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RESEARCH PAPER

Synergistic cellular toxicity and uptake effects of iodixanol conjugated to anionic linear globular dendrimer G2

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ABSTRACT

Objective(s): Early diagnosis of cancer using noninvasive imaging techniques has been discussed in several recent studies. The present study aimed to assess the synergistic effects of iodixanol-conjugated polyethylene glycol (PEG)-citrate (anionic linear globular) dendrimer G2 on MCF-7 breast cancer cells and human embryonic kidney 293 (HEK293) cells.

Materials and Methods: PEG-citrate dendrimer G2 was synthesized and purified. The product was characterized using atomic force microscopy (AFM), electron energy loss spectroscopy (EELS), dynamic light scattering (DLS). At the next stage, the product was conjugated to iodixanol, purified and lyophilized. The cytotoxic effects of the iodixanol, plain PEG-citrate dendrimer G2, and iodixanol-PEG-citrate dendrimer G2 complex were evaluated using methylthiazole-tetrazolium (MTT) assay on the MCF-7 and HEK293 cells. Inductively coupled plasma mass spectrometry (ICP MS) is a mass spectrometry technique, which applies inductively coupled plasma to ionize samples.

Results: According to the obtained results, the uptake of PEG-citrate dendrimer G2 iodixanol increased significantly compared to iodixanol alone ($P<0.05$), indicating the importance of lack of significant in-vitro toxicity. Moreover, in the particle size and higher negative zeta potential confirmed the loading of iodixanol in dendrimer G2. Increase, the loading of iodixanol in dendrimer was confirmed by the chemical shifts in HNMR.

Conclusion: Therefore, it was concluded that the addition of anionic linear globular dendrimer G2 to iodixanol affected the cellular uptake of the drug with no significant toxicity. Recent findings also confirmed that this novel complex could be applied as an effective cancer imaging agent for molecular biology and molecular imaging applications.

Keywords: *Cellular Toxicity, Iodixanol, Linear Globular Dendrimer*

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INTRODUCTION

Having struggled with cancer for many years, scientists have perennially been concerned with finding early diagnostic techniques to help cure cancer in the early stages. According to annual cancer statistics, cancer is one of the five leading causes of death across the world [1]. Early cancer diagnosis has been discussed in several recent studies, as well as the methods used by scientists for cancer diagnosis, such as physical examination [2], genetic tests [3], endoscopy [4], biopsy [5], and imaging modalities[6].

Among various diagnostic methods, imaging modalities have advanced dramatically in recent years. With the advent of molecular imaging, studies have been focused on the development of new imaging agents with lower toxicity and higher accuracy compared to the conventional methods. In this regard, various types of nanoparticles have been employed [7].

Nanoparticles are synthesized, Nano-sized particles that are used for drug delivery and disease diagnosis or treatment in recent pharmaceutical studies. Among various nanoparticles, dendrimers have been reported to have antiviral [8], antibacterial [9], imaging [10], and anticancer applications [11] in biomedical nanotechnology.

Dendrimers are composed of a core and

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sidelong branches [12] and could be charged positively (e.g., PAMAM [13]) or negatively (e.g., polyethylene glycol [PEG]-citrate dendrimer G2 [14]). In the present study, a negatively charged, globular dendrimer with a PEG core and citric acid side chains, known as PEG-citrate dendrimer G2, was employed. The dendrimer has great biodegradability and could be degraded in cells, while its citric acid side chains are used as an energy source in the citric acid cycle as one of the subsidiaries of the cycle. Moreover, the dendrimer is highly biocompatible, and the PEG core is excreted from the body through the kidneys with no changes after degradation in cells. Furthermore, the synthesis of this dendrimer is simple and cost-efficient, and the use of the dendrimer is associated with lower toxicity, higher monodisperses and purity, and lower molecular weight and hydrophilicity [15-17].

Iodixanol is a non-ionic iodinated contrast media, which is available on the market with the trademark Visipaque. It is also applied in computed tomography (CT)-scan [18]. However, this contrast media could cause various side-effects, ranging from mild allergic reactions to severe complications such as seizure and fainting [19-22].

Evaluation of the additive effects of PEG-citrate dendrimer G2-iodixanol on cancerous and noncancerous cells is considered to be the first step toward the assessment of the early diagnosis and prognosis of cancer.

The present study aimed to assess the additive effects of iodixanol-PEG-citrate (anionic linear globular) dendrimer G2 on MCF-7 breast cancer cell line and human embryonic kidney 293 (HEK293) cells.

MATERIALS AND METHODS

Materials

Iodixanol (VISIPAQUE™) was purchased from GE Healthcare Inc. (Cork, Ireland), and PEG 600, citric acid, dimethylformamide (DMF), and N, N'-dicyclohexylcarbodiimide (DCC) were supplied from Merck (Germany). In addition, N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride were obtained from Sigma-Aldrich (USA), and the HEK293 and MCF-7 cell lines were purchased from the Pasteur Institute (Iran). Dynamic light scattering (DLS), zeta potential, and electron energy loss spectroscopy (EELS) were measured using Malvern Nano ZS.

Atomic force microscopy (AFM) images, which were captured using the JPK Nano Wizard II. All other chemical reagents were of an analytical grade and used without additional purification.

METHODS

Synthesis of PEG-Citrate-dendrimer G2

Second-generation anionic PEGylated citric-based dendrimer (PEG-citrate-dendrimer G2) was synthesized based on the method proposed by Haririan I. et al. (2010) [23]. To do so, PEG (4 mL) was diluted in DMF (10 mL) followed byu the addition of DCC (15 g).

The reaction mixture was stirred for 30 minutes at room temperature. Afterwards, citric acid (1.42 g) was added, and the reaction mixture was stirred at 25°C for one hour.

Following that, DCC (4.4 mL) and DMF (10 mL) were added, and the reaction mixture was stirred for 15 minutes. At the next stage, citric acid (4.2 g) was added again, and stirring continued for 10 days at 25°C. After this period, PEG-citrate-dendrimer G2 was purified using the Sephadex G-50 column (GE Healthcare Life Sciences, UK), and the PEG-citrate-dendrimer G2 was freeze-dried.

Assessment of hydrodynamic size distribution, zeta potential, and EELS of PEG-citrate-dendrimer G2 using DLS

At this stage, DLS and EELS of PEG-citrate-dendrimer G2 were measured with the optical density of 0.2-0.3 at the wavelength of 633 nanometers using helium-neon laser under ambient conditions.

AFM images of PEG-citrate-dendrimer G2

The AFM images of the PEG-citrate-dendrimer G2 were recorded in double distilled water on a microscope slide in the intermittent contact mode (air) using the Nano Wizard II (JPK Instruments, Germany) at room temperature (25°C).

Iodixanol and PEG-citrate-dendrimer G2 mixture

In order for the quantitative *evaluation* of the synergistic effects of iodixanol and PEG-citrate-dendrimer G2, the agents were mixed gently on a shaker (model: Unimax 1010/Incubator 1,000; Heidolph Instruments, Germany) for 10 minutes at the temperature of 25°C (iodixanol:PEG-citrate-dendrimer G2: 1:2 and 1:1 molar ratios).

Quantification of cell viability and proliferation based on MTT assay

Human embryonic kidney (HEK293) and human breast cancer (MCF-7) cell lines were obtained from the national cell bank of the Pasteur Institute (Iran). The cells were cultured on either DMEM or RPMI meda supplemented with 10% fetal bovine serum in a humidified incubator containing 5% CO_2.

The cytotoxicity of PEG-citrate-dendrimer G2-iodixanol (at two different ratios) and free iodixanol on MCF-7 and HEK293 cell lines was assessed at the concentrations of 50, 100, 400, 500, 600, and 700 mg/ml using MTT assay 48 hours after treatment at the wavelength of 570 nanometers using a microplate reader.

Cellular uptake of PEG-citrate-dendrimer G2-iodixanol using inductively coupled plasma-mass spectrometry (ICP-MS)

The uptake of PEG-citrate-dendrimer G2-iodixanol (2:1 molar ratio) and free iodixanol into the MCF-7 and HEK293 cell lines was assessed using the inductively coupled plasma mass spectrometry (ICP-MS; model: Elan 6100 DRC-e, Perkin-Elmer, USA) based on the method proposed by Mohammadzadeh P. et al. (2017) [15] by measuring the amount of unabsorbed iodine by the cells. In brief, PEG-citrate-dendrimer G2-iodixanol (2:1 molar ratio) and free iodixanol were incubated with HEK293 and MCF-7 cells (30,000 cells per well) in cell plates for six hours. Afterwards, the cell medium was collected, and the cells were washed three times with phosphate buffered saline and collected each time. The iodine content of the samples was measured via ICP-MS (Table 1) and compared to the control samples (equivalent iodixanol without cells) in order to determine the level of unabsorbed iodixanol by the cells.

The amount of absorbed iodixanol by the cells was determined based on the difference between the total samples (PEG-citrate-dendrimer G2-iodixanol [2:1 molar ratio] and free iodixanol on the cells and free iodixanol without cells) and unabsorbed samples.

Statistical analysis

Data analysis was performed in Prism 5 software using one-way analysis of variance (ANOVA) and Tukey's post-hoc test. In all the statistical analyses, P-value of less than 0.05 was considered significant (n=3).

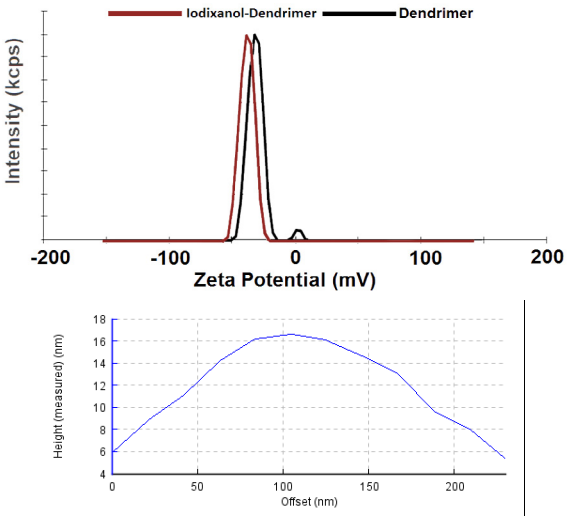

Fig 1. Zeta Potential (-31.3 mV) and Hydrodynamic Size Distribution (113 nm) of PEG-Citrate Dendrimer G2

RESULTS

Size, zeta potential, EELS, AFM, and H^1-NMR of PEG-Citrate-Dendrimer G2

Figs 1, 2, 3 and 5 shows the DLS, EELS, AFM, and H1-NMR of PEG-citrate-dendrimer G2 and iodixanol-dendrimer G2. Based on the AFM images, PEG-citrate-dendrimer G2 had narrow size distribution, globular structure, and smooth surface. Furthermore, the AFM analysis (Fig 3) confirmed the DLS and zeta potential results (Fig 1).

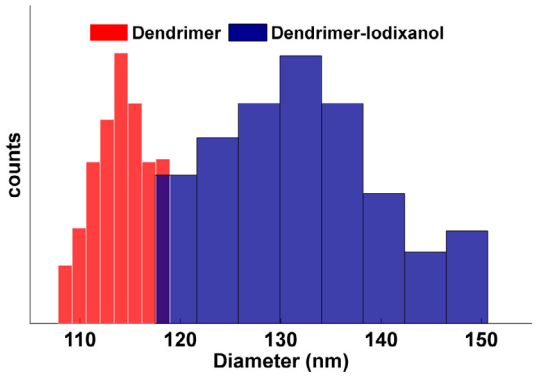

Fig 2. Electron Energy Loss Spectroscopy (EELS)

Accordingly, the mean size and zeta potential were 113 nanometers and -31.3 mV, respectively. Increase in the particle size and higher negative zeta potential confirmed the loading of iodixanol in dendrimer G2 (Figs 1).

Moreover, the loading of iodixanol in dendrimer was confirmed by the chemical shifts in HNMR: 2.1(C-H, methyl), 3 (C-H, diastereotopic),

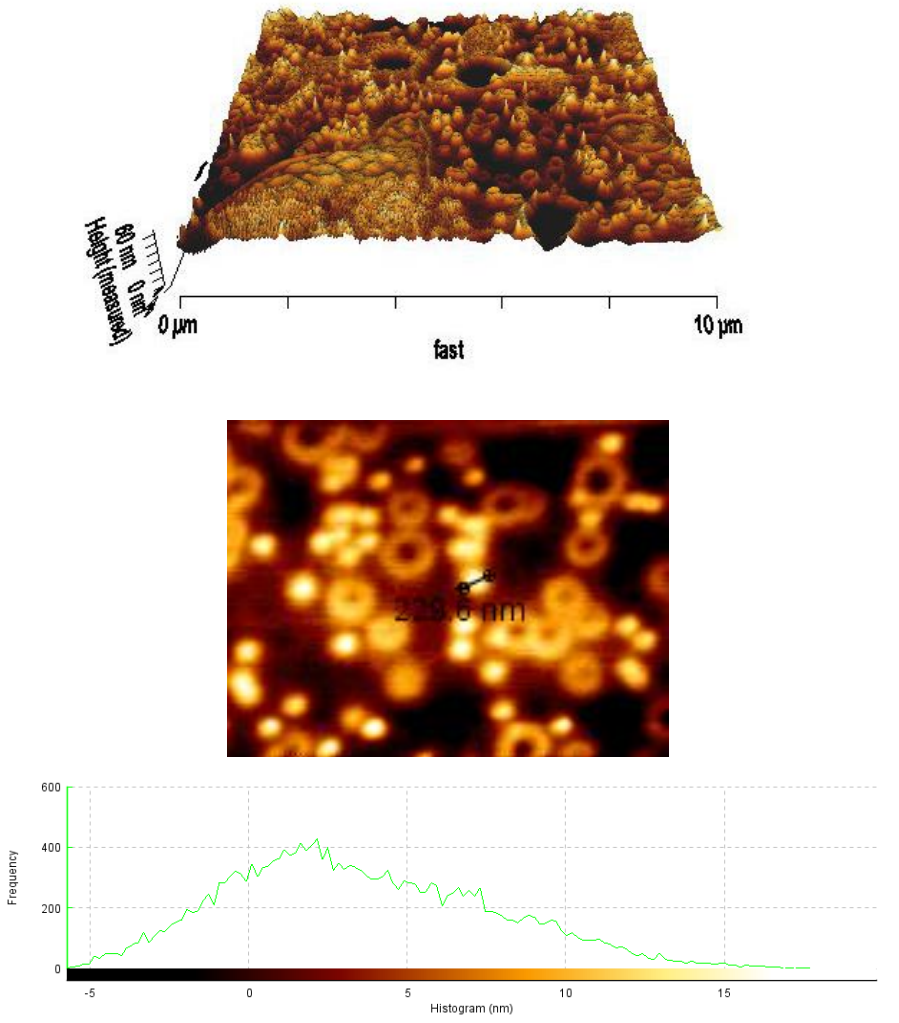

Fig 3. 2D and 3D AFM Images of PEG-Citrate-Dendrimer G2 (Histogram and cross-section graphs determined properties of particles in samples; physical size: 10.00x10.00 µm, mean value: 2.655 nm, mean roughness Ra: 4.566 nm, RMS roughness Rq: 6.153 nm, peak-to-valley roughness Rt: 65.68)

3.10 (C-H, diastereotopic), 3.65 (esteric linker), 5.1(C-H, methylene), 5.2 (O-H, methylene), 5.6 (O-H, methyn), 8.55 (N-H amide) ppm (Figs 5). Moreover, chemical shifts appeared at 5.60, 5.10, 3.65, and 3.10 ppm confirmed the successful synthesis of dendrimer (Fig 5).

Quantification of cell viability and proliferation using MTT assay

The anti-proliferative effects of PEG-citrate-dendrimer G2-iodixanol (at two different ratios) and free iodixanol on MCF-7 and HEK293 cell lines were assessed using MTT assay.

According to the findings, both PEG-citrate-dendrimer G2-iodixanol (at two different ratios) and free iodixanol on MCF-7 and HEK293 cell lines had no significant (P-value of less than 0.05) toxicity after 48 hours of treatment (Fig4).

Table 1. Indication of Unabsorbed Iodixanol Concentration in MCF-7 and HEK293 Cell Lines based on ICP-MS in Free Iodixanol and Iodixanol-PEG-Citrate-Dendrimer G2 (Data expressed as ppm; limit of quantity: 15 ppm)

	Iodixanol	PEG-Citrate-Dendrimer G2-Iodixanol
MCF-7	53 ppm	21 ppm
HEK293	52 ppm	21 ppm

Note: Data in table indicate unabsorbed iodixanol in specific cell types

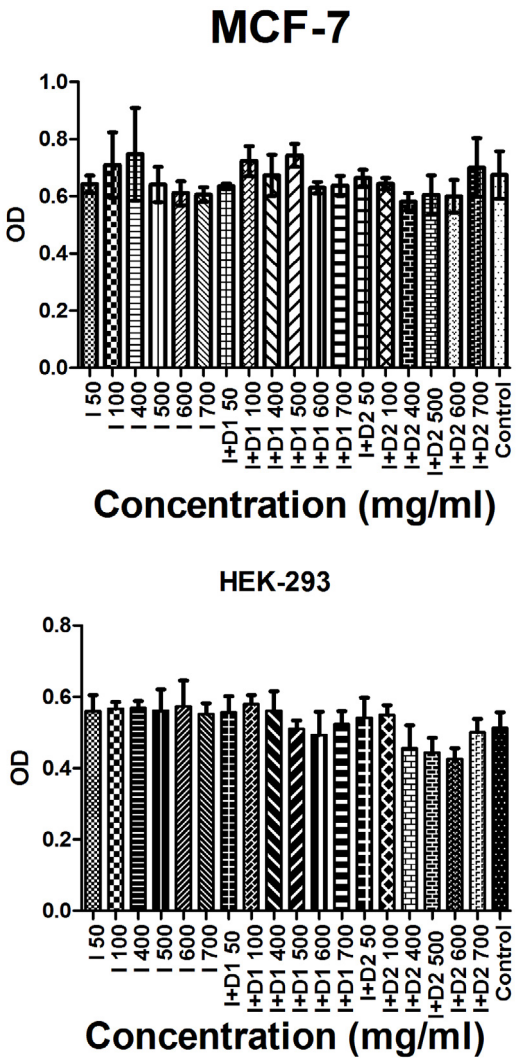

Fig 4. MTT Assay Results (n=4; P<0.05; Graphs showing no significant difference in toxicity of treated cells [iodixanol, PEG-citrate-dendrimer G2, and iodixanol-PEG-citrate-dendrimer G2] compared to control cells in cancerous and noncancerous samples

Evaluation of cellular uptake via the ICP-MS

According to the information in Table 1, the uptake of iodixanol by the dendrimer complex was significantly higher than compared to the uptake of free iodixanol.

Moreover, similar to the uptake of free iodixanol, the uptake of iodixanol-dendrimer conjugate remained similar in both HEK293 and MCF-7 cell lines.

On the other hand, the free iodixanol uptake by cells was considered insignificant when compared to the total iodixanol present in the medium without cells (Table 1).

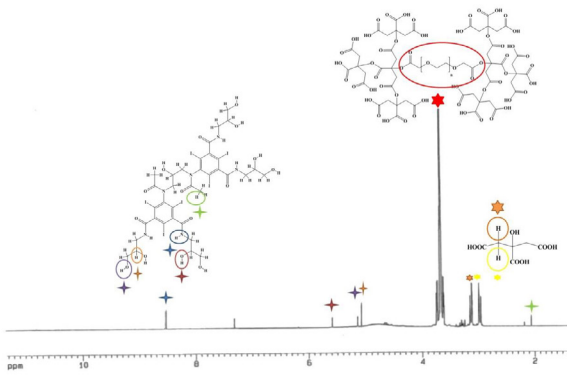

Fig 5. NMR spectroscopy

DISCUSSION

This was the first research to investigate the diagnostic and therapeutic applications of conjugated iodixanol to PEG-citrate dendrimer G2 in cancer diagnostic. To meet the objectives of the current research, PEG-citrate dendrimer G2 was synthesized based on the protocol reported by Haririan et al (2010) [23]. Following that, the product of the synthesis was confirmed using AFM, DLS, zeta potential, and EELS. In order to determine the synergistic effects of PEG-citrate dendrimer G2 and iodixanol, they were mixed. Finally, cellular tests were conducted on cancerous MCF-7 and normal HEK293 cell lines for the comparison of the effects, including in-vitro cellular uptake test via the ICP-MS and in-vitro cytotoxicity test.

In a study in this regard, Namazi et al. (2005) reported the first successful synthesis of the second generation of an anionic linear globular dendrimer (PEG-citrate dendrimer G2) [24]. In 2010, the research procedure involving various methods to investigate the characterization and effects of this new generation in comparison with the first generation (anionic linear globular dendrimer G1) was reported. According to the obtained results, the second generation of dendrimers (concentration up to 0.5 mg/ml) exerted no significant cytotoxic effects on normal cells. However, the first generation of dendrimers was reported to have higher toxicity compared to the second generation, among other reported properties were higher flexibility and polydispersity in size and lower negative charge. Furthermore, it has been confirmed that nanoparticles could be applied extensively in nanomedicine research. PEG-citrate dendrimer

G2 has been of great interest in several studies and applied as a carrier of antiviral, antibacterial, and imaging agents, as well as anticancer drugs [25]. Comparison of PEG-citrate dendrimer G2 with other dendrimers (e.g., PAMAM, a positively charged nanoparticle) has demonstrated its negatively-charged nature could prevent toxicity. Moreover, the disruptive interaction of positively charged nanoparticles with the cell membrane of healthy cells has been confirmed.

In another research in this regard, Behrouz et al. (2017) reported that PAMAM dendrimer in conjugation with AS1411 aptamer carrying 5-FU could be used in cancer treatment [26]. On the other hand, Mohammadzadeh et al. (2017) reported the conjugation of AS1411 aptamer with PEG-citrate dendrimer G2 carrying iodixanol for the first time. Previous studies have also investigated the in-vitro and in-vivo studies of this agent [15]. Comparison of two studies in this regard indicates that negatively charged dendrimers because lower toxicity to healthy cells (noncancerous). Other superior properties of these agents include biocompatibility, biodegradability, easy synthesis compared to other nanoparticles (e.g., PAMAM and gold nanoparticles), and higher permeability in cancer cells due to the PEG core and hydrophilicity, making PEG-citrate dendrimer G2 a promising carrier in cancer diagnosis and therapy.

In another study, Ghoreishi et al. (2017) reported the use of ^{99m}Tc- labeled PEG-citrate dendrimer G2-chlorambucil in an in-vivo biodistribution study as a noninvasive method. According to the obtained results, the new nanoparticle could be successfully labeled with ^{99m}TC, and the biological assessments were acceptable as well [16]. In 2016, a research group used magnetic iodixanol in conjugation with magnetic nanoparticles, and their radio-opacity was examined. In the mentioned research, it was asserted that the conjugate could be applied as a proper contrast agent [27]. Similarly, Hainfeld et al. [28, 29] reported that gold nanoparticles could easily overcome the limitations of iodinated contrast agents, such as toxicity, fast clearance, and short imaging time. Newly reported research has also indicated that nanoparticles are frequently used along with iodinated contrast media (e.g., iodixanol), which may decrease the side-effects and in-vivo limitations of other contrast agents.

In the current research, the synthesis of PEG-citrate dendrimer G2 was confirmed by AFM, DLS, zeta potential, and EELS. The obtained results demonstrated a new dendrimer with negative charge, spherical shape, and smooth structure. On the other hand, the in-vitro cellular toxicity results showed no statistically significant difference in toxicity between the unloaded dendrimer and dendrimer-iodixanol conjugate on both cancerous and normal cells.

According to the findings of the current research regarding *in vitro* cellular uptake, cellular uptake significantly enhanced after conjugating iodixanol with the dendrimer in the normal cells. Since in vivo imaging applications require complexes with lower toxicity, our findings indicated that a new complex which could be used for cancer imaging to reveal the optimal media for diagnostic imaging.

In the present study, the significant synergistic effects of iodixanol-PEG-citrate dendrimer G2 on the cancerous and noncancerous cells indicated that it is a nontoxic, agent which is strongly taken up by cancer cells. Therefore, it could be concluded that the new complex is a promising diagnostic media in vitro, while it could also be applied in future in vivo experimentations.

CONCLUSION

In this study, the synergistic effects of iodixanol-PEG-citrate dendrimer G2 on cancer cells were compared to that of normal cells. Interestingly, the designed combination (iodixanol-PEG-citrate dendrimer G2) could be a promising imaging agent for cancer diagnosis with no cytotoxic effects against human normal cells.

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