Mitochondria-Mediated Protein Regulation Mechanism of Polymorphs-Dependent Inhibition of Nanoselenium on Cancer Cells

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The present study was (i) to prepare two types of selenium nanoparticles, namely an amorphous form of selenium quantum dots (A-SeQDs) and a crystalline form of selenium quantum dots (C-SeQDs); and (ii) to investigate the nano-bio interactions of A-SeQDs and C-SeQDs in MCF-7, HepG2, HeLa, NIH/3T3, L929 cells and BRL-3A cells. It was found that A-SeQDs could induce the mitochondria-mediated apoptosis, necrosis and death of cells, while C-SeQDs had much weaker effects. This polymorphs-dependent anti-proliferative activity of nano-selenium was scarcely reported. Further investigation demonstrated that A-SeQDs could differentially regulate 61 proteins and several pathways related to stress response, protein synthesis, cell migration and cell cycle, including “p38 MAPK Signaling”, “p53 Signaling”, “14-3-3-mediated Signaling”, “p70S6K Signaling” and “Protein Ubiquitination Pathway”. This was the first report to demonstrate the involvement of protein synthesis and post-translational modification pathways in the anti-proliferative activity associated with NMs. Compared with previously fragmentary studies, this study use a nanomics approach combining bioinformatics and proteomics to systematically investigate the nano-bio interactions of selenium nanoparticles in cancer cells.

Over the past several decades, a large number of nanomaterials (NMs) with metallic, non-metallic, and organic natures, are widely used in therapeutics, nanoimaging, and biosensing1–6. It has been shown that DNA conjugated gold nanoparticle networks have a potent antiviral ability and protect host cells from attack of respiratory syncytial virus7. Some non-metallic inorganic nano-species such as silica and carbon have been widely applied in drug delivery, biological imaging, and photothermal therapy8–12. Polyaniline and polypyrrole organic nanoparticles can be used as near-infrared light-absorbing agents for photothermal therapy of cancer13,14. In addition, NMs can be emitted into environment and food chains because of the combustion of fossil fuels and industrial production, raising a health concern about the potential toxicities of NMs15–19. Therefore, it is critical to understand the molecular mechanisms underlying the biomedical activities or the health risks of non-metallic inorganic and organic NMs.

Nanomics, aiming to study the interactions of nanomaterials with genes, proteins and other biomolecules in biological systems by integrating the proteomics, genomics, and metabonomics with bioinformatics, is a more and more attractive research field. Through protein classification and annotation, enrichment analysis and correlation analysis from omics and bioinformatics analysis, the data can be scientifically interpreted and the biological significance can be pinpointed20. In this regard, several studies about the nano-bio interactions based on omics techniques have been reported21–26. In addition, fascinating polymorphs-dependent bioactivity of NMs has been reported recently, such as copper sulphide nanoparticles27. However, the underlying mechanisms still remain unexplored. Meanwhile, selenium compounds attracted more and more attention in the past decade due...
to their high bioavailability and antioxidant activities, low toxicity, and novel therapeutic properties. But the polymorphs-dependent anti-proliferative activity of nano-selenium was scarcely reported.

In the current study, two nanoparticles were firstly prepared, namely amorphous selenium quantum dots (A-SeQDs) and crystalline selenium quantum dots (C-SeQDs). The subsequent anti-proliferative effects on cancer cells were investigated, demonstrating that SeQDs exhibited anti-proliferative effects on cancer cells in a polymorphs-dependent manner. A-SeQDs rather than C-SeQDs could specifically inhibit the proliferation of cancer cells. Finally, we explored the mechanisms underlying the polymorphs-dependent anti-proliferative activity of nanoscaled selenium. The results suggested that A-SeQDs was effective in inducing mitochondria-mediated apoptosis and necrosis (Fig. 1). It was speculated that A-SeQDs could enter directly mitochondria of cancer cells and regulate apoptosis-associated pathways including "p38 MAPK Signaling", "p53 Signaling" and "14-3-3-mediated Signaling". Moreover, the changes of mitochondria could further affect the endoplasmic reticulum (ER) through the interaction of mitochondria with ER and regulate the pathways involved in protein synthesis and post-translational modification including "p70S6K Signaling" and "Protein Ubiquitination Pathway". This was the first report to demonstrate the involvement of protein synthesis and post-translational modification pathways in the anti-proliferative activity associated with NMs.

Results and Discussion
Preparation and characterization of SeQDs. In this study, two polymorphs of SeQDs were prepared through self-redox decomposition of selenosulfate precursor in the presence of bovine serum albumin. From the X-ray diffraction (XRD) pattern (lower curve in Fig. 2a), the sample prepared under 20 °C did not exhibit any obvious diffraction peaks, indicating the successful formation of amorphous phase (A-SeQDs). The energy-dispersive X-ray (EDX) spectrum (inset of Fig. 2a) indicated that the composition of the sample is selenium. Compared with A-SeQDs, the XRD pattern of the sample prepared under 80 °C (upper curve in Fig. 2a)
presented the sharp diffraction peaks corresponding to the (100), (101), (110), (102), (111), (201), (112), (202) and (210) planes of the hexagonal Se (JCPDF 06-0362), respectively, indicating the formation of crystalline phase (C-SeQDs). These results demonstrated that two SeQDs with different polymorphs could be prepared through the facile adjustment of the preparation conditions.

The high resolution-transmission electron microscopy (HRTEM) observations indicated the different sizes and polymorphs of the different SeQDs. From the results shown in Fig. 2c–e, the two samples were both the well-dispersed QDs with the average diameters of $2.25 \pm 0.19$ nm for A-SeQDs (Fig. 2c) and $4.10 \pm 0.04$ nm for C-SeQDs (Fig. 2e). In addition, the existence of a diffused halo ring rather than any detectable rings or spots in the selected-area electron diffraction (SAED) pattern of A-SeQDs (inset of Fig. 2c) further revealed the formation of the amorphous product. In comparison, the SAED pattern of C-SeQDs exhibits the obvious diffraction spots (inset of Fig. 2e), further confirming the formation of the crystalline product.

Results from the $\xi$-potential measurement revealed that both SeQDs samples had the good stability under the physiological conditions. The $\xi$-potentials of the samples in H$_2$O, phosphate buffer solution (PBS), and Dulbecco’s modified Eagle medium (DMEM) were determined to be $-38.5$, $-21.3$, and $-23.8$ mV for A-SeQDs (Fig. 3a) and $-35.5$, $-17.1$, and $-18.3$ mV for C-SeQDs (Fig. 3b), respectively, proving that two SeQDs possessed good stability and carried net negative charges under the physiological conditions. The formation of the stable and clear dispersions of two SeQDs in PBS further confirmed the stability of SeQDs under the physiological conditions (inset of Fig. 3a,b).

Optical properties of SeQDs were determined. The UV-Vis spectra showed that A-SeQDs exhibited stronger absorbance between 200–377 nm and weaker absorbance between 378–800 nm than C-SeQDs (Fig. 4a). The solid photoluminescence (PL) determination revealed that SeQDs had the good PL properties (right in Fig. 4b). Results demonstrated that QDs exhibited the emission peak centered at 605 nm for A-SeQDs and 614 nm for C-SeQDs when excited with light at a wavelength of 475 nm. In order to further test the PL properties of two SeQDs in PBS further confirmed the stability of SeQDs under the physiological conditions (inset of Fig. 3a,b).

Detection of cytobiology activity and internalization of two SeQDs. The anti-proliferative effects of two SeQDs forms on various cells were determined using thiazolyl blue tetrazolium bromide (MTT) assay. The results indicated that SeQDs exhibited the polymorphs-dependent anti-proliferative effects on various cancer cells. In Fig. 5, the red bands represent the anti-proliferative effects of A-SeQDs on different cells with different treatment times, while the green bands represent those of C-SeQDs. $IC_{50}$ values of A-SeQDs in Hep G2 (Fig. 5d), MCF-7 (Fig. 5e), and HeLa cells (Fig. 5f) are $0.0757$ mM, $0.0953$ mM, and $0.2754$ mM, respectively. However, the $IC_{50}$ values in normal cells including NIH 3T3, L929, and BRL-3A cells were undetectable (Fig. 5a–c), suggesting that A-SeQDs selectively inhibited the proliferation of cancer cells. Furthermore, with the increase of doses ($0$ nM to $5$ nM) and treatment time ($24$ to $72$ h), the anti-proliferative effects of A-SeQDs significantly increased in a
dose-dependent and time-dependent manner. In contrast, C-SeQDs just slightly inhibited the proliferation of all the test cell lines and the IC₅₀ values were undetectable, suggesting that the anti-proliferative activity of SeQDs is polymorphs-dependent.

Given the important functions of internalization for the anti-proliferative effects of NMs, the internalization of two SeQDs were determined by transmission electron microscopy (TEM) observations using Hep G2 cells as an example. Results showed that A-SeQDs (dark spots in Fig. 5g,h) and C-SeQDs (dark spots in Fig. 5I,j) could be both effectively internalized into Hep G2 cells via vacuole-like vesicles (or membrane-bound vesicles), and tended to form clusters. Furthermore, the cellular uptake efficiencies of SeQDs were evaluated by ICP-MS analysis. From the results shown in Fig. 6, the intracellular concentrations of SeQDs in Hep G2 cells are significant different. The intracellular concentration of C-SeQDs (0.0123 pg/cell) is about 3.5 times higher than that of the A-SeQDs (0.0027 pg/cell) and 15.4 times higher than that of the control (0.0008 pg/cell). It is surprising that the higher intracellular concentration of C-SeQDs than A-SeQDs does not lead to the stronger anti-proliferative effect on cancer cells, suggesting the intersample dose-independent anti-proliferative effects.

Cell Cycle and apoptosis determination by flow cytometric analysis. To determine the possible mechanisms of the polymorphs-dependent anti-proliferative activity of SeQDs, effects of two SeQDs on the cell cycle distribution were analyzed by flow cytometric analysis. As shown in Fig. 7a, Hep G2 cells were easier to be arrested in S phase after A-SeQDs treatment. When treated by 0.16 mM of A-SeQDs for 72 h, the percentage of the S phase increased from 24.61% of the control group to 39.70%. However, the percentage of the S phase treated by C-SeQDs was 24.36%, almost as same as the control group. Moreover, the cyan peak of the apoptotic and necrotic cells of the A-SeQDs treatment group was significantly higher than that of the C-SeQDs treatment group (Inset of Fig. 7a), most likely attributable to the stronger inhibition effects of A-SeQDs on mitosis and proliferation of cancer cells. These results were well in agreement with the above anti-proliferation results.

To distinguish the effects of two SeQDs on apoptosis and necrosis of cancer cells, the percentages of the dead, viable, apoptotic and necrotic cells of Hep G2 cells were determined by Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double-staining flow cytometric analysis. Results showed that A-SeQDs rather than C-SeQDs could significantly induce the late apoptosis (upper right quadrant in inset of Fig. 7b) of cancer cells. After treated by 0.16 mM of A-SeQDs for 72 h, the percentage of the late apoptotic cells significantly increased to 11.5%, much higher than those of the control group (1.82%) and the C-SeQDs treatment group (2.79%) (insert of Fig. 7b). These results suggested that the different anti-proliferative effects of A-SeQDs and C-SeQDs on cancer cells were polymorphs-dependent at late apoptosis stage.

Co-localization with the mitochondria by confocal laser scan microscopy. Considering the significant apoptosis-inducing effect of A-SeQDs on cancer cells and the important roles of mitochondria in the regulation of apoptosis, the mitochondria of the model cells exposed to A-SeQDs were visualized by confocal laser scan microscopy (CLSM) to investigate whether A-SeQDs could co-localize with the mitochondria. Meanwhile the co-localization coefficient was evaluated by Pearson’s correlation factor. From the results (Fig. 8), the fluorescence of A-SeQDs(Fig. 8a2) and the red fluorescence of the Mitochondria Tracker (Fig. 8a1) overlapped well each other. This clearly indicates that A-SeQDs could co-localize with the mitochondria of Hep G2 cells. The Pearson’s correlation factor was determined as 0.80 (Fig. 8a4), indicating the preferential accumulation of A-SeQDs mainly in the mitochondria of Hep G2 cells. From Fig. 8b–d, A-SeQDs can also preferentially accumulate in the mitochondria of MCF-7, HeLa, and BRL-3A cells. The Pearson’s correlation factors were determined as 0.77, 0.78, and 0.83, respectively. From the results shown in Figure S1, similar to A-SeQDs, C-SeQDs can also preferentially accumulate in the mitochondria of the model cells. However, from Fig. 8e and Figures S2 and S3, the fluorescence of A-SeQDs and C-SeQDs and the red fluorescence of the Lyso-Tracker just slightly overlapped each other. The Pearson’s correlation factor for Hep G2 cells was determined as 0.13. These results suggest that SeQDs could not preferentially accumulate in the lysosome of model cells. Furthermore, Δψₘ of Hep G2 cells exposed to SeQDs were measured. The results show that SeQDs could lead to the dose-dependent loss of Δψₘ (see Supplementary Figure S4). More importantly, the effect of A-SeQDs on the loss of Δψₘ was higher than that of C-SeQDs, correlated well with...
their apoptosis-inducing effects. This revealed that A-SeQDs could efficiently breakdown the mitochondrial membrane of cancer cells and led to the apoptosis. Therefore the mitochondria-induced endogenous apoptosis should play the important role in the inhibition of the cancer cells proliferation by A-SeQDs.

Based on the above results, A-SeQDs could inhibit the proliferation of cancer cells through the cellular uptake and mainly localization to mitochondria, breakdown of the mitochondrial membrane, depletion of the mitochondria potential, and induction of the apoptosis and the cell cycle arrest in S phase.

Figure 5. The anti-proliferative effects of A-SeQDs and C-SeQDs on different cells. (a) NIH 3T3 cells; (b) L929 cells; (c) BRL-3A cells; (d) Hep G2 cells; (e) MCF7 cells; (f) HeLa cells. Red bands show the results of A-SeQDs, green bands show the results of C-SeQDs. TEM images of Hep G2 cells treated with (g, h) A-SeQDs and (i, j) C-SeQDs. Panels h and j show the higher-magnification images of the rectangle framed area in panels g and i. Dark spots show the SeQDs. Scale bar in e, g: 2 μm; scale bar in (f, h): 500 nm.

Figure 6. Intracellular SeQDs concentrations in Hep G2 cells.
Proteomics analysis based on bioinformatics. To systematically and deeply study the molecular mechanisms of the polymorphs-dependent anti-proliferative effects of SeQDs on cancer cells, proteins regulated by SeQDs were detected using nanomass. The representative 2D gel images for whole cell proteins extracted from Hep G2 cells were shown in Fig. 9. The proteomic maps of the control and the treated cells were compared using ImageMaster 2D Platinum 7.0 software to identify the protein spot variations. After treatment, significantly differentially expressed protein spots (p < 0.05) with 1.5-fold change in intensity were scored. Based on the differentially expressed proteins for A-SeQDs treatment, 61 proteins including 29 up-regulated and 32 down-regulated proteins could be identified. Tables 1 and S1 (see Supplementary Information) presented the identified proteins.

The identified proteins are categorized based on their cellular locations, namely cytoplasm (37.71%), cell membrane (18.03%), mitochondrion (16.39%), nucleus (13.11%), endoplasmic reticulum (ER, 8.19%), and extracellular matrix (6.56%), respectively (Fig. 10a). Furthermore, in order to better understand the biological relevance of the changes in protein expression in response to SeQDs, the proteins are classified into 7 groups based on their functions, including stress response, signaling pathway and transduction, protein biosynthesis and metabolism, cell cycle, cell adhesion and migration, metabolism, and oxidation-reduction process.

To systematically and comprehensively study the anti-proliferative mechanisms of A-SeQDs and C-SeQDs, bioinformatics including Ingenuity Pathway Analysis (IPA) and hierarchical clustering analysis were used to compare the protein expression profiles between the samples. The studies indicated that different SeQDs exhibited discriminatively prominent effects on biological functions and canonical pathways of Hep G2 cells. The biological function patterns of the identified proteins were clustered and the results were shown in Fig. 10b. Firstly, A-SeQDs induced apoptosis, necrosis and death of the tumor cells, while C-SeQDs had much weak effects on these cellular processes, which were consistent with the above results. Secondly, it was logical that the functional enrichment and clustering analysis was mainly focus on death and survival of the tumor cells.

To further clarify the important signaling pathways related to the anti-proliferative activities of SeQDs in Hep G2 cells, pathway analysis was performed to connect the differentially expressed proteins with canonical biological pathways by IPA software. A Benjamini-Hochberg corrected Fischer’s exact test was utilized to calculate the p-value associated with a canonical pathway (Fig. 10c). Results showed that A-SeQDs exhibited more pronounced and global impact on various canonical pathways than C-SeQDs, especially the pathways in relation to cell survival such as the apoptosis pathways “p53 Pathway”, “p38 MAPK Signaling”, and “Protein Ubiquitination Pathway”, and protein synthesis such as “PI3K/AKT-mTOR/p70S6K Signaling” and “Protein Ubiquitination Pathway”. Although previous studies indicated that the anti-proliferative activities of selenium-containing compounds can be attributed to the Akt/Mdm2/AR controlled apoptosis or induction of S phase arrest, our results reveal that cooperation of multiple pathways generates functional difference between different Se samples.

Subsequently, the proteins with the important roles in multiple pathways are summarized and discussed. As suggested by IPA results (see Supplementary Table S2), some differentially expressed proteins may play multiple roles in mediating multiple pathways. For example, the level of 14-3-3 protein in Hep G2 cells exposed to A-SeQDs showed significant down-regulation over 7-fold (no significant change exposure to C-SeQDs) in comparison with control group. As a family member of multifunctional phosphoserine binding molecules, 14-3-3 protein serves as an effector of survival signaling. This study demonstrates that when treated by A-SeQDs, down-regulation of 14-3-3 protein could exert direct or indirect influence on differential changes of multiple pathways such as “p70S6K Signaling”, “p53 Pathway”, “14-3-3 mediated Signaling”, “DNA damage-induced 14-3-3c Signaling”, and “Cell Cycle: G2/M DNA Damage Checkpoint Regulation” (see Supplementary Table S1). It means that A-SeQDs might exhibit the anti-proliferative activity through the regulation of the 14-3-3 expression and the related multiple pathways, not just limited to the previous reports. In addition, thioredoxin reductase...
1 (TRXR1) showed approximately 3-fold up-regulation upon exposed to A-SeQDs, which was confirmed by western blotting analysis (Fig. 11, see Supplementary Figure S5 for full-length blots online). As a key element of the thioredoxin system, this protein applies to the detoxification of reactive oxygen metabolites and the signaling processes. The overexpression of TrxR implied the disorder of the intracellular redox homeostasis, which might induce the significant apoptosis of the target cells.

Our analysis showed that A-SeQDs exhibited significant influence on heat shock proteins (HSPs) (Table 1). The levels of HSPs family members (HSP90, HSP70, and HSP27), except for the heat shock factor-binding protein (HSPBL), were significantly down-regulated upon exposure to A-SeQDs rather than to C-SeQDs. However, HSPBL was significantly up-regulated upon exposure to A-SeQDs rather than C-SeQDs. It is well-known that HSPs are one of the most conserved cytoprotective warriors against apoptosis and oxidative stress. The down-regulations of HSPs induced by A-SeQDs in Hep G2 cell indicated the dysfunction of protection.

Figure 8. CLSM images of the different cells incubated with different samples. (a) Hep G2 cells incubated with A-SeQDs; (b) MCF-7 cells incubated with A-SeQDs; (c) HeLa cells incubated with A-SeQDs; (d) BRL-3A cells incubated with A-SeQDs; (e) Hep G2 cells incubated with C-SeQDs; (f) Mitochondria Tracker; (g) A-SeQDs; (h) Dark-field image of Mitochondria Tracker + A-SeQDs; (i) Intensity correlation plot of Mitochondria Tracker and A-SeQDs. Scale bar: 20 μm.
contributing to the anti-proliferative effects. It was worthy to notice that the changes of HSPs expression in comparison with the control group were related to the changes of multiple pathways especially “Protein Ubiquitination Pathway” and “p38 MAPK Signaling” (see Supplementary Table S2). It could be therefore concluded that HSPs might serve as an indicator of potential anti-proliferation targets or effector of A-SeNPs.

Additionally, ER stress associated proteins 78 kDa glucose-regulated protein (GRP78) and ER resident protein 29 (ERP29) were significantly up-regulated in both A-SeQDs and C-SeQDs treated groups (Table 1). Meanwhile, protein disulfide-isomerase A3 (PDIA3), namely ERP57, showed significant upregulation after A-SeQDs treatment. These results agree well with western blotting analysis results (Fig. 11, see Supplementary Figure S5 for full-length blots online). The overexpression of these proteins could be regarded as the indicator of ER stress response42. As reported, over-expression of ERP29 was inversely correlated with tumor progression due to induction of apoptosis and inhibition of cell cycle in a variety of cancer cells43. When ER stress happens, ER chaperon (GRP78) dissociates from the ER transmembrane proteins, leading to the activation of transmembrane proteins. Therefore, high-expression of ER-associated proteins can be considered as the self-protective mechanism of HepG2 cell against SeQDs. However, recent findings uncovered that the junction between the ER and the mitochondria played a crucial role in cell death regulation 44. Under ER stress condition, mitochondria-associated membrane induces apoptosis via the ER–mitochondria interaction and further the mitochondria dysfunction44. Equivalently differential ER stress response to two polymorphs of SeQDs would be helpful to understand equivalently loss of \( \Delta \psi_m \) (see Supplementary Figure. S1).

**Conclusion**

This research was to study the nanomics of two polymorphs of SeQDs including A-SeQDs in cancer cells. It was the first time to demonstrate the anti-proliferative activity of SeQDs was polymorphs-dependent with A-SeQDs being much more effective than C-SeQDs in cancer cells. This was most likely A-SeQDs had more effective influence on breaking the mitochondrial membrane, depleting the mitochondria potential, inducing the apoptosis and the cell cycle arrest in S phase. Nanomics approach was used to study the underlying mechanisms. Results showed that A-SeQDs could alter 61 proteins functionally related to metabolism, protein synthesis and post-translational modification, and involved in various signaling pathways, such as “p53 Pathway”, “p38 MAPK Signaling”, “14-3-3 mediated Signaling”, “p70S6K Signaling”, and “Protein Ubiquitination Pathway”. The protein synthesis and post-translational modification pathways including “p70S6K Signaling” and “Protein Ubiquitination Pathway” were first reported to be involved in the anti-proliferative effects of NMs. These findings propose a molecular basis for further unraveling the mechanisms of the anti-proliferative effects of NMs on cancer cells. The idea employing nanomics as a tool to study the interaction between a nanoparticle and cancer cells is powerful.
| Protein name            | Accession No. | Cellular function                        | Fold changesa |
|------------------------|---------------|------------------------------------------|---------------|
|                        |               | A-SeQD | C-SeQD |
| **Stress response**    |               |        |        |
| Thioredoxin reductase 1| Q16881_TRXR1  | Oxidoreductase                           | 2.994 | 1.335 |
| Heat shock 70kDa protein 1A | P08107_HSP71   | Stress response                          | 0.2542 | 0.7892 |
| Heat shock protein beta-1| P04792_HSPB1   | Stress response                          | 0.0741 | 0.5671 |
| Heat shock factor-binding protein 1-like protein 1| C9CN9_HS8PL | Stress response                          | 2.095 | 1.114 |
| 78kDa glucose-regulated protein | P11021_GRP78   | Endoplasmic reticulum stress process     | 5.382 | 2.234 |
| Quinone oxidoreductase-like protein 1| O95825_QORL1   | Oxidoreductase                           | 1.616 | 1.0289 |
| Annexin A4              | P09525_ANXA4  | Stress response                          | 0.4103 | 0.6749 |
| Annexin A2              | P07355_ANXA2  | Stress response                          | 0.5883 | 0.905 |
| Activator of 90kDa heat shock protein ATPase homolog 1| O95433_AHSA1 | Stress response                          | 1.777 | 1.0832 |
| Heat shock protein HSP 90-beta | P08238_HS90B | Stress response                          | 0.659 |  |
| Endoplasmic reticulum resident protein 29| P30040_ERP29  | Endoplasmic reticulum stress process     | 6.354 | 7.422 |
| **Signal pathway and transduction** | |        |        |
| Protein Wnt-7a          | O00755_WNT7A  | Signal pathway                           | 0.1193 | 0.5286 |
| Regulator of G-protein signaling 3 | P49796_RGS3  | Signal pathway                           | 0.1279 | 1.038 |
| Rho GDP-dissociation inhibitor 1 | P52565_GDI1R1 | Signal transduction                      | 0.6514 | 0.3019 |
| T-cell-specific surface glycoprotein CD28 | P10747_CD28 | Signal pathway                           | 2.399 | 1.977 |
| Glycoprotein hormone beta-5 | Q86YW7_GPHB5 | Signal pathway                           | 0.1876 | 0.9034 |
| 14-3-3 protein sigma    | P31947_1433S | Signal transduction                      | 0.141 | 1.008 |
| **Protein biosynthesis and metabolism** | |        |        |
| Eukaryotic translation initiation factor 3 | O15371{EIF3D} | Protein biosynthesis                     | 0.1859 | 0.7888 |
| Glycyl-tRNA synthetase   | P41250_SYG   | Protein biosynthesis                     | 5.73 | 1.313 |
| Ubiquitin carboxyl-terminal hydrolase isozyme L5 | Q9YSK5_UCHL5 | Transcription regulation                 | 15.37 | 1.403 |
| T-complex protein 1 subunit alpha | P17987_TCPA | Protein folding                          | 1.653 | 1.266 |
| Reticulocalbin-1         | Q15293_RCN1  | Endoplasmic reticulum stress process     | 0.2422 | 0.6512 |
| 39S ribosomal protein L17 | Q9NRX2_RM17  | Protein translation                      | 3.724 | 1.036 |
| 39S ribosomal protein L38 | Q96DV4_RM38  | Protein translation                      | 1.599 |  |
| Sorting and assembly machinery component 50 homolog | Q9YS12_SAM50 | Protein metabolism                       | 0.6034 | 1.055 |
| Diphthine methyl ester synthase | Q9H2P9_DPH5 | Protein metabolic process                | 14.65 | 1.382 |
| Elongation factor 2      | P13639_EF2   | Protein biosynthesis                     | 0.6907 | 0.185 |
| Tyrosine-protein phosphatase non-receptor type 4 | P29074_PTN4 | Protein metabolism                       | 2.142 | 0.8319 |
| T-complex protein 1 subunit theta | P50990_TCPQ | Proteins folding                         | 0.6905 | 0.9746 |
| Ras-related protein Rab-3D | O95716_RAB3D | Protein transport                        | 0.6783 |  |
| RILP-like protein 2       | Q9690_R1PL2  | Protein transport                        | 1.6921 | 0.2279 |
| Mitochondrial 2-oxodicarboxylate carrier | Q9BQT8_ODC | Protein transport                        | 0.452 | 0.2889 |
| **Cell cycle**           |               |        |        |
| Histone-binding protein  | Q09628_RBBP4 | Cell cycle regulation                    | 0.6532 | 0.2111 |
| Proliferating cell nuclear antigen | P12004_PCNA | DNA repair                               | 0.05689 | 1.0010 |
| tRNA (cytosine(34)-C(5))-methyltransferase | Q08J23_NSUN2 | Cell division                            | 0.1782 | 0.6879 |
| E3 SUMO-protein ligase NSE2 | Q96MF7_NSE2  | Cell division                            | 1.895 | 0.6007 |
| Proteasome subunit alpha type-2 | P25787_PSA2 | Mitotic cell cycle                       | 3.287 | 7.424 |
| Mitotic spindle assembly checkpoint protein MDA1 | Q9Y6D9_MD1L1 | Cell division                            | 2.675 | 0.9113 |
| **Cell adhesion and migration** | |        |        |
| Morsin                  | P26038_MOES  | Cell adhesion                            | 0.629 | 1.035 |
| Flotillin-1             | O75955_FLOT1 | Cell scaffolding protein                 | 3.134 | 0.8988 |
| Myosin-13               | Q9UKX3_MYH13 | Cell adhesion and migration              | 0.8122 | 0.5214 |
| Ezrin                   | P15311_EZRI | Cell adhesion                            | 0.593 | 0.9917 |
| Angiopoietin-related protein 2 | Q9UK99_ANG12 | Cell autocrine                           | 0.327 | 0.6654 |
| **Metabolism**          |               |        |        |
| Delta(3,5)-Delta(2,4)-dieneoyl-CoA isomerase | Q13011_ECH1 | Lipid metabolism                        | 0.2481 | 0.7873 |
| Enoyl-CoA hydratase     | P30084_ECHM  | Lipid metabolism                        | 0.1501 | 1.017 |
| Thiosulfate sulfurtransferase | QST7W7_TSTD2 | Small molecule metabolism              | 2.362 | 1.053 |

Continued
Protein name | Accession No. | Cellular function | Fold changes  \\
---|---|---|---
S-formylglutathione hydrolase | P10768_ESTD | Cellular metabolic process | 2.76 1.809  \\
Oxysterol-binding protein 2 | Q969R2_SERA | Lipid Transport | 1.90 0.7726  \\
D-3-phosphoglycerate dehydrogenase | O43175_MPP | Glycolysis | 5.79 1.546  \\
Mitochondrial-processing peptidase subunit beta | O75439_MPP | Cellular metabolic process | 0.1289 0.9633  \\
Methylthioribose-1-phosphate isomerase | Q8BV20_MTNA | Small molecule metabolic process | 0.326 1.0174  \\
V-type proton ATPase subunit B(Endomembrane) | P21281_VATB | Energy metabolism | 1.492 1.015  \\
UDP-glucose 6-dehydrogenase | O60701_UGDH | Small molecule metabolism | 0.6014 1.461  \\
Bifunctional coenzyme A synthase | Q13057_COASY | Lipid metabolism | 1.343 0.6184  \\

| Protein name | Accession No. | Cellular function | Fold changes  \\
---|---|---|---
Protein disulfide-isomerase A3 | P30101_PDIA3 | Cell redox homeostasis | 4.967 1.349  \\
Glycolase domain-containing protein 4 | Q9HC38_GLOD4 | Isomerase | 1.855 1.073  \\
Glutathione S-transferase omega-1 | P78417_GSTO1 | Oxidoreductase | 1.669 1.536  \\
Microtubule-associated protein tau | P10636_TAU | Microtubule assembly | 1.5862 1.488  \\
Echinoderm microtubule-associated protein-like 2 | O95834_EMAL2 | Microtubule assembly | 0.6186 0.8546  \\
Ribose-phosphate pyrophosphokinase 2 | P11908_PRPS2 | Nucleotide biosynthesis | 3.255 0.575  \\
Ribose-phosphate pyrophosphokinase 1 | P60891_PRPS1 | Nucleotide biosynthesis | 1.586 3.208  \\

| Protein name | Accession No. | Cellular function | Fold changes  \\
---|---|---|---
Oxidation-reduction process | | |  \\

Table 1. List of the differentially expressed proteins and the quantitative changes after SeQDs treatment.  
Protein spots were quantified based on the normalized average percentage of volume derived from ImageMaster 2D Platinum 7.0 software analysis. The data shown in the A-SeQDs and C-SeQDs column are the ratios of different protein expression levels in the treated cells to those in the control cells.

Figure 10. (a) The cellular distributions of the identified proteins. (b) The clustered biological function patterns of the identified proteins. The red color represents the promotion effect on the related process. The green color represents the inhibition effect on the related process. The grey and black colors represent the nonsignificant influence. (c) The significantly enriched canonical pathways of the identified proteins. The red color represents the significant effects on the canonical biological pathways. The brighter the red color is, the stronger the effect is. The grey and black colors represent the nonsignificant influence.
Preparation of SeQDs. For the preparation of A-SeQDs, black selenium powder was added into aqueous solution of sodium sulfite (50 mM) at 95 °C. Then BSA (70 mg) was added into the reaction system and the pH was adjusted to 6.0. The reaction system instantly changed to red color. Subsequently, the reaction system was incubated at 20 °C for 12 h. Finally, the dispersion was centrifuged, washed, and freeze-dried. The preparation of C-SeQDs was performed under the similar conditions to those used in the preparation of A-SeQDs, except for the elevated incubation temperature (80 °C) and time (24 h).

Characterization of SeQDs. The size and morphology of the SeQDs were characterized by HR-TEM (JEOL JEM-2100) with the acceleration voltage of 200 kV. The crystal phases were determined by XRD using a D8ADVANCE X-ray diffractometer (Bruker axs Com.) with graphite monochromatized Cu Kα radiation (λ = 0.15406 nm) in the 2θ range of 20–80°. EDX spectrum was recorded on a GENESIS system (EDAX Inc.) attached to the JEM-2100 microscope. The photoluminescence (PL) measurements were carried out on a HITACHI FP-6500 spectrophotometer. ζ potential was measured on a Nano-ZS instrument in triplicate in H2O, PBS, or DMEM.

Cell lines and cell culture. The cell lines used in this study were all purchased from American Type Culture Collection (ATCC, Manassas, VA), including MCF-7 breast adenocarcinoma cells, Hep G2 human hepatoblastoma cells, HeLa human cervical carcinoma cells, and NIH/3T3 mouse fibroblast cells. They were cultured in RPMI-1640, DMEM, or EMEM medium supplemented with 10% heat-inactivated FBS, penicillin (100 units/mL), Streptomycin (100 μg/mL), amphotericin B (0.25 μg/mL) at 37 °C in a humidified incubator at fully humidified atmosphere at 37 °C. 5% CO2 and 95% room air.

Detection of apoptosis by flow cytometric analysis. Apoptotic and necrotic cell levels were determined by flow cytometric analysis after double staining with Annexin V-FITC and PI using an assay kit. After incubated with SeQDs for 72 h, the medium was removed and the cells were washed twice with ice-cold PBS. After stained, the cells were collected, washed with ice-cold PBS, and suspended in binding buffer. The cells were analyzed by a FACSCalibur flow cytometer (Becton Dickinson & Co., NJ) and the percentages of apoptotic and necrotic cells were quantified.

Cell cycle analysis. Well-cultivated Hep G2 cells were treated with SeQDs (0.02, 0.06, and 0.16 mM) for 72 h. Subsequently, cells were collected, washed with ice-cold PBS, and fixed with cold 70% ethanol at 4 °C. Then, cells...
were washed with ice-cold PBS and resuspended in ice-cold PBS. Finally, cells were incubated with RNase and PI and analyzed by a FACSCalibur flow cytometer.

**CLSM analysis.**  Hep G2 cells cultured in 35 mm confocal dish with cover glass bottom were stained with Mitochondrial Tracker (1 μg/mL). After washed twice with PBS, cells were cultured in medium containing A-SeQDs for 4 h on a thermo-cell culture FCS2 chamber and analyzed by Carl Zeiss Cell Observer (Jena, Germany).

**Evaluation of loss of Δψm.**  The mitochondrial membrane potential (Δψm) was determined with the assistance of fluorescent probe JC-1. Hep G2 cells exposed to A-SeQDs (0.02, 0.06, and 0.16 mM) were collected and washed with PBS. Cells were incubated with JC-1 (5 μM), washed and re-suspended in PBS at a density of 1 × 10^6 cells/mL. The fluorescence intensity at the wavelength of 595 nm was measured at an excitation wavelength of 515 nm using a FACSCalibur flow cytometer.

**Inductively Coupled Plasma-Mass Spectrometry (ICP-MS).**  Intracellular Se concentration was determined by ICP-MS. Briefly, collected cells were digested with 3 mL of concentrated nitric acid and 1 mL of H2O2 in an infrared rapid digestion system at 180 °C for 1.5 h. The digested solution was reconstituted to 10 mL with Milli-Q H2O and used for ICP-MS analysis (ELAN DRC-e, Perkin–Elmer Sciex).

**TEM observation of cells.**  Hep G2 cells (5 × 10^4 cells/mL) were treated with SeQDs for 72 h, washed with PBS, and fixed with glutaraldehyde (2.5%). Cells were postfixed in osmium tetroxide, dehydrated in ethanol, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate by TEM.

**Protein extraction.**  Cells were washed with PBS, collected, and re-suspended in lysis buffer containing urea (7 M), thiourea (2 M), CHAPS (4%), dithiothreitol (20 mM), Pharmalyte pH 3–10 (0.5%), protease inhibitor mix, and nuclease mix. Protein concentrations were determined using non-interference protein assay kit (SK3071, Sangon Biotech., China) according to the manufacturer’s instruction.

**Two dimensional gel electrophoresis.**  For analytical and preparative gels, protein samples (350 μg) were loaded into the rehydrated 24 cm non-linear Immobilized pH gradient (IPG) strip (pH 3–10, nonlinear). The first dimensional isoelectric focusing (IEF) run was carried out using the following conditions: (i) 30 V, 10 h; (ii) 60 V, 3 h; (iii) 100 V, 1.5 h; (iv) 500 V, 1.5 h; (v) 1000–10000 V, 3 h; (vi) 1000 V–140000 V.h. This was followed by the second dimensional SDS-PAGE, performed on 1.0 mm polyacrylamide gels (12.5%) at a constant power of 10 W per gel at 16 °C by using Ettan DALTsix electrophoresis system (Amersham Biosciences). All samples were run in triplicate to ensure reproducibility.

**Protein visualization and image analysis.**  2D gels were visualized by staining with Coomassie blue. The stained gels were scanned by an image scanner and all the triplicate gels were then analyzed with ImageMaster 2D Platinum 7.0 software (GE Healthcare).

**MALDI-TOF/MS and MS/MS.**  The differentially expressed protein dots were cut from the gels to prepare dry extract. Dry extract was re-dissolved in matrix solution (1 mL) containing a cyano-4 hydroxydeminic acid (CHCA, 5 mg/mL) in trifluoroacetic acid (0.1%) and acetonitrile (50%). The extract was spotted onto the MALDI target plate and allowed to dry in air prior to MS analysis using an MALDI-TOF/TOF Mass Spectrometer (Applied Biosystems 4800 Proteomics Analyzer, Framingham). The peptides and proteins were identified by using GPS explorer software Version 3.6 (Applied Biosystems) and MASCOT search engine (Version 2.1; Matrix Science).

**Western blotting.**  Protein extracted from cell lysates as described above was resolved on SDS-PAGE gel and transferred onto PVDF membrane via semidy transfer (BioRad). Membranes were blocked in 3% BSA and washed in Tris-buffered saline with 0.1% Tween. Membranes were incubated with 1:500 primary antibody (anti-β-actin, anti-Grp78, anti-PDIA3, anti-TRXR1, purchased from Abcam) followed by the corresponding secondary antibody with 3 washing steps in between. Protein bands were developed with chemiluminescence kit.

**Bioinformatics analysis.**  Categorization of protein functions was performed based on Uniprot/Trembl database search. Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com) was used to determine the distribution of identified proteins and their participation in molecular networks related to carcinogenesis. Detailed information of the identified proteins including accession number and expression changes were imported to IPA software to perform the analysis based on Ingenuity Knowledge Base. The functions and involved pathways of each protein obtained from IPA analysis was then clustered by Cluster 3.0 and TreeView software.

**Statistical analysis.**  All the biological experiments were carried out at least in triplicate, and the results were expressed as mean ± SD. Differences between two groups were analyzed by one-way analysis of variance (ANOVA) multiple comparisons.

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Author Contributions
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. G.Y. and L.Y. designed the experiment. G.Y., L.Y. and G.W. prepared the manuscript. G.W. prepared the samples and performed the proteomics measurements and bioinformatics analysis. G.W. and G.Y. carried out the anti-proliferation experiments. L.Z. and J.S. performed the flow cytometric analysis. X.W. performed the TEM characterization of the QDs and the QDs-treated cells. H.Z. carried out the CLSM observations. G.Y., L.Y., G.W., X.M. and K.W. interpreted the results and wrote the manuscript. All authors discussed the results and commented on the manuscript.

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