Effect of BSA-coated Superparamagnetic Iron Oxide Nanoparticles on Granulosa Cells

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Abstract. *Background: Since superparamagnetic iron oxide nanoparticles (SPION) possess unique features, they provide a huge platform for medical applications, especially for cancer diagnosis and therapy (e.g. imaging, and drug targeting). However, heterogeneous effects on mammalian cells with regard to reproductive tissue are described. An experimental study was carried out to study the effects of SPIONs on both the expression of steroid hormone receptor and viability of granulosa cells, which play a key role in ovarian health and fertility. Materials and Methods: Human granulosa cells were cultured in vitro and incubated with different concentrations of SPIONs. After 48 h, steroid receptor expression and cell viability were evaluated. Results: Treatment of granulosa cells with SPIONs did not affect estrogen receptor β1 or progesterone receptor-A expression and had no significant effect on cell viability. Conclusion: Nanoparticles precoated with bovine serum albumin (BSA) do not alter granulosa cell phenotype, whereas literature suggests that other nanoparticles induce apoptosis and reduce steroid receptor expression. Our data indicate an overall better outcome using SPIONs coated with BSA.*

Superparamagnetic iron oxide nanoparticles (SPION) have provoked increasing interest for medical and scientific purposes due to their exceptional characteristics including magnetic, electronic and optical properties. Among the most promising medical applications are contrast enhancement in magnetic resonance imaging, magnetic hyperthermia treatment and drug delivery, that are of special interest in cancer therapy and diagnosis. Furthermore, a combination of those two enables SPIONs to become theranostic agents (1-7). SPIONs are already in clinical use for magnetic resonance imaging as a negative contrast agent for diagnosis; and for therapy by hyperthermia, where SPIONs are heated by a magnetic field; as well as in magnetic drug targeting by loading SPIONs with an active substance, an exceptionally promising approach in medicine (8).

Since these potential applications for SPIONs are performed on living cells, it is very important to evaluate their biocompatibility and possible toxic effects. Because small-dimensioned nanoparticles have a high surface area, they are presumed to be more active and therefore have more side-effects when brought into biological surroundings. This includes reactions with chemical or biological components causing severe cellular damage, as well as production of reactive oxygen species or interaction with DNA (9). One of the possible reasons for this is that due to their charge, whenever nanoparticles enter a biological environment they become covered with proteins in which they then induce conformational changes; this may affect the bioreactivity of nanoparticles and could lead to revealing of new epitopes, transformed functions and effects on avidity (10). Thus, pre-coating SPIONs with protein would circumvent this problem, thereby establishing biocompatibility, by limiting induction of dynamic changes (11).

Nanoparticles have been shown to cross reproduction-relevant biological barriers including blood–testicular as well as the placental barrier (12). Especially for young people undergoing cancer diagnosis and therapy who wish to have children, it is of the utmost importance to ensure the safety of their reproductive health. While in somatic cells injuries from nanoparticles may cause inflammation or transformation, reproductive cells may suffer from impaired fertility or

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undergo alterations leading to congenital defects. Steroid receptor production is one of the key parameters for evaluating the functions of germ cells and the reproductive system is regulated by steroid hormones. Therefore, we analyzed the effect of SPIONs coated with protein on steroid receptor expression in granulosa cells, which play a major role in maintaining ovarian function, health and female fertility, while these cells are protectively located around the oocyte, maintaining steroid hormone homeostasis. The two steroid hormones estrogen and progesterone function through different hormone receptors. Estrogen and progesterone bind to a nuclear receptor, estrogen furthermore to an intracellular transmembrane receptor, which is the G-protein-coupled receptor GPR30 (13). Herein we focused on their nuclear receptors, estrogen receptor (ER) and progesterone receptor (PR). Different ER and PR isoforms have been described, ER-α/-β and PR-A/-B, with only slight differences in receptor composition. Herein we showed for the first time the enormous advantages of bovine serum albumin-coated SPIONs ($SEON^{LA\text{-}BSA}$) established using granulosa cells (Figure 1).

Materials and Methods

Nanoparticles. SPIONs were synthesized and characterized extensively in-house, as described before by Zaloga *et al*. (14). Briefly, lauric acid-coated SPIONs ($SEON^{LA}$) were synthesized by co-precipitation in aqueous media and subsequent *in situ* coating with lauric acid. They were then additionally coated with bovine serum albumin ($SEON^{LA\text{-}BSA}$) by dilution in excess protein solution and the unbound protein was removed by ultrafiltration (15).

Cell culture and immunocytology. Primary human granulosa cells and the human luteinized granulosa cell-line, HLG-5, collected from infertile women undergoing *in vitro* fertilization pre-embryo transfer treatment (Department for Obstetrics and Gynaecology Ludwig Maximilian University, Munich, Germany) (16), were used for *in vitro* experimentation. They were and are cultured in Dulbecco's modified Eagle's medium (DMEM) (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany) and were seeded at 5×10^4/500 μl medium in an 8-chamber slide (Becton Dickinson, New Jersey, USA) overnight. Cells were treated with 10, 25 and 50 μg/ml $SEON^{LA\text{-}BSA}$ or with a equivalent amount of distilled water for 48 h. Immunohistochemistry was then performed using a combination of pressure cooker heating and the standard streptavidin–biotin– peroxidase complex with the use of the mouse-IgG-Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) (17). Primary antibodies used for immunocytologycal staining were anti-ER-β1 and anti-PR-A. Detailed immunocytologycal staining is described elsewhere (18).

Staining for cell viability using flow cytometry. In order to examine the viability of HLG-5 cells treated with $SEON^{LA\text{-}BSA}$, 2×10^5 cells/ml were treated with 10, 25 and 50 μg/ml $SEON^{LA\text{-}BSA}$ and incubated at 37˚C in 95% humidified air and 5% CO_2. After 24, 48, and 72 h, cells were trypsinized and resuspended in 1 ml DMEM. A volume of 75 μl was then incubated with 300 μl of freshly prepared staining solution containing 1 μg/ml annexin V-fluorescein isothiocyanate (FITC) (AxV; Life Technologies, Darmstadt, Germany), 20 μg/ml propidium iodide (PI; Sigma Aldrich, Taufkirchen, Germany), 1 μg/ml Hoechst 33342 (Hoechst; Life Technologies, Darmstadt, Germany), and 5.1 μg/ml DiIC1(5) (1,1',3,3,3',3'-hexamethylindodicarbocyanine iodide (Dil; Life Technologies, Darmstadt, Germany) in Ringer's solution for 20 min at 4˚C.

Flow cytometry was performed employing a Gallios cytofluorometer (Beckman–Coulter, Pasadena, CA, USA). Electronic compensation was used to eliminate bleed through fluorescence. The data analysis was performed with Kaluza software version 2.0 (Beckman Coulter). All flow cytometric experiments were conducted in triplicates, and the results were averaged.

Statistical analysis. The intensity and distribution patterns of specific immunocytological staining were evaluated using the semiquantitative immunoreactive score (IRS) to assess the expression pattern of steroid receptors (19). The IRS score was calculated by multiplication of the optical staining intensity score (graded as none=0; weak=1; moderate=2; and strong staining=3) and the score for the percentage of positively stained cells (no staining=0; <10% of cells=1; 11-50% of cells=2; 51-80% of cells=3; and >81% of cells stained=4). The slides were recorded by two observers by consensus. Sections were examined using a light microscope (JVC; Victor Company of Japan, Yokohama, Japan) equipped with a color camera (Leitz, Wetzlar, Germany). The results were evaluated using the non-parametric Mann–Whitney-*U*-test as well as Student's *t*-test. Significance was assumed at $p<0.05$. Tests for statistical significance of differences in flow cytometric data were carried out in SPSS (IBM® SPSS® Advanced Statistics).

Results

$SPION^{LA\text{-}BSA}$ do not interfere with expression of steroid hormone receptors. In order to show effects of BSA-coated SPIONs on steroid hormone receptor expression, primary granulosa cells were treated with 25 μg/ml $SEON^{LA\text{-}BSA}$ and the human luteinized granulosa cell-line HLG-5 was treated with 10 μg/ml, 25 μg/ml or 50 μg/ml $SEON^{LA\text{-}BSA}$. For each concentration, an equivalent amount of distilled water was added to control cells and treated cells were compared individually to their respective controls. After 48 h, ER-β1 and PR-A expression were determined in treated and control cells using immunocytochemistry. After analyzing positive cells statistically using the IRS under light microscopy, no significant changes in ER-β1 or PR-A expression were observed in primary (Figure 2A) granulosa cells when treated with 25 μg/ml $SEON^{LA\text{-}BSA}$ ($p=0.05$ and $p=0.7$, respectively, compared to controls; Figure 2B). Similarly, nor were there any significant differences found between HLG-5 granulosa cells when treated with different concentrations of $SEON^{LA\text{-}BSA}$ and the controls (Figures 3 and 4) regarding the IRS for ER-β1 ($p=0.3$, $p=0.1$ and $p=0.7$ for treatment at 10, 25 and 50 μg/ml $SEON^{LA\text{-}BSA}$, respectively; Figure 5A) or in the IRS for PR-A ($p=0.1$, $p=0.06$ and $p=0.05$ at 10, 25 and 50 μg/ml $SEON^{LA\text{-}BSA}$, respectively; Figure 5B).

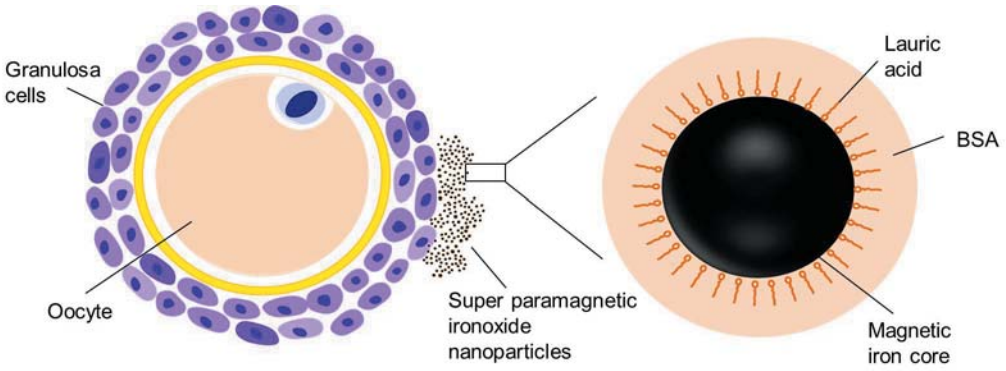

Figure 1. *A: Granulosa cells treated with super paramagnetic iron oxide nanoparticles (SPIONs). B: SPION coated with lauric acid and a protein layer of bovine serum albumin (BSA) to provide biocompatibility (SEON^{LA-BSA}).*

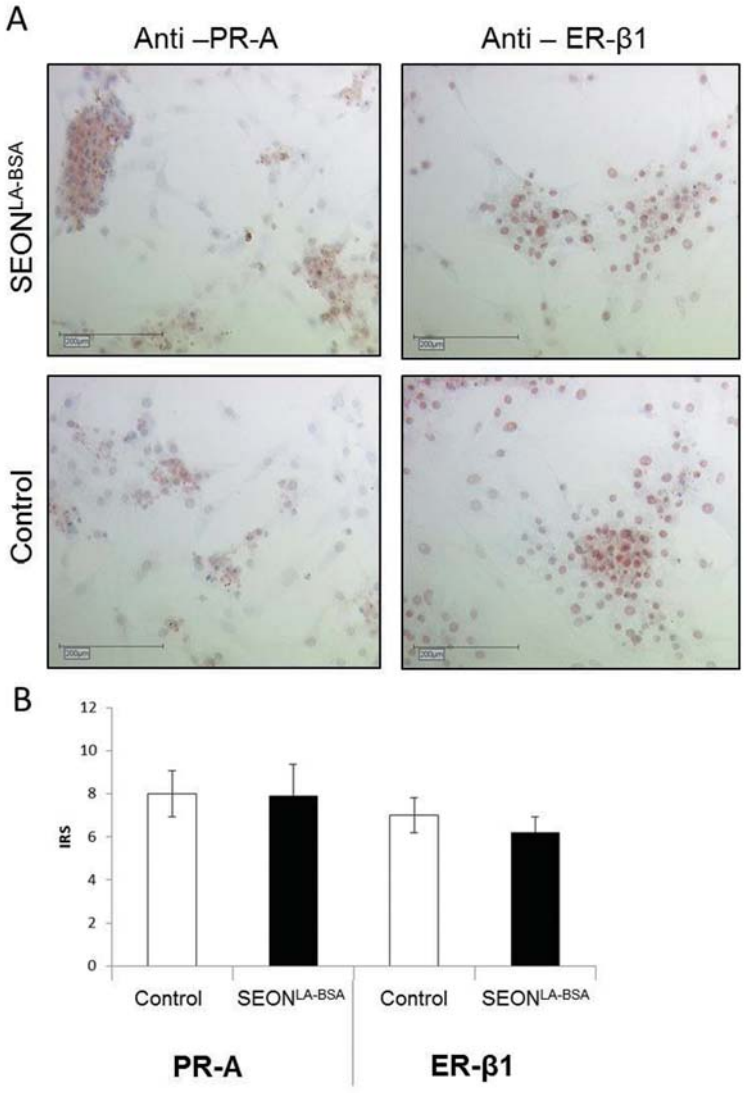

Figure 2. *A: Brown nuclear staining with anti-progesterone receptor A (PR-A) (left panel) and anti-estrogen receptor β1 (ER-β1) (right panel) in primary granulosa cells treated with 25 μg/ml SEON^{LA-BSA} for 48 h. B: The immunoreactive score (IRS) for ER-β1 and PR-A staining was calculated by multiplication of the score for the staining intensity and the score for the percentage of positively stained cells. Data are the mean±SD (n=3).*

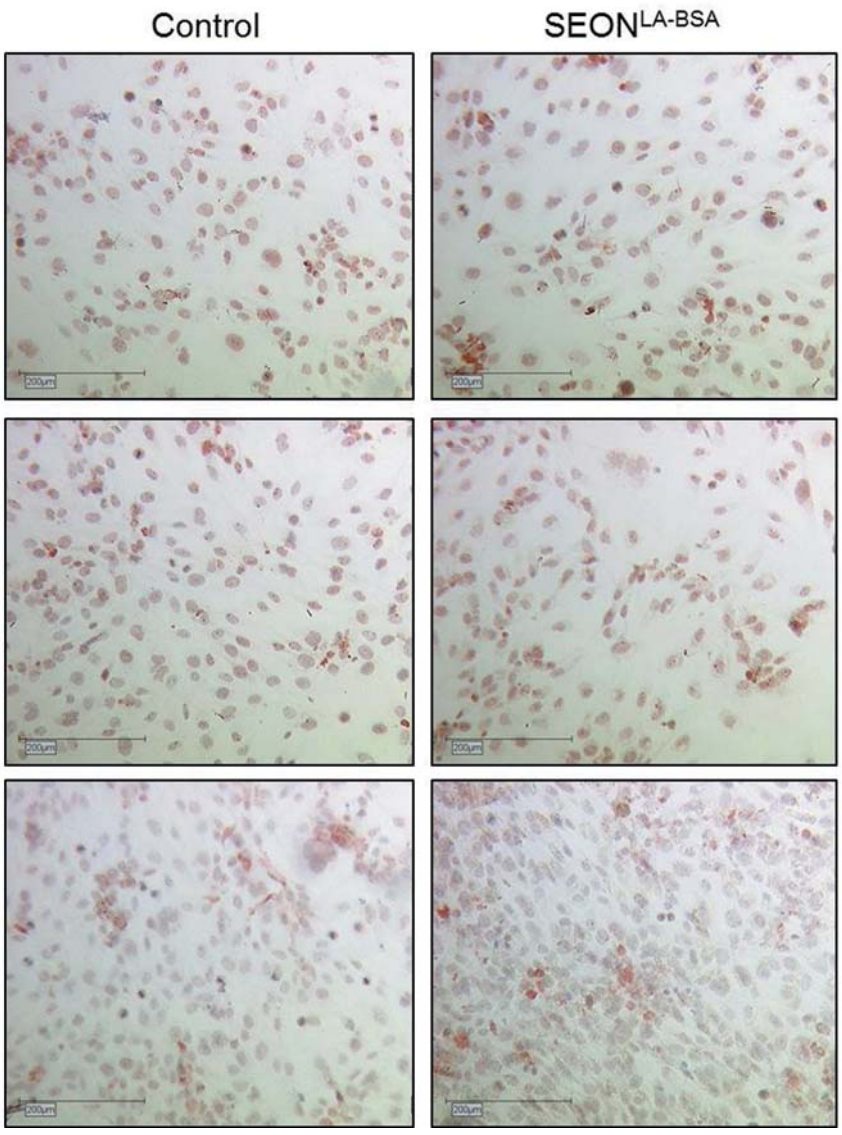

Figure 3. *Brown nuclear staining with anti-estrogen receptor β1 in HLG-5 cells treated with 10, 25 and 50 μg/ml SEON^{LA-BSA} for 48 h.*

Viability of granulosa cells is not affected by SEON^{LA-BSA}. In viable cells, phosphatidylserine (PS) is found primarily in the inner surface of the plasma membrane. During apoptosis, PS is exposed on the cell surface. Re-distribution of PS in apoptosis and the loss of plasma membrane integrity in necrosis were monitored by AxV/PI staining. HLG-5 cells were incubated with SEON^{LA-BSA} at different concentrations (10, 25 and 50 μg/ml) for 24 h, 48 h (Figure 6) and 72 h and the rate of viable (AxV^-/PI^-), apoptotic (AxV^+/PI^-) and necrotic cells (PI^+) was analyzed. Mock-treated cells served as controls. Untreated and SEON^{LA-BSA}-treated cells maintained a viable phenotype throughout the experiment with about 70% viable cells (Figure 6A). SEON^{LA-BSA} did not significantly induce cell death of HLG-5 cells at any concentration. To further visualize viability, cells were stained with Dil and Hoechst (DNA staining). Dil diffuses across cellular plasma membranes, resulting in uniform staining of the entire cell. It is used to measure mitochondrial membrane potential in apoptotic cells, with a loss in membrane potential being reflected in a loss of fluorescent signal, thus indicating a loss of viability. SEON^{LA-BSA} uptake had no notable effect on viability after 24 h and 48 h (Figure 6B).

Discussion

From the provided data, we conclude that protein pre-coated nanoparticles are highly biocompatible and appear to have

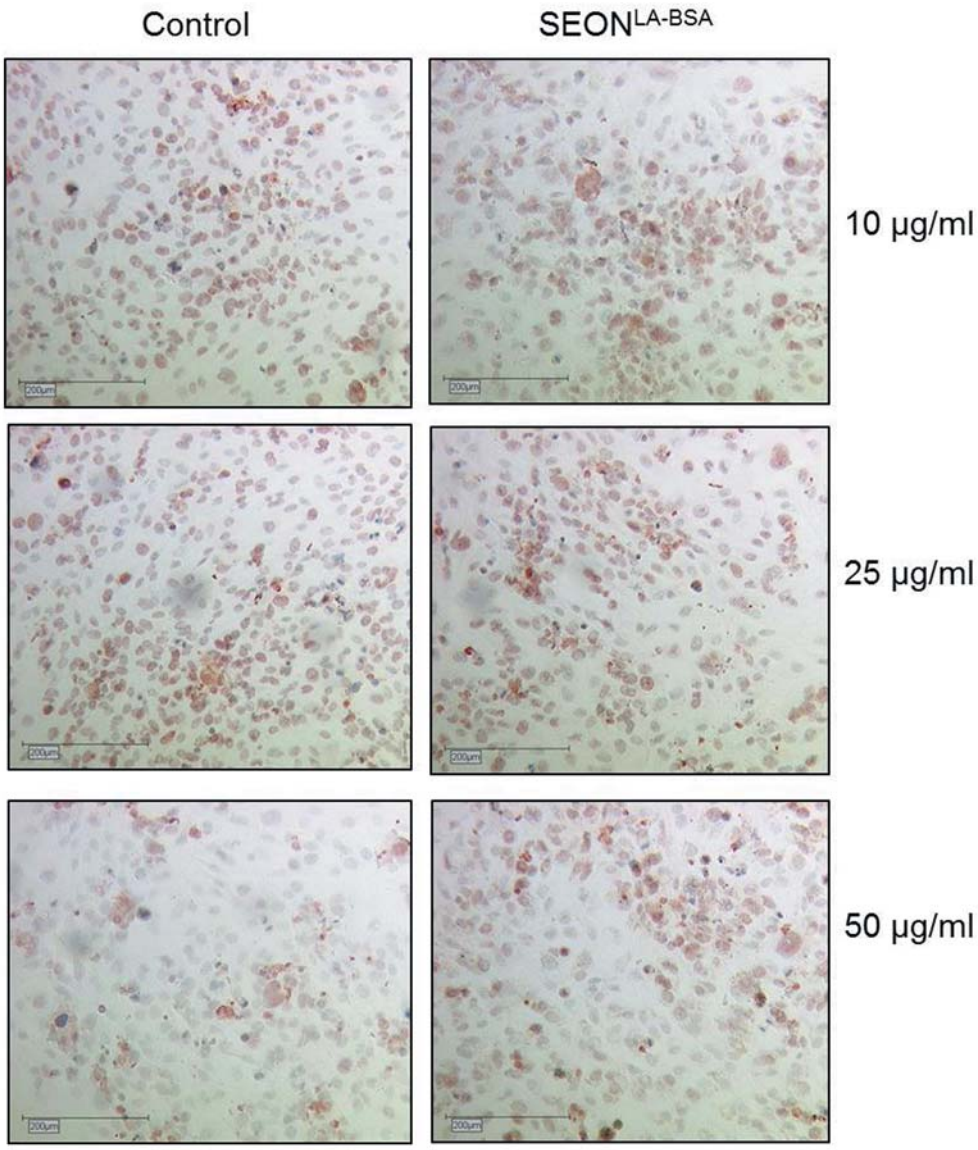

Figure 4. *Brown nuclear staining with anti-progesterone receptor A in HLG-5 cells treated with 10, 25 and 50 µg/ml SEON^{LA-BSA} for 48 h.*

no negative effects on reproductive tissue, compared to other types of nanoparticles (20-22). Thus, for the first time we have shown that nanoparticles pre-coated with protein, SEON^{LA-BSA}, do not affect granulosa cell steroid receptor expression and viability. In contrast, literature refers to calcium phosphate nanoparticles promoting apoptosis and inhibiting cell growth at different concentrations (10 µg/ml and 100 µg/ml) (20). Study of gold nanoparticles showed they travel across the granulosa cell membrane and thereby alter estradiol secretion and accumulation (21), which is a negative outcome since the reproductive system is regulated by steroid hormones. The uptake of quantum dot with transferrin bioconjugates induced reproductive toxicity and consequently disturbed oocyte formation of antrum cavity and disturbed steroid biosynthetic pathways, reducing estradiol production (22).

Several studies on the improvement of biocompatibility of nanoparticles agree on the importance of understanding of the protein corona and consequently how nanoparticles interact with cells, since these proteins manage the specific cellular receptors used by the protein–nanoparticle complex (23), the cellular internalization pathway (24), as well as the immune response (25, 26). Nanoparticle uptake in cells depends on their coating and this in turn influences the level of uptake and

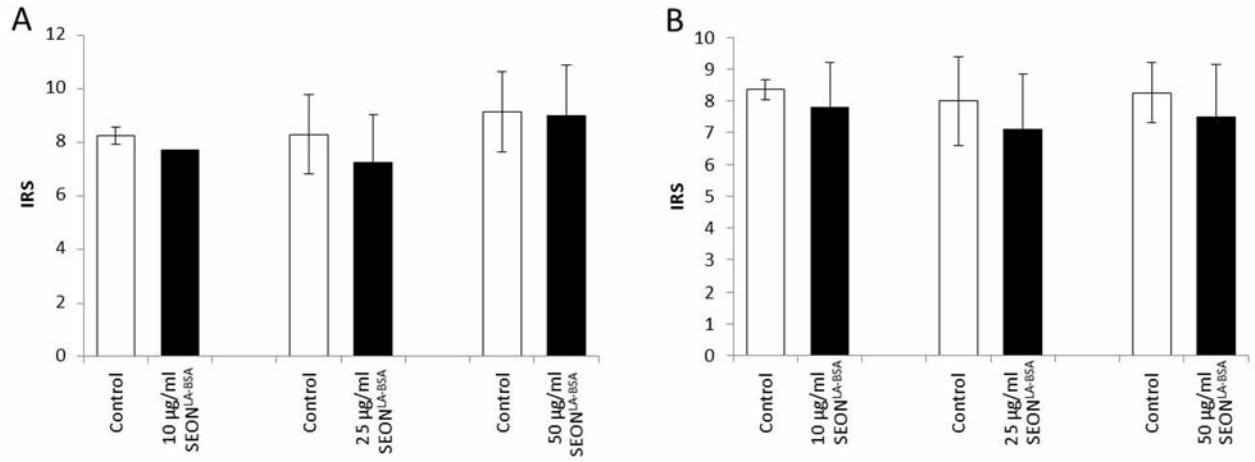

Figure 5. *The immunoreactive score (IRS) for staining with anti-estrogen receptor β1 (A) and anti-progesterone receptor-A (B) in HLG-5 cells treated with different concentrations of SEON^{LA-BSA} was calculated by multiplication of the score for the staining intensity and the score for the percentage of positively stained cells. Data are the mean±SD (n=3).*

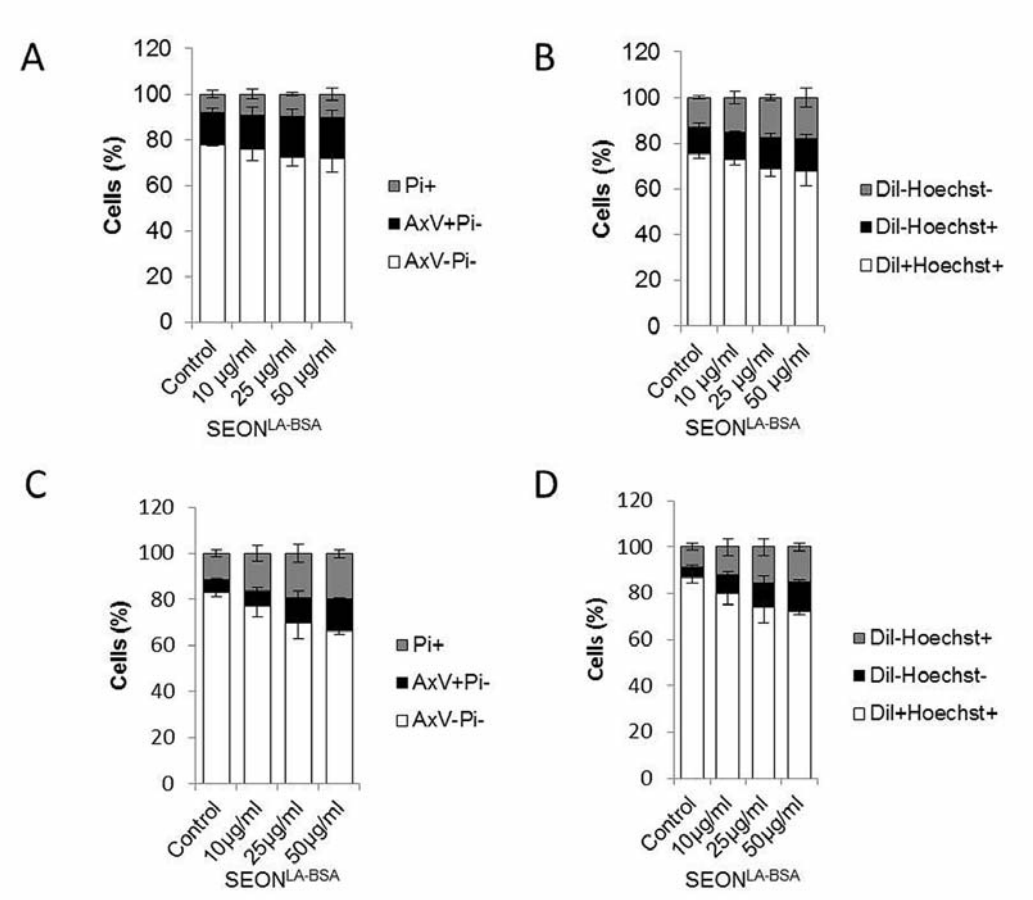

Figure 6. *Flow cytometric analysis of HLG-5 cell viability after treatment with SEON^{LA-BSA} for 24 h (A, B) and 48 h (C, D). A and C: Annexin V (AxV)/propidium iodide (PI) staining, showing viable (AxV$^-$/PI$^-$), apoptotic (AxV$^+$/PI$^-$) and necrotic cells (PI$^+$). B and D: Staining with 1,1',3,3,3',3'-hexamethylindodicarbo-cyanine iodide (Dil) (reflecting mitochondrial potential, and viable cells) and Hoechst 33342 (Hoechst) (nuclear dye, indicating cell viability). Data are the mean±SD (n=3).*

intracellular distribution. For example, nanoparticles with an existing protein corona are always seen in vesicles without cell damage, whereas nanoparticles without pre-coating are either found free in the cytosol or in vesicles or lysosomes and are more prone to aggregation and causing cell destruction (27).

Our data indicate better outcome using SEON^{LA-BSA} overall, including cell viability as well as steroid hormone receptor expression, thus making them more biocompatible and less toxic and less destructive, since a BSA coating on nanoparticles abrogates the high surface activity on interacting cells and tissue. This study proposes that the effects of nanoparticles on ovarian function should be intensively investigated, since alteration in the microenvironment of oocytes is a very potent platform for exploring the influence of nanomaterials on reproductive tissue.

Conflicts of Interest

The Authors report no conflicts of interest in regard to this work.

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