High-Contrast Magnetic Resonance Imaging and Efficient Delivery of an Albumin Nanotheranostic in Triple-Negative Breast Cancer Xenografts

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Triple-negative breast cancer (TNBC) is a fast growing and strong metastasizing tumor, which presents almost no cellular receptors that can be addressed by targeted therapeutics. In addition, TNBC is often characterized by high tumor grading, fast growth rates, and early metastasis. Therefore, multifunctional drug carriers allowing efficient drug delivery and bioimaging to treat and track TNBC tissue in vivo would be highly desirable. A human serum albumin-based polyethylene glycol copolymer (dcHSA-Gd-Dox) is synthesized combining multiple copies of the chemotherapeutic drug doxorubicin and gadolinium (III) (Gd(III))-based magnetic resonance imaging (MRI) contrast agent. The biodegradable albumin-based nanocarriers reveal high-contrast tumor imaging and efficient drug delivery in a preclinical TNBC xenograft model, where the xenografts are grown on the chorioallantoic membrane of fertilized chick eggs. dcHSA-Gd-Dox is injected intravenously, and the distribution of the compound is monitored by MRI and inductively coupled optical plasma emission spectrometry. dcHSA-Gd-Dox is rapidly taken up into MDA-MB-231 cells and exhibits significant cytotoxic efficacy. dcHSA-Gd-Dox combines high tissue enrichment with low systemic toxicity and high-contrast MRI rendering it an attractive nanotheranostic for TNBC.

1. Introduction

Among women, breast cancer (BC) is the most frequently diagnosed cancer worldwide and every year, it accounts for more than 400 000 cases of death.[1] It is well known that BC is not a homogeneous disease, but it consists of different subtypes.[2,3] Four major intrinsic subtypes have been identified by genomic studies: the luminal subtypes A and B, which express hormone receptor-related genes, triple-negative/basal-like BC, and human epidermal growth factor receptor 2 (HER2)-positive BC.[2–4] Triple-negative breast cancer (TNBC) accounts for about 15% of BC cases and is associated with a poor prognosis as TNBC cells do not express estrogen, progesterone, and HER2 receptors that are usually addressed in targeted BC therapies.[2] The heterogeneity of the disease and lack of receptors has limited the successful development of receptor-specific therapeutics and currently, there are no approved targeted therapies available for TNBC.[1,2]

Today, only a few systemic drug treatment options exist for TNBC like taxanes (e.g., paclitaxel), anthracyclines (e.g.,...
doxorubicin (Dox), and platinum agents (e.g., cisplatin) for adjuvant and neoadjuvant therapies. However, their high cellular toxicity affects healthy human cells as well, resulting in severe and partially dose-limiting adverse effects. For example, anthracyclines like the widely used drug Dox frequently induce nausea and vomiting, myelosuppression, alopecia, mucositis, and cardiotoxicity.

Therefore, various nanocarrier-drug complexes have been reported aiming at the treatment of TNBC. Among these, protein-based therapeutics are a promising class of nanocarriers, which earned great interest as translational drug delivery platform with increasing numbers of clinical trials in different phases. In particular, the abundant blood plasma protein human serum albumin (HSA) has been applied as drug delivery system, and albumin nanoparticles, albumin-drug conjugates, albumin-binding drug derivatives, albumin-coated nanoparticles, and prodrugs have been developed and advanced into clinical trials. Nanoparticle albumin-bound (Nab) formulations consist of denatured albumin aggregates that form nanoparticles with sizes of ≈100–200 nm with encapsulated lipophilic drug molecules. Abraxane, a Nab formulation of paclitaxel, has been approved by the FDA to treat metastatic BC, and other cancers such as non-small-cell lung cancer and pancreatic cancer. Nab formulations showed enhanced permeability and retention effect (EPR) in cancer tissue compared to the free drug. Hence, in contrast to individual HSA proteins, HSA-based denatured nanocarriers benefit from the EPR effect due to their increased sizes of 100–200 nm. Recently, cell- or tissue-targeted therapeutics have received much attention, and monoclonal antibodies or recombinant fusion proteins functionalized with drug molecules have been approved and marketed. It has been proposed that albumin nanocarriers accumulate in the tumor space by both passive and active drug targeting and follow therapeutic progress. To date, there is no albumin carrier available that targets TNBC and allows concurrent in vivo imaging.

In routine clinical diagnostics and screenings, magnetic resonance imaging (MRI) is the method of choice due to its noninvasiveness and high spatial resolution for soft tissue. Compared to computed tomography (CT), it is devoid of radiation or other health risks, and it offers diagnostic accuracy of various diseases, for example, acute and chronic cerebral hemorrhage, that is, stroke. In breast cancer imaging, MRI plays an essential role with multiple indications and has shown superior sensitivity compared to other modalities like ultrasound and mammography. Therefore, attempts were made to fabricate albumin nanoparticles for tumor-targeted imaging by MRI, for example, in combination with the photosensitizer chelator chlorin e6 that binds MRI-active Mn2+. Recently, albumin nanoparticles have been fabricated for MR/fluorescence imaging and photothermal therapy by attaching gadolinium (Gd) (III) and indocyanine green. Gd-based bioimaging still represents a method of choice as it features high relaxivity, stability, and safety and albumin-based formulations containing (Gd(III)) were applied for MR tumor imaging in an avian in vivo model. However, there are still several challenges associated with the design of an in vivo theranostic for treating TNBC. There is the high risk that nanocarriers build up by noncovalent adsorption of the active compounds (IGC, chlorine e6, GD-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (Gd-DOTA), and cytotoxic drugs) to HSA show rapid loss of imaging signals and a high systemic toxicity due to leakage of active compounds during in vivo circulation. Therefore, TNBC tissue selective and effective albumin nanocarriers with combining high cytotoxicity and high-contrast imaging with minimized leakage of the drugs or imaging agents are still elusive.

Herein, we designed an albumin-based nanocarrier dCHSA-Gd-Dox, which provides multiple copies of covalently attached MRI contrast agent Gd(III)-DOTA and the chemotherapeutic agent Dox that has been linked via a pH-controlled hydrazone linker to facilitate drug release at the target tissue and minimize systemic drug leakage. dCHSA-Gd-Dox exhibits TNBC tissue selectivity and low systemic toxicity combined with high antitumor efficacy in a preclinical TNBC in vivo model. High-contrast bioimaging due to covalent loading of high numbers of MRI contrast agents and toxicity due to multiple Dox molecules and their controlled pH-triggered release in the lysosomal or endosomal compartments of the tumor cells paves the way to effective TNBC theranostics with great potential for personalized disease monitoring and translational applications.

2. Results and Discussion

2.1. Synthesis and Characterization of dCHSA-Gd-Dox

HSA provides many attractive features as a nanocarrier such as high numbers of amino groups (−NH2 = 60 per HSA) and carboxylic acid groups (−COOH = 98 per HSA) of the lysine, glutamic, and aspartic acid side chains that allow the introduction of various functionalities. First, the carboxylic acid side chains were converted into primary amino groups by reacting HSA with ethylenediamine yielding cationized HSA (cHSA, Figure 1A, step 1; see details in Figure S7A, Supporting Information) as reported. The additional positive surface charge at physiological pH enhances cellular uptake of HSA and allows further chemical modifications of the amine groups. Almost all of the carboxylic acid side chains were converted into primary amino groups as quantified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF; Figure S6, Supporting Information, cHSA) yielding monodisperse cHSA. In the next step, about 20 PEG chains with molecular weight of 2000 g mol−1 were attached to the cHSA as indicated by the increase in molecular weight of 40 kDa yielding cHSA-PEG (Figure 1A, step 2; see for details Figures S7A and S6, Supporting Information, cHSA-PEG). In order to facilitate sensitive MRI of the nanocarrier, multiple copies of the complex agent DOTA were introduced by reacting DOTA-N-hydroxysuccinimide (DOTA-NHS) with the remaining primary amino groups at the surface of cHSA-PEG (Figure 1A,B, step 3). The molecular weight increased by 19 kDa indicating the introduction of about 49 DOTA

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Figure 1. Synthesis of dcHSA-Gd-Dox. A) General scheme of dcHSA-Gd-Dox synthesis. dcHSA-Gd-Dox was synthesized starting from native HSA in seven steps. Amino acids with a carboxylic group as side group and the C-terminus were cationized by addition of ethylenediamine (step 1 “cationization”). Afterward PEG-NHS was added to form chSA-PEG (step 2 “PEGylation”). To enable MRI, DOTA-NHS reacted with the chSA-PEG (step 3 “DOTA-NHS,” see details in B top panel) and then, DOTA complexed Gd(III) (step 4 “GdCl₃,” see details in B top panel). After denaturation and reduction of the disulfide bonds (step 5 “denaturation,” see details in B bottom panel), Dox-maleimide (MI) was conjugated to the free thiols (step 6 “Dox-MI,” see details in B bottom panel). After changing to a physiological buffer (step 7 “phosphate buffer”), dcHSA-Gd-Dox formed spherical nanocarriers. At tumor sites, dcHSA-Gd-PEG is digested by enzymes and the acid-responsive linker of the Dox-maleimide is cleaved due to the acidic environment resulting in release of Dox. B) Detailed scheme of dcHSA-Gd-Dox synthesis, steps 3–6.

units on average (Figure 2E top; Figure S6, Supporting Information, chSA-DOTA). Gadolinium cations were complexed by about 44 of the attached DOTA groups by addition of GdCl₃ to chSA-DOTA in Mill-Q at 60 °C and overnight stirring forming chSA-Gd (Figure 1A,B, step 4 and Figure 2E center; Figure S6, Supporting Information, chSA-Gd). chSA-Gd was separated from free gadolinium by ultracentrifugation (molecular weight cut off [MWCO] 30 kDa). Even though the PEG and DOTA groups were attached in a statistical fashion to chSA, the synthesis procedures are robust, allow upscaling, and possess only negligible batch-to-batch variations in the attached functionalities. Furthermore, quality control is feasible by applying mass spectrometry.

As the number of drug molecules is particularly crucial for dosing, we have attached Dox in a site-specific manner to the thiols of the cysteine residues at the polypeptide backbone after denaturation and reduction of chSA-Gd (Figure 1A,B, steps 5 and 6). HSA provides 17 disulfide groups and 1 unpaired cysteine. We have reported previously that after denaturation, about 27–28 cysteines of the total number of 35 cysteines of HSA are accessible for Michael reactions.[19,44] About 7–8 cysteines of
the HSA sequence are colocalized (see Figure S7C, Supporting Information) and due to steric hindrance, no second Michael addition occurs after the first thiol has reacted.\[^{[19,44]}\] Therefore the number of drug molecules per modified protein is consistent and very reproducible with little batch-to-batch variations.

The (6-maleimidocaproyl)hydrazone of Dox, a pH-cleavable hydrazone linker connecting Dox to maleimide, was synthesized according to the literature.\[^{[45]}\] cHSA-Gd was denatured in a 5 M urea buffer and the disulfide bridges were reduced by TCEP. About 27 available thiol groups of the denatured cHSA-Gd were reacted with Dox-maleimide according to MALDI-TOF (Figure 2E bottom; Figure S6, Supporting Information, dcHSA-Gd-Dox) yielding dcHSA-Gd-Dox in quantitative yield. A pH-responsive release has many advantages over nanocarriers with noncovalently adsorbed drug molecules as unwanted leakage of the drugs during biodistribution in vivo is minimized. We have demonstrated previously that dcHSA-Dox conjugates connected via the hydrazone linker revealed a controlled two-step release of Dox.\[^{[19]}\] The first step has to be triggered by proteases such as trypsin and cathepsin B, which digest the polypeptide backbone of the protein carrier. These proteases are usually located in the endosome or lysosome and overexpressed in various cancer types.\[^{[19]}\] Only then, release of the Dox is triggered by the acidic pH value in the endosome or lysosome resulting in the cleavage of the hydrazone bond.\[^{[19]}\]

2.2. Size Determination of dcHSA-Gd-Dox

The dimensions of the nanocarriers were determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM). DLS was measured in a 0.5 mg mL\(^{-1}\) solution of dcHSA-Gd-Dox in Milli-Q, Dulbecco’s Phosphate Buffered Saline (DPBS), and phosphate buffer (PB, 50 mm, pH 7.4). In Milli-Q water, dcHSA-Gd-Dox showed a diameter of about 151.2 ± 3.5 nm (n = 3) with a polydispersity index (PDI) of 0.41 ± 0.08. Under more physiological conditions (DPBS), dcHSA-Gd-Dox formed nanostructures with diameters of about 87.8 ± 2.3 nm (n = 3) and a PDI of 0.31 ± 0.05. In the presence of a higher concentrated PB (50 mm, pH 7.4), nanobubbles of about 86.8 ± 1.6 nm (n = 3) with a PDI of 0.31 ± 0.04 (Figure 2A) were detected. Noteworthy, no smaller or larger particle populations were detected. TEM images showed spherical nanostructures of dcHSA-Gd-PEG with a diameter of about 99.2 ± 17.7 nm (n = 66, Figure 2B,C). The contrast in TEM results only from the high load of Dox and gadolinium as no protein stains were applied. Previously, nanoparticles with mean diameters of around 100 nm revealed a significant EPR effect.

2.3. Relaxivity Measurements of dcHSA-Gd-Dox

dcHSA-Gd-Dox showed favorable predispositions for MRI. As shown in Figure 2D, a linear correlation between the relaxation rate \( r_1 \) and dcHSA-Gd-Dox concentration was observed, yielding a T1 relaxivity of \( r_1 = 13.75\text{ mm}^{-1}\text{s}^{-1} \). Compared to the \( r_1 \) relaxivity of clinically used Gd(III) contrast agent Gd-BOPTA (MultiHance, 4.6 mm\(^{-1}\)s\(^{-1}\)), dcHSA-Gd-Dox (13.75 mm\(^{-1}\)s\(^{-1}\)) exhibited significantly improved enhancement indicating higher sensitivity compared to other gadolinium-based macromolecular contrast agents. The paramagnetic properties were at high levels comparable to published gadolinium-based macromolecular complexes.\[^{[46–48]}\] Overall, the high number of Gd(III) per polymer provided a high relaxation time and therefore a high contrast.
2.4. Uptake and Cytotoxicity of dcHSA-Gd-Dox in Breast Cancer Cells In Vitro

The fluorescence of Dox was exploited to visualize dcHSA-Gd-Dox by fluorescence microscopy. Breast cancer cells were incubated with dcHSA-Gd-Dox or Dox for 1 h and unbound nanocarriers were removed by washing. Subsequent fluorescence microscopy showed uptake of dcHSA-Gd-Dox into MDA-MB-231 breast cancer cells (Figure 3A). MDA-MB-231 cells were incubated for 24–72 h and cell viability was analyzed by monitoring mitochondrial dehydrogenase activity (Figure 3B). After 72 h, the IC_{50} of dcHSA-Gd-Dox (77.5 nm) appeared slightly higher than the IC_{50} of conventional Dox (17.9 nm), but was still in the nanomolar range (Figure 3B).

2.5. dcHSA-Gd-Dox Accumulates in Tumor Xenografts

The application of dcHSA-Gd-Dox was studied in vivo in tumor xenografts grown on the chorioallantoic membrane (CAM) of fertilized chick eggs as a preclinical in vivo model (Figure 4A). Because of its simplicity and direct accessibility, the CAM is an attractive preclinical in vivo model with proven applicability in tumor-related research to explore, for example, tumor biology, growth, and angiogenesis.\[49,50\] It enables rapid screening of substances with reliable assessment of substance activity, distribution, and toxicity in an in vivo environment\[51\] and it has been widely implemented for the evaluation of drug delivery and kinetics of, for example, drug conjugates,\[52\] prodrugs,\[53\] or nanoparticles.\[54\] We have previously confirmed that data obtained from the CAM model correlate with in vivo experiments in mice.\[55–57\] Therefore, the CAM model represents an intermediate form between isolated cultured cells and animal experiments in rodents and contributes to animal welfare by saving mammalian laboratory animals and matches the 3Rs recommendations.

Previously, MRI and the measurement of tumor growth in the CAM model has been combined based on our MRI protocols.\[41,58,59\] Compared to the standard gadolinium-based MRI contrast agent MultiHance, dcHSA-Gd-Dox showed a much higher signal-to-noise ratio (SNR) in tumor tissue 1–15 h after intravenous injection indicating dcHSA-Gd-Dox accumulation in tumor specimens (Figure 4B,C and Table 1). Only after 35 h, no contrast enhancement was detectable anymore (Figure 4B,C). Moreover, we did not observe significant contrast enhancement.

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**Figure 3.** Tumor cell uptake and cytotoxicity of dcHSA-Gd-Dox. MDA-MB-231 breast cancer cells were incubated with dcHSA-Gd-Dox or equimolar Dox, washed, and the fluorescence of Dox was exploited to visualize dcHSA-Gd-Dox in tumor cells by fluorescence microscopy. A) Representative fluorescent images of MDA-MB-231 cells incubated with 0.36 µM dcHSA-Gd-Dox or equimolar Dox for 1 h and stained with Hoechst 33342 (from left to right: DIC, DAPI, Cy3, and merged fluorescent image). Original magnification 200×. B) Cell viability of MDA-MB-231 cells in vitro, incubated with dcHSA-Gd-Dox or Dox for 24, 48, and 72 h. XTT assay, mean ± SEM of n = 3 (each experiment performed in triplicates).
Figure 4. dcHSA-Gd-Dox accumulation in breast cancer xenografts in vivo. $1 \times 10^6$ MDA-MB-231 stably expressing firefly luciferase were grafted onto the chorioallantoic membrane (CAM) of the chick embryo. Six days later, 50 µL of Dox covalently linked to gadolinium (dcHSA-Gd-Dox) or a commercial gadolinium-containing MRI contrast agent (MultiHance) were intravenously injected at equimolar concentrations of gadolinium (3.6 µmol kg$^{-1}$, egg weight 50 g). MRI scans were conducted before and after the injection up to 40 h. A) MDA-MB-231 xenograft on the CAM (first and third pictures from left), intravenous application of dcHSA-Gd-Dox (second from left) and xenograft after collection (rightmost picture). B) MRI images of representative breast cancer xenografts before and after intravenous injection of MultiHance (first row) or dcHSA-Gd-Dox (second row) and the same tumors after histochemical preparation (HE, magnification 50×). C) Mean signal to noise ratio (SNR) in tumor tissue after injection of dcHSA-Gd-Dox or MultiHance, measured over time. Mean ± SD of five tumor-bearing eggs/group; Mann–Whitney rank sum test, **p < 0.001, *p < 0.05. D) Gadolinium in tumor, liver, and blood plasma 6 h after i.v. injection of dcHSA-Gd-Dox or MultiHance as measured by inductively coupled plasma optical emission spectrometry (ICP-OES). Data are mean ± SEM of 3–4 embryos per group; Mann–Whitney rank sum test, *p < 0.05.

Table 1. Substance doses used for in vivo diagnostic and therapeutic applications.

|             | dcHSA-Gd-Dox [µmol kg$^{-1}$] | Gd(III) [µmol kg$^{-1}$] | Dox [µmol kg$^{-1}$] |
|-------------|-------------------------------|---------------------------|-----------------------|
| MRI         | 0.08                          | 3.60                      | 2.21                  |
| Therapy, low dose | 0.03                          | 1.22                      | 0.75                  |
| Therapy, high dose | 0.09                          | 4.1                       | 2.50                  |

in any other organ or embryonic compartment but only in the tumor xenografts (Figure S8, Supporting Information).

To further analyze dcHSA-Gd-Dox biodistribution, we collected breast cancer xenografts, chick embryo blood, and livers 6 h after injection of dcHSA-Gd-Dox or MultiHance and analyzed the gadolinium contents in tissues using inductively coupled plasma optical emission spectrometry (ICP-OES). This technique is highly sensitive and accurately quantifies single elements in tissue samples. Gadolinium analysis confirmed the preferable accumulation of dcHSA-Gd-Dox in tumor tissue (Figure 4D). Furthermore, dcHSA-Gd-Dox was detectable in chick embryo livers, but liver distribution did not differ significantly from the standard gadolinium-based contrast agent MultiHance (Figure 4D). At that time point, dcHSA-Gd-Dox was not detectable in embryonic blood samples (Figure 4D). Thus, both MRI and ICP-OES analyses revealed dcHSA-Gd-Dox enrichment in breast cancer xenografts. Therefore, dcHSA-Gd-Dox seems to overcome intrinsic limitations associated with low relaxivity and unspecific tissue uptake of small molecule Gd(III) complexes, and proves applicability as an MR contrast agent for breast cancer xenograft imaging in ovo.
2.6. Biocompatibility of dcHSA-Gd-Dox

Embryos are highly predictive for compound toxicity because living organisms in the embryonic stage are highly vulnerable to external toxic influences. Three days after intravenous administration, dcHSA-Gd-Dox was well tolerated by chick embryos as embryo survival rates did not significantly differ from embryos injected with physiological sodium chloride solution; 95% and 77% of embryos injected with dcHSA-Gd-Dox 0.75 and 2.5 µmol kg\(^{-1}\), respectively, survived indicating high biocompatibility of dcHSA-Gd-Dox. In contrast, injections of equimolar doses of commercial Dox resulted in significantly reduced survival rates, that is, 66% and 59% after low and high dosages, respectively (Figure 5A and Table 1). Thus, dcHSA-Gd-Dox exhibits much lower systemic toxicity than conventional Dox.

Often, divergent concentrations of nanocarriers are being used for diagnostic and therapeutic applications because the therapeutic concentration of the nanocarrier is below the imaging threshold, and, on the other hand, the concentration used for imaging is accompanied by high toxicity. However, we could clearly demonstrate that dcHSA-Gd-Dox provides low toxicity in embryos at therapeutic concentrations allowing MRI at a comparable concentration (Table 1).

2.7. dcHSA-Gd-Dox Inhibits the Growth of Tumor Xenografts

In addition to its diagnostic applications, we investigated the therapeutic efficacy of dcHSA-Gd-Dox in tumor tissue. The transparency of the CAM facilitates continuous observation of the tumor site and in vivo monitoring after treatment.\(^{(51)}\) In our studies, we used luminescence-based tumor monitoring that has previously been validated.\(^{(61,62)}\) Recently, we have shown antitumor efficacy of multidomain protein complexes by luminescence monitoring of tumor viability in vivo.\(^{(63)}\)

Breast cancer growth was assessed in vivo by measuring tumor-cell-derived bioluminescence upon topical application of d-luciferin (Figure 5B). Mean bioluminescence of xenografts treated with dcHSA-Gd-Dox was significantly reduced compared to control or Dox-treated samples (Figure 5C and Table 1). These results were confirmed by subsequent immunohistochemical analysis of the cell proliferation marker Ki-67 (Figure 5D,F). This nuclear protein is indicative of mitotic phases of the cell cycle and peaking in the M phase, whereas it is not detectable in resting G\(_0\) cells.\(^{(64)}\) A cohort study including more than 3500 breast cancer patients identified Ki-67 expression of tumor tissue as a prognostic parameter correlated with lower disease-free and overall survival.\(^{(65)}\) In our study, dcHSA-Gd-Dox significantly reduced the proliferative Ki-67-positive fraction in breast cancer xenografts compared to the vehicle control group, whereas conventional Dox had a minor effect on this parameter. Furthermore, TUNEL staining revealed that dcHSA-Gd-Dox and conventional Dox induced apoptosis in cancer xenografts (Figure 5E,F). Thus, in the preclinical CAM in vivo model, the theranostic dcHSA-Gd-Dox inhibits the growth of breast cancer xenografts and exerts higher biocompatibility and better antitumor efficacy than conventional Dox.

In vivo measurements of tumor bioluminescence and immunohistochemical analysis of cell proliferation (Ki-67) and apoptosis (TUNEL) demonstrated dose-dependent inhibition of tumor growth by dcHSA-Gd-Dox. The therapeutic efficacy of dcHSA-Gd-Dox was significantly stronger compared to free Dox at the same molar concentrations.

3. Conclusion

dcHSA-Gd-Dox nanocarriers were designed based on denatured, cationized, and PE Gyalted HSA biopolymers and about 44 Gd(III)-DOTA groups providing MRI with high relaxation time and imaging contrast. 27 Dox molecules were attached via a pH-cleavable hydrazone linker that releases Dox in acidic environments thus yielding nanocarriers with high toxicity (IC\(_{50}\) of about 77.5 nm) in MDA-MB-231 TNBC cells. dcHSA-Gd-Dox significantly reduced the proliferative Ki-67-positive fraction in breast cancer xenografts, whereas conventional Dox only had a minor effect. Both MRI and ICP-OES analyses revealed dcHSA-Gd-Dox enrichment in tumor tissue, where it rapidly accumulated and persisted for at least 15 h. Thus, the accumulation of dcHSA-Gd-Dox in tumor tissue facilitated higher local concentrations, which could account for the observed significant antitumor potency, which was even outperforming equimolar dosages of conventional Dox.

The preferential enrichment of dcHSA-Gd-Dox within tumor xenografts facilitates its simultaneous usage for diagnostic and therapeutic purposes and implies a lower risk of systemic side effects. Nanocarriers exhibiting tumor selectivity and low systemic toxicity combined with high antitumor efficacy are not available for the treatment of TNBC. We envision that in TNBC patients, MR imaging after dcHSA-Gd-Dox application could be useful i) to evaluate if the chemotherapeutics reach the tumor site and ii) to assess tumor response to therapy over time. Therefore, we believe that dcHSA-Gd-Dox represents a promising theranostic awaiting further exploration and translational applications.

4. Experimental Section

Statistical Analysis: SigmaPlot software 12.0 (Systat Software GmbH, Erkrath, Germany) was used for statistical analysis. IC\(_{50}\) values were calculated by nonlinear regression analysis. Two-group comparisons were analyzed using Mann–Whitney rank sum test, whereas multi-group comparisons were performed by Kruskal–Wallis one-way analysis of variance (ANOVA) with Newman–Keuls post hoc test, alpha = 0.05. For the analysis of qualitative data (embryo survival rate), Chi-square test (Fisher’s exact test) was performed. p-values were indicated as *p < 0.05, **p < 0.01, and ***p < 0.001. Experimental results were usually presented as means ± SEM (standard error of the mean) or SD (standard deviation) of n independent experiments or samples (n ≥ 3). n is defined in the main text for each experiment. Data of cytotoxicity, embryo survival, and tumor luminescence were normalized to the values under control conditions upon vehicle treatment as 100%. Further details on data presentation and sample size of each data set can be found in the respective figure legend.

Reagents and Equipment: Dox hydrochloride from Cayman Chemical (Ann Arbor, MI) was used for in vitro studies. For in vivo experiments, Dox hydrochloride from Teva or NeoCorp (both 2 mg mL\(^{-1}\)) was purchased. d-Luciferin potassium salt (Biomol, Hamburg, Germany) was dissolved in phosphate-buffered saline and stored in aliquots at −20 °C. Gadolinium (III) chloride hexahydrate was purchased from Alfa Aesar, REactus
Figure 5. dcHSA-Gd-Dox inhibits the growth of breast cancer cells xenografts in vivo. $0.5 \times 10^6$ MDA-MB-231 stably expressing firefly luciferase were grafted onto the chorioallantoic membrane (CAM) of the chick embryo. Three days later, treatment commenced by i.v. injection of 50 µL of either Dox (Dox, low dose, $0.75 \mu$mol kg$^{-1}$, and high dose, $2.5 \mu$mol kg$^{-1}$) or Dox covalently linked to dcHSA-Gd-Dox (containing the same amount of Dox molecules as the positive control). Tumor luminescence was analyzed 72 h after i.v. injection, 10 min after addition of d-luciferin ($0.75 \text{mg mL}^{-1}$, 10 µL) with integration time 1 s. A) Survival rates of chick embryos 72 h after treatment (16–39 eggs per group). Chi-square test, Fisher’s exact test. B) In vivo luminescent imaging of breast cancer xenografts before and after the addition of d-luciferin as indicated. C) Representative pictures of luminescent tumors in vivo (left panel) and mean luminescence of MDA-MB-231 cancer xenografts 72 h after treatment (right panel). Mean ± SEM of two experiments, $n = 6$ tumors/group. Newman–Keuls test. Immunohistochemical analysis of breast cancer xenografts. Three days after treatment, tumor xenografts were collected, paraffin embedded, and stained to analyze the number of proliferating and apoptotic MDA-MB-231 breast cancer cells. D) Percentage of Ki-67$^+$ proliferating breast cancer cells. E) Percentage of apoptotic TUNEL$^+$ cells in tumor xenografts. In every tumor, 205–619 cells were analyzed. Data are mean ± SEM of 3–4 tumors per group, Newman–Keuls test. F) Representative pictures of tumor sections after immunohistochemical staining. Upper row: HE, hematoxylin and eosin, original magnification 25×; center row: Ki-67 antigen, red-brown nuclei are indicative of proliferating cells, original magnification 200×; lower row: TUNEL staining (brown) to detect cells with fragmented DNA as apoptosis marker, original magnification 400×. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, $#p < 0.05$. 

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(99.9% purity). HSA was bought from Sigma-Aldrich. PEG-2000-NHS was purchased from Rapp Polymere and Vivaspin20 from Sartorious.

Absorbance, fluorescence, and luminescence were measured in vitro using an Infinite M1000 PRO plate reader (Tecan Group, Maenndorf, Switzerland), whereas an IVIS in vivo imaging system (PerkinElmer, Waltham, MA) was used for bioluminescence analysis of breast cancer xenografts in vivo. ICP-OES was performed by using an Ultima 2 (Horiba, Kyoto, Japan). For MRI and ICP-OES measurements, conventional MRI gadolinium contrast agent Gd-BOPTA (MultiHance, Bracco Imaging, Konstanz, Germany) served as a control. MALDI-TOF was performed on a Bruker Daltonics Reflex III spectrometer. Saturated solution of sinapinic acid dissolved in 1:1 water:acetonitrile with 0.1% trifluoroacetic acid was used as the matrix solution.

**Cell Lines:** MDA-MB-231 breast adenocarcinoma cells (ACC 732) were obtained from the Leibnitz Institute, German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

MDA-MB-231 cells were cultured in DMEM medium (high glucose) supplemented with 2 mM glutamine, 0.1 mM MEM nonessential amino acids (NEAA), 10% heat-inactivated fetal calf serum, and 1% penicillin/streptomycin with regular passaging 2–3 times per week.

**Synthesis and Characterization of cHSA-Gd-Dox:** The synthesis of 2,2′,7′,7′-tetramethoxy-1,10-phenanthroline (PMA) was performed according to the literature with slight modifications. The crude product was purified by Vivaspin 20 ultracentrifugation tubes (30 kDa MWCO) and subsequently reacted with methoxy-P2EG2000-NHS (28 equiv.) in degassed phosphate buffer (pH 8.0) to obtain cHSA-PEG containing around 20 PEG units.

**Synthesis of cHSA-DOTA:** A 10-DOTA-NHS (501 mg mol⁻¹, 28 equiv.) in degassed phosphate buffer (pH 8.0) was added to dissolve cHSA-PMA in 5 mL dimethyl sulfoxide was added and the mixture was stirred at 120 °C for 24 h.

**MDA-MB-231 breast adenocarcinoma cells (ACC 732) were obtained from the Leibnitz Institute, German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).**

MDA-MB-231 cells were cultured in DMEM medium (high glucose) supplemented with 2 mM glutamine, 0.1 mM MEM nonessential amino acids (NEAA), 10% heat-inactivated fetal calf serum, and 1% penicillin/streptomycin with regular passaging 2–3 times per week.

**Cell Viability Analysis and Fluorescent Microscopy In Vitro:** For in vitro studies of dCHSA-Gd-Dox cytotoxicity, MDA-MB-231 were seeded in 96-well plates the day before the experiment (5000 cells per well). After 24 h, medium was replaced by medium containing dCHSA-Gd-Dox or Dox at equimolar concentrations. After the indicated incubation time, XTT tetrazolium salt was added to the wells. In viable cells, mitochondrial dehydrogenases reduce XTT to formazan and the colorimetric measurement of formazan dye yields the relative amount of viable cells compared to control cells treated with vehicle only. The XTT cell proliferation assay kit was purchased from Roche Diagnostics (Filderstadt, Germany).

In addition, cells seeded in ibidi slides were incubated with dCHSA-Gd-Dox for 1 h at 37 °C, stained with Hoechst 33342 (Molecular Probes, 1 µg mL⁻¹) and analyzed by fluorescent microscopy using a Tri-E inverse fluorescence microscope (Nikon, Düsseldorf, Germany) with 20x objective and a filter set with broad 75-nm emission passband (573–648 nm).

**Anticancer Activity in an Avian Breast Cancer Xenograft Model In Vivo:** All experiments were conducted in compliance with the European directive for “Protection of Animals Used for Experimental and Scientific Purposes” and the respective German juridical implementation. For in vivo experiments, fertilized eggs from Gallus domesticus were bought from a hatchery (LSL Rhein-Main Geflügelvermehrungsbetriebe GmbH & Co.KG, Dieburg, Germany), cleaned, and incubated at 37 °C. Four days after fertilization, a circular part of the egg shell was carefully removed to uncover the subjacent CAM. The area was covered with tape to avoid evaporation. Three days later, a silicone ring (interior diameter 5 mm) was placed onto the CAM and MDA-MB-231 cells stably expressing firefly luciferase were resuspended in matrigel/medium (1:1) and xenotransplanted inside the ring onto the CAM (0.5 × 10⁶ cells in 20 µL per egg). Matrigel was purchased from BD Biosciences (Heidelberg, Germany). In our studies, avian embryos were sacrificed no later than 8 days after hatching.

Three days after cancer cell xenotransplantation, 50 µL of either Dox (low dose, 0.75 µmol kg⁻¹, or high dose, 2.5 µmol kg⁻¹) or Dox covalently linked to dCHSA-Gd (low dose, 0.03 µmol kg⁻¹; or high dose, 0.09 µmol kg⁻¹ containing the same amount of Dox molecules as the positive control, see Table 1) were intravenously injected. Compounds were dissolved in 0.9% NaCl solution for infusion.

To measure tumor growth, tumor luminescence was analyzed 72 h after intravenous injection of dCHSA-Gd-Dox and 10 min after addition of luciferin (0.75 mg mL⁻¹, 10 µL with an integration time of 1 s, using an IVIS in vivo imaging system. Afterward, tumor xenografts were extracted from the CAM, fixed, and embedded in paraffin, and cut into 5 µm sections for immunohistological analysis.

Antibody against the human proliferation antigen Ki-67 was used to analyze the proportion of proliferating cells within cancer xenografts (Dako Cytomation, Glostrup, Denmark). To detect apoptotic tumor cells, the TUNEL method to visualize DNA strand breaks by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling was applied.

**MR Imaging and Contrast Analysis:** The longitudinal relaxation time T₁ was derived from quantification of the resulting T₁ relaxation constants as a function of Gd(III) concentration. A dilution series of dCHSA-Gd-Dox and Gd-BOPTA (MultiHance) resulting in Gd(III) concentrations from 150 to 760 µm (dCHSA-Gd-Dox) and 1 µm to 1 mM (MultiHance) diluted in 0.9% NaCl aqueous solution was measured at room temperature at 11.7 Tesla field strength. T₁ values were derived by fitting a monoexponential...
MR imaging and intravenous injection of compounds was performed as previously described.[41] In short, MRI in vivo was performed on a small-animal MRI device (11.7 Tesla Bruker Biospec 117/16, Bruker Biospin, Ettlingen, Germany). A 72 mm quadrature volume T/R resonator was used for signal reception. A T₁-weighted 3D FLASH (3D-TFLASH) sequence was used for assessment of the contrast agent distribution (acquisition parameters: TR/TE = 6/2 ms, matrix = 400 × 439 × 96, spatial resolution = 100 × 100 × 560 μm³ and NSA = 2). Additionally, anatomic images were acquired using a multi-slice RARE sequence (acquisition parameters: TR/TE = 4320/45 ms, matrix size = 650 × 650, in-plane resolution = 77 × 91 μm², slice thickness = 0.5 mm, no inter-slice gap, RARE factor = 8, and NSA = 4). During scanning, the eggs were reproducibly positioned in a polystyrene holder after cooling at 4 °C for 110 min to avoid motion artifacts. For acquisition and visualization, ParaVision 6.01 software (Bruker Biospin, Ettlingen, Germany) was used. MRI scans were conducted before and after the injection of 50 μL of dcHSA-Gd-Dox or a commercial gadolinium-containing MRI contrast agent (MultiHance) at equimolar concentrations of gadolinium (3.6 μmol kg⁻¹, respective egg weight 50 g). Consecutive MRI scans were performed starting 30 min after injection and continued for up to 40 h. To analyze SNR, regions of interest (ROIs) were manually selected at the same position at different time points. The value was corrected for the SNR of the background using the formula: $\text{SNR} = \frac{(S_{ROI} - S_{BG})}{\delta_{BG}}$ with $S_{ROI}$ and $S_{BG}$ being the mean value over the ROI and background and $\delta_{BG}$ the standard deviation of the background.

**Inductively Coupled Plasma Optical Emission Spectrometry Measurement of Gadolinium:** To follow dcHSA-Gd-Dox distribution, embryos were sacrificed 6 h after intravenous injection, breast cancer xenografts and chick embryo organs (blood, liver) were collected, weighed, and the content of gadolinium was analyzed by ICP-OES. To facilitate elemental analytics of gadolinium, tissue was digested in aqua regia (nitrohydrochloric acid) by a microwave-assisted procedure (Microwave 3000, Anton Paar, Graz, Austria).

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

gadolinium-DOTA, human serum albumin nanocarrier, theranostic, triple-negative breast cancer

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1. Jerlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D. M. Parkin, D. Forman, B. Bray, Int. J. Cancer 2015, 136, E359.
2. Collignon, L. Lousberg, H. Schroeder, G. Jerusalem, Breast Cancer (Dove Med Press) 2016, 8, 93.
3. C. M. Perou, T. Sorlie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, C. A. Rees, J. R. Pollack, D. T. Ross, H. Johnsen, L. A. Akslen, O. Fluge, A. Pergamenschikov, C. Williams, S. X. Zhu, P. E. Lonning, A. L. Borresen-Dale, P. O. Brown, D. Botstein, Nature 2000, 406, 747.
4. T. Sorlie, C. M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, T. Thorsen, H. Quist, J. C. Matese, P. O. Brown, D. Botstein, P. E. Lonning, A. L. Borresen-Dale, Proc. Natl. Acad. Sci. U. S. A. 2001, 98, 10869.
5. R. H. G. Teles, H. F. Moralles, M. R. Corinneti, Int. J. Nanomed. 2018, 13, 2321.
6. J. Lao, J. Madani, T. Puertolas, M. Alvarez, A. Hernandez, R. Pazo-Cid, A. Artal, A. Anton Torres, J. Drug Delivery 2013, 2013, 456409.
7. L. Liu, Y. Wang, L. Miao, Q. Liu, S. Musetti, J. Li, L. Huang, Mol. Ther. 2018, 26, 45.
8. B. Darvishi, L. Farahmand, A. K. Majizadeh, Mol. Ther. – Nucleic Acids 2017, 7, 164.
9. D. Yang, X. Yao, J. Dong, N. Wang, Y. Du, S. Sun, L. Gao, Y. Zhong, C. Qian, H. Hong, Bioconjugate Chem. 2018, 29, 2776.
10. M. W. Kim, H. Y. Jeong, S. J. Kang, I. H. Jeong, M. J. Choi, Y. M. You, C. S. Lee, I. H. Song, T. S. Lee, J. S. Lee, A. Y. S. Park, Theranostics 2019, 9, 837.
11. R. Goyal, C. H. Kapadia, J. R. Melamed, R. S. Riley, E. S. Day, Cell Mol. Bioeng. 2018, 11, 383.
12. K. Greish, A. Mathur, R. Al Zaharni, S. Elkaissi, M. Al Jishi, O. Nazzal, S. Taha, V. Pittala, S. Taurin, J. Controlled Release 2018, 291, 184.
13. C. Mei, N. Wang, X. Zhu, K. H. Wong, T. Chen, Adv. Funct. Mater. 2018, 28, 1805225.
14. V. Bellat, R. Ting, T. L. Southard, L. Vandat, H. Molina, J. Fernandez, O. Aras, T. Stokol, B. Law, Adv. Funct. Mater. 2018, 28, 1803969.
15. N. Zhang, X. Liang, C. Gao, M. Chen, Y. Zhou, C. J. Krueger, G. Bao, Z. Gong, Z. Dai, ACS Appl. Mater. Interfaces 2018, 10, 29385.
16. B. Elsadek, F. Kratz, J. Controlled Release 2012, 157, 4.
17. H. B. Ruttala, T. Ramasamy, B. S. Shin, H. G. Choi, C. S. Yong, J. O. Kim, Int. J. Pharm. 2017, 519, 11.
18. S. Chakraborty, M. Sison, Y. Wu, A. Ladenburger, G. Pramanik, J. Biskupek, J. Extermann, U. Kaiser, T. Lasser, T. Weil, Biomater. Sci. 2017, 5, 966.
19. Y. Wu, S. Ihme, M. Feuring-Buske, S. L. Kuan, K. Eisele, M. Lamla, Y. Wang, C. Buske, T. Weil, Adv. Healthcare Mater. 2013, 2, 884.
20. J. Gao, S. Jiang, X. Zhang, Y. Fu, Z. Liu, Bioorg. Chem. 2019, 83, 154.
21. Y. Wu, K. Eisele, M. Doroshenko, G. Algara-Siller, U. Kaiser, K. Koynov, T. Weil, Small 2012, 8, 3465.
22. L. Pes, S. D. Koester, J. P. Magnusson, S. C. Matese, P. O. Brown, D. Botstein, P. E. Lonning, A. L. Borresen-Dale, Proc. Natl. Acad. Sci. U. S. A. 2001, 98, 10869.
23. H. B. Ruttala, T. Ramasamy, B. S. Shin, H. G. Choi, C. S. Yong, J. O. Kim, Int. J. Pharm. 2017, 519, 11.
24. F. Kratz, I. A. Müller, C. Ryppa, A. Warnecke, ChemMedChem 2008, 3, 20.
25. B. Elsadek, R. Graeser, A. Warnecke, C. Unger, T. Saleem, N. El-Melegy, H. Madkor, F. Kratz, ACS Med. Chem. Lett. 2010, 1, 234.
26. D. J. Bharali, M. Khalil, M. Gurbuz, T. M. Simone, S. A. Mousa, NanoMedicine 2009, 4, 1.
27. M. A. Socinski, C. J. Langer, I. Okamoto, M. E. Polanski, D. Page, J. Orsini, H. Zhang, M. F. Renschler, Ann. Oncol. 2013, 24, 314.
28. W. W. Ma, M. Hidalgo, Clin. Cancer Res. 2013, 19, 5572.
29. R. Kinosita, Y. Ishima, V. T. G. Chuang, H. Nakamura, J. Fang, H. Watanabe, T. Shimizu, K. Okuhira, T. Ishida, H. Maeda, M. Otagiri, T. Maruyama, Biomaterials 2017, 140, 162.
30. R. Chen, C. Liang, C. Wang, Z. Liu, Adv. Mater. 2015, 27, 903.
