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PHARMACEUTICAL TECHNOLOGY

TRASTUZUMAB-DENDRIMER-FLUORINE DRUG DELIVERY SYSTEM

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Abstract: Breast cancer is the most frequently occurring cancer in women worldwide with more than one million new cases diagnosed each year. The objective of this study was to develop a Trastuzumab-dendrimer-fluorine drug delivery system by covalent attachment of Trastuzumab to a fluorinated PAMAM-G5 dendrimer. The Trastuzumab-dendrimer-fluorine drug delivery system was used to treat MCF-7 with Her-2 overexpression. The use of PAMAM-G5, which bears 128 primary amine surface groups, enables covalent attachment of both antibody and fluorinated functional groups for enhancement of cellular uptake. Thus, Trastuzumab was covalently attached to fluorinated PAMAM-G5 dendrimers and used as a vehicle for drug delivery to three dimensional (3D) cultured cells. The efficiency of Trastuzumab-dendrimer-fluorine drug delivery system binding to Her-2 receptors was measured by cell viability. The Trastuzumab-dendrimer-fluorine drug delivery system was found to have a higher efficiency in the treatment of Her-2 overexpressing MCF-7 cells than Trastuzumab alone. The incorporation of ^{19}F by addition of heptafluorobutyric acid anhydride (HFAA) to PAMAM-G5 increased lipophilicity and hydrophobicity of the drug delivery system.

Keywords: Trastuzumab, drug delivery system, Her-2 receptor, fluorine, PAMAM-G5

The main disadvantage of conventional chemotherapy is a nonspecific drug delivery and toxicity towards healthy cells. The development of drug delivery systems is one approach that is commonly used to avoid or lessen this disadvantage. Drug delivery systems have the potential to improve cancer cell targeting where precision of delivery is needed in the treatment. In oncological therapy, one major class of drugs used to target cancer are monoclonal antibodies. By definition, monoclonal antibodies are proteins secreted by specific white blood cells of the animal or human origin which have the ability to recognize specific targets called antigens. Therapeutic antibodies conjugated with fluorinated dendrimers could be used as alternatives to native antibodies for tumor targeting applications. Antibodies are engineered to track a specific tumor antigen and bind themselves to the surface of cancer cells. Thus far, limited clinical benefit has been observed with antibody-drug conjugates although the potential exists for the development of improved antibody-drug delivery systems.

Human epidermal growth factor receptor-2 (Her-2) is one of the most promising targets in immunotherapy. Her-2, which is a member of the ErbB family of receptors, is overexpressed on the surface of tumors. The family of ErbB consists of EGFR (ErbB-1), Her-2 (ErbB-2), Her-3 (ErbB-3), and Her-4 (ErbB-4) (1, 2). Studies have shown that up to 30% of breast cancer patients and 20% of ovarian carcinomas overexpress the Her-2 receptor (3, 4). Her-2 overexpression in breast cancer confers increased tumor aggressiveness (3, 4) and is a key molecule in the regulation of apoptosis in breast cancer cells (5). The low density of Her-2 receptors on cancer cells limits targeting detection. Therefore, monitoring of Her-2 receptor treatment requires a highly sensitive technique. Her-2 overexpression protects breast cancer cells from apoptosis and is identified as a target for breast cancer therapy (6). The Trastuzumab-dendrimer-fluorine drug delivery system may offer the possibility of increasing antibody delivery to Her-2 receptors in Her-2 overexpressed breast cancer cell culture. The Trastuzumab-dendrimer-fluorine drug delivery system (Fig. 1) molecular probe has been designed to improve Her-2 treatment as evaluated by a determination of treatment efficacy (1). The advantages of this application are that dendrimer-monoclonal antibody conjugates selectively target cancer cells avoiding delivery to

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organs such as the spleen. Dendrimer-monoclonal antibody conjugates have been shown to target Her-2 overexpressing cells but in the absence of antibody, the dendrimers alone do not bind to Her-2 targets (7). In vivo results in mice using dendrimer-monoclonal antibody conjugates show no hair loss, no weight loss and reduction of tumor size with low concentrations of delivered Her-2 monoclonal antibody (7). We wish to explore the potential of fluorinated dendrimers as vehicles for drug delivery in Her-2 directed Trastuzumab immunotherapy model studies. Studies of drug delivery to cancer cells using dendrimers have the unexpected benefit of enhanced cellular uptake of the transported drug. The use of dendrimers in drug delivery may be found to enhance drug efficacy since they offer several modes of drug attachment including monomolecular micelle-like encapsulation, covalent and ionic bonding. Peripheral groups on commercially available dendrimers such as poly(amidoamine) (PAMAM) are easily functionalized with fluorine-containing groups. The coupling of fluorinated dendrimers with Trastuzumab will allow for tracking, and quantification using ^{19}F MRI and ^{19}F NMR. The uptake of Trastuzumab-dendrimer-fluorine conjugates can be monitored by various methods in ^{19}F MRI and ^{19}F NMR. By monitoring ^{19}F SI intensity, the number of treated cells can be determined where an increase in ^{19}F signal intensity corresponds to an increase in cell death. The use of 3D breast cancer cell cultures will provide tissue density that mimics in vivo tumor conditions. This project presents an innovative approach towards the synthesis and application of new Trastuzumab derivatives that are expected to improve efficacy and non-invasive visualization. It is anticipated that the results from this research will provide the impetus for the development of an in vitro clinical protocol. Currently, nanoparticles such as hyperbranched polymers, liposomes, micelles or dendrimers are believed to be good candidates for drug delivery systems (7). The reason for the construction of the Trastuzumab-dendrimer-fluorine drug delivery system was to obtain a system that is easy to track by ^{19}F NMR due to the presence of specific ^{19}F nuclei and to enhance antibody lipophilicity.

EXPERIMENTAL

Materials

Human Adenocarcinoma MCF-7 Cell and Human Mammary Epithelial Cell (HMEC) lines for cell cultures were supplied from ATCC (American Type Culture Collection (ATCC®), MANASSAS, VA, USA) and were delivered from (LGC, Łomianki, Poland). All compounds for cell cultures were supplied by Fisher Scientific (Oakland, ON). Trastuzumab (Herceptin) was purchased from Genetech Inc. (San Francisco, CA, USA) and delivered from the company Roche Polska (Warsaw, Poland). Collagen bovine type Lyophilized Fibrous Powder from Tendon (Advanced BioMatrix, USA),

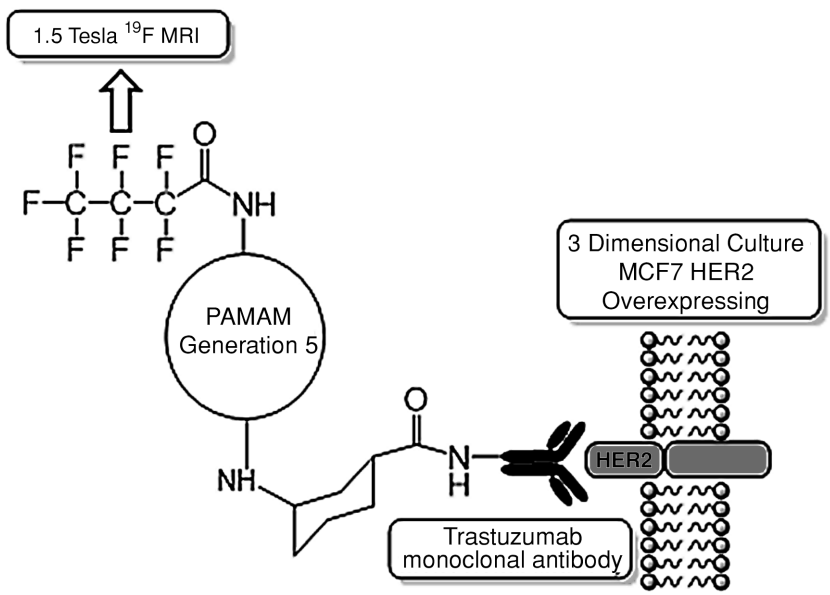

Figure 1. A Trastuzumab-dendrimer-fluorine molecule docking to a Her-2 receptor expressed on the cell surface.

Penicillin – Streptomycin – Neomycin Solution Stabilized, Fetal Bovine Serum (FBS), Epidermal growth factor (EGF) was from Sigma Aldrich, (Sigma -Aldrich, USA). Growth media for cells were prepared under sterile conditions in a laminar airflow chamber manufactured by Alpina (Konin, Poland).

Cell cultures

MCF-7, HER-2 producing cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Her-2 overexpression of these cell lines was confirmed by flow cytometric analysis according to the manner reported by Chan et al. (8). For mimicking in vivo conditions, maintenance of physiological pH under atmospheric CO_2 (< 0.5%) conditions was required. To accomplish this, CO_2 independent medium (Gibco/Invitrogen Corp.) supplemented with 10% FBS, 1% L-Glutamate, and 1% antibiotic (penicillin/streptomycin) was used. Prior to bioreactor placement, the passaged cells were re-suspended in the CO_2 independent medium and plated (25 mL, 1×10^6 cells/mL) on a 6-well tissue culture polystyrene (TCPS). Each well was then filled with 2 mL of CO_2 independent medium and cultured at 37şC under atmospheric CO_2 for 24 h. The cells were maintained in tissue culture flasks and were cultured as monolayers until cells number reached 0.5×10^7 cells/mL and then the cells were seeded in a bioreactor. A total of 16 bioreactors were used for the study.

Human Mammary Epithelial Cells (HMEC)

HMEC cells (GIBCO Invitrogen, Rockville, MD) were isolated from mammoplasty tissue and cultured in culture flask until cell number reached 0.5×10^5 cells/mL. Medium171 supplemented with 0.4% bovine extract, 5 mg/L bovine insulin, 0.5 mg/L hydrocortisone, and 3 µg/L human epidermal growth factor were used. A total of 16 bioreactors were used for the study.

3D cell culture

MCF-7 and HMEC cells were seeded in the bioreactor device. The bioreactor system is a closed-loop system consisting of a porous hydrophilic hollow fiber with 0.1 µm size pores in a glass tube connected to polysulfone tubing for media flow. For this ex vivo study, we used a single fiber in the bioreactor cartridge. The cell growth medium was fed from a reservoir bottle. We used a collagen solution to create an extracellular matrix between the cells and the fiber. The polysulfone fiber was coated with protein by flushing with 10 mL of coating solution containing 1 mg collagen per 1 mL of phosphate-buffered saline (PBS). After inoculation, the bioreactor was perfused using a peristaltic pump. The flow of medium started at a rate of 5 mL/min and was gradually increased to 14 mL/min. The pH was maintained in the extra-capillary space throughout the duration of experiments between 6.8 and 7.0. The pH of the cell growth medium was monitored using a pH meter, and the measurements were taken every 1 h for the first 12 h and every 24 h for the next 5 weeks. The perfusion medium was changed weekly when the glucose level reached 2 g/L measured with a glucometer. The breast cancer cells were allowed to grow in the bioreactor until the density of the cells reached 10^9 cells/mL.

MTT assay

The MTT assay was used to assess cell viability. This assay is a colorimetric reaction that can easily be measured from cell monolayers that have been plated in 35 mm dishes or multi-well plates. Typically, 10,000 cells suspended in 100 µL of media are incubated with 10 µL of MTT reagent (Cat # 30-1010K; ATCC) for approximately 3 h, followed by the addition of a detergent solution to lyse the cells and solubilize the colored crystals.

Cell cultures were incubated for 3 h in a culture medium containing 0.5 mg mL MTT reagent. After 2 h, the incubation buffer was removed and the blue MTT–formazan product was extracted with acidified isopropyl alcohol (0.04 N HCl). After 30 min extraction at room temperature, the absorbance of the formazan solution was read spectrophotometrically at 570 nm.

Synthesis of Trastuzumab-dendrimer-fluorine drug delivery system

Trastuzumab was conjugated to fluorinated dendrimers by the following protocol (Fig. 2): Dendrimer **1** (G5-PAMAM) was dissolved in anhydrous methanol followed by addition of triethylamine (TEA) and excess heptafluorobutyric acid anhydride (HFAA) to obtain ca. 80% surface coverage yielding fluorinated dendrimer **2**. ^{19}F NMR analysis provided confirmation and yield of the conversion of **1** to **2**. Product **2** was reacted with sulfo-succinimidyl 6-[3'-(2-pyridyldithio)-propionamido]hexanoate (Sulfo-LC-SPDP) to introduce a disulfide group to give **3**. The disulfide group was cleaved with dithiothreitol (DTT) to form the highly reactive thiolate **4**. All fluorinated dendrimers were purified by chromatography and characterized by ^{19}F NMR. Trastuzumab was modified by reaction with

sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) in PBS at room temperature providing a thiol-reactive maleimide group to give product **5.** The maleimide linked trastuzumab **5** was then reacted with fluorinated dendrimer thiolate **3** in a PBS-EDTA buffer to give the thioether cross-linked dendrimer conjugate **6.**

Synthesis of product 2 Fluorinated Dendrimers (fluorinated PAMAM G5):

To 5 mL of methanol, 50 mg of PAMAM G5 (1.27 mL of a 5% solution in x, 1.73×10^{-3} mmol) and 21 µL of trimethylamine (15.2 mg, 0.15 mmol) were added and the mixture was stirred at room temperature for 30 min. After 30 min, 32 µL of heptafluorobutyric anhydride (0.053 g, 0.13 mmol) in 2.6 mL of methanol was added to the PAMAM G5-Et_3N mixture and this solution was allowed to stir for 24 h at room temperature. After 24 h, the mixture was dialyzed (dialysis bags 10 kD cutoff) against PBS buffer (pH = 7.4) for 24 h and again for 24 h against distilled water. The solution was transferred to a test tube and the water was removed by heating at 66°C in a test tube heating

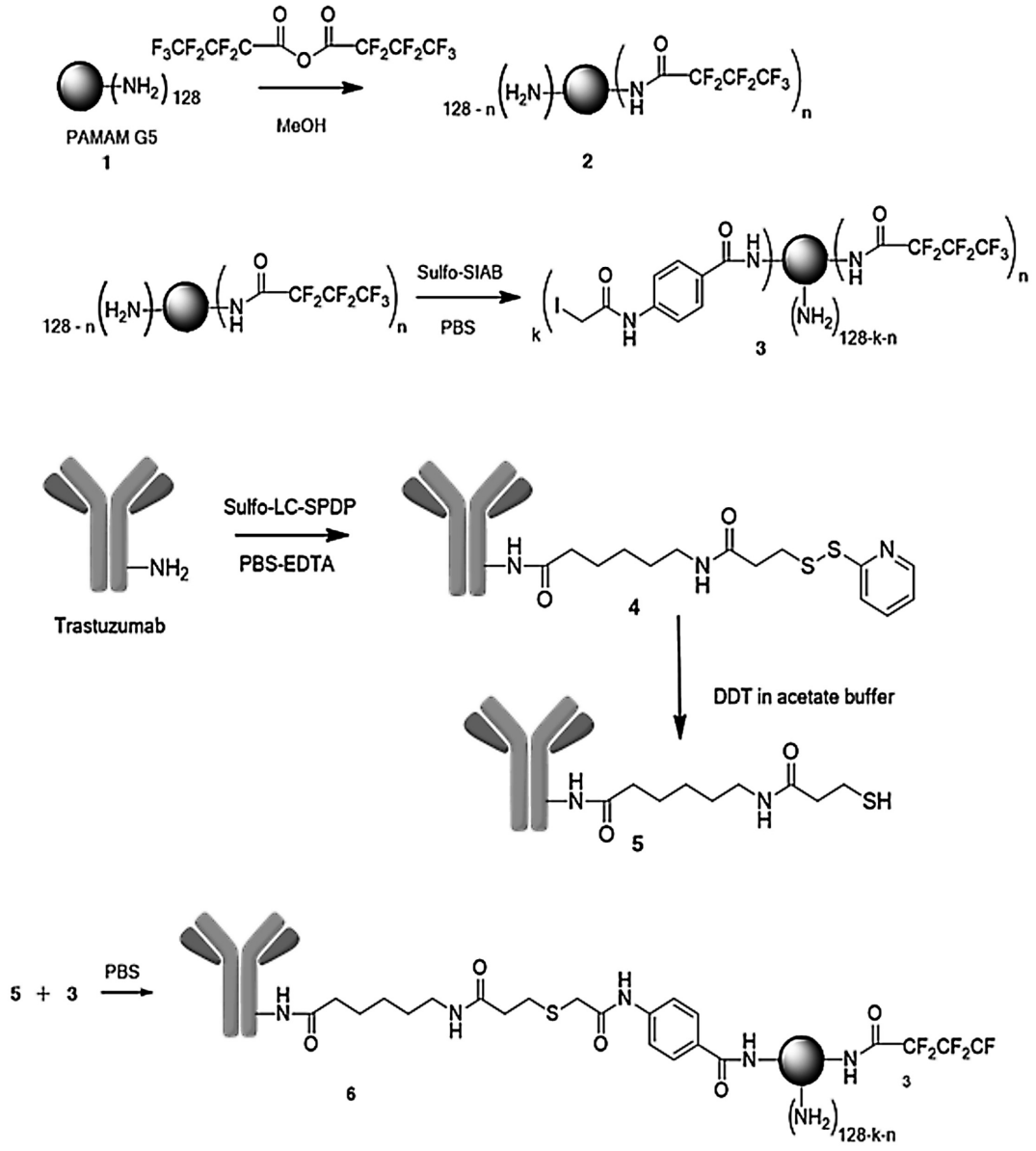

Figure 2. Synthesis of Trastuzumab-dendrimer-fluorine conjugates.

Table 1. Concentration of Trastuzumab in 6.

Sample	6a	6b	6c	6d	6e	6f	6g
Product 6 in µg/mL	0.067	0.66	66.25	132.5	265	662.5	1325
Trastuzumab concentration in µg/mL	0.05	0.5	50	100	200	500	1000

Table 2. Bioreactor setup.

HMEC	B1	B2	B3	B4	B5	B6	B7
HMEC	B8	B9	B10	B11	B12	B13	B14
MCF-7	B15	B16	B17	B18	B19	B20	B21
MCF-7	B22	B23	B24	B25	B26	B27	B28

block under a stream of nitrogen gas. The number of fluorine atoms was determined by a ninhydrin test. The extent of fluorination was quantified by comparison of the CF_3 relative integrated peak areas for an internal standard and the fluorinated dendrimer. We expect ca. 714 ^{19}F per PAMAM dendrimer **2**. The degree of fluorination will be adjusted to optimize ^{19}F signal intensity and water solubility of **2**.

Synthesis of product 3 (fluorinated PAMAM G5-Sulfo-SIAB)

Ten milligrams of **2** was dissolved in 8 mL of PBS buffer. A stock solution was prepared by adding 1.7 mg of Sulfo-SIAB in 1 mL of distilled water (1.7 mg Sulfo-SIAB, 504.20 g mol^{-1}, 3.4×10^{-6} mol, 3.4×10^{-3} mM). 1 mL of 3.4×10^{-3} mM Sulfo-SIAB is then added to 30 mg of **2** in 8 mL of PBS. The solution is allowed to stir for 30 min. After 30 min, the mixture was passed through a Sephadex 25 column and used as-is. The absorbance at 343 nm of the reduced sample was recorded.

Synthesis of product 4 (Trastuzumab Sulfo-LC-SPDP)

20 mg of Trastuzumab (4 terminal amines, mw = 148 kD, 0.005g = 3.37×10^{-8} mol, at most 4 (3.37×10^{-8} mol) = 1.34×10^{-7} mol terminal amine) was dissolved in 1 mL of PBS-EDTA (20 mM sodium phosphate, 150 mM sodium chloride, 1 mM EDTA, 0.02% sodium azide, pH = 7.4). A stock solution was prepared by dissolving 2 mg Sulfo-LC-SPDP in 200 µL of distilled water (2 mg Sulfo-LC-SPDP, 527.57 $gmol^{-1}$, 3.8×10^{-6} mol, 0.38 mmol, 19 mM, excess). 50 µL of 19 mM Sulfo-LC-SPDP (25 µL of 19 mM Sulfo-LC-SPDP = 4.75×10^{-7} mol) was then added to trastuzumab and the reaction was allowed to stand (no stirring) for 60 min.

Synthesis of product 5 (reduction of 4)

After 60 min, 23 mg of DTT was dissolved in 1 mL of acetate buffer (acetate buffer: 100 mM sodium acetate, 100 mM NaCl). The solution is then added to 5 and allowed to stand for 30 min. After 30 min, the mixture was passed through a Sephadex-25 column.

Synthesis of product 6

The resulting solution of **5** was then added to a solution of **3**. The mixture was allowed to stir for 24 h. After 24 h, the mixture was dialysis (dialysis bags 10 kD cutoff) against PBS buffer (pH = 7.4) for 24 h and again for 24 h against distilled water. The solution was transferred to a test tube and the water was removed by heating at 66°C in a test tube heating block under a stream of nitrogen gas. The final conjugate **6** was purified by ultrafiltration. The Trastuzumab-dendrimer-^{19}F conjugate was analyzed by HPLC and UV-vis spectroscopy.

^{19}F NMR of 6: To quantitate ^{19}F concentration in product **6** before use in cell treatment, **^{19}F NMR** was acquired**.** Suspensions of product **6** at 5 mg/mL was prepared in each of three 20 mM sodium citrate buffers prepared at pH 7.0, 5.0, and 2.0, respectively.

***Ex vivo* treatment of 3D**

Application of product 6 to Her-2 Overexpressing Cells: To optimize the binding of product **6** with the cell surface Her-2 receptor, cell cultures grown in the device was treated for 24, 48 and 72 h. The treatment was performed with several concentrations of **6**. Trastuzumab (m.w. 145531 g/mol) is administered at a dose of 4 mg/kg (4 µg/mL = 27 nM) in the first week of clinical treatment. When one Trastuzumab is present in **6**, the corresponding concentration is 5.2 µg/mL assuming

a molecular weight of 194372 g/mol. Based on this calculation, aqueous solutions of **6** was used at concentrations of 0.067 µg/mL (**6a**), 0.66 µg/mL (**6b**), 66.25 µg/mL (**6c**), 132.5 µg/mL (**6d**), 265 µg/mL (**6e**), 662.5 µg/mL (**6f**) and 1325 µg/mL (**6g**) administered to 10^9 cells/mL in the 3D culture. The corresponding concentration of Trastuzumab in each sample of **6** is shown in Table 1. Control treatments will be performed to compare the efficiency of **6** with Trastuzumab.

For the treatment of 10^9 HMEC cells/mL, Trastuzumab in µg and media in mL were mixed to obtain concentration of 0.05 µg/mL, 0.5 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, 500 µg/mL and 1000 µg/mL were used in bioreactors **B1, B2, B3, B4, B5, B6** and **B7,** respectively.

For the treatment of 10^9 HMEC cells/mL, product **6** at concentrations of 0.067 µg/mL, 0.66 µg/mL, 66.25 µg/mL, 132.5 µg/mL, 265 µg/mL, 662.5 µg/mL and 1325 µg/mL were injected into bioreactors **B8**, **B9**, **B10**, **B11**, **B12**, **B13** and **B14**, respectively.

For the treatment of 10^9 MCF-7 cells/mL, Trastuzumab at concentration of 0.05 µg/mL, 0.5 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, 500 µg/mL and 1000 µg/mL were used in bioreactors **B15, B16, B17, B18, B19, B20 and B21** respectively.

For the treatment of 10^9 MCF-7 cells/mL, **6** at concentrations of 0.067 µg/mL, 0.66 µg/mL, 66.25 µg/mL, 132.5 µg/mL, 265 µg/mL, 662.5 µg/mL and 1325 µg/mL were injected into bioreactors, **B22, B23, B24, B25, B26, B27** and **B28,** respectively. The experimental setup is presented in Table 2.

For control, two HMEC bioreactors **B0 (1-2)** and MCF-7 bioreactors **B0 (3-4)** were grown. To determine the efficacy of the Trastuzumab-dendrimer-fluorine drug delivery system we measured MCF-7 cell viability 24 h, 48 h and 72 h after treatment. ^{19}F MRS was performed to determine the efficacy of **6.** A positive treatment effect will increase ^{19}F signal intensity (SI) on spectra. An increase in SI was a linear function with increasing cellular uptake of **6**. The concentration of ^{19}F was estimated using ^{19}F (SI) based on reference TFA. ^{19}F MRS was acquired using a one-pulse sequence (flip angle 60?; repetition time (TR) = 800 ms; number of average 2; echo time (TE) = 6 ms). Due to different chemical environments, fluorine signal varies for fluorine alone, fluorinated dendrimer alone, and Trastuzumab-dendrimer-fluorine conjugates attached to the Her-2 receptor.

Calibration 1: For the calibration 8 samples were prepared as follows 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 MCF-7 cells with Her-2 overexpressing. The cells were treated with 10 mL of **6a-6g (Table 1),** respectively. The ^{19}F SI was measured for each number of cells and each concentration. The calibration curves were prepared as ^{19}F SI is linearly related to the number of cells treated in a bioreactor device (9, 10).

Calibration 2: 10 mL of **6 a-g** (Table 1) was introduced into a separate bioreactor device without cells

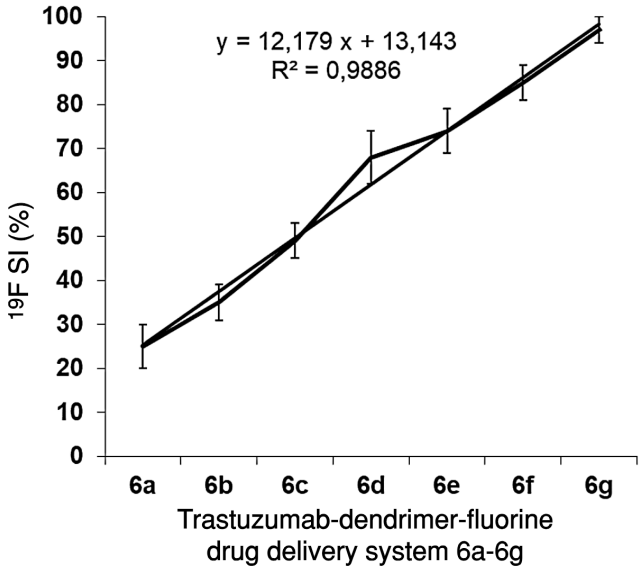

Figure 3. ^{19}F SI of 6a-6g. The presented data are mean values ± standard deviation of six independent experiments.

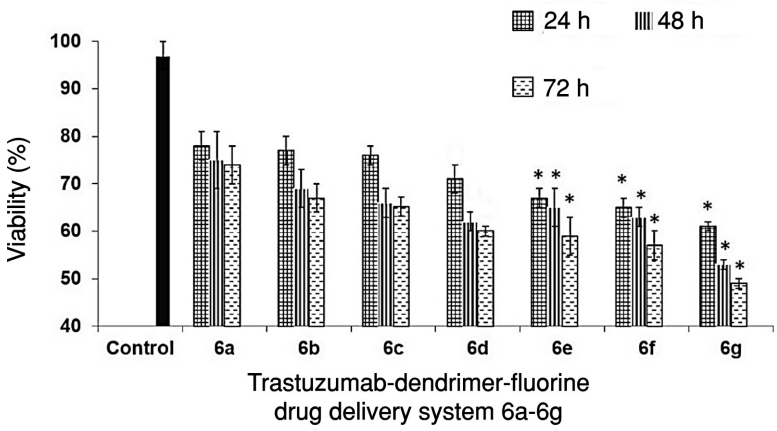

Figure 4. Viability of MCF-7 cells after 24, 48 and 72 h of treatment with 6a-6g. The presented data are mean values ± standard deviation of six independent experiments.

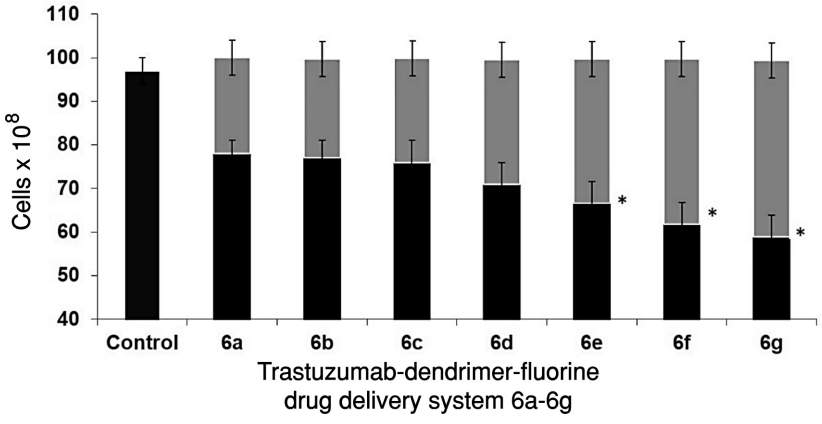

Figure 5. Number of control cells and cells treated with 6a-6g. Black color represents live cells and grey color is the number of killed cells after 24 h of treatment. The presented data are mean values ± standard deviation of six independent experiments.

and MRS was performed. ^{19}F SI was measured directly from axial slice of the bioreactor device. The changes in ^{19}F SI at different **6a-6g** concentrations were observed and the calibration curves were prepared as ^{19}F SI is linearly related to the amount of ^{19}F molecules. The ^{19}F SI of pure HFAA was considered as 100% of SI. Percentage change in ^{19}F SI will be normalized to pure HFAA using the following equation: ^{19}F SI (% change) = [(U-L)/U]×100%, where L = ^{19}F SI of ^{19}F in emulsions **6a-6g** (9, 10).

Calibration **1** was used to find the exact number of treated cells. Data collected in calibration **2** was used to visualize the concentration of **6**.

The presented data are mean values ± standard deviation of six independent experiments.

Statistical analysis

The results are presented as the mean ± standard deviation (mean ± SD). All study data were analyzed using analysis of variance followed by Dunnett's test for pairwise comparison. An asterisk indicates that the experimental values are significantly different from those of the control (*$P < 0.05$).

RESULTS AND DISCUSSION

The important challenge of this study was to determine 3D growth of cancer cell culture in the bioreactor device. The 3D cell culture was grown over a 4-week time period. The cell density was not

varied within the bioreactors due to consistent media flow. Cell counting was done using an MTT assay. To create an extracellular matrix between the cells and the fiber the collagen was used. The cell counting results showed an increase in cell numbers in the bioreactor device during four weeks.

^{19}F SI of the ^{19}F moiety in Trastuzumab-dendrimer-fluorine drug delivery system and ^{19}F SI of heptafluorobutyric anhydride alone was measured. The heptafluorobutyric anhydride alone has a single peak at -81 ppm. The ^{19}F MRS study of Trastuzumab-dendrimer-fluorine drug delivery system showed that the ^{19}F occurred as a single peak with the resonance at -81 ppm and is acquired with maximum SI after 60 min, as compared to TFA standard. The acquired spectra of the **6a-g** showed a single peak at -93 ppm. It was taking about 15 min while single ^{19}F signals from **6a-g** showed about 50% of SI. The levels of HFBA were 6% for **6a-g** as measured from ^{19}F SI. The study was repeated six times (Fig. 3).

MCF-7 Cell viability after treatments with Trastuzumab

After 24 h of treatment with Trastuzumab at concentrations 0.05 µg/mL, 0.5 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, 500 µg/mL and 1000 µg/mL, the MCF-7 cell viability was 83 ± 3%, 82 ± 4%, 81 ± 4%, 76 ± 4%, 71 ± 4%, 66 ± 3% and 70 ± 3%, respectively. The treatment with concentrations 100 – 1000 µg/mL of Trastuzumab, significantly decreased the MCF-7 growth when compared to control.

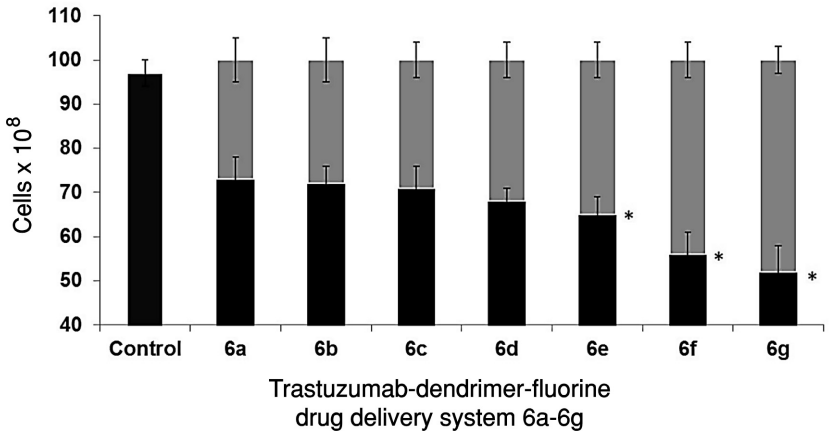

Figure 6. Number of control cells and cells treated with 6a-6g. Black color represents live cells and grey color is the number of killed cells after 48 h of treatment. The presented data are mean values ± standard deviation of six independent experiments.

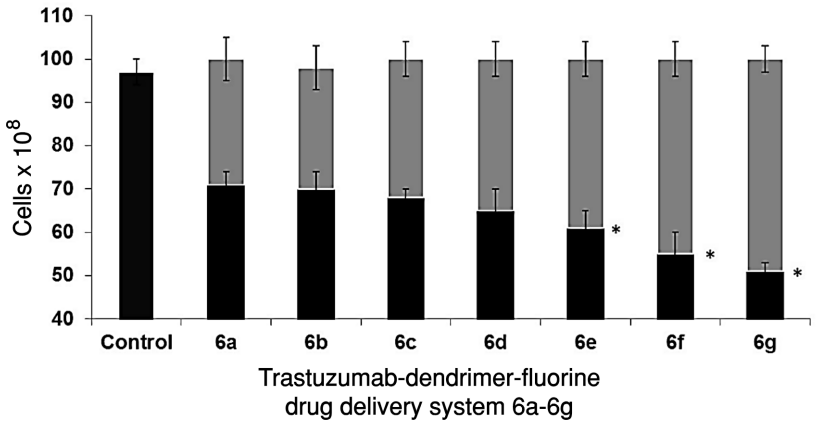

Figure 7. Number of control cells and cells treated with 6a-6g. Black color represents live cells and grey color is the number of killed cells after 72 h of treatment. The presented data are mean values ± standard deviation of six independent experiments.

Cell viability after 48 h was, 80 ± 2%, 78 ± 2%, 76 ± 3%, 73 ± 3%, 69 ± 3%, 57 ± 2% and 61±3%, respectively. At the same time, significant changes in cell viability were found for concentrations equal and higher than 100 µg/mL of Trastuzumab.

Within 72 h the viability decreased to 77 ± 4%, 73 ± 3%, 71 ± 3%, 68 ± 3%, 64 ± 4%, 56 ± 2% and 54 ± 3%, respectively. There were no significant changes in cell growth for Trastuzumab concentrations lower in **6a**-**6d** (Fig. 4).

Cell viability after 24 h treatments with 6a-6g

After 24 h, the MCF-7 cells treated with **6a-6g** showed viability equal to 78 ± 3%, 77 ± 2%, 76 ± 6%, 71 ± 8%, 67 ± 9%, 61 ± 1% and 48 ± 5%, respectively. MCF-7 cells exposure to **6e-6g** showed a significant decrease in the cell growth and cells damage. The normal HMEC cells treated with **6a-6g**, showed the following decreases in cells growth 3 ± 1%, 2.7 ± 1.1%, 3.3 ± 0.9%, 3.2 ± 2%, 4 ± 1%, 7 ± 2%, 6 ± 2.1% and 7.3 ± 1.9%, after 24 h (Fig. 5).

Cell viability after 48 h of treatment with 6a-6g

After 48 h, the MCF-7 cells treated with **6a-6g** showed viability equal to 73 ± 2%, 72 ± 4%, 71 ± 2%, 68 ± 5%, 65 ± 5%, 56 ± 7% and 52 ± 2%, respectively (Fig. 6). We did not observe significant decreases in the growth of MCF-7 control cells treated with **6** for concentration lower or equal **6d**. The normal HMEC cells treated with **6a-6g** showed the following decreases in cells growth 3.1 ± 1.5%, 2.9 ± 1.3%, 3.5 ± 0.7%, 3.4 ± 1%, 4.3 ± 2%, 7.2 ± 3%, 6.2 ± 0.5% and 7.5 ±0.9%, for the same probes respectively.

Cell viability after 72 h of treatment with 6a-6g

After 72 h, the viability of MCF-7 cells decreased to 71 ± 3%, 70 ± 4%, 69 ± 2%, 65 ± 5%, 62 ± 5%, 55 ± 7%, 50 ± 2%, respectively, while treated with **6a-6g**. There were no significant changes in cell growth for Herceptin concentrations lower than **6b**. The normal HMEC cells treated with **6a-6g** showed the following decreases in cells growth 3.1 ± 1.5%, 2.9 ± 1.3%, 3.5 ± 0.7%, 3.4 ± 1%, 4.3 ± 2%, 7.2 ± 3%, 6.2 ± 0.5% and 7.5 ±0.9%, for the same probes respectively (Fig. 7).

Table 3. Calibrations in MCF-7 cells.

Product	Function
6a	SI = 10 × number of cells -5.3
6b	SI = 10 × number of cells +5
6c	SI = 12 × number of cells -6
6d	SI = 11 × number of cells +2
6e	SI = 17 × number of cells -5.1
6f	SI = 21 × number of cells +3.2
6g	SI = 23 × number of cells +6.7

^{19}F MRS for cell density quantification

The detection limit of heptafluorobutyric anhydride in MCF-7 cells was determined using the same MRS procedure. The absolute number of treated cells was estimated from the viability and compared with ^{19}F MRS SI of Trastuzumab-dendrimer-fluorine drug delivery system delivered to the cells. The uptake of Trastuzumab-dendrimer-fluorine drug delivery system was determined, using calibration **1**. The advantage of ^{19}F MRS is coming from the selectivity of a signal which is detected only from cells treated with fluorine derivatives of Trastuzumab. The 1000 cells treated with **6a** is delivered the minimal ^{19}F MRS signal. The calculated standard curves were linear. As excepted, the level Trastuzumab-dendrimer-fluorine drug delivery system was dependent on the numbers of targeted cells. After 48 h of treatment with Trastuzumab-dendrimer-fluorine drug delivery system ^{19}F signal obtained from the region of the cells was higher than the signal detected after 24 h. The ^{19}F SI was noted to be higher after 72 h. post-injection of Trastuzumab-dendrimer-fluorine drug delivery system. In summary, the concentration of ^{19}F in Trastuzumab-dendrimer-fluorine drug delivery system was high enough to be detectable with ^{19}F MRS and showed efficient intracellular accumulations that corresponded to cell viability.

^{19}F MRS signal and cell quantification

The absolute number of treated cells was estimated from the ^{19}F MR spectra using the signal intensity of cells treated with a fluorinated drug delivery system. The quantification of cell numbers was possible due to the calibration of SI performed with a known number of cells treated with the fluorinated drug delivery system. Table 3 presents equations obtained during calibration 1 in MCF-7 cells.

The 1000 cells are the minimal cell number treated with **6g** and detectable with ^{19}F MRS at 1.5 Tesla. The calculated standard curve was linear and given by equation $y = 23x + 6.7$. As expected, the level ^{19}F SI was increased with the numbers of targeted cells.

After 24 h of treatment with **6g,** the number of killed cells reached the number 3.9×10^8 cells/mL and after 48 h increased to 4.8×10^8 cells/mL. A maximum of dead cells was measured after 72 h. of

treatment and was equal to 5.1×10^8 cells/mL. The results of all treatments are presented in Table 3.

DISCUSSION

The cells in the bioreactor device were perfused with media to allow sufficient oxygen and glucose concentration to avoid hypoxia. We did not observe the concentrations of Trastuzumab-dendrimer-fluorine drug delivery system in media reservoir after 72 h. Therefore, most Trastuzumab-dendrimer-fluorine drug delivery system was delivered to the cells. The nutrition and waste were delivered in a controlled manner through the fibers. The polysulfone fiber was flushed with 10 mL of a solution containing 1 mg collagen per 1 mL PBS.

Trastuzumab docking in cancer cells was possible with covalent attachment of fluorinated dendrimers. In this study, Trastuzumab was attached to fluorinated fifth-generation (G5) polyamidoamine (PAMAM). This study was an attempt to develop a new methodology to quantify Trastuzumab-dendrimer-fluorine location and efficiency noninvasively.

One study has demonstrated ^{19}F MRI at 4.0 Tesla imaging after injection of 200 µL of 20 mg/mL PAMAM-G3 dendrimers modified with 15 to 38 heptafluoroacyl substituents in female B6 mice (11). Images showed that particulates remain in circulation and are efficiently filtered by the liver (11). Another study described a synthesis of PAMAM(G5) dendrimers conjugated to anti-Her-2 monoclonal antibodies (12). Here, the efficiency of the conjugates after administration to MCA207 cells *in vitro* was detected by flow cytometry and confocal microscopy with a fluorescent probe. In addition, 2.4 nmol of the conjugate was injected into mice intravenously and the dendrimer-monoclonal antibody conjugate was predominantly internalized into Her-2 expressing tumor cells. The advantages of this application are that dendrimer-monoclonal antibody conjugates selectively targets cancer cells and does not go to the organs such as spleen. Dendrimer-monoclonal antibody conjugates have been shown to target Her-2 overexpressing cells but in the absence of antibody, PAMAM(G5) dendrimer does not bind to the Her-2 target (11). *In vivo* results in mice obtained in this study using dendrimer-monoclonal antibody conjugates showed no hair loss, no weight loss and reduction of tumor size with low concentrations of Her-2 monoclonal antibody (11). Confocal microscopy also demonstrated that the conjugate is present in the tumors, attached to and internalized by many of the tumor cells (11). One more promising study described the application of ^{19}F MRI at 1.5 Tesla to image large fluorinated dendrimers. The lowest detectable concentration for ^{19}F MRI reported in this study was 18.5 µM of fluorinated dendrimer (or 10 mM in ^{19}F concentration) with a scan time of only 150 seconds (13). Artemow has developed superparamagnetic nanoparticles with Trastuzumab (14) to treat Her-2 overexpressing breast cancer cells. Trastuzumab was conjugated with particles that are easy to track by ^{1}H MRI. The superparamagnetic nanoparticles were directed to Her-2 receptors pre-labeled with a biotinylated monoclonal antibody and generated strong T_2 contrast. The contrast observed in the images was proportional to the expression level of Her-2. Based on the expression efficiency of the receptor in the tested cells, the estimated MRI detection limit is in the range of 10^6 Her-2 receptors per one cancer cell. Therefore, ^{19}F MRI is able to detect ^{19}F compounds delivered only to the tissue of interest since the natural content of ^{19}F (10^{-3} µmol/g wet tissue) is below the detection limit. This is of particular importance for ^{19}F studies as ^{19}F content is low and signal averaging is always needed to provide a high signal-to-noise ratio (SNR). As the SNR increases with the root square of scan time, long scan times are needed. To shorten relaxation time T_1, a paramagnetic material such as lanthanide ion may be placed in the proximity of ^{19}F. The most common applications of ^{19}F MR in cancer research are imaging and spectroscopy of ^{19}F labeled anticancer agents when applied to cell cultures as they offer a controlled and systematic way to investigate cellular and molecular properties associated with disease (15).

This research presented here is focused on the development of anticancer therapeutics that can be monitored by MRS. The coupling of fluorinated dendrimers with Trastuzumab allows for tracking and quantification using ^{19}F MRS. The uptake of Trastuzumab-dendrimer-fluorine conjugates can be monitored by various methods in ^{19}F MRS.

CONCLUSIONS

In this study Trastuzumab conjugated with fluorinated dendrimers to improve efficacy was developed. The addition of fluorine to Trastuzumab enables the SI to be measured by ^{19}F MRS. Trastuzumab has been effectively used in fluorine-rich emulsions to treat Her-2 overexpressing breast cancer cells. In this project, we presented an innovative approach towards the synthesis and in vitro application of new Trastuzumab derivatives. This methodology could be applied to study the efficien-

cy of other immunotherapeutic antibodies and receptors. This research will be continued with the use of ^{19}F MRI to improve Trastuzumab delivery to breast cancer cells.

Conflict of interests and Financial disclosure.

There is no conflict of interest.

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