oxygen. The analyses of the curves are shown in Table 1.

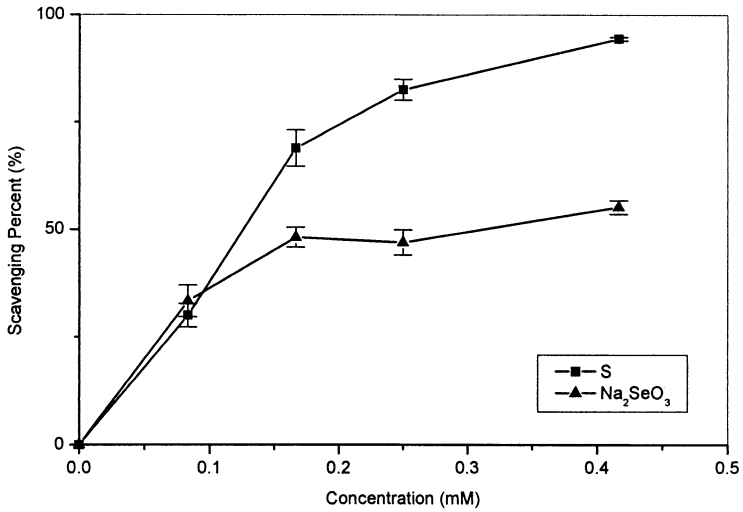

Fig. 7. Scavenging effect of S on nitric oxide. The data are shown as the mean ± SD ($n = 3$).

In order to make clear if the effects of Nano-Se come mainly from the red elemental Se nano-particles and not from BSA, we studied the effects of BSA. As shown in Figs. 9 and 10, at low concentrations of BSA, M and L still have quite high activities, which means that the main effects of Nano-Se really do come from red elemental Se nano-particles and their different sizes.

However, the data also suggest that there could be interactions between BSA and the red elemental Se nano-particles. These interactions may decide the thermodynamic and kinetic functions of Nano-Se on scavenging free radicals. The influences of BSA could not be simply subtracted. The free radical scavenging effects of the Nano-Se may be enhanced by the interactions between nanoparticles and BSA. So we may assume that nanoparticles could interact with the other proteins in vivo and result in some harmful consequences.

Nanotechnology is nowadays thought to be a highly promising field just like biotechnology [15]. The most attractive aspect is the special characteristics resulting from the nanometer scale producing huge surface areas and the quantum effects.

Nano-Se is a newly discovered material that is constructed by BSA and red elemental Se nano-particles. In this article, on the one hand, we proved that the elemental Se nanoparticles have significant effects both on scav-

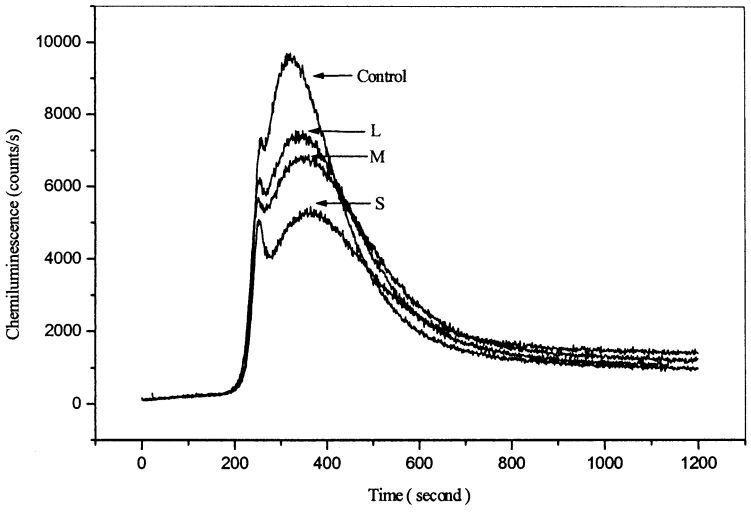

Fig. 8. Protection of DNA against oxidative damage by Nano-Se. At the same concentration of 33 μM Se, there is one size-dependent effect of Nano-Se: S > M > L.

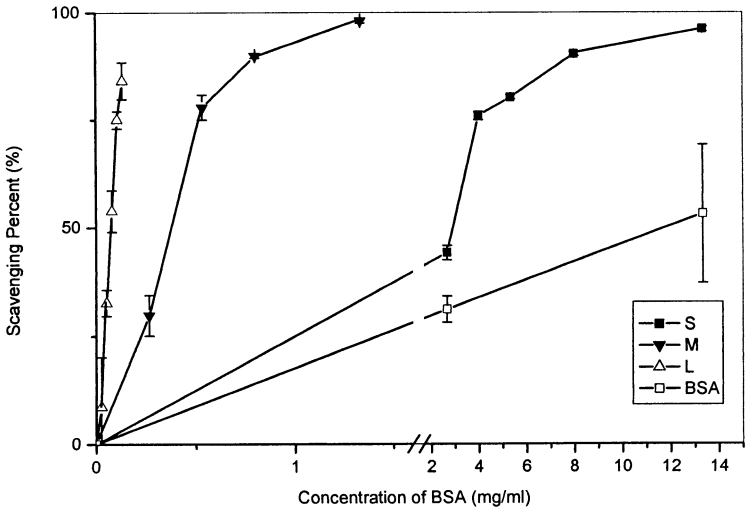

Fig. 9. Comparison with pure BSA of scavenging free radicals generated from AAPH. The X-axis here represents the concentrations of BSA. The content of BSA in the sample S, M, and L of 5 mM is 40 mg/ml, 4 mg/ml, and 0.4 mg/ml, respectively. S is influenced the most by BSA, M less and L the least.

enging the free radicals and protecting DNA from oxidation size dependently: the smaller, the better. On the other hand, these data could give several lines of implication for the interactions between nanoparticles and proteins in biological systems:

1. The sizes of red elemental Se nano-particles are dependent on the concentration of BSA: we found that BSA could be substituted by other proteins. The BSA/Se (w/w) ratio in S was 100:1, it is postulated that there could be an even higher ratio in vivo, which may lead to the formation of smaller Nano-Se with enhanced efficiency in scavenging free radicals. So far the putative anticarcinogenic effect of Se could be partially explained by the selenoproteins [16]. But selenoproteins can not explain the effect wholly: even though a daily dose of 50 μg Se for adults is sufficient to saturate selenoproteins, an additional 200 μg Se supplement is still necessary to achieve an obvious chemproventive effect [17]. Here we can give one optional hypothesis to this question: the supra-nutritional dose Se may be necessary partially as Nano-Se, which can stick to proteins in vivo and increase the local concentrations of Se on proteins, which leads

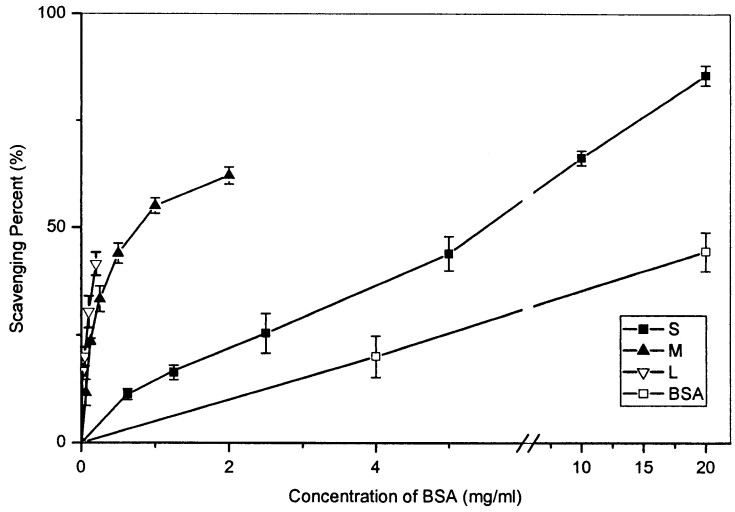

Fig. 10. Comparison with pure BSA on the scavenging of DPPH. The X-axis here represents the concentrations of BSA. S is influenced the most by BSA, M less and L the least.

to more efficient protective effects against free radicals and the subsequential anticarcinogenetic effects.

2. In addition to the potential advantages of Nano-Se in vivo, there are also potential disadvantages of Nano-Se. Because the adhesion of Nano-Se onto proteins is nonspecific, the adhesion may reduce or even disable the normal functions of the proteins, which will result in disturbed cellular processes. Thus, the safety aspect of the therapeutic use of nanoparticles needs to be considered.

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ABBREVIATIONS

AAPH—2,2'-azo-bis-(2-amidinopropane) hydrochloride
BSA—Bovine serum albumin
DETC—Diethyldithiocarbamate
DPPH—1,1-diphenyl-2-picryhydrazyl
ESR—Electron spin resonance
HP—Hemoporphyrin
IC_{50}—Fifty percent inhibition concentration
Luminol—5-amino-2,3-dihydro-1,4-phthalazinedione
Nano-Se—Nano red elemental selenium
OP—1,10-phenothroline
PBS—Phosphate-buffered saline
4-POBN—α-(4-pyridyl-1-oxide)-N-t-butylnitrone
Trolox—6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TEMPONE—2,2,6,6-tetramethyl-4-piperidone
X—Xanthine
XO—Xanthine oxidase