Chapter

Tumor Necrosis Factor Related Apoptosis Inducing Ligand-conjugated Near IR Fluorescent Iron Oxide/Human Serum Albumin Core-shell Nanoparticles of Narrow Size Distribution for Cancer Targeting and Therapy

Itay Levy, Igor Grinberg, Benny Perlstein, Enav Corem-Salkmon and Shlomo Margel*

Department of Chemistry, Bar Ilan University, Israel

**Corresponding Author:** Margel S, Department of Chemistry, Bar Ilan University, Ramat Gan, Israel, Email: Shlomo.Margel@biu.ac.il

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Abstract

Although much progress has been made in the field of cancer therapy, cancer remains one of the leading causes of death in the western world. Here we have designed and studied a unique type of composite multi-functional near IR (NIR) fluorescent iron oxide (IO) nanoparticles (NPs) of narrow size distribution for tumor targeting and therapy. These NPs were prepared by nucleation followed by controlled growth of thin films of IO onto Cy7-conjugated gelatin nuclei and coated with human serum albumin (HSA) by a thermal precipitation process. The hydrodynamic diameter of these core-shell NPs could be easily controlled by altering the precipitation reaction temperature. For targeting and an anti-cancer effect, we conjugated the Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) cytokine to the surface of the NIR fluorescent IO/HSA NPs via a polyethylene glycol (3 kDa) linker. The conjugated TRAIL exhibited enhanced and prolonged anti-cancer activity in both human glioblastoma multiforme and colon cancer cell lines. Further, the combination of these IO/HSA-TRAIL NPs with the commonly used chemotherapeutic drug doxorubicin resulted in a synergistic anti-cancer effect on these cancer cell lines. In addition, we also clearly demonstrated by topically and IV administrations the specific targeting effect and the synergistic therapy effect of the NIR fluorescent NPs in-ovo, by using a chicken embryo model of tumors derived from the various human cancer cell lines.

Keywords

Iron Oxide Nanoparticles; Near IR Fluorescent Iron Oxide Nanoparticles; Cancer Targeting; Cancer Therapy

Introduction

During the last decades, much has been learned about cancer which has led to the development of new and effective therapies.
However, despite these advances, cancer remains a leading cause of death in the western world [1].

Nanoparticles (NPs) as a delivery systems have attracted much attention as both passive and active tumor-targeting vehicle. Due to the “enhanced permeability and retention” (EPR) effect NPs are preferentially concentrated and retained in the leaky vasculature of tumor tissue [2,3]. In addition, functionalized NPs with targeting groups can promote specific receptor-mediated endocytosis, limiting nonspecific uptake into normal cells [4,5]. Iron Oxide (IO) NPs can also be used for detection purpose, as contrast agents for MRI or spectrally, by the attachment of a fluorescent dye. Indeed, studies performed by our group, have shown that it is possible to mark the IO NPs with a fluorescent dye which further improves the probe capabilities [6,7]. Recent developments in near IR (NIR) fluorescent dyes and instrumentation allow for an additional improvement to the NPs probe capabilities, due to low autofluorescence and high tissue penetration which enables in-vivo tracking [8-10].

NPs can be designed according to an intended purpose. For example, NPs can be conjugated to a α,ω-polyethylene glycol (PEG) which can stabilize and prolong the NPs’ stay in blood by reducing the interaction with plasma proteins [11-15]. For therapeutic purposes, NPs can be conjugated to anti-cancer compounds such as the cytokine Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL).

TRAIL is a member of the tumor necrosis factor (TNF) family of cytokines capable of initiating apoptosis through engagement with death receptors (DRs) [16]. Most tumor cells widely express TRAIL DRs. This enables TRAIL to specifically target tumor cells while sparing healthy cells. Therefore TRAIL is expected to exert minimal toxic effects, as demonstrated in clinical trials [16-18].

Despite its great promise, TRAIL and TRAIL-related therapy failed to exhibit effective treatment either as mono-therapy or combined therapy [17]. Upon systemic administration, TRAIL is rapid-
ly eliminated, with a plasma half-life of less than 30 minutes, thus it is unable to reach its target in sufficient concentrations [19,20]. In the past few years, much effort has been put in an attempt to develop TRAIL-mimicking therapy. For example, preclinical studies on TRAIL containing additional structures (tags) in order to stabilize TRAIL. However, no clinical development of tagged TRAIL was performed due to their toxicity [18,21]. Another type of TRAIL mimicking therapy is antibodies that can bind to a specific DR. However, it turned out that antibodies against one type of DR are not enough to elicit the desired anti-tumor effect [20].

In this study, we chose to focus on two types of cancers: colon cancer and glioblastoma multiform (GBM), as a model. Colon cancer accounts for approximately 8% of all cancer related deaths in the United States in 2014, however, survival rates can improve when the cancer is detected and treated in the early stages [1,22]. GBM belongs to the most aggressive human brain cancers because of the rapid proliferation, resistance to treatment, diffuse growth and invasion into distant brain areas. Patients with GBM have a poor prognosis since brain tumors respond poorly to the conventional treatments i.e., radiation or chemotherapy [23,24].

The main purpose of the present article was to study the anti-cancer activity and stability of TRAIL conjugated to the NIR fluorescence IO/HSA-PEG NPs. Furthermore, we were interested in studying the anti-cancer synergetic effect of IO/HSA-PEG-TRAIL combined with Doxorubicin (DOX). In-vitro experiments were carried out on GBM or colon cancer cell lines and in-ovo experiments were performed on a chicken embryo model producing tumors from the same cell lines.

**Materials and Methods**

All materials were purchased from commercial sources and used without further purification, as follows: Bicarbonate buffer (BB; 0.1 M, pH 8.3), ferric chloride hexahydrate, hydrochloric acid (1 M), sodium hydroxide (1 M), sodium nitrite, paraformaldehyde (4%), Triton X-100, gelatin from porcine skin, human serum albumin
(HSA), Cy7, poly ethylene glycol (PEG) of MW of 3000 g/mol with N-hydroxysuccinimide (NHS) groups at both ends (NHS-PEG-NHS), divinyl sulfone (DVS), triethylamine (TEA), D-glucose, doxorubicin (DOX) and Matrigel were purchased from Sigma-Aldrich (Israel); Recombinant human TRAIL was purchased from PeproTech (Israel); Midi-MACS magnetic columns was purchased from Miltenyi Biotec (Germany); Cytotoxicity detection kit (LDH) was purchased from Roche Applied Science (Germany); Phosphate-buffered saline (PBS; free of Ca$^{+2}$ and Mg$^{+2}$; 0.1 M, pH 7.4), Dulbecco’s modified Eagle’s medium–nutrient mixture (DMEM), Eagle’s minimal essential medium (EMEM), McCoy’s 5A modified medium, L-15 medium, fetal calf serum (FCS), fetal human serum (FHS), glutamine, trypsin–EDTA solution (0.25%) and cell proliferation kit (XTT) were purchased from Biological-Industries (Israel); Tissue culture plates (24 and 96 wells) and 35 cm culture plates were purchased from Greiner bio-one (Germany); Human cell lines A172, U87, HCT116 and SW620 were purchased from American Type Culture Collection (Manassus, VA); Fertile chicken eggs were obtained from a commercial local supplier (Israel); Human TRAIL ELISA kit, quantikine, was purchased from R&D systems (Biotest, Israel). Water was purified by passing deionized water through an Elgastat Spectrum reverse osmosis system (Elga, High Wycombe, UK).

### Preparation of the NIR Fluorescent IO Core NPs

NIR fluorescent IO NPs were prepared by nucleation followed by controlled growth of IO thin films onto NIR fluorescent gelatin/iron oxide nuclei. First, Cy7-NHS, a NIR dye, was covalently conjugated to gelatin by adding 2 mL DMSO containing 5 mg dye to 20 mL aqueous solution containing 1% (w/v) gelatin. The pH of the aqueous solution was then raised to 8.5 by adding NaOH aqueous solution (1 N). Then, the solution was shaken for 2 h at 60°C. The volume of the solution was then adjusted to 80 mL by adding H$_2$O [25].

Core NIR IO NPs of narrow size distribution were prepared by adding FeCl$_2$ solution (10 mmol/5 ml H$_2$O, 1 N HCl 0.5 ml) to 80 ml aqueous solution containing 240 mg of the fluorescent gelatin
(during the whole procedure, the aqueous suspension is agitated at 60°C), followed by NaNO$_3$ solution (7 mmol/5 ml H$_2$O). Next, 1 N NaOH aqueous solution was added up to pH 9.5. This procedure was repeated three times with 10 min intervals. The formed magnetic NPs were then washed from excess reagents using high gradient magnetic field (HGMF) technique. A washing solution was added to the magnetic column in order to remove undesired excess reagent. As soon as the wash was completed, the column was removed from the magnetic field and the NPs were eluted by adding an aqueous bicarbonate buffer (BB, 0.1 M, pH=8.3) [25].

HSA Coating of the IO Core NPs

HSA coating was performed by shaking the aqueous suspension of the NIR fluorescent IO core NPs with 20% (w/w) HSA (MW ~66,000) at 75°C for 12-24 h. The HSA coated NPs were then washed by magnetic columns as described in previous section. Eventually the core-shell IO/ HSA fluorescent NPs were eluted with PBS (pH 7.4) using the micro magnetic columns.

Activation of the IO/HSA Core-Shell NPs

First, 1 ml of NHS-PEG-NHS dissolved in PBS (1 mg/ml) was added to 0.2 ml of the fluorescent IO/HSA NPs (5 mg/ml) dispersed in an aqueous phase. Next, the dispersion was then shaken for about 3-5 min at room temperature and the remaining non-conjugated NHS-PEG-NHS molecules were then washed with PBS from the obtained NHS-PEG-conjugated NPs using high gradient magnetic field (HGMF) technique. The activated NIR fluorescent IO/HSA-PEGNHS NPs were then eluted from the magnetic columns by applying an aqueous solution and were then immediately conjugated to a desired ligand through its amine group.

Conjugation of TRAIL to PEG Activated NIR Fluorescent IO/ HSA NPs

Briefly, 200 μl of dissolved TRAIL (0.1 mg/ml H$_2$O) were mixed with 200 μl of the activated fluorescent IO/HSA-PEG-NHS NPs (5
mg/ml) dispersed in PBS (volume was completed to 1 ml with BB). Next, the dispersion was shaken at room temperature for 60 min followed by blocking of residual activated NHS groups by adding glycine (1% w/v) and mixing the dispersion for additional 30 min at room temp. Excess of non-conjugated ligands were then removed with PBS by high gradient magnetic field (HGMF) technique and then the TRAIL-conjugated IO/HSA NPs were eluted.

Conjugation of TRAIL to DVS Activated IO NPs

In previous publication by our group the TRAIL was conjugated to IO NPs via divinyl sulfone (DVS; \( H_2C=CH-SO_2-CH=CH_2 \)) activation [26]. Briefly, 20 μl of DVS was added to 1 ml of the IO NPs (5 mg/ml) dispersed in aqueous phase. The solution was then shaken for 12 h at 60°C and the remaining free DVS was then washed from the obtained DVS conjugated NPs using high gradient magnetic field (HGMF) technique with BB.

Characterization Methods

Dry size measurements: NIR fluorescent IO and IO/HSA NPs were both diluted with \( H_2O \) to a concentration of 1 mg/ml and dried over a TEM grid. The diameters of these dried NPs were then measured by a TEM (JEM-1200EX, JEOL, Japan). Samples for TEM were prepared by placing a drop of a diluted dispersion of the NPs on a 400-mesh carbon-coated copper grid. Measurements of the NPs’ size were performed using ImageJ software, and the results represent an average of 200 NPs.

Hydrodynamic size and size distribution measurements: Samples of the NIR fluorescent IO and IO/HSA NPs synthesized in three different temperatures (55, 65 & 75°C) were dispersed in \( H_2O \) and its wet size and size distribution were determined using NANOPHAX (SympatecGmbH, Germany). Each sample was measured 5 times and the size average is presented.
Quantification of the conjugated TRAIL: To determine the concentration of the conjugated TRAIL, an Enzyme-Linked Immuno-Sorbent Assay (ELISA) method was applied. The testing procedure was performed according to the manufacturer instructions (R&D Systems, Quantikine, Human TRAIL/TNFSF10)[27]. Each measurement was tested in triplicates.

**Cell Cultures**

A172 cell line (human GBM) was maintained in Dulbecco’s modified eagle’s medium (DMEM), U87 cell line (human GBM) was maintained in Eagle’s minimal essential medium (EMEM), HCT116 cell line (human colon cancer) was maintained in McCoy’s modified medium and SW620 cell line (human colon cancer) was maintained in L-15 medium. All cell lines’ mediums were supplemented with 10% fetal calf serum (FCS), 1% of glutamine and 1% of penicillin/ streptomycin, incubated at 37°C, 5% CO₂.

**Cell Cytotoxicity Analysis**

LDH analysis [28] was used to determine and compare the in vitro activity and stability of free and NIR fluorescent TRAIL-conjugated NPs. Free and conjugated TRAIL were put in a non-heated activated human serum, in two concentrations (5 & 10 ng/ml of TRAIL), and incubated at 37°C, 5% CO₂. In all experiments, non-conjugated IO/HSA NPs were used as control (with a similar NPs’ concentration as the highest concentration of the applied IO/HSA-TRAIL NPs). During the incubation, in selected time points (0.1, 12, 60, 108, 156, 228 and 324 h), a sample was taken out of the free and the conjugated TRAIL and kept frozen at -20°C. Next, the stored samples were added into a 24 wells plate, which was seeded with 105 A172 cells per well. The plate was then incubated at 37°C, 5% CO₂ for 24 h and the medium from each well was transferred into Eppendorf tubes, centrifuged and transferred in triplicates into 96 wells plate to proceed with the LDH procedure, according to manufacturer instructions (Roche)
Applied Science). Absorption was measured with TECAN spectrophotometer to determine the cells cytotoxicity.

**Cell Proliferation Analysis**

XTT analysis [29] was performed, according to manufacturer instructions (Biological Industries). In brief, SW620, A172, U87 & HCT116 cells, respectively, were seeded onto 96 well plates and incubated for 24 h at 37°C, 5% CO₂. Next, DOX was dissolved in H₂O then diluted with a medium (according to the medium of the cell line) to produce several concentrations (detailed in the results section) of the NIR fluorescent TRAIL-conjugated IO/HSA NPs and anti-cancer drug DOX, alone or in combinations were added into the wells which were seeded with cancer cells. In all experiments, non-conjugated IO/HSA NPs were used as control (with a similar NPs’ concentration as the highest concentration of the applied IO/HSATRAIL NPs). The plate was then incubated at 37°C, 5% CO₂ for 48 h and the XTT procedure continued according to manufacturer instructions. Absorption was measured for all samples, each in triplicates, with TECAN spectrophotometer to determine the cells viability.

**Tumor Treatment in a Chicken Embryo Model**

Chicken embryo model was used to allow cancer cell lines an ability to form a tumor on the external chorioallantoic membrane (CAM), as shown in Figure 1. On embryonic day 8, a window was opened in the egg shell exposing the CAM. Then cells, A172, U87 & HCT116 suspended in 30 μl Matrigel were dripped on top of the exposed CAM. The window was sealed with cellotape and the eggs were incubated for 4 days at 37°C and 50-60% humidity. There are two types of treatment available when using this model: i.v. injection and topical administration. The i.v. injection model mimics the intended i.v. clinical treatment and the topical administration mimics the local administration treatment in humans. For the i.v. injection treatment, a total of 100 μL of 0.01 mg/mL of the NIR fluorescent TRAIL-conju-
gated IO/HSA NPs, the control NIR fluorescent IO/HSA NPs (with a similar NPs’ concentration as the highest concentration of the applied IO/HSA-TRAIL NPs) and free TRAIL (equivalent amount of TRAIL-conjugated NPs) dispersed in PBS were injected intravenously into a large CAM blood vessel exposed by an additional window in the egg shell. After the injection, the window in the egg's shell was sealed with cellotape, and the chicken embryos were returned to incubation for 96 h. Each experiment group contained 7 embryos. For the topical administration treatment, each tumor was treated, with either NIR fluorescent TRAIL-conjugated IO/HSA NPs, control NIR fluorescent IO/HSA NPs (with a similar NPs’ concentration as the highest concentration of the applied IO/HSA-TRAIL NPs), DOX or combination treatments in different concentration depended on the cell type (detailed in the results section), by dripping directly on the tumor. In both treatment types, 4 days after treatment, the tumors were cut out and then weighted. All the experiments with chicken embryos were repeated twice with similar results. To decrease animal suffer, the experiment with chicken embryos were terminated after 16 days. Experiments were performed according to the protocols of the Israeli National Council for Animal Experiments.
The fluorescence intensity of the TRAIL-conjugated IO/HSA NPs and the control NPs was measured by the Maestro II in-vivo imaging system, 2D planar fluorescence imaging of small animals (Cambridge Research & Instrumentation, Inc., Woburn, MA). A NIR excitation/ emission filter set was used for our experiments (ex: 710–760 nm, em > 750 nm). The Liquid Crystal Tunable Filter (LCTF) was programmed to acquire image cubes from 790 nm to 860 nm with an increment of 10 nm per image. The tumor samples were transferred onto black paper and then imaged.

Calculation of the synergistic effect: To examine the synergistic effect, the partial effect of the drugs was calculated according to the Bliss independence model, which is defined by the following equation [30]:

\[
\text{Eff}_{(\text{drug A + drug B})} = \text{Eff}_{(\text{drug A})} + \text{Eff}_{(\text{drug B})} - \text{Eff}_{(\text{drug A})} \times \text{Eff}_{(\text{drug B})}
\]
Where, $\text{Eff}_{(\text{drug A} + \text{drug B})}$ is the additive effect of drugs A and B as predicted by their individual effects ($\text{Eff}_{\text{drug A}}$ and $\text{Eff}_{\text{drug B}}$). For calculation purpose, in this article, the drugs’ anti-cancer effect was defined as complementary to the obtained results ($100 - \%\text{Eff}_{(\text{drug A} + \text{drug B})}$).

Results and Discussion

The present manuscript describes the synthesis and characterization of a unique NIR fluorescent IO/HSA core-shell NPs, by nucleation followed by controlled growth of thin films of IO onto Cy7-conjugated gelatin nuclei. A thin HSA coating around the NIR fluorescent IO core NPs was produced by a thermal precipitation process according to the description in the experimental part. For targeting and therapy of tumors, TRAIL was covalently bound to the HSA shell of the NIR fluorescent core-shell NPs via a PEG spacer (3 kDa). This process of TRAIL binding is different from that described in previous publications [26] wherein the TRAIL was bound directly to the IO core NPS via a DVS spacer.

Size and Size Distribution

TEM measurements (Figure 2A and 2B) indicates that the size of the NIR fluorescent IO and IO/HSA NPs are 16 ± 2 nm and 20 ± 3 nm, respectively. These results indicate that the dry average diameter of the HSA coating is approximately 4.0 nm. On the other hand, DLS measurements (Figure 2C) indicate that the average diameter of the IO and IO/HSA NPs are 103 ± 14 nm and 43 ± 5 nm, respectively. It should also be noted that after about 2 weeks the IO/HSA NPs hydrodynamic diameter did not change (data not shown) and no apparent agglomeration of the NPs was detected. Similar differences between the hydrodynamic size and the dry size of various NPs were previously reported by Margel et al. [31]. The size differences between TEM and DLS measurements can be attributed to the fact that TEM measures the dry diameter while DLS determines the hydrodynamic diameter, which takes into account the hydrated layers on the particle surface. The core IO NPs are composed of gelatin and
IO nuclei and a thin layer of surface gelatin which protects the core NPs from agglomeration. Gelatin is known for its ability to swell by absorbing water molecules up to 10 times its original size [32], these water absorbing layers may be the main reason for the significant size difference between the dry and the water dispersed IO core NPs (16 ± 2 and 103 ± 14 nm, respectively). We also assume that the coating of the IO core NPs with HSA partially prevents water molecules from interacting with the gelatin belonging to the core NPs, resulting in a decreased hydrodynamic diameter, from 103 ± 14 nm to 43 ± 5 nm. More interest in the HSA precipitation reaction has rose, assuming different precipitation may produce smaller hydrodynamic diameter.

Figure 2: TEM images of the NIR fluorescent IO (A) and IO/HSA (B) NPs. DLS measurements of the hydrodynamic diameter and size distribution of the IO (C) and the IO/HSA NPs, prepared in 3 different HSA coating temperatures.
We further investigated into the ability to control the hydrodynamic diameter of the Cy7-IO/HSA NPs, by changing the temperature of the coating process of the NIR fluorescent IO core NPs with HSA. Figure 2C shows that decreasing the temperature of the HSA coating reaction from 75°C to 65°C and 55°C causes an increase of the average hydrodynamic diameter from 43 ± 5 nm to 63 ± 9 nm and 89 ± 12 nm, respectively. This indicates that the HSA coating decreases the swelling of the gelatin caused by interacting of water with the NPs, in a temperature depended manner. We hypothesize that this phenomenon is due to the HSA coating becoming more compacted as the coating temperature increases. These results demonstrate that it is possible to control the hydrodynamic size of the NPs by simply changing the coating reaction temperatures. However, it should be noted that no change in the dry diameters of the core-shell NPs prepared at different HSA coating temperatures was found.

**Quantification of the TRAIL Conjugated to the IO/HSA NPs**

The concentrations of the TRAIL conjugated to the NIR fluorescent core-shell NPs were determined, using an ELISA assay. The ELISA assay exhibited that the concentration of the TRAIL bound to 1 mg NIR fluorescent IO/HSA NPs via a PEG linker was 4.3 ± 0.3 μg while the concentration of the TRAIL bound to the IO NPS via a DVS linker, as was done in our previous publication [26] was 3.0 ± 0.2 μg. These results show a significant increase of nearly 50% in the concentration of the conjugated TRAIL in favor of the IO/HSA-PEG-TRAIL NPs. Increased concentration of conjugated TRAIL clearly implies better conjugation efficiency, and may improve the biological efficacy of the NPs.

**NIR fluorescent IO/HSA-PEG-TRAIL NPs Exhibit Prolonged in-vitro Cytotoxic Effect**

A stability study was designed to compare the cytotoxic effect of the NIR fluorescent IO/HSA-PEG-TRAIL NPs relative to free
TRAIL, on A172 GBM cells. In addition, we compared the IO/HSA-PEG-TRAIL with a previously studied type of NPs synthesized by our group, the IO-DVS-TRAIL NPs [26]. Samples of NIR fluorescent IO/HSA-PEGTRAIL NPs, IO-DVS-TRAIL NPs and free TRAIL were adjusted to contain similar concentration of TRAIL (10 ng/ml) by diluting with PBS and non-heated human serum (75% v/v). The non-heated human serum was used to accelerate the TRAIL degradation process due to the presence of proteolytic enzymes. Samples were incubated (37°C, 5% CO₂) for 0.1, 12, 60, 108, 156, 228 and 324 h and then applied on A172 cells (see experimental part). Using LDH assay, the cytotoxicity of the samples was determined relative to treatment with Triton –X-100 as positive control and without (w/o) treatment as negative control.

As shown in Figure 3, at the first measured time point (0.1 h), the cytotoxicity of the IO/HSA-PEG-TRAIL NPs was almost similar to that of free TRAIL, with 98 ± 3% and 94 ± 3%, respectively. Following 324 h incubation, the cytotoxicity of the IO/HSA-PEG-TRAIL NPs decreased from 98 ± 3% to 29 ± 2%, whereas the cytotoxicity of free TRAIL decreased from 94 ± 3% to 13 ± 1%, showing a significant differences between the groups (P<0.01; t-test). Similar results were obtained by applying a lower concentration of TRAIL (5 ng/ml, data not shown). The cytotoxicity of the non-conjugated NIR fluorescent IO/HSA NPs was also tested and found to be similar to that of the untreated control (data not shown).
These results demonstrate that the TRAIL conjugated to the IO/HSA NPs remains active for a longer period. It may be that the conjugation of TRAIL to the IO/HSA NPs inhibits the proteolytic enzymes in the serum and therefore increase TRAIL stability. Another possible reason is that the conjugated TRAIL mimics naturally displayed TRAIL. TRAIL molecules are often conjugated to exosome in the body and are the physiological form of Apo2L/TRAIL release. Indeed, a study performed on TRAIL bound to artificial lipid vesicles with a similar size and composition as natural exosomes, showed enhanced bioactivity [33]. We think that our TRAIL conjugated NPs may also resemble the natural form of the physiological TRAIL.

Moreover, Figure 3 exhibits that the most pronounced difference between the NPs cytotoxic effect was observed after 0.1 h (p<0.01; t-test). At this time point, while upon TRAIL conjugation to the IO NPs via DVS, TRAIL partly loses its activity, were as conjugation via the PEG spacer to the IO/HSA NPs, no loss of activity is exhibited (cytotoxic effect of 65 ± 2% and 98 ± 3%, respectively). In addition,
after 324 h incubation, in non-heated human serum, the cytotoxic effect of the IO/HSA-PEG-TRAIL NPs is significantly higher (p<0.01; t-test) than that of the IO-DVS-TRAIL NPs (29 ± 2% and 9 ± 1%, respectively). These results demonstrate the improvement made in TRAIL conjugation process as well as the improvement in prolonging the activity of TRAIL. Therefore, from here on out, in the rest of the study presented in this article, TRAIL was conjugated to the IO/HSA NPs only via a PEG spacer.

The Combined in-vitro Effect of NIR Fluorescent IO/HSA-TRAIL NPs and the Chemotherapeutic Drug DOX

Proliferation experiments using XTT method were performed to determine the anti-cancer activity of the IO/HSA-TRAIL NPs in the absence and presence of the known anti-cancer drug DOX. We chose to perform a proliferation assay instead of cytotoxicity assay due to the anti-cancer drugs mechanism which prohibits cell proliferation. In brief, 0.1 X 10^5 A172 GBM cells were seeded into a 96 well plate, in 100 μL medium and incubated for 24 h at 37°C, with 5% CO₂. Next, samples were added to the cells medium as follows: IO/HSA-TRAIL (5 ng/ml of TRAIL) NPs, DOX (31, 62 or 125 ng/ml) alone or in combination. All the combination treatments were performed by applying the DOX 24 h prior to the IO/HSA-TRAIL treatments. Non-treated cells were used as control. It should be clarified that due to space difficulties in Figures 4, 5 and 6 IO/HSA-TRAIL NPs was marked as IO-TRAIL NPs. Figure 4A illustrates that when the treatments were applied combined, there was a significant reduction in A172 cell viability. Treatment with either IO/HSA-TRAIL (5 ng/ml of TRAIL) or DOX (31, 62 or 125 ng/ml) separately resulted in cells viability of 71 ± 7%, 73 ± 5%, 52 ± 5% and 13 ± 1%, respectively. In contrast, when the treatments were applied in combination, a significant reduction in cells viability was noticed. Combined treatment of IO/HSA-TRAIL (5 ng/ml of TRAIL) with DOX (31, 62 or 125 ng/ml) resulted in a reduction of cells viability to 47 ± 4%, 9 ± 1% and 0.5 ± 0.5%, respectively
Treatment with the NPs control (IO/HSA) resulted in no apparent toxicity. These results exhibit a synergetic anti-cancer effect of the combination of DOX together with IO/HSA-TRAIL.

**Figure 4:** Viability of: (A) A172 GBM cells after treatment with IO/HSA control NPs, IO/HSA-TRAIL (5 ng/ml of TRAIL) NPs, DOX (31 ng/ml), DOX (62 ng/ml), DOX (125 ng/ml) or in combinations; (B) U87 GBM cells after treatment with IO/HSA control NPs, IO/HSA-TRAIL NPs (100 or 10 ng/ml), DOX (80 or 20 ng/ml) or their combinations; (C) HCT116 colon cancer cells after treatment with IO/HSA control NPs, IO/HSA-TRAIL NPs (5 ng/ml), DOX (15, 31 or 62 ng/ml) or their combinations; (D) SW620 colon cancer cells after treatment with IO/HSA control NPs, IO/HSA-TRAIL NPs(4 or 20 ng/ml), DOX (31 ng/ml) or their combinations as determined using XTT assay (n=4, mean ± SD). **, P<0.01 from IO/HSA-TRAIL.
Figure 5: (A) A172 GBM cells were grafted onto the CAM of a chicken embryo and grown to form tumors. The tumors were then treated topically with IO/ HSA control NPs, DOX (2 μg), IO/HSA-TRAIL NPs (50 ng of TRAIL) alone or in combination. (B) U87 GBM cells were grafted onto the CAM of a chicken embryo and grown to form tumors. The tumors were then treated topically with IO/HSA control NPs, DOX (200 ng), IO/HSA-TRAIL NPs (100 ng of TRAIL) alone or in combination. (C) HCT116 colon cancer cells were grafted onto the CAM of a chicken embryo and grown to form tumors. The tumors were then treated topically with IO/HSA control NPs, DOX (2 μg), IO/HSA-TRAIL NPs (20 ng of TRAIL) alone or in combination. Tumor weight was determined for each treatment group (n=6, mean ± SD). *, P<0.05; **, P<0.01 from IO/ HSA-TRAIL. Abbreviations: w/o treatment, without treatment; IO-TRAIL, IO/HSA-TRAIL; SD, standard deviation.
Next, U87 glioma cells were tested in which 0.1 × 10⁵ cells per well were seeded. Samples were added to cells as follows: IO/HSA-TRAIL (100 or 10 ng/ml of TRAIL), DOX (80 or 20 ng/ml) alone or in combinations. Figure 4B illustrates that as a result of the combined treatment there was a significant reduction in U87 cell viability. Cells treated with either IO/HSA-TRAIL (100 ng/ml of TRAIL), IO/HSA-TRAIL (10 ng/ml of TRAIL), DOX (80 ng/ml) or DOX (20 ng/ml) separately exhibited a reduction in viability to 47 ± 3%, 87 ± 4%, 57 ± 3% and 76 ± 3% respectively. However, a combined treatment with either DOX (80 ng/ml) and IO/HSA-TRAIL (100 ng/ml of TRAIL) or with DOX (80 ng/ml) and IO/HSA-TRAIL (10 ng/ml of TRAIL), resulted in a larger reduction in viability 7 ± 1% and 27 ± 3%, respectively (P<0.01; t-test). In addition, a combined treatment with either
DOX (20 ng/ml) and IO/HSA-TRAIL (100 ng/ml of TRAIL) or with DOX (20 ng/ml) and IO/HSA-TRAIL (10 ng/ml of TRAIL), also exhibited a reduction in viability to 26 ± 3% and 55 ± 3%, respectively (P<0.01; t-test). Treatment with the control NPs (IO/HSA) resulted in no apparent toxicity. These results demonstrate a synergetic anticancer effect of the combination of DOX and IO/HSA-TRAIL. Both intrinsic and acquired resistance to TRAIL poses a huge problem in establishing clinically efficacious TRAIL therapies. Thus, the success of using TRAIL alone as a therapeutic agent, is modest [20]. From recent literature, it is known that several chemotherapeutic agents sensitize resistant cancer cells to TRAIL induced apoptosis [20]. For example, Convection-enhanced delivery of TRAIL protein combined with systemic administration of temozolomide proved more effective than either therapy alone in mouse models. These studies suggest that DOX, as well as temozolomide, may sensitize cells to TRAIL-induced apoptosis; however, the underlying signaling mechanisms remain poorly understood [34].

Positive results with the brain cancer cells encouraged testing other cancer cells and therefore colon cancer was chosen. The activity of the IO/HSA-TRAIL NPs in the absence and presence of DOX was tested. The colon cancer cell line, HCT116, was seeded into a 96 well plate, with 0.02 X 106 cells per well and incubated for 24 h at 37°C, with 5% CO₂. Cells were treated as follows: IO/HSA-TRAIL (5 ng/ml of TRAIL), DOX (15, 31 or 62 ng/ml) alone or in combinations. Untreated HCT116 cells were used as control. Figure 4C illustrates that treatment with either IO/HSA-TRAIL (5 ng/ml of TRAIL) or DOX (15, 31 or 62 ng/ml) resulted in a reduction of cells viability to 45 ± 4%, 79 ± 7%, 91 ± 4% and 59 ± 7%, respectively. In contrast, the combined treatment of IO/HSA-TRAIL (5 ng/ml of TRAIL) with DOX (15, 31 or 62 ng/ml) resulted in a greater reduction in cell viability to 21 ± 5%, 14 ± 4% and 9 ± 1%, respectively (P<0.01; t-test). Treatment with the NPs control (IO/HSA) resulted in no apparent toxicity. These results demonstrate that the combination of the two drugs increases
the toxic effect compared with the separated drugs, resulting in a synergistic effect for colon cancer cells as well.

Finally, the human SW620 colon cancer cells were tested with 0.01 X 10^6 cells seeded in each well. Cells were treated as follows: DOX (31 ng/ml), IO/HSA-TRAIL (20 or 4 ng/ml of TRAIL) alone or in combinations. Untreated SW620 cells were used as control. Figure 4D demonstrate that all of the separate treatments resulted in an almost similar effect as the untreated control. Treatment with either DOX (31 ng/ml) or IO/HSA-TRAIL (4 or 20 ng/ml of TRAIL) resulted in a minor reduction of cells viability to 90 ± 5%, 92 ± 3% and 82 ± 4%, respectively. In contrast, the combined treatment with DOX (31 ng/ml) and IO/HSA-TRAIL (4 ng/ml of TRAIL), as well as treatment with DOX (31 ng/ml) and IO/HSA-TRAIL (20 ng/ml of TRAIL) exhibited a greater reduction in viability to 81 ± 9% and 69 ± 1% respectively (P<0.01; t-test). Treatment with the NPs control (IO/HSA) resulted in no apparent toxicity. Compared to the other tested cell lines, the SW620 cells are less sensitive to both DOX and IO/HSA-TRAIL treatment. Indeed, from other studies it is known that some cells are resistance to TRAIL and chemotherapy, while others are more sensitive [35]. However, the synergistic effect of the combination treatment may turn these cells to be more responsive to the treatment.

**Anti-Tumor Effect of Topically Administered NIR Fluorescent IO/HSA-TRAIL NPs Combined with DOX in a Chicken Embryo Model**

To evaluate the anti-tumor effect of IO/HSA-TRAIL NPs combined with the anti-cancer drug DOX, a chicken embryo GBM xenograft tumor model was used. In order to study the direct effect of the drugs, tumors were grown on the external CAM of the chicken embryo and the drugs were applied topically. Initially, 2 × 10^6 human A172 cells, suspended in a matrigel, were grafted into each of the chicken embryos. The A172 GBM cells grown on the external CAM of the chicken embryo, however, the grow rate was very slow and therefore the tumor size was not optimal for this type of model.
Following tumor formation IO/HSA-TRAIL (50 ng of TRAIL), DOX (2 μg) alone or in combination was topically applied. In all the combination treatments, DOX was applied 24 h prior to the NPs. After 96 h the tumors were extracted and weighed. The weight of the treated tumors was determined relative to untreated tumor which presents normal tumor growth. As shown in Figure 5A, treatment with DOX, IO/HSA-TRAIL NPs separately and untreated control resulted in a tumor weight of 6 ± 1 g, 6 ± 1 g and 7 ± 1 g, respectively, whereas the combination treatment resulted in a significant decrease of tumor weight to 5 ± 1 g (P<0.05; t-test). Next, we decided to repeat this chicken embryo model experiment with the GBM cell line U87. To do so, we needed to optimize the conditions to fit this type of cell line, in terms of number of grafted cells (only 106 cells per embryo) and the concentration of tested compounds. The tumors formed from the U87 GBM cell line were fast to grow and reached a higher mass than the tumors formed from the A172 GBM cells, which allowed the measurements to be more accurate. The following treatments were topically applied on the chicken embryo CAM: IO/HSA-TRAIL (100 ng of TRAIL), DOX (200 ng) alone or DOX combined with IO/HSA-TRAIL (200 ng and 100 ng of TRAIL, respectively). As shown in Figure 5B, treatment with DOX, IO/HSA-TRAIL NPs separately and untreated cells produced a tumor weight of 39 ± 17 g, 43 ± 16 g and 63 ± 14 g, respectively, whereas the combination treatment resulted in a significant decrease in tumor weight to 23 ± 10 g (P<0.01; t-test). These results suggest that the combination of the two drugs increases the therapeutic effect compared with the separated drugs, exhibiting a synergetic affect.

Next, the anti-tumor effect of IO/HSA-TRAIL NPs combined with the anti-cancer drug DOX, on colon cancer cell line, HCT116, was tested. In each of the chicken embryos, $1 \times 10^6$ human HCT116 cells were grafted. The tumors were topically treated as follows: IO/HSA control NPs (in an equivalent NPs concentration to IO/HSA-TRAIL NPs), DOX (2 μg), IO/HSA-TRAIL (20 ng of TRAIL) or combination of DOX with IO/HSA-TRAIL (2 μg with 20 ng, respectively).
For the combination treatment, DOX was applied 24 h prior to the NPs. As shown in Figure 5C treatment with DOX, IO/HSA-TRAIL separately and untreated control NPs resulted in a tumor weight of $35 \pm 3 \, \text{g}$, $40 \pm 4 \, \text{g}$ and $47 \pm 3 \, \text{g}$, respectively, whereas the combination treatment resulted in a significant decrease of tumor weight to $23 \pm 4 \, \text{g}$ ($P<0.01$; t-test). These results reinforce the observation that the combination of the two drugs increases the therapeutic effect compared with the separated drugs, showing a significant synergistic affect.

It should be noted that we were interested in repeating this experiment with the colon cancer cell line SW620. However, severe bleedings prevented us from executing this experiment.

**Anti-Tumor Effect of NIR Fluorescent IO/HSA-TRAIL NPs i.v. Administered in a Chicken Embryo Model**

To determine the in-ovo anti-tumor activity of the i.v. injected IO/ HSA-TRAIL NPs in comparison to the free TRAIL, chicken embryos were grafted with HCT 116 human colon cancer cells (as described in the experimental part). After 4 days of incubation time, IO/HSA-TRAIL NPs (1 μg of TRAIL), control IO/HSA NPs (in an equivalent NPs concentration to IO/HSA-TRAIL NPs) or free TRAIL (1 μg of TRAIL), all diluted with PBS, were injected into the chicken embryo blood stream. All treated embryos were placed in incubation for an additional 4 days period. The weight of the treated tumors was determined relative to tumors w/o treatment as control.

As shown in Figure 6, the control tumors average weight was $59 \pm 11 \, \text{g}$, while the weights of the tumors treated with free TRAIL or IO/ HSA control NPs were $55 \pm 10 \, \text{g}$ and $54 \pm 9 \, \text{g}$, respectively. However, the average weight of the tumors treated with IO/HSA-TRAIL was only $45 \pm 10 \, \text{g}$, significantly lower than the control group ($P<0.05$; t-test). These results suggest that the TRAIL conjugated to the IO/ HSA NPs has reached its target and remained more active than the free TRAIL. Indeed, previous studies showed that NPs with PEG coating...
remain longer in the blood stream and accumulate in tumors due to the tumor leaky vessels [11,12]. Hence the conjugation of TRAIL to the IO/HSA NPs inhibits the TRAIL degradation by proteolytic enzymes, and thereby contributes to the TRAIL stability. Injection of the combination treatment with IO/HSA-TRAIL and DOX was not performed, considering the possibility of severe bleeding in the chicken embryo due to the need in two injections instead of one.

Next, since the NPs were labeled with the near IR Cy7, the fluorescence of the tumors was measured, to verify the uptake of both the non-conjugated and the TRAIL conjugated to the NIR fluorescent NPs in the tumors. As can be seen in Figure 7A and 7B the presence of the NIR fluorescent IO/HSA-TRAIL and IO/HSA NPs (with a similar NPs’ concentration as the concentration of the applied IO/HSA-TRAIL NPs) was well noticeable. The fluorescent intensity of the tumors treated with IO/HSA-TRAIL NPs was higher, by 60%, than the intensity of the IO/HSA control NPs. This exhibits the significant increase in concentration at the tumor site of the IO/HSA-TRAIL NPs relative to that of the control NPs. Hence, due to the conjugation of TRAIL to the IO/HSA NPs, the NPs exhibit a tumor targeting ability.
Summary and Conclusions

The present study is focused on a unique functionalized core/shell NIR fluorescent IO/HSA NPs of narrow size distribution. These NPs were produced by nucleation and controlled growth of IO thin films onto Cy7-conjugated gelatin nuclei. The NPs were then coated with HSA by a thermal precipitation process. A difference between...
the dry and hydrodynamic diameter of the IO/HSA NPs was found, however the hydrodynamic diameter could be controlled and reduced by changing the temperature of the HSA precipitation reaction.

We then showed in-vitro that TRAIL can be stabilized against degradation and thereby increase its anti-cancer activity by covalently binding TRAIL to the NIR fluorescent IO/HSA core-shell NPs via a PEG linker. Furthermore, the PEG linker also produced a better conjugation efficiency of TRAIL to the IO/HSA NPs. In addition, we demonstrated, in-vitro and in-ovo using a chicken embryo model, that combined treatment of IO/HSA-TRAIL NPs with the commonly used chemotherapeutic drug doxorubicin, topically administrated, resulted in a significant synergistic anti-cancer effect in both human glioblastoma multiforme and colon cancer cell lines. The tumor targeting ability was demonstrated by i.v injection of the IO/HSA-TRAIL, which exhibited a greater fluorescence compared to the non-conjugated IO/HSA NPs and induce a better anti-tumor effect than TRAIL.

Future research is needed to further examine our NPs with other models such as Patient Derived Xenograft (PDX) models in mice, as the next model towards clinical trials. To further exploit the synergistic effect of the conjugated TRAIL and DOX, we intend to synthesis coreshell Cy7-IO/HSA NPs containing DOX and conjugate them to TRAIL. This may allow a better passive and active tumor targeting and into tumor cells. It is possible to design this type of NPs to contain other drug combinations as well and use them to target other cancer types.

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