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Gd^{3+}-Anionic Linear Globular Dendrimer-G_2-C595 A Dual Novel Nanoprobe for MR Imaging and Therapeutic Agent: An In Vitro Study

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Abstract

Early cancer detection using MR imaging is of high global interest as a powerful non-invasive modality. The aim of this study was to synthesize a nano-dendrimer and its conjugate with C595 mab against breast cancer cell, followed by its chelating with Gd^{3+}. At the end, its ability as a nanosized probe for detection of breast cancer cells was investigated. For this reason, anti-MUC-1 mab C595 was coupled to a biocompatible anionic linear globular dendrimer G2 ($ALGDG_2$) (Poly Ethylene Glycol PEG core and citric acid shell) followed by loading with Gd^{3+}. MR imaging parameters of the prepared nanoconjugate was investigated *in vitro* performing several studies such as ^{1}H-NMR and ^{17}O-NMR, MUC-1 antigen. The obtained data showed a powerful relaxations as well as selective MUC-1 antigen and cell binding. Findings of this study showed that Gd^{3+}-ALGDG2-C595 nano-probe is potentially, a selective breast molecular imaging agent.

Keywords: MR imaging; Anionic linear glubolar dendrimer; C595; MUC-1; Breast cancer detection

Introduction

An important method for observing of nanosized contrast agents and their delivers into the tumor is selective imaging methods such as MR imaging for better targeting cancer. The fact highly depends on the specific carrier for the cancer cells that might be distinguished with attractive agents such as monoclonal antibodies or ligands. In MR imaging, contrast agent using nanoparticle plays an important role in this modality. Because of the size of cellular or subcellular units, macrosize has many obvious artifacts comparing to the nanosize in MR imaging [1-6]. For example, conventional microsize cancer contrast delivery suffers some insufficiencies of delivery, inappropriate targeting, toxicicological effects or impaired transport to cancerous site [5-8].

However, microsized cell entering carriers cannot traverse in a diffusion pattern through cells or pores including tumoric cells with pore sizes up to 380–780 nm. In confirmation, the best complex for tumor MR imaging applications would be a targeted nano-carrier complex [7,9-11]. Drug delivery using polymeric carriers is of global interest. In this regard, dendrimers (nanosized polymers) have been applied for delivering of contrast agents in MR imaging.

The biochemical properties of dendrimers, including their monodispersity, water solubility, drug loading ability and large number of functionalizable peripheral groups make these macromolecules suitable candidates for evaluation as both diagnostic and therapeutic tools. Using of safe nanocarriers such as anionic linear globular dendrimers is interesting in this regards [10-13]. Biocompatible properties of dendrimers such as log P, biodegradability, non-cytotoxicity and non-immunogenicity characteristics must be checked both under *in vitro* and *in vivo* conditions for application in MR imaging.

One generations of a highly water soluble anionic linear-globular dendrimer G2 (MW<2000 Da), (Poly Ethylene Glycol core) and citric acid periphery surface, was selected for the synthesis and subsequent MR imaging. This dendrimer has shown good capacity of drug loading in cancer therapy previously [12,13]. Monoclonal antibodies are among the best selective cancer MR imaging carrier of pharmaceuticals [14,15]. One of the targets, MUC-1, a high molecular weight transmembrane glycoprotein antigen is breast specific membrane antigen and are molecular target for a novel imaging for breast cancer [1-5].

C595 is an IgG_3, a Monoclonal Antibody against human MUC [12,13]. Several studies has shown that Mab C595 is a useful antibody either alone or incorporated with other therapeutic methods to treat the human breast cancer [8,14,15].

MR imaging is a precise more sensitive and noninvasive diagnostic pattern based on differences between relaxation ratios of protons in water and provides important graphical images. Current MR imaging contrast agents such as Gd^{3+}-DTPA (Magnevist®) improve tissue elucidation in MR images but not as well as radiopharmaceuticals which act more specifically [12,14-17].

This is the first experiment to describe synthesis and *in vitro* assessment of novel nanoconjugate contains ALGDG2 loaded Gd^{3+} and monoclonal antibody C595 as a selective breast molecular imaging. In fact, conjugation of dendrimer to C595 and Gd^{3+} loading makes a novel nanocontrast agent with potential imaging for detection of breast cancer cells.

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Materials and Methods

Preparation of the Gd^{3+}-$ALGDG_2$-C595 nanoconjugate

Anionic Linear Globular Dendrimer-G2 (ALGDG2) was synthesized based on literature [12,13]. Briefly, PEG-600 was chosen as the core and reacted with citric acid in the presence of excess amounts of thionyl chloride or EDC/DCC and the dialysis bag (cut off 2000 Da, Spectrum®, USA) was used for the purification. To synthesize the C595-dendrimer conjugate, 75 µmole ALGDG2 was reacted with 0.01 µmole EDC and 0.05 µmole Sulfo-NHS in 2 ml PBS or DDW for at least 5 minutes at pH=5.5-6 and the reaction allowed to reach to room temperature.

Thereafter, activated dendrimer was added dropwise to the solution containing 1 µmole C595 in 2 ml PBS medium in the presence of 1 mmole tri-ethyl-amine and the pH was adjusted to 7.5-8 and the reaction allowed continuing for 12 hrs at room temperature. To purify the conjugate, the reaction mixture was dialyzed with cut off of 10 KDa (Sigma,USA). To further purify the dialyzed solution was eluted through a Sephadex G-25 Fine® (Pharmacia-Fine Chemicals, Sweden) and the tubes containing the conjugate were selected for the next step (e.g., lyophilization, Gd^{3+} loading). All steps were monitored by Thin Layer Chromatography (TLC) method. Finally, 15 mmole $GdCl_3$ was added to 1 µmole of conjugate at room temperature and the reaction mixture was allowed to stir at pH=7-7.5 for at least 2 hrs.

Afterwards, to remove excess free Gd^{3+} ions the reaction pH was increased to 9 and free Gd^{3+} ion was precipitated and filtered. To increase the purity the reaction mixture was dialyzed.

Purification

To purify dendrimer-antibody conjugate sephadex G-25 fine® (gel filtration chromatography) was used. Each external ml of eluent buffer of reaction mixture was collected separately in a numbered tube and its UV-OD at 280 nm was monitored to find antibody-dendrimer conjugate as well as nonconjugated antibody. To find dendrimer conjugated C595 monoclonal antibody TLC was performed. High Pressure Liquid Chromatography (HPLC) was also performed at small sample amounts using 10 mm×250 mm pre-packed column (at room temperature). Preparative RP-HPLC experiments were also performed using Kromasil C8, 13 m, 100 A° silica media pre-packed in 10 mm×250 mm column (Eka Chemicals, Bohus, Sweden).

Gd^{3+}-FTIR Spectroscopy

To investigate whether or not the changes occurred after Gd^{3+} loading Fourier Transform Infrared (FTIR) spectroscopy (Thermonicolet, NEXUS 870, and Waltham, MA, USA) was employed by performance on nanoconjugate before and after Gd^{3+} loading.

Protein measurement

The assay performed in microplates based on micro Lowry assay [13-15] that has been adapted to microplates and a standard curve was prepared as following procedure. Briefly, bovine serum albumin (BSA) powder was dissolved in distilled water and diluted to a concentration of 1 µg/µl. A series of dilutions (5, 10, 20, 40, 60, 80, and 100 µg/well) were prepared and used in tetraplicate/100 µl. Different concentarions of protein were prepared (0-100 µg/100 µl) and a standard curve was obtained. The same procedure was performed for Gd^{3+} loaded nano conjugate and C595 alone.

Size measurement and TEM/SEM imaging

Nanosize was determined using a Brookhaven ZetaPALS (Malvern, Instrument) system for size and zeta potential distribution for both dendrimer and conjugate before and after conjugation. Beckman Multisizer Coulter equipped with a 1 nm or 100 nm apertures has been performed to depict the particle size distribution. The conjugates were also characterized by TEM (JOEL 1230; accelerating voltage, 100 kV). For TEM/SEM of samples, one drop of Gd^{3+}-$ALGDG_2$-C595 aqueous suspension was dispersed on copper grid and the excess volume was removed. The samples were used after drying at room temperature.

Relaxivity measurement

1H-NMR: Water proton relaxivity ratio at different lamor frequencies (MHz) was determined by using a spinmaster spectrometer (Stelar, Mede, Italy) set at 0.5 Tesla; the inversion recovery method was used (Number of experiments 16 and 4 scans). 90°-pulse width was set 3.5 milliseconds (ms), and the reproducibility error of the T_1 data was ± 0.75%. The temperature was monitored by an air-flow heater equipped with a copper-constantan thermocouple. The proton relaxivity ($1/T_1$) profiles were measured on a Koenig-Brown field-cycling relaxometer over a continuum of magnetic field strengths from 0.00024 to 1.2 T (corresponding to 0.0-50 MHz proton Larmor frequencies). The relaxivity measurement performed under computer monitoring with $1/T_1$ of ± 1.2%.

^{17}O-NMR: Variable-temperature ^{17}O-NMR determinations were obtained on JEOL EX-90 (2.1 Tesla) equipments containing with a 5 mm probe. D_2O used as external lock and 2.6% ^{17}O isotope solutions were used as well. The obtained transverse relaxivity ratios were calculated from signal width at 0.5 heights. More explanations were reported in supplementary materials [17,18].

***In vitro* cell binding and imaging:** To elucidate the specific binding of C595 antibody, encountering the conjugation with Gd^{3+}-ALGD-G2, to MUC-1 over expressing cells T47D, the experiment was designed and performed. The experiment was performed on two different cancer cell lines T47D (MUC-1 over-expressed) and SKOV3 (not MUC-1 over-expressed). Before the experiments Gd^{3+} loaded mab C595-dendrimer was conjugated covalently to a fluorescent agent red alexa fluor® 610 dyes. The cells were incubated with 20 nM of nanoconjugate for at least 60 min and thereafter cells were washed twice with PBS and the washed cells were placed under a Fluorescent Research Biological Microscopy System (BW OPTICS, China) and the images were taken from the cells at due time intervals.

Immunoreactivity: To ensure the right conjugation of dendrimer to antibody C595, antibodies detection (intact C595 and conjugated C595 to dendrimer) reactive with the MUC1 antigen was performed by ELISA considering some modifications [19,20]. Briefly, 100 µl/well of 1 µl/ml BSA were dispensed onto Falcon III PVC 96 wells plates and permitted to dry during the night at 37°C. Then the solution was washed and 50 µl containing MUC-1 core protein (100 µ/ml) and 50 µl of carbodiimide (1-cycloexil-3-[2 morpholinoethyl] carbodiimide metho-p-sulphonate (Sigma,USA) were suspended in duplicates. Then after, the wells were washed and 150 µl (blocking buffer) was exposed for 1 hour at room temperature, plates were then again washed/diluted samples (C595 and C595-conjugated dendrimer-Gd^{3+}) in blocking buffer (1:40 for IgG measurement) were performed with incubation at 4°C all over the night. After washing, 50 µl of peroxidase conjugated goat anti-human IgG in PBS Tween-BSA 0.1% were exposed and with an incubation time of 1 hour, the reaction was advanced with 50 µl aqueous solution of ABTS in 0.1 M citric-acid/0.2 M Na_2PO_4H (pH=5). Finally after 1 hour incubation in dark room, (OD) Optical Density

was recorded at 405 nm. Based on the obtained data the comparisons were performed.

Results and Discussion

Purification and protein assay

Purification assay performed by RP-HPLC and the data shown a retention time of 7, 12 and 13 min for dendrimer, C595 alone and dendrimer-C595 conjugate, respectively with the purity of above 95% (Figure 1A). For protein assay each 100 µg nanoconjugate contained 84.3 ± 4 µg protein regarded to Mab C595 while each 100 µg intact Mab

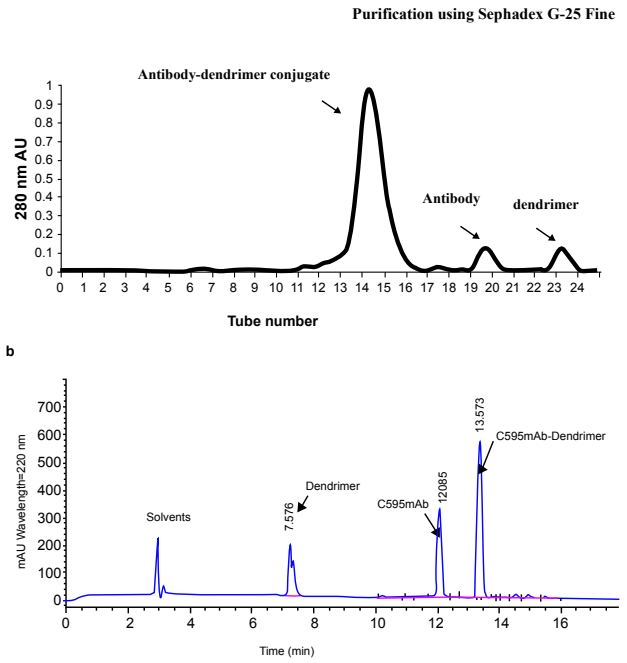

Figure 1: Schematic illustration of nano-conjugate dendrimer-C595 purification and b: Analytical RP-HPLC chromatogram (The data were superimposed). The purity of the compounds was obtained more than 95% according to AUC, area under the curve of each peak.

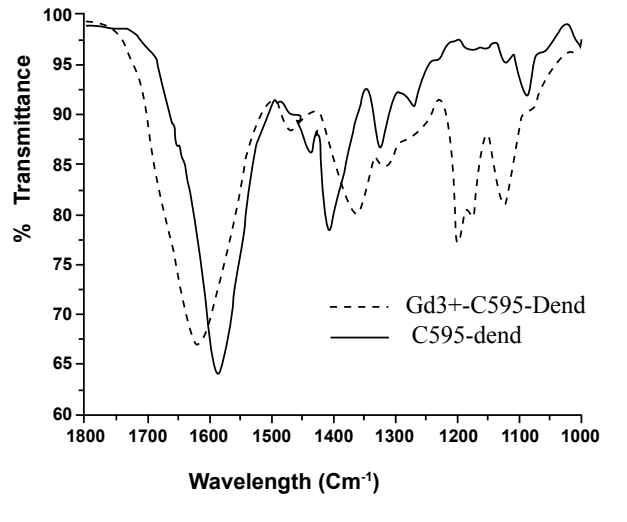

Figure 2: Changes occurred before and after Gd^{3+} loading on ALGDG_2-C595 nanoconjugate.

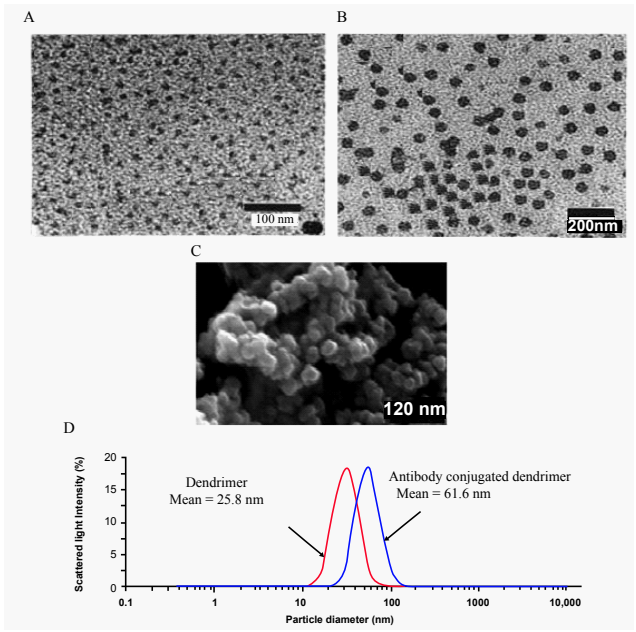

Figure 3: Illustration of dendrimer TEM (A), Gd-dendrimer-C595 (B), Gd-dendrimer-C595 (SEM) (C) and size distribution of dendrimer before and after Gd^{3+} and C595 bioconjugation (D).

C595 contained >98 µg protein. Analytical RP-HPLC chromatogram was shown in figure 1B (The data were superimposed). As can be seen from this figure, the purity of the compounds was obtained more than 95% according to AUC, area under the curve of each peak (Figure 1).

FTIR spectroscopy

Clear changes in FTIR peaks before and after Gd^{3+} loading was occurred and this indicates the essential interaction of Gd^{3+} ions and dendrimer-C595 conjugate (Figure 2).

Gd^{3+} content and Size/TEM/SEM results

Inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer Optima 3100XL) data showed 11.01 ± 4% (n=3) Gd^{3+} in each 100 µg nanoconjugate. Size distribution shows that dendrimer alone has an average size of 25 nm and after conjugation and gadolinium loading the average aggregation size reached to 61 nm. The apparent view of nanoparticles was also shown in SEM and TEM images. Figures 3A-3D shows TEM illustration of dendrimer, Gd-dendrimer-C595, Gd-dendrimer-C595 and size distribution of dendrimer before and after Gd^{3+} and C595 bioconjugation. The TEM picture of Gd-dendrimer-C595 showed high average more than 2 nm (Figure 3).

Relaxivity studies

The relaxivity data were illustrated in figures 4 and 5. As it can be found gadolinium loaded nanoconjugate showed an extreme temperature, concentration and frequency intendancy. Increase in the temperature, concentration or frequency increased the paramagnetic potential of the nano-probe. Because of low dose, the conjugate showed a less paramagnetic activity than that of standard agent like Magnevist® but, it is interesting that by increasing the temperature, concentration and frequency the relaxivity is increased similar to Magnevist® at high concentration. Both (figures 4 and 5) showed that nanosize contrast agent produced enough relaxivity (^{1}H-NMRD and ^{17}O-NMR profiles) for breast cancer cells and the results showed its higher relaxivity in comparison with Magnevist®.

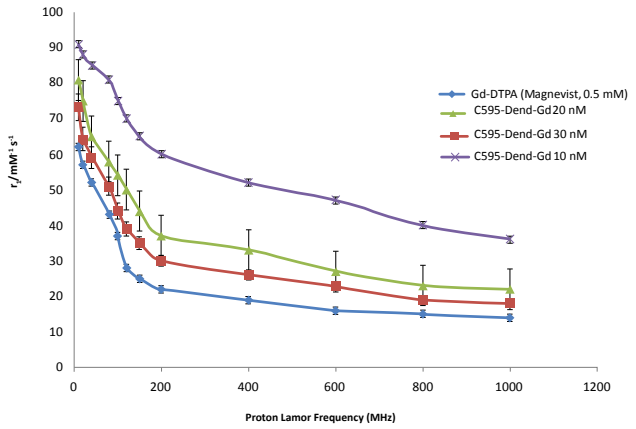

Figure 4: Proton Lamor relaxivity at different doses of nanoconjugate and comparison with standard drug Magnevist®.

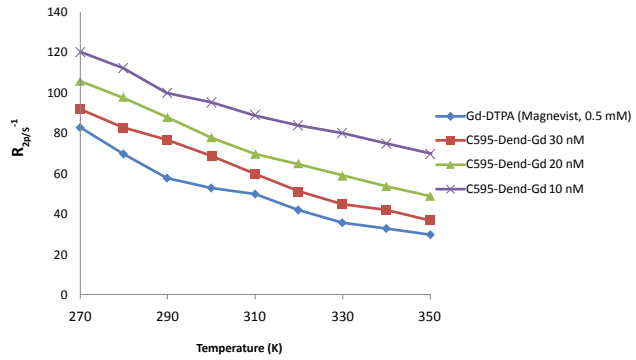

Figure 5: Illustration of ^{17}O-NMR profiles of different concentrations or temperature of nano-probe in comparison with Magnevist.

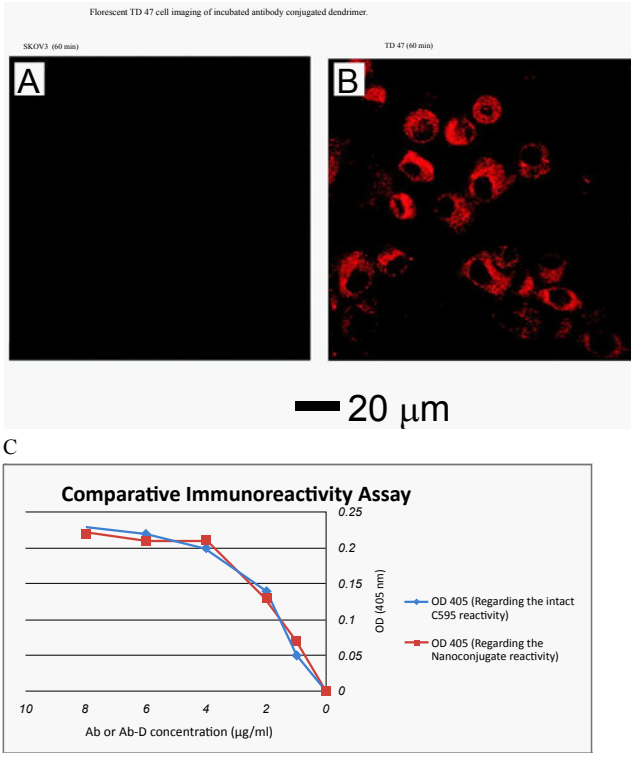

Figure 6: A-B:Flourescent images obtained from treated SKOV3 and T47D cells with 20 nM. Gd-dendrimer-C595 bio-nanoconjugate after 60 min exposure. Images were taken after cell wash. C: Immunoreactivity assay showed a not altered MUC-1-Gd-dendrimer-C595 interaction diactivity comparison with C595.

Cell binding and immunoreactivity assay

As indicated in (figure 6), flourescent images obtained from treated SKOV3 and T47D cells with 20 nm. Images were taken after cell wash. Immunoreactivity assay showed also no alteration MUC-1-Gd-dendrimer-C595 interaction diactivity comparison with C595. Interesting results were obtained while Gd^{3+}-ALGDG$_2$-C595 did not bind to SKOV3 cells but a good T47D cell binding were recorded after 60 min exposure to MUC-1 receptor expressed cells. This fact confirms characteristics of MR imaging nano-generated molecular probe. In other word, specific MUC-1 binding and specific activity of nano-probe and also dendrimer conjugation with Gd^{3+} loading to C595 mab did not produce any dynamic complications. Immunoreactivity assay also showed a good MUC-1 reactivity between Gd-dendrimer-C595 nanoprobe in comparison with unbound C595 (Figure 6A and B).

The interesting capability of anionic linear dendrimer G2 is regarding to its citric acid shell which raise potent Gd^{3+} complex formation as well as Gd^{3+} loading. The negative charge of dendrimer G2 protect nanoprobe from any surface-surface toxic interactions between the normal cell body and the conjugate.

One of the main criteria of biomolecular conjugation is regarding to not producing any biological activity complications. The complications occur while one or both biomolecules active site (covering kinetic) suffers an inactivation and this is observed sometimes occasionally [16,18-22]. Based on the obtained *in vitro* observations here, C595 anti-MUC-1 serves its activity after dendrimer conjugation and this fact leads to next nano-conjugate *in vivo* success. In confirmation, there are some reports on the anticancer activity of unbound C595 which suggests anticancer effects for C595 [20-22].

Conclusions

The present study shows a successful strategy in generating a powerful nanosize probe considering the biocompatibility and appropriate Gd^{3+} loading capability to find and detect breast cancer cells. The capability of nanosize contrast agent to produce enough relaxivity (^{1}H-NMRD and ^{17}O-NMR profiles) and specific binding to MUC-1 receptor showed that Gd^{3+}-ALGDG$_2$-C595 nano-probe is potentially, a selective breast molecular imaging contrast agent. Further *in vivo* investigations and subsequent clinical trials appear warranted.

References

1. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, et al. (2003) Cancer statistics. CA Cancer J Clin 53: 5-26.
2. Eccles SA, Box G, Court W, Sandle J, Dean CJ (1994) Preclinical models for the evaluation of targeted therapies of metastatic disease. Cell Biophys 25: 279-291.
3. Walther HE (1951) Simultaneous occurrence of malignant tumors and systemic diseases. Radiol Clin 20: 405-412.
4. Mettlin CJ, Murphy GP, Ho R, Menck HR (1996) The National Cancer Data Base report on longitudinal observations on prostate cancer. Cancer 77: 2162-2166.
5. Galasko C (1981) The anatomy and pathways of skeletal metastases. Bone Metastases. Boston: GK Hall 49-63.

6. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, et al. (1994) Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 79: 315-328.

7. Wang L, Chen H, Pourgholami MH, Beretov J, Hao J, et al. (2011) Anti-MUC1 monoclonal antibody (C595) and Docetaxel markedly reduce tumor burden and ascites and prolong survival in an *in vivo* ovarian cancer model. PLoS ONE 6: e24405.

8. Yang DJ, Kim CG, Schechter NR, Azhdarinia A, Yu DF, et al. (2003) Imaging with 99m-Tc ECDG targeted at the multifunctional glucose transport system: feasibility study with rodents. Radiology 226: 465-473.

9. Geraldes CF, Laurent S (2009) Classification and basic properties of contrast agents for magnetic resonance imaging. Contrast Media Mol Imaging 2009 4: 1-23.

10. Haririan I, Shafiee-Alavidjeh M, Khorramizadeh MR, Shafiee-Ardestani M, Ghane ZZ, et al. (2010) Anionic linear-globular dendrimer-cis-platinum (II) conjugates promote cytotoxicity in vitro against different cancer cell lines. Int J Nanomed 5: 63-75.

11. Alavidjeh MS, Haririan I, Khorramizadeh MR, Ghane ZZ, Ardestani MS, et al. (2010) Anionic linear-globular dendrimers: biocompatible hybrid materials with potential uses in nanomedicine. J Mater Sci Mater Med 21: 1121-1133.

12. Amanlou M, Siadat SD, Ebrahimi SE, Alavi A, Aghasadeghi MR, et al. (2011) Gd^{3+}-DTPA-DG: novel nanosized dual anticancer and molecular imaging agent. Int J Nanomed 6: 747-763.

13. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275.

14. Shahbazi-Gahrouei D, Roufeh M, Tavakoli MB (2006) Gadolinium-diethylenetriaminepenta-acetic acid conjugated with monoclonal antibody as new magnetic resonance imaging contrast agents for breast cancer (MCF-7) detection. Iranian Biomed J 10: 209-213.

15. Shahbazi-Gahrouei D (2009) Novel MR imaging contrast agents for cancer detection. J Res Med Sci 14: 141-147.

16. Shahbazi-Gahrouei D, Williams M, Allen BJ (2001) *In vitro* study of relationship between signal intensity and gadolinium-DTPA concentration at high magnetic field strength. Australas Radilo 45: 298-305.

17. Shahbazi-Gahrouei D, Williams M, Rizvi S, Allen BJ (2001) *In vivo* studies of Gd-DTPA-monoclonal antibody and Gd-porphyrins: potential magnetic resonance imaging contrast agents for cancer. J Magn Reson Imag 14: 169-174.

18. Koenig, SH, Brown, RD III (1987) NMR Spectroscopy of Cells and Organism; CRC Press: Boca Raton, FL. 756.

19. Von Mensdorff-Pouilly S, Gourevitch MM, Kenemans P (1989) An enzyme-linked immunosorbent assay for the measurement of circulating antibodies to polymorphic epithelial mucin (MUC-1).Tumor Biol 23: 186-195.

20. Parnaud G, Taché S, Peiffer G, Corpet DE (1999) Polyethylene-glycol suppresses colon cancer and causes dose-dependent regression of azoxymethane-induced aberrant crypt foci in rats. Cancer Res 15: 5143-5147.

21. Giray S, Bal T, Kartal AM, Kızılel S, Erkey C (2012) Controlled drug delivery through a novel PEG hydrogel encapsulated silica aerogel system. J Biomed Mater Res A 100: 1307-1315.

22. Fischer-Durand N, Salmain M, Rudolf B, Dai L, Juge L, et al. (2010) Site-specific conjugation of metal carbonyl dendrimer to antibody and its use as detection reagent in immunoassay. Anal Biochem 2010 15: 211-219.