Arabinogalactan−Folic Acid−Drug Conjugate for Targeted Delivery and Target-Activated Release of Anticancer Drugs to Folate Receptor-Overexpressing Cells

Roy I. Pinhassi,[#,‡] Yehuda G. Assaraf,[§] Shimon Farber,[||] Michal Stark,[§] Diana Ickowicz,[||] Stavit Drori,[§] Abraham J. Domb,[||] and Yoav D. Livney*[,#,‡]

Laboratory of Biopolymers and Food Nanotechnology, Department of Biotechnology and Food Engineering, The Russell Berrie Nanotechnology Institute, and The Fred Wyszkowski Cancer Research Laboratory, Department of Biology, The Technion, Israel Institute of Technology, Israel, and Institute of Drug Research, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Israel

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Folic acid (FA) is a high affinity ligand ($K_d = 0.1-1$ nM) of folate receptors (FRs) responsible for cellular uptake of folates via receptor-mediated endocytosis. FRs are frequently overexpressed in malignant epithelial cells including ovary, brain, kidney, breast, colon, and lung. FR has emerged as a target for the differential-delivery of anticancer chemotherapeutics with several FA-linked therapeutic agents currently undergoing clinical trials. Here we show that by tethering both FA and the anticancer drug methotrexate (MTX) to arabinogalactan (AG), a highly branched natural polysaccharide with unusual water solubility, a targeted biomacromolecular nanovehicle is formed, which can differentially deliver a cytotoxic cargo into FR-overexpressing cells. Moreover, by linking MTX via an endosomally cleavable peptide (GFLG), we demonstrate a target-activated release mechanism. This FA-AG-GFLG-MTX drug conjugate displayed 6.3-fold increased cytotoxic activity to FR-overexpressing cells compared to their FR-lacking counterparts. These findings establish a novel FA-tethered polymeric nanoconjugate for the targeted delivery of antitumor agents into cancer cells overexpressing FR.

1. Introduction

Chemotherapy of neoplastic diseases is often restricted by adverse systemic toxicity, which consequently limits the dose of antitumor agents that can be administered, or by the frequent emergence of anticancer drug resistance. The insufficient selectivity of antitumor agents is only one obstacle currently hindering drug efficacy. Other impediments include inaccessibility of the target, premature drug metabolism and multidrug resistance phenomena.[1] Hence, there is a growing demand for innovative targeted drug delivery systems that can selectively deliver anticancer drugs and overcome drug resistance.[2−8] The most promising approaches in the delivery of anticancer chemotherapeutics include the use of long-circulating nanodrug carriers, including polymeric drug carriers,[9−11] nanoparticles,[8,12] nanogels,[13] or liposomes coated with hydrophilic polymers that can evade elimination by the immune system.[14,15] Polymer-conjugated drugs[11] generally exhibit prolonged half-life, higher stability, water solubility, lower immunogenicity, and antigenicity and possibly a specific targeting to certain tissues or cells. This approach, explored for the first time in the 1950s, is recently gaining growing attention due to the introduction of new polymers and to advanced chemical strategies of drug coupling.[14,15] An ideal polymer for drug delivery should be characterized by biodegradability or adequate molecular weight that allows favorable pharmacokinetics and pharmacodynamics facilitating elimination from the body, hence, avoiding accumulation in vivo, low polydispersity to ensure an acceptable homogeneity of the final conjugates, and longer body residence time either to prolong the conjugate action or to allow distribution and accumulation in the target tumors. In this respect, arabinogalactan (AG), a highly branched natural polysaccharide with unusual water solubility (70% w/w in water) satisfies these strict requirements. AG is extracted from the *Larix* tree and is available at 99.9% purity with reproducible molecular weight (MW) and physicochemical properties.[16] The high water solubility, biocompatibility, biodegradability, and ease of drug conjugation in an aqueous solution render AG attractive as a potential biomacromolecular nanovehicle for folic acid-based targeted anticancer drug delivery, which has not yet been proposed for this task. While most nanovehicles are passive, active targeting modalities based on molecular recognition are rapidly advancing, using antibodies or ligands, for example, folic acid (FA), for which certain malignant tumors overexpress specific receptors, known as folate receptors (FRs).[17−19] An example is FA−drug conjugates, which have been shown to undergo receptor-mediated endocytosis into acidic endosomes in cancer cells, thereby offering a highly effective route for the delivery of anticancer drugs.[20,21] The FR constitutes a useful target for tumor-specific drug delivery primarily because (i) it is up-regulated in many human carcinomas, including malignancies of the ovary, brain, kidney, breast, colon, myeloid cells, and lung, and (ii) FR density appears to increase as the stage/grade of the cancer is more advanced, including in metastatic disease.[22] Thus, solid tumors that are currently most difficult to treat by classical therapeutic modalities may be readily targeted with FA-linked therapeutics.[23] To exploit these unique characteristics of differential FR overexpression in various

* To whom correspondence should be addressed. Phone: 972-4-8294225. Fax: 972-4-8293399. E-mail: livney@technion.ac.il.
[#] Department of Biotechnology and Food Engineering, Israel Institute of Technology.
[‡] The Russell Berrie Nanotechnology Institute, Israel Institute of Technology.
[§] Department of Biology, Israel Institute of Technology.
[||] School of Pharmacy, The Hebrew University of Jerusalem.

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carcinomas, FA has been tethered to both low molecular weight drugs as well as macromolecular complexes as a means of targeting the attached chemotherapeutic cargo molecules to malignant cells. Conjugation of FA to macromolecules has been shown to enhance their delivery to FR-expressing cancer cells.[24−29] To further maximize the efficacy of antitumor agents and minimize untoward side effects, nanoscopic drug delivery systems have been developed by introducing target-activated triggering mechanisms for drug release. These should increase the feasibility of obtaining local high-dose therapy in malignant tissues and at intracellular compartments, while minimizing premature drug release in the blood. Such an approach requires sophisticated strategies and systems that perform the desired chemical/physical functions depending on the triggering signals. For example, polymer−drug conjugates linked by a peptide bond such as the peptide linker Gly-Phe-Leu-Gly (GFLG), which is known to be stable in serum yet cleavable by endolysosomal peptidases,[10,30] bears promise for an efficient target-activated drug release mechanism inside tumor cells.

Herein we devised a new targeted delivery system for anticancer drugs based upon the naturally occurring polymer AG and characterized its ability to differentially recognize, penetrate, and eliminate tumor cells overexpressing FRα. The mode of synthesis employed here may be easily adapted to prepare therapeutic conjugates with several different cytotoxic drugs which may act synergistically to exploit the complete potential of the polymeric nanocarrier. Using an in vitro model cell line overexpressing FRα, we show here for the first time that this novel antitumor drug conjugate can differentially target and eliminate cells overexpressing FRα.

2. Experimental Section

Materials. All reactants employed in this study were of analytical grade: ethylenediamine (EDA), propane-1,3-diamine (PDA; Biolab, Jerusalem, Israel), methotrexate (MTX; Iffect Chemphar Co., Ltd., Shenzhen, China), dimethyl sulfoxide (DMSO), and 4-(dimethylamino) pyridine (DMAP; Merck and Co., Inc.). 1,3-Dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), diisopropylethylamine (DIEA), triethylamine, folic acid (FA), and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich Inc., Rehovot, Israel. The peptide Gly-Phe-Leu-Gly (GFLG) was tailor-synthesized by BioSight Ltd., Israel. Arabinogalactan (AG) was purchased from Lonza Inc. (Allendale, NJ). Growth media for cell culture were obtained from Gibco, U.S.A., and Beth-Haemek, Israel.

Methods. *Determination of Molecular Weight of Polymers and Conjugates.* Molecular weights of starting polymers and synthetic conjugates were estimated using a size exclusion chromatography (SEC) system (Spectra Physics instrument, Darmstadt, Germany) consisting of an isocratic pump (HP-1050, Waldbronn, Germany), methylmethacrylate-gel size exclusion column (Shodex KB-803 HQ, Phenomenex, Japan), refractive index (RI) detector, and Breeze 3.20 software (Waters Corp., Milford, U.S.A.). The eluent used was 0.05 M $NaNO_3$. Average molecular weights were determined according to pullulan standards (PSS, Mainz, Germany) with molecular weights between 5800 and 212000. A sage-metering pump model 365 (Orion, NJ) was used for slow and reproducible addition of reactants.

Oxidation of Arabinogalactan. AG (10 g, 62.5 mmol of monomer saccharide units) was dissolved in 100 mL of doubly deionized water (DDW). To this solution potassium periodate was separately added at 1:1 mol ratio (IO_4^-/AG units), and the mixture was stirred in the dark at room temperature for 2 h. The resulting polyaldehyde was purified from iodate (IO_3^-) and unreacted periodate (IO_4^-) by Dowex-1 (acetate form) anion exchange chromatography, followed by extensive dialysis against DDW (5 L × 4; 12000 Da cutoff cellulose tubing) at 4 °C for 3 days. Purified polyaldehyde was freeze-dried to obtain a white powder at an average yield of 75% ± 5% (w/w). FT-IR (KBr) =1724 cm^{-1} (C═O). The aldehyde content was determined by the hydroxylamine hydrochloride method.[31]

Synthesis of NHS Esters of FA or of MTX (General Method). Lyophilized FA (1.0 g, 2.26 mmol) dissolved in 20 mL of distilled dimethyl sulfoxide (DMSO) was activated with 3 equiv of 1,3-dicyclohexylcarbodiimide (DCC) coupling agent (1.4 g, 6.78 mmol) for 2 h at room temperature. Then 0.78 g of *N*-hydroxysuccinimide (NHS; 3 equiv, 6.78 mmol) and 0.14 g of 4-dimethylaminopyridine (DMAP; 0.5 equiv, 1.14 mmol) were added to the flask. The mixture was stirred in a light-protected container overnight at 40 °C under a nitrogen atmosphere. The resulting precipitate (dicyclohexylurea (DCU)) was discarded by filtration and the filtrate was concentrated by partial removal of the solvent under reduced pressure at 40 °C. Precipitation of the product was achieved by dropwise addition of the concentrated solution into a cold solution of acetone/diethyl ether (30:70 v/v ratio) followed by filtration. The precipitate was washed with 100 mL of acetone/diethyl ether (30:70 v/v ratio) solution and diethyl ether (3 × 100 mL) and vacuum-dried over P_2O_5 to yield 60% (mol/mol) of the product.

^{1}H NMR (FA-NHS, DMSO, ppm): 2.069 (m, 2H, $-CH_2-$, FA hydrogens), 2.391 (m, 2H, $-CH_2-$, FA hydrogens), 2.791 (m, 4H, $-CH_2-$, NHS hydrogens), 4.467 (d, 1H, CH, FA hydrogen), 4.605 (s, 1H, CH, FA hydrogen), 4.938 (m, 2H, NH_2, FA hydrogens), 5.012 (m, 2H, NH_2, FA hydrogens), 6.643 (t, 1H, CH, FA benzyl hydrogen), 6.962 (t, 1H, NH, FA hydrogen), 7.655 (t, 1H, CH, FA benzyl hydrogen), 8.149 (d, 1H, NH, FA hydrogen), 8.657 (d, 1H, CH, FA benzyl hydrogen), 11.511 (broad, 1H, COOH, FA carboxylic hydrogen).

^{1}H NMR (MTX-NHS, DMSO, ppm): 1.244 (m, 2H, $-CH_2-$, MTX hydrogens), 1.683 (m, 2H, $-CH_2-$, MTX hydrogens), 2.790 (t, 4H, $-CH_2-$, NHS hydrogens), 3.291 (s, 3H, CH_3, MTX hydrogens), 4.467 (d, 1H, CH, MTX hydrogen), 4.777 (s, 1H, CH, MTX hydrogen), 4.846 (m, 2H, NH_2, MTX hydrogens), 6.589 (s, 1H, CH, MTX benzyl hydrogen), 6.821 (m, 1H, NH, MTX hydrogen), 7.518 (m, 1H, CH, MTX benzyl hydrogen), 7.736 (d, 1H, NH, MTX hydrogen), 8.603 (d, 1H, CH, MTX benzyl hydrogen), 12.324 (broad, 1H, COOH, MTX carboxylic hydrogen).

Synthesis of Folic Acid−Propaneamine Derivative. Volumes of 0.62 mL (7.4 mmol) of propane-1,3-diamine and 0.2 mL of DIEA were dissolved in 1 mL of dry DMSO. Then 400 mg FA-NHS (0.74 mmol) dissolved in 6 mL of dry DMSO was added dropwise to the clear solution. The mixture was stirred in a light-protected container overnight at room temperature under a nitrogen atmosphere. The clear solution was then concentrated by partial removal of the solvent under reduced pressure at 40 °C. The solution was poured into a cold solution of acetone/diethyl ether (30:70% v/v ratio) resulting in precipitation of the product. The obtained precipitate was washed with 50 mL of acetone/diethyl ether solution (30:70% v/v ratio) and diethyl ether (3 × 50 mL) to remove traces of unreacted reagents and diisopropylethylammonium-NHS salt. The product was dried in vacuum over P_2O_5 overnight and the yield was 60% (mol/mol).

Methotrexate-propaneamine derivative was synthesized by the same method resulting in 58% (mol/mol) yield.

^{1}H NMR (FA-propaneamine, DMSO, ppm): 1.902 (m, 2H, $-CH_2-$, PDA hydrogens), 2.087 (m, 2H, $-CH_2-$, FA hydrogens), 2.405 (m, 2H, $-CH_2-$, FA hydrogens), 2.734 (m, 2H, $-CH_2-NH_2$, PDA hydrogens), 3.333 (m, 2H, $-CH_2-$NH, PDA hydrogens), 4.475 (d, 1H, CH, FA hydrogen), 4.622 (s, 1H, CH, FA hydrogen), 5.002 (m, 2H, NH_2, FA hydrogen), 6.611 (t, 1H, CH, FA benzyl hydrogen), 7.636 (t, 1H, CH, FA benzyl hydrogen), 8.188 (d, 1H, NH, FA hydrogen), 8.671 (d, 1H, CH, FA benzyl hydrogen).

Synthesis of MTX−GFLG Conjugate. MTX-NHS active ester (MTX-NHS) (100 mg, 0.18 mmol) was dissolved in 3.5 mL of dry DMSO. GFLG (trifluoroacetate form; 1.2 equiv, 110 mg, 0.216 mmol) dissolved in 3.5 mL of dry DMSO and 0.15 mL of triethylamine (TEA) was added to the flask. The mixture was stirred in a light-protected container overnight at room temperature under a nitrogen atmosphere. The resulting solution was then concentrated by partial removal of the

solvent under reduced pressure at 40 °C. Precipitation of the product was achieved by dropwise addition of the concentrated solution into cold solution of acetone/diethyl ether (30:70 v/v ratio) followed by filtration. The precipitate was washed with 100 mL of acetone/diethyl ether (30:70 v/v ratio) solution and diethyl ether (3 × 100 mL) and vacuum-dried over P_2O_5 to yield 78% (mol/mol) of the product.

^{1}H NMR (MTX-GFLG, DMSO, ppm): 0.821 (dd, 6H, 2 CH_3, Leu hydrogens), 1.452 (m, 2H, $-CH_2-$, Leu hydrogens), 1.890 (s, 1H, CH, Leu hydrogen), 2.246 (m, 2H, $-CH_2-$, MTX hydrogens), 2.476 (m, 2H, $-CH_2-$, MTX hydrogens), 2.714 (d, 1H, $-CH_2-$, Phe hydrogen), 3.193 (d, 1H, $-CH_2-$, Phe hydrogen), 3.402 (s, 3H, CH_3, MTX hydrogens), 4.471 (d, 1H, CH, MTX hydrogen), 4.228 (d, 2H, $-CH_2-$, Gly hydrogens), 4.301 (d, 2H, $-CH_2-$, Gly hydrogens), 4.507 (m, 1H, CH, Leu hydrogen), 4.773 (s, 1H, CH, MTX hydrogen), 4.862 (s, 1H, CH, Phe hydrogen), 6.561 (m, 1H, CH, MTX benzyl hydrogen), 6.811 (m, 1H, CH, MTX benzyl hydrogen), 7.141 (m, 1H, CH, MTX benzyl hydrogen), 7.180 (m, 5H, CH, Phe benzyl hydrogens), 7.486 (m, 1H, CH, MTX benzyl hydrogen), 7.716 (m, 2H, CH, MTX benzyl hydrogens), 8.013 (m, 1H, CH, MTX benzyl hydrogen), 8.215, 8.561 (m, 2H, NH, GFLG hydrogens).

Synthesis of MTX-GFLG-NHS Ester. Previously prepared MTX-GFLG conjugate (100 mg, 0.12 mmol) dissolved in 5 mL of dry DMSO was activated with 3 equiv of dicyclohexylcarbodiimide (DCC; 75 mg, 0.36 mmol) for 2 h at room temperature. Then 41.6 mg (NHS; 3 equiv, 0.36 mmol) and 7.3 mg of DMAP (0.5 equiv, 0.06 mmol) were added to the flask. The mixture was stirred in a light-protected container overnight at 40 °C under a nitrogen atmosphere. The resulting precipitate dicyclohexylurea (DCU) was discarded by filtration and the filtrate was concentrated by partial removal of the solvent under reduced pressure at 40 °C. Precipitation of the product was achieved by dropwise addition of the concentrated solution into a cold acetone/diethyl ether solution (30:70 v/v ratio) followed by filtration. The precipitate was washed with 100 mL of acetone/diethyl ether solution (30:70 v/v ratio) followed by diethyl ether (3 × 100 mL) and vacuum-dried over P_2O_5 to yield 60% (mol/mol) product.

^{1}H NMR (MTX-GFLG-NHS, DMSO, ppm): 0.811 (m, 6H, 2 CH_3, Leu hydrogens), 1.457 (m, 2H, $-CH_2-$, Leu hydrogens), 1.891 (s, 1H, CH, Leu hydrogen), 2.192 (m, 2H, $-CH_2-$, MTX hydrogens), 2.481 (m, 2H, $-CH_2-$, MTX hydrogens), 2.711 (d, 1H, $-CH_2-$, Phe hydrogen), 2.791 (m, 4H, $-CH_2-$, NHS hydrogens), 3.193 (d, 1H, $-CH_2-$, Phe hydrogen), 3.337 (s, 3H, CH_3, MTX hydrogens), 3.601 (d, 1H, CH, MTX hydrogen), 4.229 (d, 2H, $-CH_2-$, Gly hydrogens), 4.295 (d, 2H, $-CH_2-$, Gly hydrogens), 4.520 (m, 1H, CH, Leu hydrogen), 4.771 (s, 1H, CH, MTX hydrogen), 4.842 (s, 1H, CH, Phe hydrogen), 6.577 (m, 1H, CH, MTX benzyl hydrogen), 6.810 (m, 1H, CH, MTX benzyl hydrogen), 7.158 (m, 1H, CH, MTX benzyl hydrogen), 7.187 (m, 5H, CH, Phe benzyl hydrogens), 7.592 (m, 1H, CH, MTX benzyl hydrogen), 7.720 (m, 2H, CH, MTX benzyl hydrogens), 8.016 (m, 1H, CH, MTX benzyl hydrogen), 8.287, 8.440, 8.573 (m, 3H, NH, GFLG hydrogens).

Synthesis of MTX-GFLG-Ethyleneamine Derivative. Ethylenediamine (41 μL, 10 equiv, 0.54 mmol) and 100 μL of triethylamine, added to facilitate the reaction, were dissolved in 1 mL of dry DMSO. MTX-GFLG-NHS (50 mg, 0.054 mmol) dissolved in 2 mL of dry DMSO was added dropwise. The mixture was stirred in a light-protected container at room temperature for 20 h under a nitrogen atmosphere. The solution was concentrated by partial removal of the solvent under reduced pressure at 40 °C. Precipitation of the product was achieved by dropwise addition of the concentrated solution into a cold solution of acetone/diethyl ether (30:70 v/v ratio) followed by filtration. The precipitate was washed with 50 mL of acetone/diethyl ether (30:70 v/v ratio) solution and diethyl ether (3 × 50 mL) and vacuum-dried over P_2O_5 to yield 93% (mol/mol) product.

^{1}H NMR (MTX-GFLG-ethyleneamine, DMSO, ppm): 0.786 (m, 6H, 2 CH_3, Leu hydrogens), 1.486 (m, 2H, $-CH_2-$, Leu hydrogens), 1.883 (s, 1H, CH, Leu hydrogen), 2.250 (m, 2H, $-CH_2-$, MTX hydrogens), 2.477 (m, 2H, $-CH_2-$, MTX hydrogens), 2.588 (m, 2H, $-CH_2-$, EDA hydrogens), 2.708 (d, 1H, $-CH_2-$, Phe hydrogen), 3.071 (d, 1H, $-CH_2-$, Phe hydrogen), 3.182 (s, 3H, CH_3, MTX hydrogens), 3.457 (m, 2H, $-CH_2-$, EDA hydrogens), 3.629 (d, 1H, CH, MTX hydrogen), 4.068 (d, 2H, $-CH_2-$, Gly hydrogens), 4.254 (d, 2H, $-CH_2-$, Gly hydrogens), 4.763 (s, 1H, CH, MTX hydrogen), 4.856 (s, 1H, CH, Phe hydrogen), 6.556 (m, 1H, CH, MTX benzyl hydrogen), 6.788 (m, 1H, CH, MTX benzyl hydrogen), 7.191 (m, 5H, CH, Phe benzyl hydrogens), 7.592 (m, 1H, CH, MTX benzyl hydrogen), 7.695 (m, 2H, CH, MTX benzyl hydrogens), 8.016 (m, 1H, CH, MTX benzyl hydrogen), 8.287, 8.440, 8.541 (m, 3H, NH, GFLG hydrogens).

Synthesis of FITC-EDA. An amount of 3.5 mg (0.009 mmol) fluorescein isothiocyanate (FITC) dissolved in 0.3 mL of dry DMSO was added dropwise to the solution of EDA (0.5 μL 0.0075 mmol) in 0.3 mL DMSO under continuous stirring. The mixture was then stirred in a light-protected container at room temperature for 20 h. The solution was added to the desired products for labeling.

Synthesis of FA-PDA-AG-EDA-MTX. A total of 400 mg (2.5 mmol) oxidized AG (7.75 mmol/g aldehyde groups) was dissolved in 40 mL of borate buffer (0.1 N, pH = 11). A total of 25 mg crude FA-propaneamine and 75 mg crude methotrexate-ethyleneamine were added to the reaction mixture, resulting in a turbid solution. The mixture was gently stirred in a light-protected container at 37 °C for 72 h. The solution was centrifuged to remove the insoluble reactants, receiving a clear yellow solution. The amine-based conjugate (reduced) was obtained after reducing 20 mL of the imine conjugate solution with excess of 95 mg (2.5 mmol) of $NaBH_4$ for 2 h at 4 °C to obtain a more stable amine form. The resulting light-yellow solution was purified by dialysis against DDW (5 L × 4), applying a 3500 Da MW cutoff cellulose dialysis tubing at 4 °C for 48 h followed by lyophilization resulting in 50% (mol/mol) overall yield.

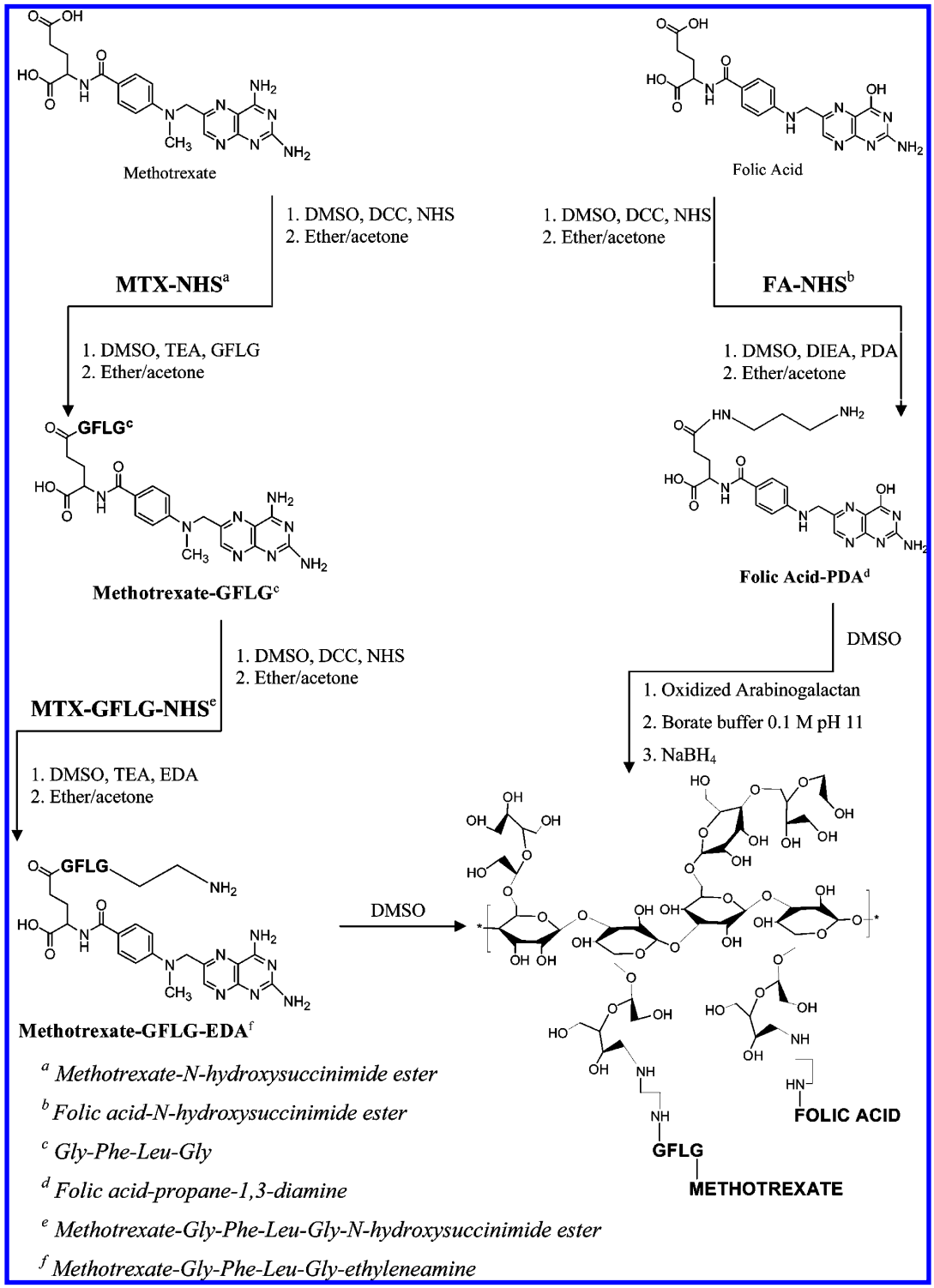
A total of 50 mg of the conjugate (imine form) was dissolved in 5 mL of the borate buffer solution (0.1 N, pH = 11). FITC-EDA (5 mg) was added dropwise to the solution. The mixture was gently stirred in a light-protected container at 37 °C for 18 h. The reduction reaction was conducted by the addition of 23.6 mg (0.62 mmol) $NaBH_4$ for 2 h at room temperature. The labeled product was purified by dialysis through 3500 MWCO dialysis tubing, against DDW (5 L × 4) for 48 h at 4 °C, followed by size exclusion column purification with Sephadex G-25, and lyophilization.

Synthesis of FA-AG-GFLG-MTX. Oxidized AG (30 mg, 0.1875 mmol; 7.75 mmol/g aldehyde groups) was dissolved in 3.5 mL of borate buffer (0.1 N, pH 11). Folate-propaneamine (4.5 mg, 0.009 mmol) and 20.7 mg EDA-GFLG-MTX (0.023 mmol) dissolved in 0.2 mL of DMSO were added to the polysaccharide solution. The mixture was gently stirred in a light-protected container at 37 °C for 72 h. Nonconjugated insoluble folate-propaneamine and EDA-GFLG-MTX were discarded by centrifugation before the reduction step. The amine-based conjugate (reduced) was obtained after reducing 1.8 mL of the imines conjugate solution with an excess of $NaBH_4$ (4.3 mg, 0.1125 mmol) at 4 °C and dark conditions for 2 h under continuous stirring. The resulting light-yellow solution was purified by dialysis against DDW (5 L × 4), applying a 3500 Da MW cutoff cellulose dialysis tubing at 4 °C for 48 h, followed by lyophilization, achieving 65% (mol/mol) overall yields.

Synthesis of AG-GFLG-MTX. The synthesis of AG-GFLG-MTX was done by the same method as the FA containing FA-AG-GFLG-MTX conjugate excluding FA addition. Yield 55% (mol/mol).

Evaluation of the Degree of Substitution with FA and MTX. The degree of substitution with FA and MTX was estimated by UV spectroscopy at 350 and 400 nm wavelengths, using Kontron Instruments Uvicon model 930 (Ms-scientific, Berlin, Germany). The samples were prepared as follows: 1 mg of the appropriate conjugate was dissolved in 100 μL of DDW and diluted with DMSO to a final

Scheme 1. Synthesis of FA-AG-GFLG-MTX Conjugate



concentration of 0.1 mg/mL. Spectral data were analyzed according to eq 1:

$$A(\lambda_i) = C_{FA}(\text{conjugate})\frac{A_{FA}(\lambda_i)(\text{pure})}{C_{FA}(\text{pure})} + C_{MTX}(\text{conjugate})\frac{A_{MTX}(\lambda_i)(\text{pure})}{C_{MTX}(\text{pure})} \quad (1)$$

where $A(\lambda_i)$ is the absorbance at wavelength λ_i, and C_{FA} and C_{MTX} are the concentrations of FA and MTX, respectively (in pure solutions or in the conjugate solution). The concentrations of FA and MTX were then calculated from the set of equations obtained for two wavelengths, 350 and 400 nm, in which the differences between the absorbance spectra of the pure compounds are relatively large, enabling to quantitatively distinguish the two compounds.

Tissue Culture and Cell Lines. Parental Chinese hamster ovary (CHO) AA8 cells and their reduced folate carrier (RFC)-null CHO subline (termed C5) were grown under monolayer culture conditions, as previously described.[32] C5 cells were stably transfected with a pcDNA3.1 expression vector harboring human folate receptor α, as detailed previously.[33] The latter cells, which highly overexpress folate

Table 1. Chemical Characteristics of the Oxidized AG and AG-MTX Conjugates

conjugate	M_w[a] (Da)	D[a]	FA[b] %w (441 Da) (mol/mol AG)	MTX[b] %w (454 Da) (mol/mol AG)
oxidized AG	15280	1.62		
FA-PDA-AG-EDA-MTX	16070	1.58	4% (1.5)	7.50% (2.7)
AG-EDA-GFLG-MTX	15530	1.49	(0.0)	11.80% (4.0)
FA-PDA- AG-EDA-GFLG-MTX	15750	1.66	4.80% (1.7)	2.40% (0.8)

[a] Average molecular weight (M_w) and polydispersity ($D = M_w/M_n$) were determined by SEC. [b] FA and MTX content (% w/w) were determined by spectrophotometric measurement of the conjugates (UV, $\lambda_1 = 350$, $\lambda_2 = 400$) using calibration curves of FA and MTX and eq 1 (determination accuracy $\pm$ 0.5%).

receptor α, were termed C5-FRα, and were routinely grown in a medium containing 0.2 nM FA.

Flow Cytometric Analysis. C5-FRα cells were routinely grown in FA-deficient RPMI-1640 medium (GIBCO) supplemented with 0.2 nM FA, 1 mM sodium pyruvate, and 10% dialyzed fetal calf serum (dFCS). Parental C5 cells were grown in RPMI-1640 medium containing 2.3 μM FA, 1 mM pyruvate and 10% FCS. All cells were grown under monolayer conditions in disposable T-75 tissue culture flasks (NUNC, Roskilde Denmark) in a humidified atmosphere of 5% CO_2 at 37 °C. A total of 24 h prior to the experiment, the growth medium was removed and cells were washed once with excess PBS to eliminate residual FA. After removing the PBS, warm FA-free medium (37 °C) was added. Cells were then incubated at 37 °C until the next day. Then cells were detached by trypsinization and resuspended in FA-free medium containing 5% dialyzed FCS, supplemented with 20 mM HEPES buffer pH = 7, setting cell concentration to 10^5 cells/mL. Portions of 1 mL of cell suspension (10^5 cells) were dispensed into 1.5 mL disposable Eppendorf tubes, followed by the addition of the tested conjugate at the predetermined concentration. Test tubes were then incubated protected from light for 90 min at 37 °C and shaken every 20 min. Then the test tubes were centrifuged (370 × g at 4 °C for 3 min) and the cell pellets were washed three times with cold (4 °C) PBS. Cells were then resuspended in cold PBS and kept on ice protected from light until flow cytometric analysis.

Analysis Using Two-Photon Confocal Scanning Laser Microscope. A total of 48 h prior to the experiment, cells were seeded in 10 mL of FA-free growth medium in 10 cm Petri dishes (Nunc, Roskilde, Denmark). On the day of the experiment, the growth medium was replaced with 5 mL of fresh FA-free medium. Cells were then subjected to various treatments and the culture plates were covered with aluminum foil to avoid photobleaching of the fluorescent samples. Draq-5 DNA dye was added 15 min prior to microscopic analysis, as recommended by the manufacturer (Alexis Biochemicals, Montreal, Quebec, Canada), and cells were incubated at 37 °C for 10 min. Cells were then washed with fresh medium and examined in a two-photon confocal laser scanning microscope (LSM510, Carl Zeiss, Germany).

Cytotoxicity Assay and Analysis of Folate Requirement for Growth. Each well of a 96-well microplate (Nunc, Roskilde, Denmark) was seeded with 2000 cells in 0.1 mL and incubated in growth medium for 24 h prior to analysis. Various treatments (depending on the specific experiment performed as detailed below) were introduced to these plates which were then incubated for 96 h in a humidified atmosphere of 5% CO_2 at 37 °C. Cell growth was followed by the XTT reagent assay (Biological Industries, Beth-Haemek, Israel), with a formazan color development incubation time of 4 h. The number of viable cells in each well was determined using a calibration curve that was prepared for this purpose based on color development obtained at that time. Absorbance at a wavelength of 490 nm was determined with a Bio-Tek plate reader spectrophotometer (Synergy HT, Bio-Tek, U.S.A.).

3. Results and Discussion

Chemistry. In the current study we have prepared a novel biomacromolecular drug delivery system by grafting a model anticancer drug MTX, as well as FA, as the targeting molecule, onto AG, a natural polysaccharide, and investigated its efficacy as a potential antitumor agent against a model cell line overexpressing FRα. MTX was conjugated to the polymer backbone either only via a short linker ethylenediamine (EDA) or also via a tetra-peptide linker, GFLG, which can be efficiently cleaved by endosomal peptidases, but is stable in the circulation.[34] The steps of synthesis performed to generate this targeted biomacromolecular vehicle are depicted in Scheme 1.

MTX-GFLG was prepared via several steps using an NHS activating group. NHS was added to the in situ activated MTX with DCC to obtain the MTX-NHS ester derivative, followed by a reaction with an excess of the tetra-peptide GFLG, thereby resulting in a MTX-GFLG derivative. The MTX-GFLG was then treated with NHS followed by addition of EDA, hence, yielding the MTX-GFLG-ethylamine derivative. FA propylamine was similarly prepared using an NHS activating group followed by addition of propanediamine.

Prior to the conjugation, arabinogalactan was oxidized by reacting the polysaccharide in aqueous medium with potassium periodate at 1:1 mol ratio (IO_4^-/AG unit) for 2 h in a light-protected container. This oxidation process results in saccharide ring-opening,[16] as shown in Scheme 1. A typical aldehyde-carbonyl peak appeared in the FTIR spectrum at 1724 cm^{-1}. The resulting polyaldehyde was purified from iodate and unreacted periodate ions by DOWEX-1 anion exchange chromatography (acetate form, pH 7).[16]

To prepare the polymeric targeted nanovehicle, oxidized AG was reacted with the FA-propylamine and the MTX-GFLG-ethylamine to form an azomethene bond (Schiff base). The drug-AG Schiff bases were then reduced to the amine bond using sodium borohydride. The unreacted aldehyde groups on the oxidized AG were reduced to the corresponding alcohols to prevent further binding of the FA-AG-GFLG-MTX conjugate to proteins and body components in vivo.

To prepare the polymeric targeted nanovehicle, oxidized AG was reacted with the FA-propylamine and the MTX-GFLG-ethylamine followed by reduction with sodium borohydride to obtain the amine-based conjugate.

A portion of this conjugate was also linked to the fluorescent molecule, FITC, and was subsequently used to visualize the membrane binding, endocytosis, and intracellular localization of the drug carrier in FRα-overexpressing cells.

Grafting of FA and MTX on the conjugates had no significant effect on the molecular weights and polydispersity index, evaluated by SEC analysis, as they were found very close to that of the oxidized AG used (Table 1). Average molecular weight of the FA-AG-GFLG-MTX conjugate was 15.75 kDa.

^{1}H NMR Analysis of the Conjugates. The conjugations were confirmed by ^{1}H NMR spectroscopy (as detailed in the Methods section). Figure 1 depicts the ^{1}H NMR spectra of FA, MTX,

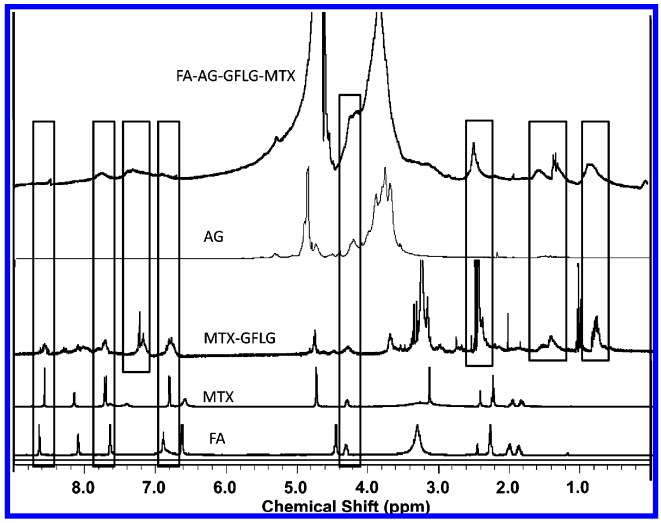


Figure 1. ^{1}H NMR spectra of FA, MTX, MTX-GFLG, oxidized AG, and the complete construct, FA-(PDA)-AG-(EDA)-GFLG-MTX. Peaks common to the components, and the final construct are marked with vertical frames. Spectra were obtained at 25 °C using a 300 Hz ^{1}H NMR.

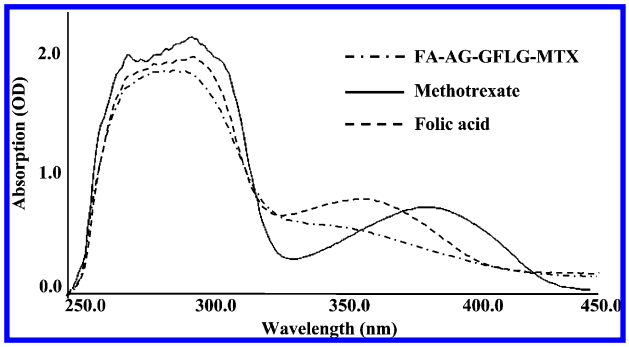


Figure 2. UV spectrograms of free FA and MTX as well as the FA-AG-GFLG-MTX conjugate.

GFLG-MTX, ungrafted AG, and grafted AG with FA and GFLG-MTX. In contrast to unconjugated AG, AG linked with FA and GFLG-MTX demonstrated common signal peaks with that of unbound drug and of FA. In a detail, the peaks which appeared at 0.8, 1.4, 1.6, and 7.2 ppm were common to both MTX-GFLG and GFLG. Further resonance recorded at 4.2, 6.9, 7.6, and 8.6 ppm is attributed to FA. Peaks obtained from MTX and FA appeared in the same region due to similar chemical structure. Glucose hydrogens of the drug conjugate were detected at 3.5−4.5 ppm.

UV Spectroscopy Results. The degrees of substitution with FA and MTX were evaluated by UV spectroscopy (Figure 2, eq 1) and the results are presented in Table 1.

Characterization of FRα-Overexpressing Cells. We have established a cell line system comprising (1) parental CHO-AA8-C5 cells (designated "C5") that are devoid of reduced folate carrier (RFC/SLC19A1) transport activity,[32] which essentially lack FRα expression, and (2) their stable transfectant cell line CHO-AA8-C5-FRα highly overexpressing FRα (designated "C5-FRα"). Flow cytometric analysis of plasma membrane expression of FRα in viable C5-FRα transfectant cells stably overexpressing FRα was performed using the anti-FRα monoclonal antibody MOv18 which recognizes an external epitope of FRα on the surface of viable cells; this analysis revealed 85-fold FRα overexpression in the stable C5-FRα transfectants cells when compared to their parental C5 counterpart (Figure 3).

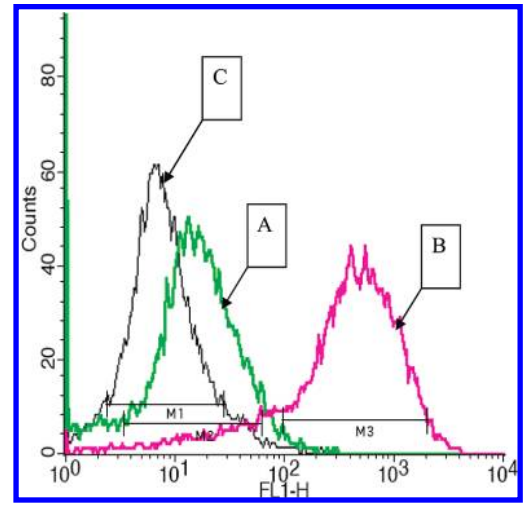


Figure 3. Flow cytometric analysis of FRα expression in parental C5 cells (A) and in FRα-overexpressing C5-FRα cells (B). Autofluorescence of both cell lines (C). FRα-expressing cells were detected by viable immunofluorescence using the anti-FRα MOv18 monoclonal antibody. This assay revealed an 85-fold FRα overexpression of the transfectant C5-FRα cell line relative to parental C5 cells.

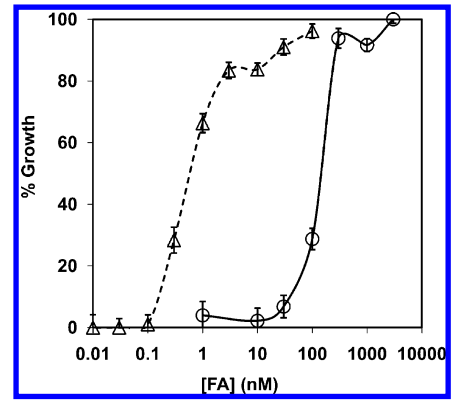


Figure 4. FA growth requirement of parental C5 cells (circles) and their C5-FRα transfectant cells (triangles). Error bars represent standard error. Cells were incubated for 7 days, and cell growth was evaluated using a colorimetric assay. Whereas the concentration of FA that supports 50% maximal growth (EC_{50}) of parental C5 cells was ∼100 nM, that of the stable C5-FRα transfectant cells was 100-fold lower (i.e., ∼1 nM).

To corroborate the functionality of FRα overexpression, we determined the FA growth requirement of both parental C5 and C5-FRα transfectant cells (Figure 4); whereas the concentration of FA that supports 50% maximal growth (EC_{50}) of parental C5 cells was ∼100 nM, that of the stable C5-FRα transfectant cells was ∼1 nM, that is, 100-fold lower. Hence, the 100-fold decrease in the FA growth requirement in transfectant cells is in good agreement with the extent of surface FRα overexpression and further corroborates the functionality of the overexpressed FRα.

We further investigated our paired cell line model to determine the exact concentrations where FRα saturation occurs; this was achieved by measuring the uptake of a FA-FITC conjugate in the presence of increasing concentrations of the latter (Figure 5A). C5-FRα transfectant cells displayed an EC_{50} of ∼2 nM of FA-FITC and saturation was reached at ∼10 nM. In contrast, parental C5 cells showed no detectable FA-FITC uptake in the concentration range tested, which emphasizes the sensitivity of this in vitro paired cell line model. This experiment also provides crucial information for future experiments regarding the effective concentration range of FA-AG-FITC conjugates

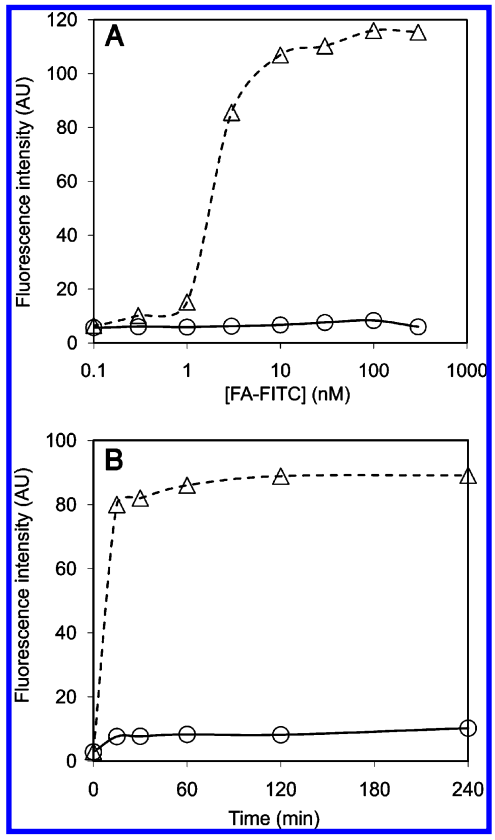


Figure 5. (A) FA-FITC conjugate titration curve for C5-FRα cells (triangle) and parental C5 cells (circles). Following a 24 h incubation in FA-free medium, cells were detached and resuspended in FA-free medium at pH 7.4 (10^5 cells/mL). To 1 mL aliquots of 10^5 cells, FA-FITC conjugate was added at each predetermined concentration, incubated for 1 h (37 °C), and analyzed by a flow cytometer (AU = arbitrary units). C5-FRα transfectant cells displayed an EC_{50} of ~2 nM of FA-FITC and saturation was reached at ~10 nM, whereas parental C5 cells showed no detectable FA-FITC uptake in the concentration range tested. (B) Time-course of cellular FA-FITC accumulation in C5-FRα transfectant cells and their parental C5 cells, at a constant FA-FITC conjugate concentration of 200 nM. Following the addition of the FA-FITC conjugate, at a fixed concentration of 200 nM, cells were incubated for predetermined times, washed with cold PBS, and immediately analyzed with a flow cytometer.

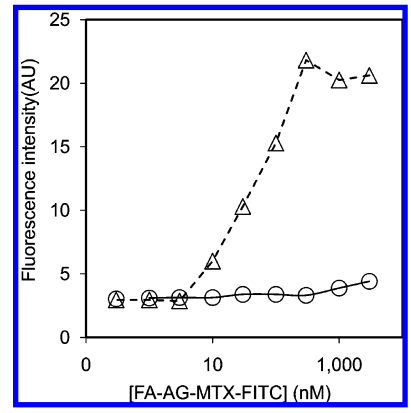


Figure 6. Dose-dependence of FA-AG-MTX-FITC conjugate accumulation in parental C5 cells (circles) and their FRα overexpressing subline, C5-FRα (triangles). Following a 24 h incubation in FA-free medium, cells were detached and resuspended in FA-free medium at pH 7.4. To 1 mL aliquots of 10^5 cells, FA-AG-MTX-FITC conjugate was added, incubated for 1 h (37 °C), and analyzed with a flow cytometer. The polymeric AG-conjugate showed an EC_{50} of ~80 nM and saturation was reached at ~300 nM. Parental C5 cells showed no conjugate uptake in the concentration range tested.

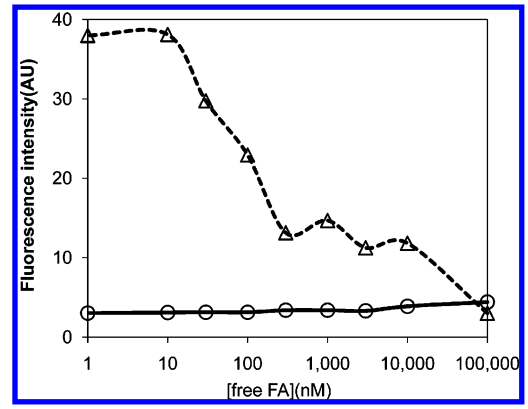


Figure 7. Relative FRα affinity assay: Competition between FA-AG-MTX-FITC conjugate and free FA for binding to C5-FRα and to parental C5 cells. Cells were coincubated for 2 h in increasing concentrations of free FA at a constant concentration (200 nM) of the FA-AG-MTX-FITC conjugate. Then fluorescence per cell was determined by flow cytometry. A 50% competition was achieved with 100 nM free FA: C5-FRα cells (triangles) and parental C5 cells (circles).

(0.1−1 μM, assuming that their affinity does not change drastically relatively to that of FA-FITC). It is noteworthy that this concentration range is exactly the one previously reported.[36]

Moreover, our data (Figure 5B) suggest that membrane binding occurs within a few minutes. This finding is in accord with a previous study by Turek et al.[35] who demonstrated that FA-protein particles bind membrane FR in KB carcinoma cells in less than 15 min. According to their study, intracellular endosomes were noticeable after an hour, and conjugate release to the cytoplasm was detectable after ~6 h. We hence conclude that the present in vitro paired cell line model is a highly sensitive and reliable system for the examination of FA-conjugate based targeted delivery systems.

Exploiting this paired cell line model, we screened the AG-based conjugates for specificity and selectivity.[37] Conjugates underwent three stages of evaluation. (a) Examination of the selectivity and specificity of binding and cell association to membrane FRα: (i) One negative control for binding specificity tested the conjugate's binding to parental C5 cells lacking FRα expression. (ii) A second negative control was coincubation of FRα-overexpressing cells with the tested conjugate along with a large molar excess of free FA. (b) Subcellular localization of fluorescent conjugates in various intracellular compartments, including endosomes by confocal microscopy, to examine internalization of the conjugate via endocytosis into FRα-overexpressing transfectant cells. (c) Evaluation of the cytotoxic activity of the conjugate: conjugates harboring drug cargo should selectively eliminate target FRα-overexpressing cells due to the intracellular release of the cytotoxic cargo.

Cell-Association and Selectivity of Cellular Uptake of a FA-AG-MTX Conjugate. Following these three complementary readouts, we first estimated the affinity of FITC-labeled FA-(PDA)-AG-(EDA)-MTX conjugate for FRα using increasing conjugate concentrations in the presence or absence of free FA. As apparent from Figure 6, the polymeric AG-conjugate showed an EC_{50} of ~80 nM and saturation was reached at ~300 nM. Notably, parental C5 cells showed no conjugate uptake in the concentration range tested, thereby underlining the selective mode in which the AG-conjugate associates with FRα.

Moreover, cell-association was competitively eliminated when excess FA was coincubated with the FA-AG-MTX-FITC conjugate. Figure 7 shows an assay estimating the relative affinity (RA) for FRα; this technique measures the ability of

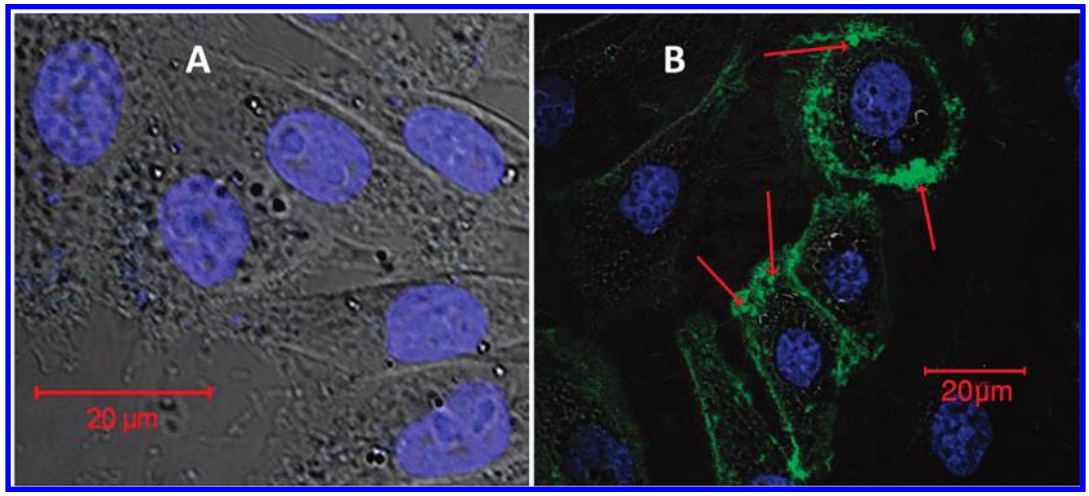


Figure 8. Confocal laser micrographs of the subcellular localization of the FA-AG-MTX-FITC conjugate in parental C5 (A) and C5-FRα ± cells (B). Cells were seeded in FA-free growth medium (10 mL/10 cm Petri dish) and incubated for 48 h prior to the experiment. Then, medium was replaced with fresh FA-free medium and cells were subjected to 0.5 μM FA-AG-MTX-FITC conjugate for 2 h in FA-free medium in the dark. Draq 5 DNA dye was added at 5 μM, and equilibrated for 10 min at 37 °C, 15 min prior to confocal microscopy analysis. Cells were then washed and examined with confocal laser microscope. Green fluorescent endosomes are denoted by red arrows, whereas the blue fluorescent structures are nuclei. Plasma membrane staining of FRα ± overexpressing cells is also apparent. Scale bar is 20 μm.

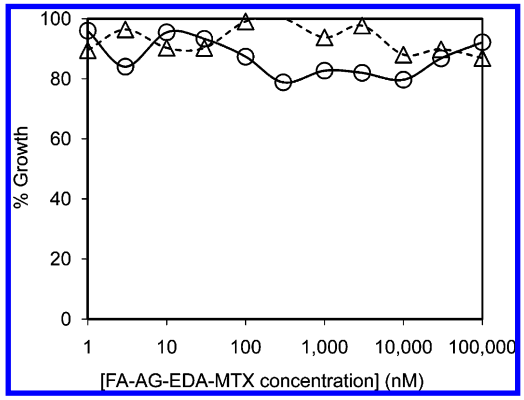


Figure 9. Cytotoxicity test of FA-AG-(EDA)-MTX conjugate to C5-FRα cells (triangles) and parental C5 cells (circles). Each of the 96 wells was seeded with 2000 cells and incubated in growth medium for 24 h prior to analysis. Cell growth was followed by the colorimetric XTT assay. Evidently, no significant growth inhibition was observed for both cell lines with this conjugate.

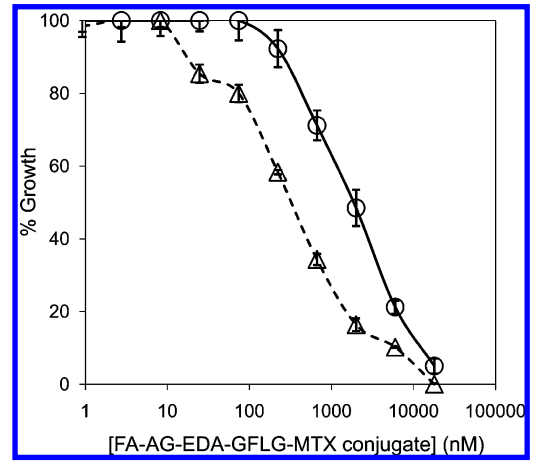


Figure 10. Cytotoxicity of FA-AG-EDA-GFLG-MTX conjugate to C5-FRα cells (triangles) and to parental C5 cells (circles). Each of the 96 wells was seeded with 2000 cells and incubated in growth medium for 24 h prior to analysis. FA-AG-EDA-GFLG-MTX conjugate was added and plates were incubated for 96 h at 37 °C. Cell growth was followed by the colorimetric XTT assay. Absorbance was determined at 490 nm. The mean IC_{50} of C5-FRα and for parental C5 cells were 320 ± 5 and 2000 ± 126, respectively. Tests were conducted in tetraplicates (error bars represent standard error).

the conjugate to directly compete with free FA for binding to cell surface FRα;[38] and when 500 nM of the AG-conjugate (in terms of the whole polymer) were coincubated with increasing concentrations of free FA, we observed that 100 nM of free FA were required to achieve 50% competition. Thus, this polymeric carrier had successfully complied with the first two criteria in the screening process, thereby demonstrating selective cell association and uptake by FRα-overexpressing cells.

Using confocal laser microscopy (Figure 8), we then confirmed that selective endocytosis of FA-AG-MTX-FITC conjugate by C5-FRα transfectant cells occurred and not by C5, wild-type cells.

Next, we tested the FA-AG-(EDA)-MTX conjugate for selective cytotoxicity. Figure 9 shows the results of the comparison between growth of FR_{α}-overexpressing cells and parental C5 cells in the presence of FA-AG-(EDA)-MTX. Evidently, no significant cytotoxic effect was observed on both cell lines.

The fact that no cytotoxicity was observed albeit the effective selective uptake, suggested the drug is not released from its polymeric carrier after endocytosis. Therefore, we added a cleavable peptide linker (GFLG), which has been reported[10,30] to be stable in the circulation but cleavable by endolysosomal proteases. Thus, the addition of such a linker constitutes a target-activated release mechanism, which may improve the safety and the targeting selectivity of drug delivery. The FA-AG-(EDA)-GFLG-MTX conjugate (that is devoid of FITC labeling) was tested for its cytotoxic activity on cells lacking or overexpressing FRα. We first compared the cytotoxicity of this construct to parental C5 cells versus that observed with C5-FRα cells. We hypothesized that the preferential accumulation of the MTX-bearing FA-conjugate to cells overexpressing FRα should result in enhanced cytotoxicity when compared to FRα-lacking parental cells and that the cleavability of the peptide linker would enable drug efficacy. Indeed, the cytotoxicity data support our hypotheses; we observed a 6.3-fold higher cytotoxicity for FRα overexpressing C5-FRα cells when compared to parental C5 cells lacking FRα expression (Figure 10). This indicates a moderate but significant selectivity. The mean IC_{50} values obtained for C5-FRα and parental C5 cells were 320 ± 5 and

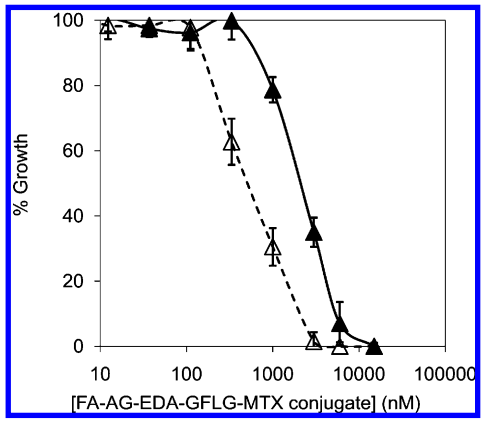


Figure 11. Cytotoxicity of FA-AG-EDA-GFLG-MTX conjugate to C5-FRα cells incubated in FA lacking medium (empty triangles) or in 50 μM FA-containing medium (solid triangles). The experiment was conducted in pentaplicates. Error bars represent standard error. Cytotoxicity test was performed as described in Figure 10 legend. A 6.3-fold higher cytotoxicity was obtained for cells incubated in FA-free medium relative to cells incubated with excess FA. The average IC_{50} values measured for FRα cells in the absence or presence of 50 μM of FA were 400 ± 13 and 2500 ± 65, respectively.

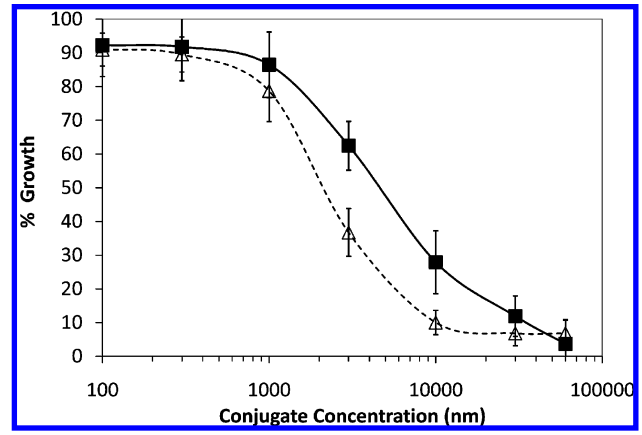


Figure 12. Evaluating the necessity for FA as a targeting moiety on the AG-GFLG-MTX conjugate. FR_{α} cells exposed to FA-AG-EDA-GFLG-MTX (triangles) compared to the same cells exposed to AG-EDA-GFLG-MTX (solid squares). Cytotoxicity tests were performed as described in Figure 10 legend.

2000 ± 126 nM, respectively. An increased ratio of the IC_{50} values observed for the above paired cell lines might be achievable by increasing the accessibility of the polymer-bound FA to FRα by conjugating more FA residues per polymer molecule or by using a longer linker arm, thereby improving the effectiveness and selectivity of this polymeric nanovehicle. Moreover, the cytotoxicity may be improved by loading the polymer with more MTX, and/or other anticancer drugs.

To further corroborate the FA-mediated uptake of this cytotoxic construct, an additional experiment was undertaken (Figure 11), where C5-FRα cells were incubated in the presence of excess free FA (50 μM). In this case, a 6.3-fold higher cytotoxicity was also obtained for C5-FRα cells incubated in FA-free medium relative to cells incubated with excess FA. The average IC_{50} values obtained for C5-FRα cells in FA-lacking medium and in medium supplemented with 50 μM FA were 400 ± 13 and 2500 ± 65, respectively.

The targeting function of FA is obvious when the vehicle is carrying drugs that have no interaction with cell membrane receptors. However, because in the current example, MTX, a FA analogue was used as the test anticancer drug, it was important to evaluate the necessity of FA, as MTX has some affinity to FRα, which may suffice for targeting, thereby serving a dual role. To test this hypothesis, an AG conjugate lacking FA was synthesized (AG-EDA-GFLG-MTX), and its cytotoxicity was tested on FRα cells in comparison with the complete conjugate (FA-AG-EDA-GFLG-MTX). These results are depicted in Figure 12. Considering also the relative compositions of these two vehicles (Table 1), the advantage of including FA as a targeting moiety is clearly demonstrated: AG-EDA-GFLG-MTX carried 4 moles of MTX per mole of AG, while FA-AG-EDA-GFLG-MTX carried only 0.8 moles MTX per mole AG, and 1.7 mole FA, and yet, the IC_{50} of the latter was 40% when compared to that of the former. This is due to the significantly higher affinity of FRα for FA, compared to its affinity for MTX.

4. Conclusions

Taken collectively, our data establish that the novel AG anticancer biomacromolecular drug carrier devised here possesses a promising potential as a targeted nanovehicle for the selective delivery and target-activated release of chemotherapeutic agents. An important potential advantage of such a polymeric vehicle is that, by loading it with several synergistically active antitumor agents at optimal stoichiometric ratios, effective treatment may be achieved toward malignant cells as well as multidrug resistant tumor cells.[1,39] Moreover, such a vehicle may be tailored for a particular disease or a particular patient as an initial step toward personalized medicine.

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