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1. Introduction

Epithelial ovarian cancer is the fifth leading cause of cancer-related death in women worldwide [1]. Most patients are diagnosed with an advanced stage of disease (Fédération Internationale de Gynécologie et d’Obstétrique [FIGO] stage III–IV) and suffer from extensive abdominal metastases [2,3]. Aggressive surgical cytoreduction and chemotherapy are used as primary treatment, but nevertheless up to 70% of these patients will develop recurrent disease and eventually succumb. Long-term survival is poor with a 5-year survival of less than 35% [2,3]. Overall survival statistics have not significantly improved over the last decades and new avenues for better treatment are clearly warranted [4].

Conventional chemotherapeutics affect proliferating cancer cells as well as normal cells, resulting in systemic adverse events that greatly affect quality of life. As a consequence, the dose administered has to be limited resulting in a suboptimal treatment that negatively affects prognosis of cancer patients. The use of drug delivery systems may be helpful to overcome these problems by improving biodistribution, resulting in high local drug concentrations at the tumor site while minimizing exposure to healthy cells [5]. Beneficial effects of drug delivery systems such as liposomal doxorubicin (Caelyx/Doxil) and albumin bound paclitaxel (nab-paclitaxel), have been reported in several (pre)-clinical studies [6–9]. Previously we described a novel class of drug delivery vehicles, lyophilisomes, which are spherical nano- to microsized biocapsules that can be prepared from various proteins (e.g. albumin, collagen, and elastin) [10,11]. Albumin-based lyophilisomes can be efficiently loaded with doxorubicin and are able to eliminate ovarian cancer cells in vitro [10]. In addition, the albumin wall of lyophilisomes offers opportunities for functional modification, e.g. by the incorporation or conjugation of components in and/or on the wall. Antibody-conjugated lyophilisomes have been shown to specifically bind to cancer cells expressing the corresponding antigen, thus enabling active cancer-targeting [12].
Although it is hypothesized that active targeting of cancer cells by drug delivery systems using specific antibodies or ligands has the potential to broaden the therapeutic index of anti-cancer drugs, the favorable effect of tumor-cell targeting over non-targeting systems was reported to be disappointing [5,6,13]. As most of these studies have focused on targeting cancer cells, other approaches such as targeting the cancer extracellular matrix (ECM) may offer valuable alternatives [14].

The ECM represents a network of proteins and proteoglycans that is abundantly remodeled during cancer development and actively contributes to cancer progression [15,16]. A large amount of intratumoral matrix correlates with poor prognosis in cancer, including ovarian cancer [17]. Major components of the ECM are collagen, laminin and proteoglycans. Proteoglycans function to a large extent through their glycosaminoglycan side chains; linear negatively charged polysaccharides built from repeating disaccharides [18]. Highly 4,6-sulfated chondroitin sulfate (CS-E), a specific class of glycosaminoglycans, is found to be abundantly expressed in the ovarian cancer stroma while being absent or present in only very small amounts in healthy stroma, thus representing an attractive target for anti-cancer therapy [19,20]. Interestingly, the amount of these targets expressed in the stroma is relatively high when compared to targets expressed at the cancer cell surface.

In this study, we present an innovative concept of an anti-cancer strategy aiming at forming a depot of chemotherapeutic-loaded lyophilisomes in the ovarian cancer ECM. Targeting the cancer ECM rather than cancer cells might be helpful to overcome hurdles observed in cell-targeting therapies as the cancer ECM is a relatively stable structure, unlike cancer cells that are characteristically genetically instable [21]. Due to intratumoral heterogeneity, cell-targeting therapies may only affect subpopulations of cancer cells, and leave other cancer cells and cancer-promoting cells (e.g. cancer-associated fibroblasts, endothelial cells, and macrophages) unaffected [22]. Release of chemotherapeutics from a depot of drug-loaded lyophilisomes in the cancer ECM may affect all cells in its vicinity including cancer cells, cancer stem cells and cancer-associated stromal cells. Collagens have been used as micro-environmental anchors for targeted anti-cancer therapy [23,24], but collagen is also abundantly present in normal tissues. Therefore, in this study we focus on CS-E as a much more cancerspecific molecular target. We describe the construction and evaluation of a lyophilisome-based drug delivery system specifically targeting highly sulfated CS-E in the ovarian cancer stroma.

2. Materials and methods

2.1. Patient material

Study approval was given by the Regional Committee for Medical Research Ethics and performed according to the Code for Proper Secondary Use of Human Tissue (Dutch Federation of Biomedical Scientific Societies, www.federa.org). Cryosections (5 μm) of advanced stage high grade serous ovarian cancer were used for immunofluorescence analysis of antibody-functionalized lyophilisome specificity. Paraffin embedded sections (4 μm) of lung, cervical, breast, renal cell, endometrial, and colon cancer were used for immunohistochemical analysis of CS-E expression.

2.2. Production of antibody-functionalized lyophilisomes

2.2.1. Modification of GD3G7 antibodies for sortase-mediated conjugation

The single chain antibody GD3G7 was previously selected against embryonic glycosaminoglycans and showed specificity for CS-E [19]. For site-selective conjugation of GD3G7 at the carboxy-terminus, leaving the antigen-binding parts of this antibody intact, the LPETG sortase A-recognition motif was introduced. To this end the GD3G7 reading frame was cloned in plasmid pHENIX-LPETG-His-VSV to yield pHENIX-GD3G7-LPETG-His-VSV. Expression of the fusion protein in E. coli strain ER2566 was induced with isopropyl β-D-thiogalactoside (IPTG) as described previously [25]. GD3G7-LPETG-His-VSV was released from the periplasmic space via osmotic lysis using 200 mM Tris-HCl, pH 8.0, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 20% (w/v) sucrose containing protease inhibitors. Purification by nickel-NTA affinity chromatography was performed as described (NTA-Ni Sepharose®, IBA Life sciences) [26].

2.2.2. Introduction of DBCO functionality to GD3G7 by sortagging

pGBMCS-SortA, a gift from Dr. Fuyuhiko Inagaki [27] was transfected into E. coli ER2566 for standard protein expression. Bacterial expression was performed as described and IPTG-induced cells were lysed by sonication at 4 °C using a Bandelin Sonopuls HD2070 sonicator. His-tagged sortase was purified with NTA-Ni Sepharose as described above.

To equip the GD3G7 antibody with a bio-orthogonal chemical click handle (Fig. 1), 16 μM GD3G7-LPETG-His-VSV was incubated overnight at room temperature with 4 mM amino-PEG4-DBCO (Click Chemistry Tools, Scottsdale, USA) in the presence of 40 μM sortase A in 50 mM Tris, 150 mM NaCl, 10 mM CaCl2, pH 7.5. Reaction product was cleared from unreacted GD3G7-LPETG-His-VSV, cleaved G-His-VSV tags and sortase A by depletion on nickel-NTA beads. Free amino-PEG4-DBCO was removed by filtration in PBS over a 10 kDa centrifugal filter device (Amicon® Ultra-4, Merck Millipore) using standard protocols. Routinely, filters were washed five times to obtain highly purified product.

The sortase mediated reaction was evaluated by applying bioorthogonal click chemistry between DBCO and azide. The sortaged product was incubated with azido-cyanine-7.5 (Lumiprobe GmbH, Hannover, Germany) for 1 h at 4 °C, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels and gel imaging at 800 nm (Odyssey® CLx imaging system). Thereafter, gels were stained for presence of proteins with 0.1% (w/v) Coomassie Brilliant Blue R-250 solution (MP Biomedicals, Santa Anna, CA) in 50% (v/v) methanol and 10% (v/v) acetic acid in water.

2.2.3. Preparation of lyophilisomes and introduction of azide functionality

Lyophilisomes were prepared from bovine serum albumin (BSA; PAA Laboratories, Linz, Austria) as described previously [10]. Briefly, droplets of 20 μl 2.5 mg/ml BSA (containing 10% FITC-labeled BSA (Sigma-Aldrich, St. Louis, MO, USA)) in 0.01 M acetic acid were snap frozen in liquid nitrogen. Capsules were formed using an annealing and lyophilization regimen [11]. Large structures were removed by centrifugation (60g).

Lyophilisomes were prepared for click chemistry by introducing azide groups to the surface of lyophilisomes. Lyophilisomes were suspended in PBS containing 0.1% tween20 (v/v) (PBST; pH 8.0), sonicated (Cycle 0.5; Amplitude 20; 10 cycles) with a Sartorius labsonic P sonicator (Göttingen, Germany), mixed with 100 times molar excess NHS-PEG4-azide (Jena Bioscience, Jena, Germany) and incubated under rotation at room temperature overnight. Next, lyophilisomes were washed three times with PBST and centrifuged at 17,000g for 5 min to remove free NHS-PEG4-azide, and stored in PBST at 4 °C.

Modification of lyophilisomes with PEG4-azide was analyzed using flow cytometry. Lyophilisomes (2.5 μg) with or without PEG4-azide were incubated with 1 μg/ml DBCO-IR dye 680RD (LI-COR Biotechnology, Bad Homburg, Germany), which binds only
azido modified lyophilisomes, in PBST under rotation at room temperature for 1 h. Control samples were incubated in PBST only. Lyophilisomes were three times washed with PBST and centrifuged at 17,000 \( g \) for 5 min, re-suspended in PBST and analyzed for their 680RD signal with a BD FACSCalibur flow cytometer (BD Biosciences, Breda, the Netherlands). Data were analyzed using FlowJo software (Version 10, Treestar, Ashland, OR).

### 2.2.4. Loading of lyophilisomes with doxorubicin

Loading of lyophilisomes with doxorubicin was performed as described previously [10]. In short, 200 \( \mu \)g of lyophilisomes with or without GD3G7 antibody were washed twice with 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) buffer (pH 7.4; Promega, Madison, WI, USA) and centrifugated at 17,000 g for 5 min at 4 °C. Next, lyophilisomes were incubated overnight with 500 \( \mu \)l 0.5 mg/ml doxorubicin (Accord Healthcare, the Netherlands) in 10 mM HEPES buffer v/v at room temperature. Non-entrapped doxorubicin was removed by centrifuging at 17,000 g, 5 min, 4 °C and collecting the supernatant. Subsequently, the lyophilisomes were washed three times with 10 mM HEPES buffer (17,000 g, 5 min, 4 °C) and the supernatants were collected. The doxorubicin concentration in 10 mM HEPES buffer of the three collected supernatants was quantified spectrophotometrically at 490 nm (Synergy BioTek 2 Plate reader, BioTek, Winooski, VT, USA). The entrapment efficiency was calculated using the following formula:

\[
\text{Entrapment efficiency (\%)} = \frac{[\text{doxorubicin}]_{\text{total}} - [\text{doxorubicin}]_{\text{free}}}{[\text{doxorubicin}]_{\text{total}}} \times 100
\]
2.2.5. Antibody functionalization of lyophilisomes

In order to achieve specific targeting of lyophilisomes to CS-E, GD3G7 antibodies were conjugated to lyophilisomes through bioorthogonal click chemistry. A 1.25× molar excess GD3G7-LPET-PEG-DBCO was reacted to azido-conjugated lyophilisomes in PBST via the scheme depicted in Fig. 1C. Reaction was allowed to proceed overnight at room temperature. In a control reaction, azide-conjugated lyophilisomes were incubated with unmodified GD3G7 antibodies. Free antibodies were removed through three washing steps with PBST by centrifugation at 17,000 g for 5 min. Conjugation was evaluated using horseradish peroxidase-conjugated Protein A that binds to the V_{H3} domain of the antibody [19]. Antibody-functionalized lyophilisomes and non-functionalized lyophilisomes (2.5 μg) were incubated with 0.1 μg/ml peroxidase-Protein A (Merck Millipore, Darmstadt, Germany) in PBST for 1 h under rotation at room temperature. Afterwards, lyophilisomes were washed three times with PBST, centrifuged at 17,000 g for 5 min and peroxidase activity in the pellets was measured by reaction in 0.0243 M citric acid, 0.0514 M K_{2}HPO_{3}, 0.012% H_{2}O_{2} (v/v) and 0.04% ortho-phenylenediamine (v/v). After 30 min at room temperature, 12.5% H_{2}SO_{4} (v/v) was added to stop the reaction. Subsequently, lyophilisomes were centrifuged and the absorbance of the supernatant was measured at 492 nm using a Synergy BioTek 2 Plate reader (BioTek, Winooski, VT, USA).

2.3. Evaluation of the targeting potential of antibody functionalized lyophilisomes

2.3.1. Binding to ovarian cancer stroma

Human ovarian cancer cryosections (5 μm) were pre-treated with 2 mM Mg\textsubscript{Ac} in 25 mM TrisHCl buffer (pH 8.0) with and without the chondroitin sulfate digesting enzyme chondroitinase-AC (30 μU/ml, 1 h, 37° C). After blocking with 2% BSA in PBS (w/v), sections were incubated with either antibody-functionalized lyophilisomes or non-functionalized lyophilisomes (0.1 mg/ml in 2% BSA in PBS) for 1 h. Nuclei were visualized by incubation with 10 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Merck, Darmstadt, Germany) and CS-E was visualized by immunofluorescence, sections were mounted with cover glasses in mowiol-488.

2.3.2. Binding to peri-cellular chondroitin sulfate E in vitro

Although CS-E in ovarian carcinomas is predominantly localized in the cancer stroma and not (peri)cellularly, we used cell lines which do or do not produce CS-E in vitro as a model for analyzing the binding properties of the delivery system. Cell lines SKOV3 (ATCC, #HTB 77) and SKOV3-F7 (overexpressing CS-E [28]) showed strong (peri)cellular CS-E expression while cell line HFF1 (ATCC, #SCRF-1041) showed no CS-E expression (Supplementary data, Fig. S1). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM)-glutamax (Gibco, ThermoFisher scientific, Waltham, MA, USA) containing 10% fetal bovine serum (PAN Biotech, Aidenbach, Germany) (v/v) and 100 IU/ml penicillin and 100 μg/ml streptomycin (Amresco, Solon, OH, USA), at 37 °C in a humidified incubator in a 5% CO\textsubscript{2} atmosphere. When 80% confluency was reached, cells were dissociated using 0.05% trypsin (v/v) in 0.53 mM EDTA in Hank’s balanced salt solution (Corning Mediatech, Tewksbury, MA, USA) and maintained as proliferating cultures. Cells were tested for mycoplasma contamination every four months using a MycoAlert™ mycoplasma detection kit (Lonza, Basel, Switzerland). After thawing, cells remained in culture for a maximum of six months.

Cells were cultured in 10-well glass slides in 75 μl medium to >90% confluency. Next, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS (v/v) for 20 min, blocked with 2% BSA in PBS (w/v) and subsequently incubated for 1 h with 0.1 mg/ml antibody-functionalized lyophilisomes or non-functionalized lyophilisomes. Cells were incubated with 10 μg/ml DAPI for nuclear staining and mounted in Mowiol-488.

2.3.3. Binding to immobilized chondroitin sulfate E

To evaluate the specificity of the antibody-functionalized lyophilisomes for CS-E, the following glycosaminoglycans were coated onto a 10-well glass slide (Thermo Fisher Scientific, Waltham, USA) all at 0.1 mg/ml: CS-A (from bovine trachea, Sigma-Aldrich), CS-B (dermatan sulfate, from porcine intestinal mucosa, Celsus Laboratories Inc.), CS-C (from shark cartilage, Sigma-Aldrich), CS-D (from shark cartilage, Seikagaku), CS-E (from squid cartilage, Seikagaku), heparin (from porcine intestinal mucosa, Sigma-Aldrich), and heparan sulfate (from bovine kidney, Sigma-Aldrich). After blocking with 2% BSA in PBS (w/v), glass slides were incubated for 1 h at room temperature with either antibody-functionalized lyophilisomes or non-functionalized lyophilisomes (0.1 mg/ml), or GD3G7 antibodies as control. Thereafter, glass slides were rinsed in PBS and mounted in Mowiol-488 (Calbiochem, La Jolla, CA, USA). Lyophilisomes were visualized using their FITC label.

Analyses were performed using a Leica DM6000B fluorescent microscope. Image processing was performed using Imagej 1.48v (National Institutes of Health, USA). For visualization purposes, brightness and contrast were adjusted similarly for all images including the controls.

2.4. Visualization of chondroitin sulfate E

The expression of CS-E was visualized by immunofluorescence or by applying the avidin-biotin complex method [29]. In brief, slides were incubated with the GD3G7 antibody (1:5) after blocking, followed by a mouse anti-VSV antibody (clone PS54, 1:10) and either a goat anti-mouse IgG Alexa Fluor 488 (Life Technologies, 1:500) or biotinylated horse-anti-mouse IgG antibody (Vector Laboratories Inc., CA, USA, 1:200) and ABC reagent (Vectastain ABC anti-mouse-IgG kit, Vector Laboratories Inc.).

2.5. Evaluation of cytotoxic potential of antibody-functionalized lyophilisomes

2.5.1. Cytotoxicity analysis in vitro

The cell eliminating potential of doxorubicin loaded, antibody-functionalized lyophilisomes was investigated using a cell viability assay. To mimic a CS-E rich extracellular matrix, 96-wells cell culture plates (Corning Costar, NY, USA) were coated overnight with CS-E (0.1 mg/ml). The next day, non-immobilized CS-E was removed by washing with PBS and wells were incubated with either: (1) culture medium, (2) Caelyx (PEGylated liposomal doxorubicin, Janssen-Cilag B.V., Tilburg, the Netherlands), (3) free doxorubicin (Accord Healthcare, Utrecht, the Netherlands), (4) empty non-functionalized lyophilisomes, (5) empty antibody-functionalized lyophilisomes, (6) doxorubicin loaded non-functionalized lyophilisomes and (7) doxorubicin loaded, antibody-functionalized lyophilisomes, all preparations were diluted in culture medium. Doxorubicin concentration was 20 μM and the amount of lyophilisomes with or without doxorubicin were equal. After incubation for 1 h at 37 °C, wells were washed with culture medium. Subsequently, 5000 SKOV3-F7 cells were seeded in 100 μl medium and cell viability was measured after 5 days. Wells were washed with medium and 100 μl medium containing 10% (v/v) Alamar blue reagents (ThermoFisher Scientific) was added with an incubation time of 4 h at 37 °C. Cell viability was measured by fluorescence (excitation at 570 nm, emission at 585 nm) using a Synergy BioTek 2 Plate reader (BioTek, Winooski, VT, USA).
2.5.2. In vitro doxorubicin release

To determine whether lyophilisomes can eliminate cells by releasing doxorubicin, but without entering cells, an in vitro experiment using 2 kDa cut-off membranes (Slide-A-Lyzer™ MINI dialysis units 2000 MWCO, Thermo Scientific) was conducted. Free doxorubicin will pass the membrane, but doxorubicin in lyophilisomes or liposomes will not. 5000 SKOV3-F7 cells were seeded in the upper compartment of the membrane, while in the lower compartment the following conditions were added: (1) culture medium, (2) 5 or 20 μM Caelyx (liposomal doxorubicin), (3) 5 or 20 μM free doxorubicin, (4) lyophilisomes, (5) 5 or 20 μM doxorubicin in lyophilisomes, (6) PBS and (7) 5 μM doxorubicin in lyophilisomes in PBS. The amount of doxorubicin loaded and empty lyophilisomes was equal. After 48 h, 30 μl medium containing 30% (v/v) Alamar blue was added to the cells, with an incubation time of 4 h at 37 °C. Cell viability was measured as described.

2.5.3. Visualization of cellular doxorubicin uptake

To visualize uptake of doxorubicin from lyophilisomes into cells (nuclei), 5000 SKOV3F7 cells were cultured on a glass slide overnight and subsequently incubated with either free doxorubicin, Caelyx (liposomal doxorubicin) or lyophilisomes with or without 5 μM doxorubicin for 24 h. Finally, cells were washed once with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS (v/v) for 10 min. Nuclei were stained with 10 μg/ml DAPI in PBS (w/v) for 10 min and cells were mounted in Mowiol-488. Analyses were performed using a Leica DM6000B fluorescent microscope.

2.6. Statistical analysis

Statistical analyses were tested by one-way analysis of variance (ANOVA) with posthoc Bonferroni’s Multiple Comparison Test using Graphpad Prism version 5.03 (Graphpad software, La Jolla, CA, USA). All tests were two-sided and p-values <0.05 were considered significant. All experiments were performed at least three times independently.

3. Results

3.1. Generation and evaluation of antibody-functionalized lyophilisomes

Because random conjugation of single chain antibodies to albumin lyophilisomes using classical 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-Hydroxysuccinimide (EDC/NHS) chemistry carries a risk of modifying amino acids that are crucially involved in antigen binding, we chose to develop a method of controlled and site-specific conjugation using sortagging (Fig. 1). GD3G7 antibodies harboring the LPETG sortase consensus motif (molecular weight of ~28.5 kDa) could be readily produced and purified and were labeled with PEG-DBCO (Fig. 2A). The product was analyzed by a click reaction between DBCO and azide-cyanine 7.5, and in line with expectation, was only seen for GD3G7-PEG-DBCO as was demonstrated by SDS-PAGE analysis (lane 3 in Fig. 2A; note that the molecular weight of the GD3G7-PEG-DBCO-N3-cyanine 7.5 is slightly lower than the unreacted GD3G7-LPETG-His-VSV in lane 2, due to loss of the G-His-VSV tag during the sortase reaction).

The conjugation of amino-PEG4-azide to the surface of lyophilisomes was evaluated using flow cytometry. After incubation with DBCO-IR dye 680RD, which only binds azide modified lyophilisomes, the median fluorescent intensity of azide-conjugated lyophilisomes was 631 (95% confidence interval [CI]: 578–684) compared to 26 (95% CI: 21–30) for non-conjugated lyophilisomes (p < 0.001) (Fig. 2B). Conjugation was further evaluated by using peroxidase-conjugated Protein A, which binds to single chain antibodies of the V3 class, as is antibody GD3G7. High levels of Protein A bound to antibody-functionalized lyophilisomes compared to non-functionalized lyophilisomes [0.71 (95% CI: 0.53–0.89) vs 0.19 (95% CI: 0.11–0.27), p < 0.001] (Fig. 2C).

Loading of lyophilisomes with doxorubicin resulted in a mean entrapment efficiency of 28% (95% CI: 18–38%), which corresponds with a mean drug loading of 0.35 mg doxorubicin/mg lyophilisomes (95% CI: 0.23–0.48).

3.2. Specificity of antibody-functionalized lyophilisomes

Targeting properties of antibody-functionalized lyophilisomes were analyzed in patient derived ovarian cancer tissues (Fig. 3A and B). Cryosections of high grade serous ovarian carcinomas were incubated with antibody-functionalized lyophilisomes and non-functionalized lyophilisomes. An abundance of antibody-functionalized lyophilisomes associated with the cancer stroma (containing CS-E), whereas almost no binding to ovarian cancer epithelial cells was observed. Non-functionalized lyophilisomes showed background reactivity with both the ovarian cancer stroma and epithelial cells (Fig. 3A). Specificity of binding was assayed by pretreatment of ovarian cancer sections with the chondroitinase-AC. Enzymatic treatment abolished the reactivity of antibody-functionalized lyophilisomes with the ovarian cancer stroma (Fig. 3A).

The specificity of antibody-functionalized lyophilisomes was further analyzed in vitro using two human ovarian cancer cell lines producing CS-E (SKOV3 and SKOV3F7) and a cell line not producing CS-E (HFF1 cells, human foreskin fibroblasts) (Fig. 3B). The antibody-functionalized lyophilisomes showed strong reactivity with the CS-E producing cell lines (SKOV3 and SKOV3F7) compared to the CS-E-negative cell line HFF1. The non-functionalized lyophilisomes showed limited reactivity with any type of cell line. Finally, the specificity of the antibody-functionalized lyophilisomes for various glycosaminoglycans was determined. Antibody-functionalized lyophilisomes showed strong reactivity with the highly sulfated CS-E subtype (Fig. 3C), while only background signal was observed with other immobilized glycosaminoglycans including CS-A, CS-B (also known as dermatan sulfate), CS-C, CS-D, heparin and heparan. The non-functionalized lyophilisomes showed no significant reactivity with any type of glycosaminoglycan (Supplementary data, Fig. S2).

3.3. Cytotoxic potential of drug loaded, antibody-functionalized, lyophilisomes in vitro

A CS-E rich environment was created to assess the effect of doxorubicin loaded antibody-functionalized lyophilisomes on ovarian cancer cells. As shown in Fig. 4A, viability of cells cultured in CS-E coated wells that were pre-incubated with doxorubicin loaded antibody-functionalized lyophilisomes was decreased to 30% (95% CI: 22–39%), and was significantly lower compared to the cell viability after pre-incubation with doxorubicin loaded non-functionalized lyophilisomes, free doxorubicin, and Caelyx (liposomal doxorubicin) of 77% (95% CI: 65–89%), 70% (95% CI: 65–75%), and 103% (95% CI: 97–109%), respectively (p < 0.001). Empty lyophilosomal control conditions (functionalized or non-functionalized) did not affect cell viability.

3.4. Mode of doxorubicin release and nuclear localization

To study whether cell death occurs by extracellular release of doxorubicin from lyophilisomes or by cellular uptake of doxorubicin loaded lyophilisomes, an in vitro study using 2 kDa cut-off membranes was performed. 2 kDa membranes allow free doxorubicin (544 Da) to pass, but not larger components such as
lyophilisomes-associated doxorubicin, Caelyx (liposomal doxorubicin), cells, and enzymes. Free doxorubicin and doxorubicin loaded lyophilisomes in culture medium containing 10% FBS decreased cell viability in the upper compartment to 46.7% (95% CI: 39.4–54.0%, p < 0.001) and 39.5% (95% CI: 25.9–53.1%, p < 0.001), respectively (Fig. 4B). In contrast, equal doses of Caelyx and empty lyophilisomes did not significantly affect cell viability, 97.9% (95% CI: 83.3–112.4%) and 93.9% (95% CI: 74.6–113.2%), respectively. Moreover, a lower dose of 5 lM of free doxorubicin and doxorubicin in lyophilisomes significantly affected cell viability, whereas the same concentration of Caelyx did not; interestingly, doxorubicin loaded lyophilisomes in PBS did not significantly affect cell viability (Supplementary data, Fig. S3).

After incubation of cells with lyophilisomes loaded with doxorubicin, the drug localized to the nuclei, the site of action (Fig. 4C). This was also observed with free doxorubicin, but not with Caelyx under the same conditions (24 h of incubation).

3.5. Generality of CS-E expression in the stroma of solid cancers

In order to explore the stromal CS-E expression associated with various solid cancers other than ovarian, we immunohistochemically analyzed five samples of lung, cervical, breast, renal cell, endometrial, and colon cancer for the expression of CS-E. In the vast majority of each cancer type, stromal overexpression of CS-E was observed (Fig. 5).

4. Discussion

In the current study, we have evaluated the feasibility of a novel concept of therapeutic tumor targeting based on an ECM-targeting drug delivery system. Largely neglected, the cancer ECM provides potential targets for therapy and may have advantages over targeting cancer cells. Due to intra-tumoral heterogeneity, targeted therapies against cancer cell specific targets may act only on a subpopulation of cancer cells whilst other subpopulations not expressing the target, and cancer-associated stromal cells (i.e. fibroblasts and endothelial cells), are left unaffected [21]. Cancer-associated stromal cells have been identified as significant contributors to cancer growth and dissemination, and the additional elimination of these cells may benefit clinical outcome [30,31]. Furthermore, expression of cancer cell specific targets may change over time resulting in resistance to the applied targeted therapy [13]. Targeting anti-cancer drugs to the more stable ECM may be helpful to overcome these hurdles.

The potency of ECM-targeting therapies is supported by recent studies which have demonstrated that delivering anti-cancer drugs to the tumor stroma can successfully eliminate tumor cells and their micro-environment in vivo [23,24]. However, the approaches that were used in these studies were of limited translational value because these were not specific for the cancer stroma. Because the antibody-functionalized lyophilisomes in our study target a unique cancer-specific stromal antigen, this may result in a more specific...
Fig. 3. Specificity of antibody-functionalized lyophilisomes for highly sulfated chondroitin sulfate E (CS-E). (A) Effect of chondroitin sulfate degrading enzymes on targeting properties of antibody-functionalized lyophilisomes and non-functionalized lyophilisomes. Ovarian cancer cryosections were pre-incubated with either chondroitinase-AC that digests CS (ChAC+), or buffer without enzyme (ChAC−). Sections were stained with GD3G7 antibodies (visualized in red by Alexa-594) to indicate presence of CS-E chains in the cancer stroma. The general histology of the ovarian carcinoma cryosection is visualized by hematoxylin and eosin (H&E) staining and show epithelial cancer cells (marked with asterisk) surrounded by cancer-associated stroma. (B) Reactivity of antibody-functionalized lyophilisomes and non-functionalized lyophilisomes with CS-E producing cell lines (SKOV3-F7, SKOV3) and a non CS-E producing cell line (HFF1); (C) Reactivity of antibody-functionalized lyophilisomes and non-functionalized lyophilisomes with immobilized glycosaminoglycans CS-E and heparin, the reactivity with GD3G7 antibodies was used as control; Abbreviations: Ab, antibody. Scale bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
tumor targeting with concomitant less exposure to healthy surrounding tissues [23,24].

Here we report that antibody-functionalized lyophilisomes, generated by a stable and specific bioorthogonal click reaction between GD3G7-PEG 4-DBCO and azido-functionalized lyophilisomes, have specific binding properties for the ovarian cancer stroma rich in CS-E motifs. In addition and focusing on targeting characteristics, ovarian cancer cells in a CS-E rich microenvironment were efficiently eliminated by antibody-functionalized lyophilisomes loaded with doxorubicin, in contrast to non-functionalized lyophilisomes and Caelyx (liposomal doxorubicin), the latter being a commonly used second line chemotherapeutic for the treatment of ovarian cancer. These results indicate the potential of the ECM-targeting drug delivery system as a novel class of targeted therapy for the treatment of ovarian cancer.

To achieve specific stroma-targeting of the drug delivery system, we applied a two-step approach comprising sortase mediated ligation and bioorthogonal click chemistry. First, we equipped single chain GD3G7 antibodies with a click chemistry handle using the recently published sortagging approach [32]. Sortase A, a transpeptidase from *Staphylococcus aureus*, recognizes a LPXTG motif and catalyses cleavage between threonine and glycine residues forming an intermediate complex [33]. The complex is then

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Fig. 4. Cell viability and doxorubicin release in vitro. (A) A chondroitin sulfate E (CS-E) rich environment was pretreated with either doxorubicin loaded lyophilisomes with/without antibody functionalization, free doxorubicin or Caelyx (liposomal doxorubicin), all preparations containing 20 μM doxorubicin. After washings, ovarian tumor cells (SKOV3F7) were cultured and viability was assayed. Antibody-functionalized lyophilisomes with doxorubicin were most effective in eliminating ovarian cancer cells. (B) Viability of SKOV3F7 cells cultured on a 2 kDa dialysis membrane separating them from a lower compartment containing various conditions. Doxorubicin loaded lyophilisomes and free doxorubicin were equally effective, whereas Caelyx (liposomal doxorubicin) did not affect the cell viability. (C) Nuclear localization of lyophilisomal derived doxorubicin in vitro. SKOV3F7 cells were incubated for 24 h either with free doxorubicin, Caelyx (liposomal doxorubicin), empty lyophilisomes (depicted in green) or doxorubicin loaded lyophilisomes (depicted in red). Nuclei were stained blue with DAPI. Free doxorubicin and released doxorubicin from lyophilisomes localized to the nuclei (colored red). *** indicates a statistical significant difference with non-asterisks marked conditions with p < 0.001. Bars represent mean ± standard deviation (n = 3). Scale bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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nano-sized particles may be of benefit since uptake of micro-sized particles from the abdominal cavity into the circulation is restricted, thus elongating intraperitoneal half life and treatment efficacy [43]. The use of a targeted drug delivery system for the intraperitoneal treatment of ovarian cancer may be of major interest since the beneficial effect of intraperitoneally administered chemotherapeutics is limited by substantial (local) toxicity [38].

Resolved by a nucleophilic attack of the N-terminal protein thus forming a covalent bond [34]. Recently, it has been demonstrated that amine groups of other compound (e.g. amino-PEG) can be substrate for the LPETG-sortase intermediate thus offering a versatile toolbox for sortase A catalyzed ligations in protein engineering [35]. By positioning the LPETG sequence upstream of purification tags, these tags can be replaced with handles for click chemistry, while allowing convenient and rapid purification of the reaction product from unreacted proteins and by-products of the reaction. Using this system we generated GD3G7-PEG4-DBCO with high purity. Of note, because the click handle is positioned at the extreme carboxyterminal end, there is a minimal risk of affecting antibody affinity, unlike the widely used approach of EDC/NHS-mediated conjugation.

Lyophilisomes were used here as biocapsules for doxorubicin, and represent an attractive class of drug delivery systems for ECM-targeting since they can be prepared in a wide range of size (100 nm–10 μm), thereby being eligible for systemic as well as intraperitoneal administration [11]. Ovarian cancer has a unique tumor biology and metastatic spread pattern since it is usually confined to the peritoneal cavity. Standard treatment regimes include debulking surgery and systemic chemotherapy. As these patients especially suffer from extensive abdominal metastases, delivering adjuvant chemotherapeutics into the peritoneal cavity in addition to systemically administered chemotherapy, has been shown to significantly improve survival rates of ovarian cancer patients and is currently the standard of care for a selected group of patients [36–39]. Lyophilisomes can be prepared with a diameter as small as 100–200 nm allowing them to extravasate from leaky intratumoral vessels and accumulate in the cancer interstitium (enhanced permeability and retention effect) [40,41]. By subsequent binding to CS-E chains located in the stroma adjacent to intratumoral vessels, longer retention of nanoparticles in the cancer tissue and increased treatment efficacy may be achieved. On the other hand, it has been proposed that micro-metastases (≤1 mm) at the peritoneum have no vasculature and consequently will not be reached by systemic therapy [42]. For an (adjuvant) intraperitoneal application, the use of micro-sized particles over nano-sized particles may be of benefit since uptake of microsized particles from the abdominal cavity into the circulation is restricted, thus elongating intraperitoneal half life and treatment efficacy [43]. The use of a targeted drug delivery system for the intraperitoneal treatment of ovarian cancer may be of major interest since the beneficial effect of intraperitoneally administered chemotherapeutics is limited by substantial (local) toxicity [38].

Fig. 5. Stromal chondroitin sulfate E (CS-E) expression (in red) in various solid cancers including lung cancer, cervical cancer, breast cancer, renal cell cancer, endometrial cancer, and colon cancer. Scale bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Intensity stromal CS-E expression has been associated with various ovarian cancer subtypes including low grade and high grade serous, clear cell, and low grade and endometrioid cancer [20]. In this study we showed that CS-E was highly upregulated in the stroma of a variety of solid tumors including breast cancer, endometrioid cancer, cervical cancer, lung cancer, colon cancer, and renal cell cancer. Accordingly, delivery systems targeting CS-E may be applicable to a large and diverse group of cancers.

Release of drugs from stroma-targeting lyophilisomes is essential to eliminate cells. We demonstrated that doxorubicin loaded lyophilisomes in fetal bovine serum enriched culture medium release a substantial part of their drug resulting in nuclear localization of the drug (the site of action) and ovarian cancer cell death, in contrast to (Caelyx) liposomal doxorubicin that did not affect cell viability. This release cannot be explained by simple diffusion as the release in a neutral buffer (PBS) was minimal and did not result in cell death. Albumin represents an important transport protein and is known for its non-covalent reversible ligand-binding capacity. Moreover, it has several binding sites for hydrophobic components which may explain the affinity of albumin for doxorubicin, maintaining the drug within lyophilisomes [10] and which may contribute to the high drug loading capacity compared to Caelyx (0.35 mg doxorubicin/mg lyophilisomes vs 0.125 mg doxorubicin/mg liposomes) [44]. We hypothesize that hydrophobic components (e.g. fatty acids) or free albumin in serum-enriched medium may compete with the albumin-doxorubicin binding and lead to drug release. In addition, proteolytic enzymes in the cancer stroma may contribute to the degradation of the albumin lyophilisomes thus enabling release of its payload [45]. In order to enhance drug release, antibody-functionalized lyophilisomes hold potential for additional functionalization, e.g. by the incorporation of substrates for proteolytic enzymes upregulated in the ovarian cancer ECM, into the albumin wall.
5. Conclusion

In this study, we constructed and evaluated a drug delivery system targeting the cancer-associated stroma, based on albumin lyophilisomes loaded with doxorubicin and functionalized with antibodies to highly sulfated chondroitin sulfates. The delivery system may contribute to a novel class of therapy, based on addressing specific components in the extracellular matrix of tumors.

Conflict of interest

All authors declare to have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejpb.2016.12.010.

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