Bioconjugate Chemistry

Article

pubs.acs.org/bc

Functionalizing Liposomes with anti-CD44 Aptamer for Selective Targeting of Cancer Cells

Walhan Alshaer,[†,‡,§] Hervé Hillaireau,[†,‡] Juliette Vergnaud,[†,‡] Said Ismail,[§] and Elias Fattal*[,†,‡]

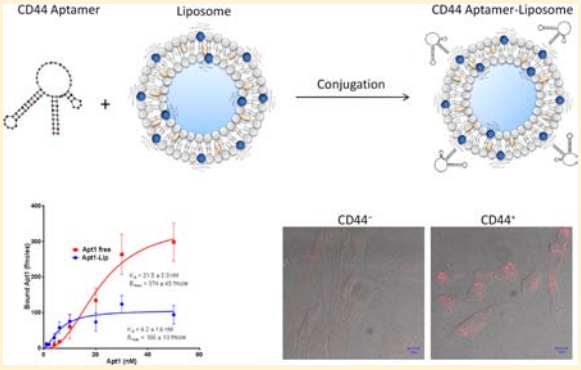
[†]Univ Paris-Sud, Institut Galien Paris-Sud, LabEx LERMIT, Faculté de Pharmacie, 5 rue J.B. Clément, 92296 Châtenay-Malabry, France

[‡]CNRS UMR 8612, Institut Galien Paris-Sud, LabEx LERMIT, Faculté de Pharmacie, 5 rue J.B. Clément, 92296 Châtenay-Malabry, France

[§]Molecular Biology Research Laboratory, Faculty of Medicine, University of Jordan, Amman 11942, Jordan

Supporting Information

ABSTRACT: CD44 receptor protein is found to be overexpressed by many tumors and is identified as one of the most common cancer stem cell surface markers including tumors affecting colon, breast, pancreas, and head and neck, making this an attractive receptor for therapeutic targeting. In this study, 2′-F-pyrimidine-containing RNA aptamer (Apt1), previously selected against CD44, was successfully conjugated to the surface of PEGylated liposomes using the thiol−maleimide click reaction. The conjugation of Apt1 to the surface of liposomes was confirmed by the change in size and zeta potential and by migration on agarose gel electrophoresis. The binding affinity of Apt1 was improved after conjugation compared to free-Apt1. The cellular uptake for Apt1-Lip was tested by flow cytometry and confocal imaging using the two CD44$^+$ cell lines, human lung cancer cells (A549) and human breast cancer cells (MDA-MB-231), and the CD44$^-$ cell line, mouse embryonic fibroblast cells (NIH/3T3). The results showed higher sensitivity and selectivity for Apt1-Lip compared to the blank liposomes (Mal-Lip). In conclusion, we demonstrate a successful conjugation of anti-CD44 aptamer to the surface of liposome and binding preference of Apt1-Lip to CD44-expressing cancer cells and conclude to a promising potency of Apt1-Lip as a specific drug delivery system.



INTRODUCTION

Most of the current conventional antitumor treatments aim to restrain proliferation and metastasis of tumor cells. However, many of these therapeutics fail to eradicate tumors, as indicated by some poorly understood clinical events including drug resistance and tumor relapse.[1,2] One of the main hypotheses to explain such events states that malignancies depend on a small population of stem-like cells that are highly resistant to conventional tumor therapeutics and are able to maintain and propagate tumors.[3,4] Those cells, to which the name Cancer Stem Cells (CSC) was given, are characterized by the ability for self-renewal, tumor initiation and proliferation, and differentiation into other tumor cells.[5−7] Therefore, targeting CSCs as well as the bulk tumor cells could be of high therapeutic potency in eliminating tumors and decreasing tumor relapse.[8,9] Many CSC surface markers have been identified such as CD133, EpCAM, and CD44.[10−12] These markers constitute potent targets for specific and selective targeting of antitumor therapeutics into CSCs.[13] Among all CSC surface markers, CD44 has been characterized as the most common biomarker, being overexpressed by many tumors including colon, breast, pancreatic, head, and neck cancers.[11,14−16] CD44 is a multistructural and functional cell surface glycoprotein known to play a role in cell communication between the adjacent cells and between the extracellular matrix. CD44 is the main receptor for hyaluronic acid (HA) and collaborates with other cellular proteins to regulate cell adhesion, proliferation, homing, migration, motility, growth, survival, angiogenesis, and differentiation.[17]

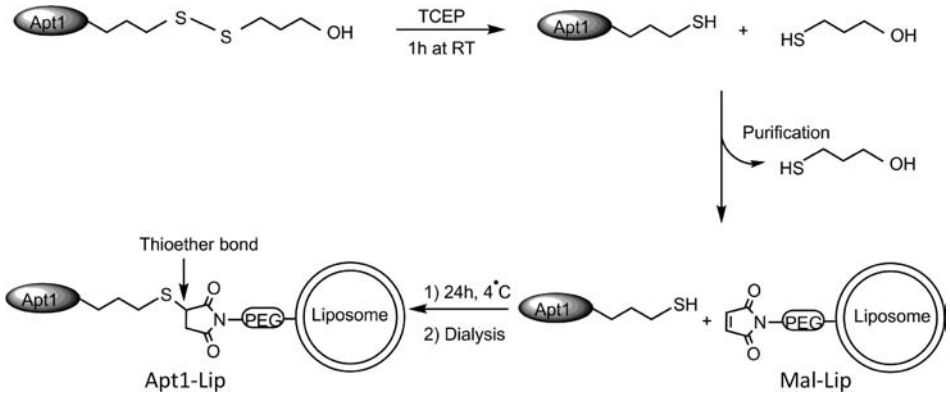
Aptamers are synthetic single-stranded oligonucleotides or peptides that can be selected against almost any target including ions, small chemical molecules, peptides, proteins, and enzymes, and even whole living cells such as bacteria and tumor cells.[18] Aptamers can be selected from a combinatorial library consisting of 10^{13} to 10^{15} different sequences by a method named systematic evolution of ligands by exponential enrichment (SELEX).[19,20] The principle of molecular binding is based on the ability of the aptamer to fold into complex three-dimensional structures and shapes and then fit and bind with

Special Issue: Biofunctional Biomaterials: The Third Generation of Medical Devices

Received: September 16, 2014
Revised: October 23, 2014
Published: October 24, 2014

dx.doi.org/10.1021/bc5004313 | *Bioconjugate Chem.* 2015, 26, 1307−1313

Scheme 1. Apt1-Liposome Functionalization Using Thiol−Maleimide Click Reaction



the selected target with high affinity and specificity. Aptamers hold several advantages over their long established competitors, monoclonal antibodies, such as their low toxicity and immunogenicity, easy chemical modifications, efficient reproduction with long shelf life, and reasonable cost. Therefore, aptamers constitute promising molecules with high potential in biomedical applications including therapeutics, diagnostics, biomarker discovery, and specific targeting ligands.[21,22] They can be clinically used since Macugen was the first successful aptamer-based therapeutic, which was approved in 2006 by the FDA for the treatment of age macular degeneration (AMD). Moreover, many aptamers are in clinical trials now.[23]

Among all nanoparticle-based drug delivery systems, liposomes are considered as one of the most successful in clinical use due to advantages including biocompatibility, stability, ease of synthesis, low batch-to-batch variation, and high drug payload with one or more different therapeutic molecules.[24] Many liposome formulations have been approved for drug delivery by passive ways based on the concept of enhanced permeability and retention. However, such passive targeting does not discriminate between normal and diseased cells. Therefore, cell-specific targeting liposomes have been developed to increase the accumulation of drugs in diseased cells, mostly cancer stem cells in the case of CD44 targeting, and thus decrease the toxic side effects to normal cells.[25] Therefore, active targeting could increase the accumulation of therapeutics around the targeted cells, compared to untargeted cells. Antibodies and hyaluronic acid (HA) have been widely and successfully used in the development of drug nanocarrier systems that target CD44-expressing tumor cells.[26,27] However, there are significant challenges in the utilization of antibodies and HA for targeting. Antibodies were shown to be immunogenic and exhibit rapid immune clearance from the peripheral circulation;[28,29] while low molecular weight HA was shown to be inflammatory, angiogenic, and immunostimulatory.[30] Therefore, anti-CD44 aptamers are attractive alternative to functionalize liposomes for selective targeting of therapeutics into CD44-expressing tumor cells.

In previous work we succeeded in selecting a modified RNA aptamer, named Apt1, against the standard isoform of human CD44 receptor protein using the SELEX method.[31] The selected aptamer was modified with 2′-F-pyrimidine to increase stability against nucleases for biological applications. In the present work, we developed an aptamer-functionalized-liposome (Apt1-Lip) as a model of a drug delivery system that selectively targets CD44-expressing tumor cells. Such functionalization was performed by thiol−maleimide conjugation chemistry between 3′-thiol modified Apt1 and the maleimide functionalized to the surface of the liposomes as shown in Scheme 1. The targeted liposomes were shown to express high affinity for CD44 positive cells without triggering any inflammatory response within these cells.

RESULTS AND DISCUSSION

Conjugation of Apt1 to Liposomes. Several aptamers have been successfully functionalized to different nanoparticles for selective targeting and drug delivery.[32] In this work liposomes functionalized with an anti-CD44 2′-F-pyrimidine RNA aptamer were developed as drug delivery systems able to selectively bind CD44-expressing tumor cells. Maleimide-functionalized liposomes (Mal-Lip) composed of DPPC:Cholesterol:DSPE-PEG-Maleimide have been prepared by the thin lipid film hydration method[33] followed by extrusion through a 100 nm polycarbonate membrane. Conjugation of Apt1 to Mal-Lip was carried out using thiol−maliemide cross-linking chemistry which is an efficient strategy to conjugate 3′-thiol-modified aptamers to the maleimide-functionalized polyethylene glycol (PEG).[34]

The efficiency and effect of Apt1 conjugation on the size and zeta potential of liposomes was investigated and data were compared to blank liposomes (Table 1). The size of Apt1-Lip showed a slight increase in the hydrodynamic diameter (140 ± 6 nm, $n = 6$) compared to blank Mal-Lip (129 ± 5 nm, $n = 6$). Further characterization to different liposomes was performed by detection of zeta potential of both Mal-Lip (−17.5 ± 0.9 mV, $n = 6$) and Apt1-Lip (−31.0 ± 2.3, $n = 6$) in a diluted PBS buffer (8 mM NaCl, pH 7.4). The increase in liposome size (∼11 nm) and decrease in zeta potential (∼ −12.5 mV) is consistent with successful conjugation of the negatively charged macromolecule Apt1 to the surface of the liposomes. Additional information about the successful conjugation was obtained by the analysis of Apt1-Lip on agarose gel electrophoresis (Figure 1). Apt1-Lip appears trapped in the well where no signal was

Table 1. Hydrodynamic Diameter and Zeta Potential Charecterization of Liposomes before and after Apt1 Conjugation (mean ± SD, $n=6$)

	Lip	Apt1-Lip
mean hydrodynamic diameter (nm ± SD)	129 ± 5	140 ± 6
polydispersity index (PDI ± SD)	0.09 ± 0.02	0.11 ± 0.03
ζ-potential (mV ± SD)	−17.5 ± 0.9	−31.05 ± 2.3

dx.doi.org/10.1021/bc5004313 | Bioconjugate Chem. 2015, 26, 1307−1313

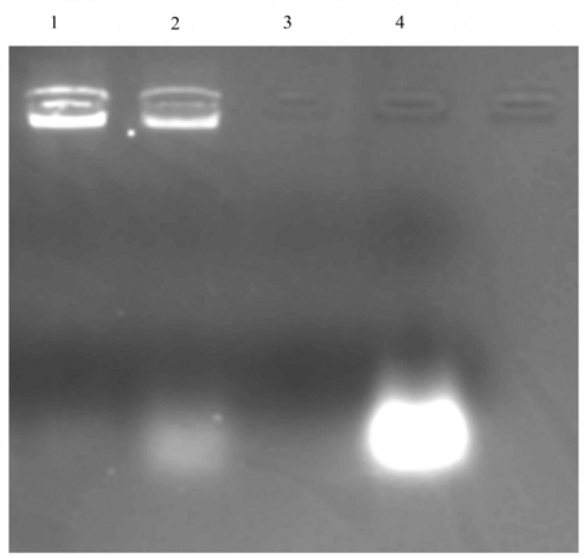


Figure 1. Characterization of liposomes before and after Apt1 conjugation using agarose gel electrophoresis. Lanes: 1, Apt1-Lip after dialysis; 2, Apt1-Lip before dialysis; 3, Mal-Lip; 4, free Apt1.

obtained for blank Mal-Lip. Moreover, electrophoresis showed the efficacy of dialysis in removing unreacted free Apt1 from the Apt1-Lip. Therefore, Apt1 functionalization could be achieved by covalent attachment to the surface of liposomes by establishing thioether bonds and not by adsorption.

Apt1 density has been determined using the fluorescent signal obtained from FITC-Apt conjugated to the surface of liposomes. The average amount of Apt1 conjugated molecules was calculated to be 6.06 nmol of Apt1 per mg of lipids and to an aptamer surface density of ~422 of Apt1 molecule per one liposome which corresponds to a conjugation efficiency of ~73 ± 3% (mean ± SD, n = 3).

Binding Affinity to CD44 Protein. Binding affinity of functionalized liposomes is an important factor that impacts the efficacy of the delivery system. Therefore, we investigated whether the conjugation of Apt1 may affect the affinity of binding to CD44 by performing a binding experiment for the free Apt1 and the Apt1-Lip with CD44 protein immobilized on magnetic beads. The binding of Apt1-Lip showed higher binding affinity (K_d = 6.2 ± 1.6 nM) compared to the affinity of free Apt1 (K_d = 21.5 ± 3.3 nM) (Figure 2). This shows that the conjugation did not negatively affect the binding affinity to CD44 receptor model. Moreover, such higher binding affinity of Apt1-Lip can be explained by multivalent binding of conjugated Apt1 to CD44 on the surface of the beads. However, free Apt1 showed superior maximum binding saturation (B_{max} = 374 ± 45 fmol) compared to Apt1-Lip (B_{max} = 106 ± 13 fmol). This can be due to the binding of Apt1-Lip covering large areas on the beads resulting in steric hindrance of the remaining free binding sites compared to free Apt1 as appears in Figure 2A.

Cellular Uptake. To investigate the binding specificity and selectivity to CD44 expressing cells, three cell lines have been selected for cellular uptake. The human lung cancer cells (A549) and the human breast cancer cells (MDA-MB-231) are known to have high CD44 expression. *p < 0.05 compared to untreated cells and Lip-Rhod. The mouse embryonic fibroblast cell line (NIH/3T3) was used as negative cells for CD44. All cell lines have been confirmed for the expression or the absence of CD44 by flow cytometry using FITC-anti-CD44 antibodies. Both A549 cells and MDA-MB-231 cells had shown a high CD44 expression level while NIH/3T3 cells were negative for CD44 expression (Supporting Information Figure S1). The same concentrations of Apt1-Lip-Rhod and Lip-Rhod were incubated with all selected cell lines and the mean fluorescent intensity was detected by flow cytometry.

Both A549 and MDA-MB-231 cells showed higher mean fluorescent intensity when treated with Apt1-Lip-Rhod compared to Lip-Rhod. No significant difference in the mean fluorescent intensity was seen between Apt1-Lip-Rhod and Lip-Rhod was obtained with NIH/3T3 negative cells (Figure 3). These results demonstrate the preference of Apt1-Lip-Rhod to bind to CD44 positive cells and such binding preference is indicated by the higher selectivity and uptake sensitivity of CD44 positive cells to Apt1-Lip-Rhod compared to Lip-Rhod CD44-negative cells. Furthermore, the cellular uptake was analyzed using confocal microscopy and the results demonstrated higher accumulation of Apt1-Lip in cytoplasm of CD44$^+$

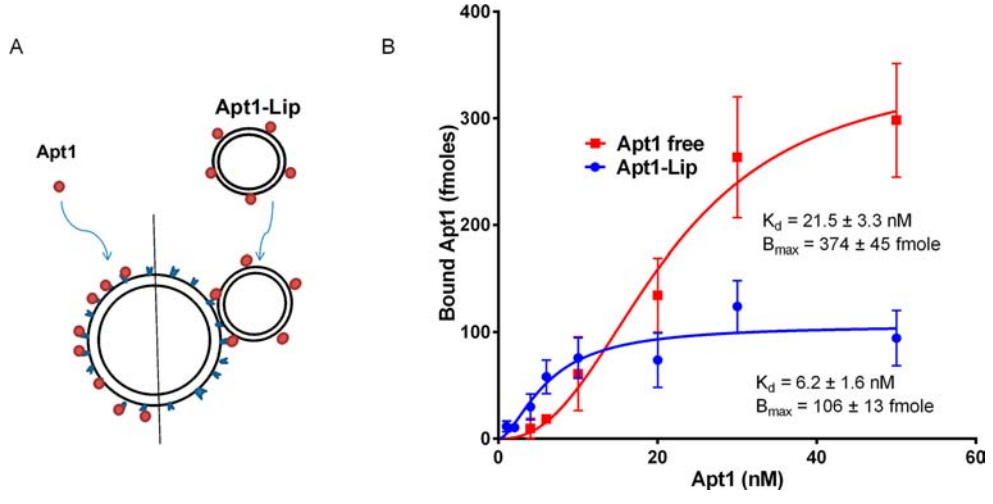


Figure 2. Binding affinity curves of free Apt1 and Apt1-Lip to CD44. (A) Different concentrations of radiolabeled Apt1 (free or conjugated) have been incubated with fixed concentration of CD44 protein immobilized on magnetic beads. (B) Specific binding was obtained after subtracting nonspecific interaction with negative beads. The data are fitted using one binding site with hill equation (mean ± SD, n = 3).

dx.doi.org/10.1021/bc5004313 | Bioconjugate Chem. 2015, 26, 1307−1313

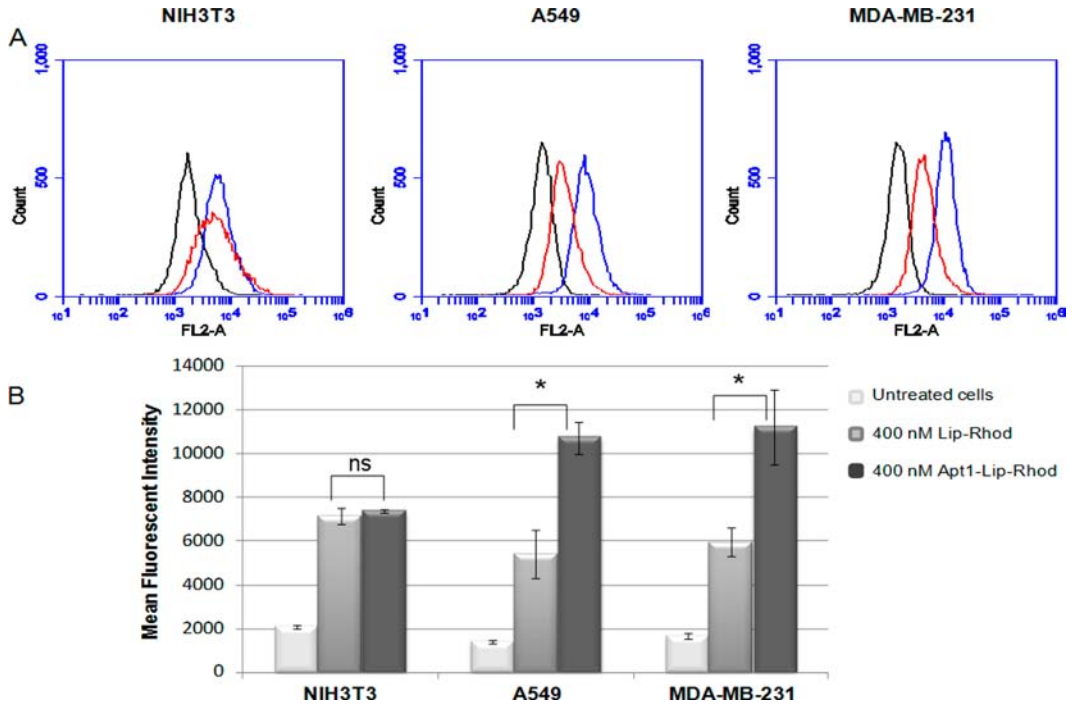


Figure 3. Cellular uptake of Apt1-Lip-Rhod and Mal-Lip-Rhod. (A) Flow cytometry analysis of the three cell lines after incubation with 400 nM of Apt1-Lip-Rhod (blue) and Mal-Lip-Rhod (red), untreated cells (Black). (B) Mean fluorescent intensity (MFI) obtained from flow cytometry analysis after treatment of cells with 400 nM of Apt1-Lip-Rhod and Mal-Lip-Rhod (mean ± SD, $n = 3$, $^*p < 0.05$ compared to Lip-Rhod cells, ns: not significant).

cells compared to $CD44^-$ cells, therefore supporting the results obtained from flow cytometry (Figure 4).

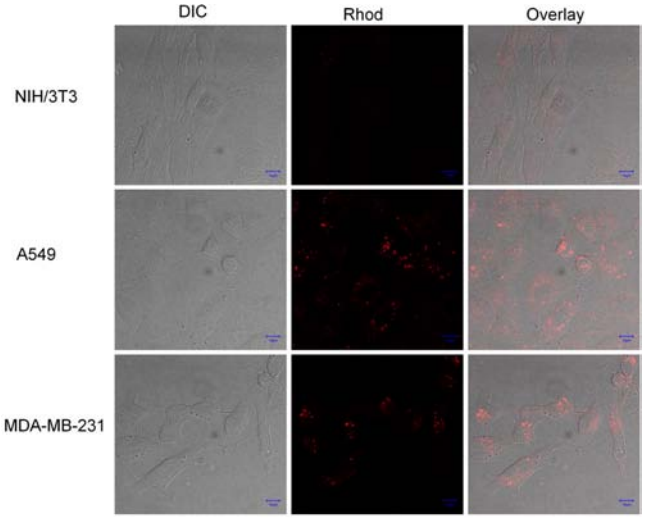


Figure 4. Confocal microscope imaging for the selected cell lines treated with 400 nM of Apt1-Lip-Rhod.

Inflammatory Response. A considerable amount of evidence illustrates the link between inflammatory cytokines and the development of tumors. There are several inflammatory cytokines that are associated with chronic inflammation including IL-1β, IL-6, and IL-8. These cytokines are found to activate signaling pathways and positive feedback loops involved in tumor maintenance, invasion, metastasis, and drug resistance.[35] Therefore, investigation of inflammatory response against drug delivery systems such as liposomes could be carefully examined, thereby enabling the control of unwanted signals which resulted from inflammatory cytokines. In this study, the inflammatory response of A549 and MDA-MB-231 cells has been investigated by detection of the inflammatory cytokines IL-12p70, TNF, IL-10, IL-6, IL-1β, and IL-8 after exposure to Apt1-Lip.

The results showed a significant increase in the secretion of IL-6, IL-1β, and IL-8 by MDA-MB-231 and IL-8 by A549 cell lines after treatment with LPS as shown in Figure 5, while no increase was detected in the cytokines IL-12p70, TNF, and IL-10 (Supporting Information Figure S2). These results highlight the importance of the cytokines IL-6, IL-1β, and IL-8 in maintaining and proliferating tumor cells. Furthermore, there was no increase in the secretion of the inflammatory cytokines IL-12p70, TNF, IL-10, IL-6, IL-1β, and IL-8 after treatment with Apt1-Lip at 400 nM concentration compared to Mal-Lip and untreated cells. These results conclude that Apt1-Lip fails to induce an inflammatory response by tumor cells realizing the safe and efficient drug delivery system for therapeutic applications. Moreover, previous research described other molecules to target CD44 receptor such as hyaluronan and specific anti-CD44 antibodies. However, some challenges face the application of these molecules for specific drug delivery purposes. For example, low molecular weight hyaluronan ($<10^6$ Da) and the monoclonal antibodies (NIH4−1) were shown to induce tumor cell survival, resistance, and escape from the immune system and induce inflammation. Therefore, choosing specific ligands such as aptamers that target CD44 receptor without further stimulation of the receptor could be of higher therapeutic potency.[36,37]

EXPERIMENTAL PROCEDURES

Materials. The lipid 1, 2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (**DPPC**) was purchased from Corden pharma

dx.doi.org/10.1021/bc5004313 | *Bioconjugate Chem.* 2015, 26, 1307−1313

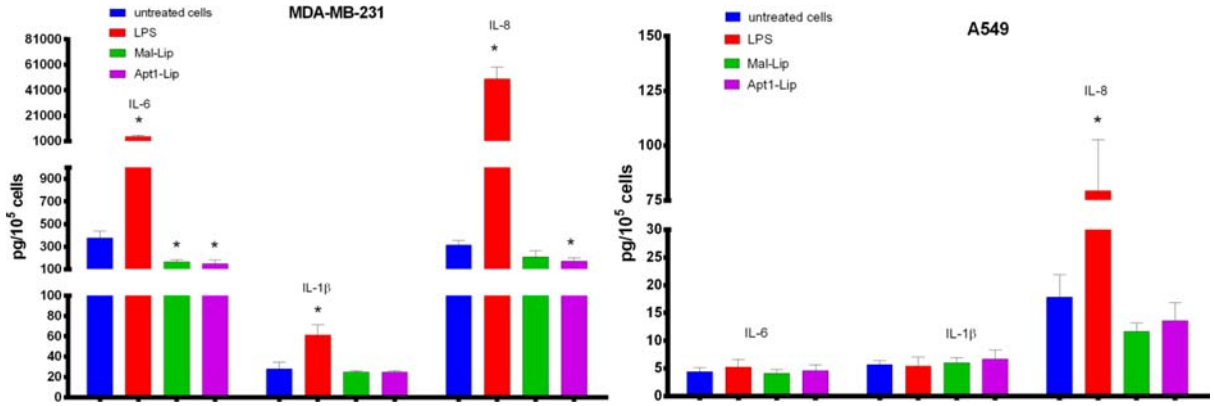


Figure 5. Inflammatory cytokine secretions IL-6, IL-1β, and IL-8. After exposure to Mal-Lip, Apt1-Lip, and 10 μg/mL LPS (mean ± SD, n = 3, *p < 0.05 compared to untreated cells).

(Germany), while 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide (polyethylene glycol)-2000] (**DSPE-PEG(2000)-Maliemide**) and L-α-phosphatidylethanolamine-*N*-(Lissamine rhodamine B sulfonyl) (**PE-Rhod**) were purchased from Avanti Polar Lipids (Alabaster, USA). Cholesterol was obtained from Sigma-Aldrich. Tris(2-carboxyethyl)phosphine hydrochloride (**TCEP**) was from Thermo Fisher Scientific (MA, USA).

Apt1 with 2′-F-pyrimidines (sequence: 5′-GGGAUGGA-UCCAAGCUUACUGGCAUCUGGAUUUGCGCGUGCC-AGAAUAAAGAGUAUAACGUGUGAAUGGGAAGCUUCG-AUAGGAAUUCGG-3′) have been chemically synthesized by cyanoethyl phosphoramidite chemistry (Midland Certified Reagent, Texas, USA). Apt1 was modified with C6-thiol SS (protected form) at the 3′-end and labeled with FITC at the 5′-end. All other materials were obtained from different resources.

Cell Lines. Human lung cancer cells (A549) and mouse embryonic fibroblast cell line (NIH/3T3) were obtained from the American Type Culture Collection (ATCC). Human breast cancer cells (MDA-MB-231) were obtained from the European Collection of Cell Culture (ECACC). A549 and NIH/3T3 cells were cultured in RPMI and DMEM medium (Lonza, Switzerland), respectively, supplemented with 10% fetal bovine serum, and 10 000 unit/mL of penicillin and 10 mg/mL of streptomycin (Lonza, Switzerland). MDA-MB-231 cells were cultured in Leibovitz's L-15 medium (Lonza, Switzerland) supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 20 mM sodium bicarbonate, and 10 000 unit/mL of penicillin and 10 mg/mL of streptomycin. All cell lines have been incubated in humidified incubator supplied with 5% CO_2 and maintained at 37 °C. Characterization of all cell lines for the expression of CD44 was tested using FITC-labeled anti-CD44 antibodies (Immunotech, Beckman-Coulter, USA).

Preparation of Maleimide-Functional Liposome. Liposomes with maleimide active group (Mal-Lip) were prepared by the thin lipid film hydration method.[33] Stock solution of DPPC, cholesterol, and DSPE-PEG(2000)-Mal in a 62:35:3 molar ratio was mixed and dissolved in 5 mL of chloroform. A thin film was obtained by evaporation of chloroform using rotary evaporator for 30 min at 50 °C with decreased pressure. This film was then hydrated with 5 mL of PBS and vortexed for 30 min followed by extrusion 13 times through a 100 nm polycarbonate membrane (Millipore) to obtain liposomes with a size around 100 nm. Rhodamine-containing liposomes have been prepared as described above with DPPC:cholesterol:DSPE-PEG(2000)-MAL:PE-Rhod at molar ratio 61:35:3:1. These liposomes were also used as blank liposomes.

Conjugation of Apt1 to Liposomes. Functionalizing Mal-Lip with Apt1 was performed using the thiol−maleimide cross-linking reaction to form thioether bonds. Before conjugation, C6-thiol-modified Apt1 (Apt1-$(CH_2)_3$-S-S-$(CH_2)_3$) has been deprotected in free nuclease water by 100 mM TCEP (pH 6.5) for 1 h at room temperature to produce Apt1-SH and then purified by precipitation using 3 volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate. After precipitation, Apt1-SH was resuspended in binding buffer (2.5 mM $MgCl_2$, 1 × PBS, pH 7.4) and folded by heating at 70 °C for 10 min followed by rapid cooling on ice for 10 min. The resulting deprotected Apt1 was conjugated to Mal-Lip at a 0.5:1 molar ratio by incubation overnight at 4 °C followed by dialysis to remove free Apt1 from the Apt1-Lip suspension.

Characterization of Apt1-Lip. *Size and Zeta Potential.* The size and zeta potential of Apt1-Lip and Mal-liposomes were measured at 25 °C by dynamic light scattering (DLS) using nano ZS (Malvern instruments, UK). Apt1-Lip or Mal-liposomes in 1 × PBS were diluted in free nuclease water to obtain 8 mM sodium chloride final concentration (pH 7.4).

Agarose Gel Electrophoresis. Agarose gel electrophoresis was performed to confirm the successful conjugation of Apt1 to the surface of liposomes. Samples of Apt1-lip (before and after dialysis), blank liposomes, and free Apt1 were loaded into 2% agarose gel (Promega, USA) supplemented with 5 μL of 2.5 mg/mL ethidium bromide followed by running electrophoresis in 1 × TAE buffer (pH 8.2) at 120 V for 20 min. Images for analysis were obtained using MF-ChemiBIS gel imaging system (DNR Bio-Imaging Systems).

Determination of Apt1 Density at the Surface of Liposomes. To quantify the amount of Apt1 conjugated to the surface of liposome, a fraction of conjugated Apt1 was quantified by detection of the fluorescence intensity obtained from FITC-Apt1-Lip (excitation 494 nm, emission 517 nm) and compared to a standard curve using a Spectrofluorometer LS 50B (PerkinElmer, USA). The number of liposomes was calculated based on the Supporting Information Equation S1. Using those two values, it was possible to divide the amount of Apt1 by the number of liposomes to obtain the density of Apt1 on the liposome surface.

Binding Experiments. *Binding Affinities.* The change in the binding affinity of Apt1 before and after conjugation was investigated using magnetic separation beads. The dissociation constants (K_d) have been determined for Apt1-Lip and free

dx.doi.org/10.1021/bc5004313 | *Bioconjugate Chem.* 2015, 26, 1307−1313

Apt1 by binding to GST-tagged CD44 protein immobilized on magnetic separation beads (Promega, USA). An amount of 50 pmol of C6-thiol Apt1 were labeled at 5′-end with radioactive phosphate of [γ-^{33}P]ATP (PerkinElmer) using T4 Polynucleotide Kinase (Promega, USA), and then reduced and conjugated following the same method described before. The binding affinity constant (K_d) were determined by incubating variable concentrations (1 to 50 nM) for both free-Apt1 and Apt1-Lip with immobilized CD44 protein (10 nM) for 1 h at 37 °C in 40 μL binding buffer. After incubation, unbound complexes were removed by washing three times with 100 μL of binding buffer. The CD44-Apt1-Lip or CD44-Apt1 complexes were then eluted from the beads using 100 μL of elution buffer (50 nM glutathione, 50 nM Tris-HCl, 1% Triton X-100). Radiaoactivity was counted in gold scintillation liquid using Scintillation Counter (LS 6000, Beckman, USA). For nonspecific binding, the same concentrations of Apt1-Lip and free-Apt1 were incubated with CD44-negative GST-tagged magnetic separation beads. The specific binding was fitted using the Hill equation

$$Y = \frac{B_{max} \times X^h}{(K_d^h + X^h)}$$

where Y is the specific binding, B_{max} is the maximum specific binding, h is the Hill slop, X is the Apt1 concentration, and K_d is the dissociation constant.

Flow Cytometry. Apt1-Lip binding specificity and uptake by cells were assessed by measuring the mean fluorescence intensity using a flow cytometer (Accuri C6, BD Biosciences, and USA). Approximately 1×10^5 cells of each selected cell line were seeded in 12-well plates and incubated for 24 h at 37 °C to reach 80% confluency. After incubation, cells were washed by PBS and incubated in 200 μL of fresh medium supplemented with 2.5 mM $MgCl_2$. Then, 400 nM Apt1-Lip-Rhod was incubated with cells for 3 h at 37 °C. The unbound Apt1-Lip was washed three times by PBS and detached by 100 μL of EDTA-accutase for 5 min. After detachment, cells were resuspended in 200 μL of binding buffer and 10 000 events were counted for each sample. Blank Mal-Lip-Rhod and the negative CD44 cell line NIH/3T3 have been used as negative controls.

Confocal Laser Scanning Microscopy. Around 5×10^4 cells have been seeded into 12-well plates containing glass coverslip and incubated under standard conditions. After 24 h of incubation, cells were washed with PBS and 200 μL of fresh medium supplemented with 2.5 mM $MgCl_2$ was added. Cells were then incubated with 400 nM Apt1-Lip-Rhod for 2 h at 37 °C. After incubation, the cells were washed three times with 500 μL of binding buffer, then fixed by 4% paraformaldehyde for 10 min at room temperature in the dark. The cells were then washed three times by PBS and the coverslip transferred onto a glass slide and dipped in mounting medium. Blank lip-Rhod was used as control for comparison. The images were then acquired by a fluorescent microscope LSM 510 (Zeiss-Meta) using the same exposure settings by 63×/1.5 oil lens.

Inflammatory Cytokines Release. To examine the inflammatory cytokines that may be released as a result of treating A549 and MDA-MB-231 cells with Mal-Lip or Apt1-Lip, 2×10^4 cells were seeded in 24-well plates for 48 h to reach 80% confluency. The supernatants were then discarded and replaced with 1 mL of fresh medium. The cells were treated with 10 μg of lipopolysaccharide (LPS) as control and with Mal-Lip or Apt1-Lip at 400 nM final concentrations. After 24 h exposure, the supernatant was collected and the cells washed with 1× PBS treated with 1× Trypsine and counted by flow cytometry. Human inflammatory cytokines IL-12p70, TNF, IL-10, IL-6, IL-1β, and IL-8 were quantified using a Cytometric Beads Array (CBA) detection kit (BD Biosciences, USA) and the results were normalized to pg/10^5 cells.

Statistical Analysis. All measurement points were repeated in three times. The values were expressed by the mean ± standard deviation. Multiple comparisons by two-way ANOVA have been used to assess the statistical significant differences between the means ($p < 0.05$).

■ ASSOCIATED CONTENT

Supporting Information

Details about the calculations of liposome number, the characterization of cell lines for the CD44 expression, and the secretion of the inflammatory cytokines IL-12p70, TNF, IL-10. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: elias.fattal@u-psud.fr. Tel: 33146835582. Fax: 33146835946.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors wish to thank Hélène Chacun for help in radioactivity experiments, Valérie Nicolas for help and advice in confocal lazer microscopy experiments, Stéphanie Denis and Nadège Grabowski for the assistance in tissue culture experiments, and Chantal AL Sabbagh for the advice in liposomes preparation. We thank AL HIKMA pharmaceutical company for funding Mr. Walhan Alshaer Ph.D. and The French Embassy in Amman (Mrs. Lisbeth Ngouanet) for their support.

■ REFERENCES

(1) Dean, M., Fojo, T., and Bates, S. (2005) Tumour stem cells and drug resistance. *Nat. Rev. Cancer 5*, 275−84.
(2) Visvader, J. E. (2011) Cells of origin in cancer. *Nature 469*, 314−22.
(3) Vermeulen, L., de Sousa e Melo, F., Richel, D. J., and Medema, J. P. (2012) The developing cancer stem-cell model: clinical challenges and opportunities. *Lancet Oncol. 13*, e83−9.
(4) Vermeulen, L., Sprick, M. R., Kemper, K., Stassi, G., and Medema, J. P. (2008) Cancer stem cells–old concepts, new insights. *Cell Death Differ. 15*, 947−58.
(5) Clevers, H. (2011) The cancer stem cell: premises, promises and challenges. *Nat. Med. 17*, 313−9.
(6) Magee, J. A., Piskounova, E., and Morrison, S. J. (2012) Cancer stem cells: impact, heterogeneity, and uncertainty. *Cancer Cell 21*, 283−96.
(7) Schwarz-Cruz-y-Celis, A., and Melendez-Zajgla, J. (2011) Cancer stem cells. *Rev. Invest. Clin. 63*, 179−86.
(8) Jones, R. J., Matsui, W. H., and Smith, B. D. (2004) Cancer stem cells: are we missing the target? *J. Natl. Cancer Inst. 96*, 583−5.
(9) Park, C. Y., Tseng, D., and Weissman, I. L. (2009) Cancer stem cell-directed therapies: recent data from the laboratory and clinic. *Mol. Ther. 17*, 219−30.

(10) Ricci-Vitiani, L., Lombardi, D. G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., and De Maria, R. (2007) Identification and expansion of human colon-cancer-initiating cells. *Nature 445*, 111−5.
(11) Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., and Clarke, M. F. (2003) Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. U. S. A. 100*, 3983−8.
(12) Eramo, A., Lotti, F., Sette, G., Pilozzi, E., Biffoni, M., Di Virgilio, A., Conticello, C., Ruco, L., Peschle, C., and De Maria, R. (2008) Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ. 15*, 504−14.
(13) Hu, Y., and Fu, L. (2012) Targeting cancer stem cells: a new therapy to cure cancer patients. *Am. J. Cancer Res. 2*, 340−56.
(14) Collins, A. T., Berry, P. A., Hyde, C., Stower, M. J., and Maitland, N. J. (2005) Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res. 65*, 10946−51.
(15) Dalerba, P., Dylla, S. J., Park, I. K., Liu, R., Wang, X., Cho, R. W., Hoey, T., Gurney, A., Huang, E. H., Simeone, D. M., Shelton, A. A., Parmiani, G., Castelli, C., and Clarke, M. F. (2007) Phenotypic characterization of human colorectal cancer stem cells. *Proc. Natl. Acad. Sci. U. S. A. 104*, 10158−63.
(16) Li, C., Heidt, D. G., Dalerba, P., Burant, C. F., Zhang, L., Adsay, V., Wicha, M., Clarke, M. F., and Simeone, D. M. (2007) Identification of pancreatic cancer stem cells. *Cancer Res. 67*, 1030−7.
(17) Zoller, M. (2011) CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? *Nat. Rev. Cancer 11*, 254−67.
(18) Jayasena, S. D. (1999) Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clin. Chem. 45*, 1628−50.
(19) Tuerk, C., and Gold, L. (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science 249*, 505−10.
(20) Ellington, A. D., and Szostak, J. W. (1990) In vitro selection of RNA molecules that bind specific ligands. *Nature 346*, 818−22.
(21) Nimjee, S. M., Rusconi, C. P., and Sullenger, B. A. (2005) Aptamers: an emerging class of therapeutics. *Annu. Rev. Med. 56*, 555−83.
(22) Keefe, A. D., Pai, S., and Ellington, A. (2010) Aptamers as therapeutics. *Nat. Rev. Drug Discovery 9*, 537−50.
(23) Foy, J. W., Rittenhouse, K., Modi, M., and Patel, M. (2007) Local tolerance and systemic safety of pegaptanib sodium in the dog and rabbit. *J. Ocul. Pharmacol. Ther. 23*, 452−66.
(24) Noble, G. T., Stefanick, J. F., Ashley, J. D., Kiziltepe, T., and Bilgicer, B. (2014) Ligand-targeted liposome design: challenges and fundamental considerations. *Trends Biotechnol. 32*, 32−45.
(25) Allen, T. M., and Cullis, P. R. (2013) Liposomal drug delivery systems: from concept to clinical applications. *Adv. Drug Delivery Rev. 65*, 36−48.
(26) Eliaz, R., and Szoka, F. (2001) Liposome-encapsulated doxorubicin targeted to CD44: A strategy to kill CD44-overexpressing tumor cells. *Cancer Res. 61*, 2592−2601.
(27) Verel, I., Heider, K. H., Siegmund, M., Ostermann, E., Patzelt, E., Sproll, M., Snow, G. B., Adolf, G. R., and van Dongen, G. A. (2002) Tumor targeting properties of monoclonal antibodies with different affinity for target antigen CD44V6 in nude mice bearing head-and-neck cancer xenografts. *Int. J. Cancer 99*, 396−402.
(28) Derksen, J. T., Morselt, H. W., and Scherphof, G. L. (1988) Uptake and processing of immunoglobulin-coated liposomes by subpopulations of rat liver macrophages. *Biochim. Biophys. Acta 971*, 127−136.
(29) Harding, J. A., Engbers, C. M., Newman, M. S., Goldstein, N. I., and Zalipsky, S. (1997) Immunogenicity and pharmacokinetic attributes of poly(ethylene glycol)-grafted immunoliposomes. *Biochim. Biophys. Acta 1327*, 181−192.
(30) Powell, J. D., and Horton, M. R. (2005) Threat matrix: lowmolecular-weight hyaluronan (HA) as a danger signal. *Immunol. Res. 31*, 207−218.
(31) Ababneh, N., Alshaer, W., Allozi, O., Mahafzah, A., El-Khateeb, M., Hillaireau, H., Noiray, M., Fattal, E., and Ismail, S. (2013) In vitro selection of modified RNA aptamers against CD44 cancer stem cell marker. *Nucleic Acid Ther. 23*, 401−7.
(32) Levy-Nissenbaum, E., Radovic-Moreno, A. F., Wang, A. Z., Langer, R., and Farokhzad, O. C. (2008) Nanotechnology and aptamers: applications in drug delivery. *Trends Biotechnol. 26*, 442−9.
(33) Bangham, A. D., Standish, M. M., Watkins, J. C., and Weissmann, G. (1967) The diffusion of ions from a phospholipid model membrane system. *Protoplasma 63*, 183−7.
(34) Da Pieve, C., Williams, P., Haddleton, D. M., Palmer, R. M., and Missailidis, S. (2010) Modification of thiol functionalized aptamers by conjugation of synthetic polymers. *Bioconjugate Chem. 21*, 169−74.
(35) Korkaya, H., Liu, S., and Wicha, M. (2011) Regulation of cancer stem cells by cytokine networks: attacking cancers inflammatory roots. *Clin. Cancer Res. 17*, 6125−9.
(36) Yasuda, M., Tanaka, Y., Fujii, K., and Yasumoto, K. (2001) CD44 stimulation down-regulates Fas expression and Fas-mediated apoptosis of lung cancer cells. *Int. Immunol. 13*, 1309−1319.
(37) Collins, S. L., Black, K. E., Chan-Li, Y., Ahn, Y., Cole, P. A., Powell, J. D., and Horton, M. R. (2011) Hyaluronan fragments promote inflammation by down-regulating the anti- inflammatory A2A receptor. *Am. J. Respir. Cell Mol. Biol. 45*, 675−683.