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Original Article

Toxicity of Thorium Oxide Nanoparticles Conjugated with Folic acid and Antibody on the Blood Mononuclear Cells from Patients with Chronic Lymphocytic Leukemia and Normal Persons

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Background and Aims: The aim of this study was to synthesize antibody-conjugated radioactive(thorium oxide) nanoparticles (ACRNPs) and folic acid-conjugated radioactive nanoparticles (FACRNPs).

Materials and Methods: After synthesis of nanoparticles, blood samples from CLL patients and normal subjects were obtained, and their mononuclear cells were isolated by Ficoll method. To evaluate cytotoxicity, serial concentrations of ACRNPs and FACRNPs were separately added to cancerous and normal mononuclear cells, incubated for 12 hours at 37 ºC, and then different assays including MTT, MTS, cell metabolic, and ATP assay were carried out. On the other hand, the mononuclear cells were captured by anti-CD20 antibody in the polystyrene tube, and then ACRNPs and FACRNPs were separately added to them. After washing, the radioactivity (counts per minute (CPM)) of each tube was read.

Results: The images obtained from electron microscopy showed that both ACRNPs and FACRNPs were spherical with the same size (near 50 nm). This study obviously demonstrated a direct relationship between CPM and concentration of ACRNPs and FACRNPs in both cancerous and normal samples. Also, there was a significant difference between CPM of cancerous and normal samples after treatment with ACRNPs or FACRNPs ($P<0.05$). However, an inverse relationship between concentration of ACRNPs or FACRNPs and their toxicity. Was found Significant difference was observed between toxicity of ACRNPs or FACRNPs on the cancerous and normal samples ($P<0.05$).

Conclusion: It can be concluded that both ACRNPs and FACRNPs have good efficacy for detection of CLL cells.

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Introduction

Based on the previous studies, radioactive atoms which bound to monoclonal antibodies or other ligands have been used in the radio-immunotherapy and radio-immunoassay [1-7]. In the brachy therapy, the radioactive microparticles such as ^{125}I, ^{103}Pd, and ^{90}Y are used for internal radiation [8-10]. It is a fact that the size of intercellular fenestrations of tumor vasculature (150–300 nm) is smaller than the size of radioactive microparticles (50–100 μm), and this leads to increased intravascular retention, high side effects, and low anticancer activity [11]. Recently, studies have revealed that the use of radioactive nanoparticles (RNPs) can improve brachy therapy [12-14] and may be applied for detection of cancers. Wang et al. showed that β-emission of ^{186}Re integrated nanoliposomes has high therapeutic effect on the squamous cell carcinoma in the nude rat xenograft model [15]. Also, Zavaleta et al. worked on pharmacokinetics and biodistribution of ^{186}Re-biotin-nanoliposomes and showed a 30% decrease in tumor volume compared with the control group [16]. Bouchat et al. showed that treatment of the highly vascularized tumors could be done by RNPs [17]. Chanda et al. demonstrated a decrease in prostate cancer size by radioactive gold nanoparticles conjugated with epigallocatechin-gallate (EGCg) [18]. It must be explained that although these nanoparticles can kill cancer cells, they can damage normal cells, too.

Among different radioactive nanoparticles, thorium oxide (ThO_2) nanoparticle is very cheap and safe. Moreover, based on our experience, different chemical modifications can be made on its surface. In this research, ThO_2 nanoparticles were selected, because of low cost, low radioactivity, and high safety compared with other commercial radioactive materials.

The aim of this study was to synthesize antibody-conjugated radioactive nanoparticles (ACRNPs) and folic acid-conjugated radioactive nanoparticles (FACRNPs). Then, the cytotoxicity of these nanoparticles was investigated on the mononuclear cells from patients with chronic lymphcytic leukemia (CLL) and from normal persons. Also, the potential use of ACRNPs and FACRNPs was studied for detecttion of cancer cells by counting of radioactivity. To date, no study has detected such effects.

Materials and Methods

ThO_2 nanoparticles were purchased from Zyst Fannafar Shargh Company, Iran. N-ethyl-N-(dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), carboxy polyethylene glycol (CPEG) with molecular weight 3,400 daltons, Hanks balanced salt solution (HBSS), 3-(4,5-dimethylthiazol-2)-2,5 diphenyltetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-tetrazolium (MTS), and CD20 antibody were obtained from Sigma-Aldrich Chemical Company, USA. Folic acid was sourced from

Aburaihan Co., Iran. Alamar Blue and ATP kit were provided from Gibco Invitrogen, UK.

Synthesis of conjugated RNPs

PEGylation

Firstly, 100 mg of CPEG was dissolved in 10 mL of phosphate buffered saline (PBS) at pH 6.0. Then, one gram of ThO_2 nanoparticles was added to 10 mL of CPEG solution, shaken gently, and incubated for 24 hours at 37 ºC. After incubation, ThO_2 nanoparticles were centrifuged at 10000 rpm for 15 minutes, the supernatant was discarded, and then nanoparticle pellets were re-suspended in PBS. The final concentration of ThO_2 nanoparticles was 0.1g/mL.

Conjugation of antibody and folic acid with RNPs

To conjugate folic acid with CPEG-coated-ThO2 nanoparticles, one mL of EDC at concentration of 50 mM, one mL of NHS at concentration of 60 mM, 5 mL of CPEG-coated-ThO_2 nanoparticles at concentration of 100 mg/mL, and one mL of folic acid at concentration of 0.2 mg/mL were mixed and incubated at 37 ºC for 1 hour. To conjugate anti-CD20 antibody with CPEG-coated-ThO2 nanoparticles, one mL of 50 mM EDC, one mL of 60 mM NHS, and 5 mL of 100 mg/mL CPEG-coated-ThO_2 nanoparticles, and one mL of 0.2 mg/mL anti-CD20 antibody were added and incubated at 37 ºC for 1 hour. After incubation, the excess EDC, NHS, antibody, and folic acid were removed by centrifugation and the pellet was re-suspended in PBS buffer. Finally, serial concentrations (25, 50, 100, 200, and 400 µg/mL) of two conjugates were prepared in RPMI1640.

To confirm the attachment of CPEG to ThO2 nanoparticles, and conjugation of antibody and folic acid, Fourier transform infrared spectroscopy (FTIR) (ELICO, India) was used. The structure and distribution size of ACRNPs and FACRNPs were studied by scanning electron microscopy (SEM) (Hitachi, S-2400, Japan) and dynamic light scattering (DLS) (Malvern Instruments, Italy), respectively.

Preparation of mononuclear cells

Five patients with CLL and five normal persons who had signed an informed consent form enrolled in this study. All were male, 30-50 years old, and non-smoker. Adequate matching between the patients and the controls was considered. The stage of CLL was confirmed by an expert pathologist. five mL of venous blood was obtained from each person, put in a heparinized plastic tube, and its mononuclear cells were separately isolated by Ficoll method. After washing with RPMI1640, the final concentration of cells was adjusted to 10^3 cells/mL.

Cytotoxicity assays

MTT assay

Firstly, 100 µL of ACRNPs and FACRNPs at serial concentrations were separately added to 100 µL of mononuclear cell suspension (10^3 cells) and then were incubated for 12 hours at 37 ºC. After incubation, cells were washed with HBSS to remove any nanoparticles, 25 µL of 5 mg/mL MTT was added and incubated at 37 ºC for 5 hours. Then, 100 µL of 70% v/v isopropanol was added to each well, and optical density (OD) of each well was read by an enzyme-linked immunosorbent assay (ELISA) reader (Novin Gostar Co., Iran) at

490 nm. Finally, all data were normalized to control, i.e., by OD of the each sample was divided to OD of negative control. The cells which were not exposed to nanoparticles were considered as negative control.

MTS assay

Briefly, 100 µL of serial concentrations of ACRNPs and FACRNPs were separately added to 100 µL of mononuclear cell suspension (10^3 cells), and incubated at 37 ºC for 12 hours. After incubation and washing with HBSS, 25 µL of 5 mg/mL MTS was added and incubated at 37 ºC for 5 hours. Then, the OD of each well was read at 490 nm by ELISA reader. Like MTT assay, all data were normalized to control, i.e., the OD of each well was divided by OD of the negative control.

Cell metabolic assay

Firstly, 100 µL of serial concentrations of ACRNPs and FACRNPs were separately added to 100 µL of mononuclear cell suspension (10^3 cells). Then it was incubated at 37 ºC fore 12 hours, and washed with HBSS. Later, 100 µL of the Alamar Blue reagent was added to each well, and incubated for 4 hours at 37 °C. After incubation, the OD of each well was read at 590 nm by ELISA reader, and was normalized to control, i.e., the OD of each well was divided by OD of the negative control that was not treated with nanoparticles.

ATP assay

At first, 100 µL of serial concentrations of ACRNPs and FACRNPs was separately added to 100 µL of mononuclear cell suspension (10^3 cells), incubated at 37 ºC for 12 hours and washed with HBSS. Then, 100 µL of ATP determination reagent and 100 µL of RPMI1640 were added, and incubated for 10 minutes at room temperature. In the final step, the OD of each well was read by a luminometer (model 9100-102, Turner Biosystems, UK), and then all data were normalized to control, i.e., the OD of each well was divided by OD of the negative control which was not treated with nanoparticles.

Detection of cancer cells

Briefly, polyethylene tubes were coated with anti-CD20 monoclonal antibody by overnight incubation at 4 ºC, and the unbound antibodies were removed by washing with PBS. Next, heparinized blood samples from patients with CLL (n=10) and normal persons (n=10) were obtained.

The mononuclear fraction of both CLL and normal samples were isolated by Ficoll method [19], washed with PBS, and then one mL of RPMI1640 was added to them (the final cell density was 1000 cells/mL).

In the next step, 100 µL of both cancerous and normal cell suspension were separately added to anti-CD20 coated tubes, incubated for 1 hour at 37 ºC, and unbound cells were washed three times by PBS. Then, one mL of ACRNPs and FACRNPs was separately added to tubes, and incubated at 37 ºC for 1 hour.

All tubes were washed with HBSS, radioactivity of each tube was determined by beta counter (Biosystem, UK), and then the mean of counts per minute (CPM) of each tube was read. The cells which were not treated with any RNPs were considered as negative control.

The study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences.

Statistical analysis

All experiments were conducted three times and the data are shown as mean ± standard deviation (SD). The SPSS software was used for statistical analysis, and P-value <0.05, obtained by Student t-test, was considered as the level of statistically significant difference.

Results

Characterization of conjugated RNPs

The SEM images of ACRNPs and FACRNPs are shown in Figure 1a and Figure 1b, respectively. As is demonstrated, both of them were spherical and had the same size (near 50 nm). The FTIR spectra of ThO_2 nanoparticles (I), folic acid (II), antibody (III), FACRNPs (IV), and ACRNPs (V) are shown in Figure 1c. As is seen, FACRNPs and ACRNPs have the specific peaks of folic acid and antibody which confirms the binding of folic acid and antibody to nanoparticles. The DLS results are shown in Figures 1d and 1e. As is seen, the size distribution of ACRNPs and FACRNPs was approximately 40-100 nm.

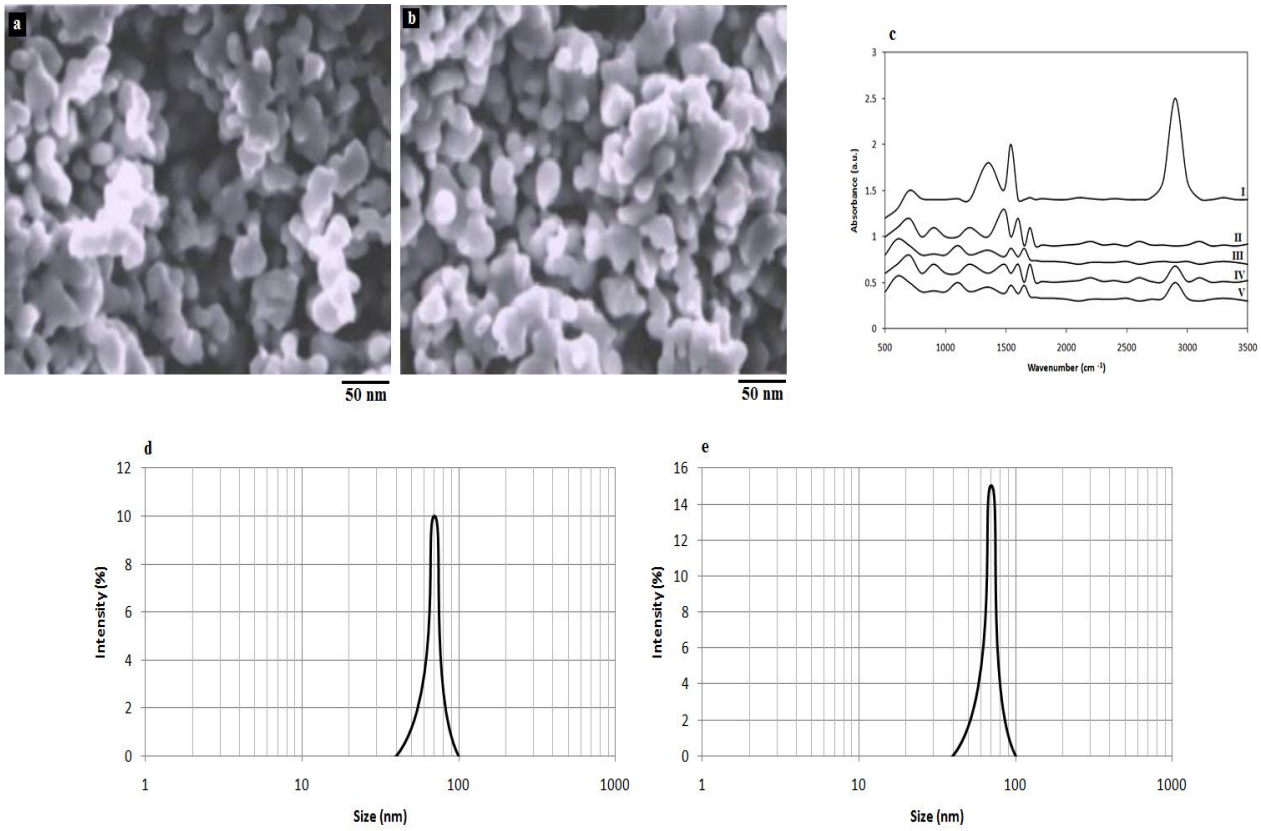

Fig. 1. The SEM images of ACRNPs (a) and FACRNPs (b); The FTIR spectrum (c) of ThO_2 nanoparticles (I), folic acid (II), antibody (III), FACRNPs (IV), and ACRNPs (V); The DLS graph of ACRNPs (d) and FACRNPs (e).

Detection of cancer cells by radioactivity measurement

Figures 2a and 2b show the CPM of tubes after treatment with ACRNPs and FACRNPs, respectively. This test obviously demonstrated a direct relationship between CPM and concentration of ACRNPs and FACRNPs in both cancer and normal samples. Also, there was a significant difference between CPM of cancer and normal samples after treatment with ACRNPs or FACRNPs ($P<0.05$). However, no significant difference was identified between ACRNPs and FACRNPs ($P>0.05$).

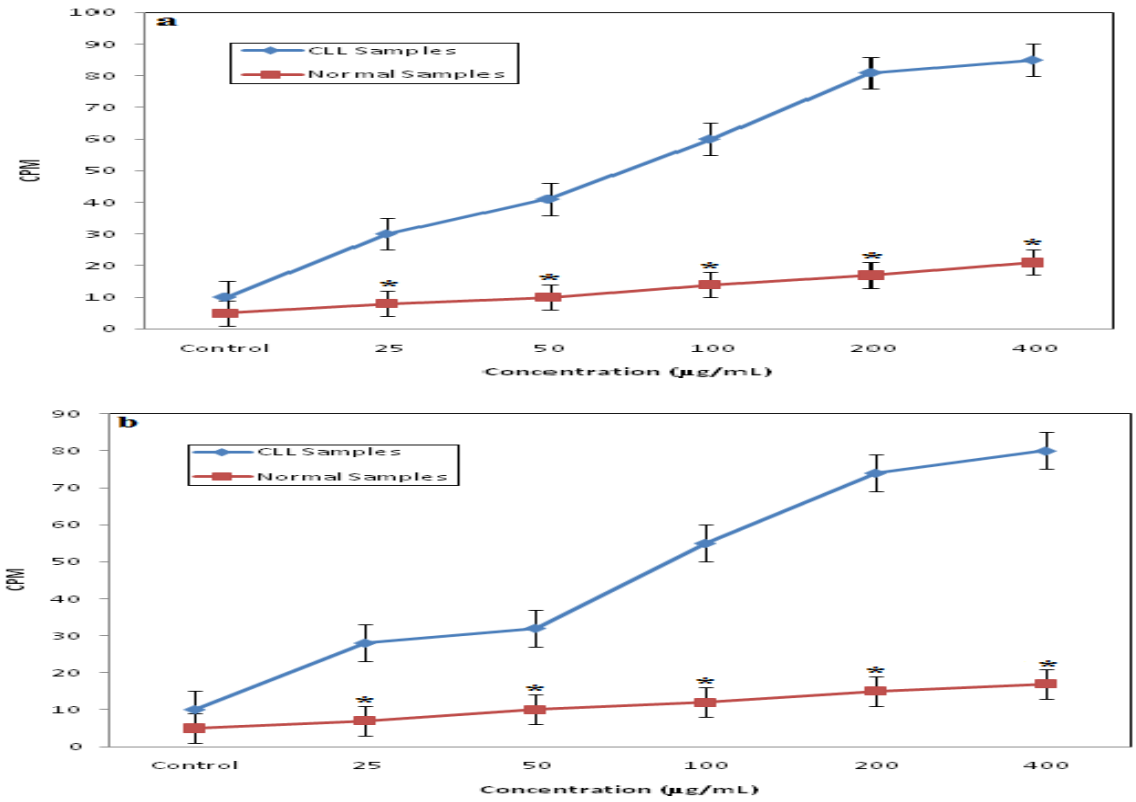

Fig. 2. The CPM of tubes after treatment with ACRNPs (a) and FACRNPs (b). * $P<0.05$ compared with CLL samples at the same concentration.

Cytotoxicity of ACRNPs and FACRNPs

To evaluate toxicity, different experiments were carried out. The results of MTT, MTS, Alamar Blue, and ATP assays are shown in Figures 3, 4, 5, and 6, respectively. Each figure has two parts: (a) is the toxicity of ACRNPs on the cancer and normal cells, and (b) is the toxicity of FACRNPs on the cancer and normal cells. There was an inverse relationship between concentration of ACRNPs or FACRNPs and their toxicity. There was a significant difference between toxicity of ACRNPs or FACRNPs on the cancer and normal samples ($P<0.05$). We did not observe any significant difference between toxicity of ACRNPs and FACRNPs ($P>0.05$).

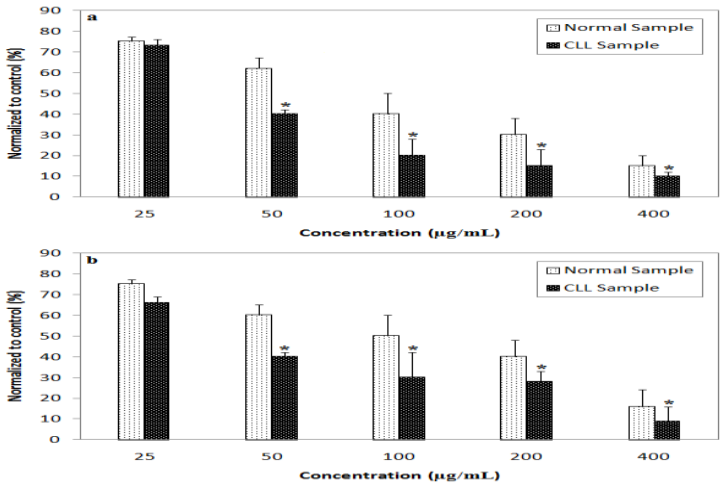

Fig. 3. The toxicity of ACRNPs (a) and FACRNPs (b) obtained by MTT assay. * P<0.05 compared with normal samples at the same concentration.

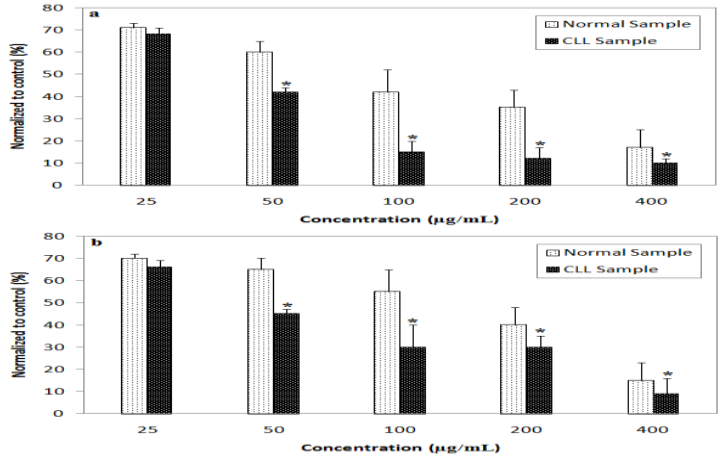

Fig. 4. The toxicity of ACRNPs (a) and FACRNPs (b) obtained by MTS assay. * P<0.05 compared with normal samples at the same concentration.

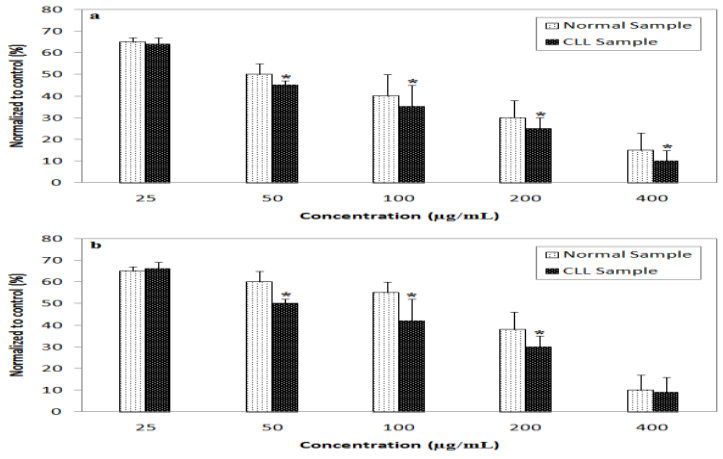

Fig. 5. The toxicity of ACRNPs (a) and FACRNPs (b) obtained by Alamar Blue assay. * P<0.05 compared with normal samples at the same concentration.

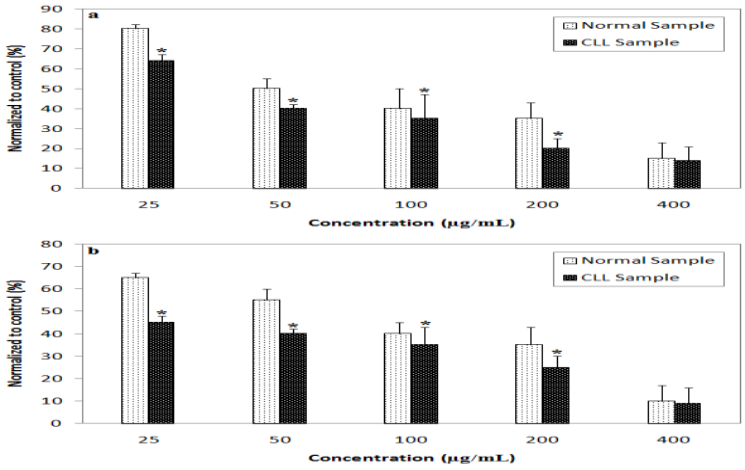

Fig. 6. The toxicity of ACRNPs (a) and FACRNPs (b) obtained by ATP assay. * P<0.05 compared with normal samples at the same concentration.

Discussion

In this study, ACRNPs and FACRNPs were synthesized, and their toxicity on the cancer and normal blood mononuclear cells was studied. ThO_2 nanoparticles which were extracted from grid of gas lamp were used because of low cost, low radioactivity, and high safety compared with other commercial radioactive materials. However, it must be mentioned that different radioactive isotopes can be used for medical applications [11,17,18]. To synthesize ACRNPs and FACRNPs, ThO_2 nanoparticles were covered with CPEG, activated by EDC/NHS, and then reacted with antibody and folic acid. This method of conjugation was introduced in the previous studies [20, 21].

This research clearly showed that the use of ACRNPs and FACRNPs leads to higher CPM in cancer samples than normal samples, but no difference was seen between CPM of ACRNPs and FACRNPs. On the other hand, it demonstrated that both ACRNPs and FACRNPs had higher toxicity on the cancer samples than normal samples.

As was demonstrated, in both radioactivity and toxicity experiments, the results were dose-dependent. This means that the highest concentration of ACRNPs and FACRNPs has the highest CPM and toxicity.

To date, there is no data on the toxicity of ThO_2 nanoparticles or conjugated ThO_2 nanoparticles. For the first time, this study proposed that conjugated ThO_2 nanoparticles have higher toxicity on the CLL cancer cells than normal cells. The authors hypothesize that the high level of folic acid receptor and CD20 on the CLL cancerous cells may be the reason for this difference. It was explained that folic acid receptor and CD20, over-expressed on CLL cancer cells, attach to ACRNPs and FACRNPs. The folate receptor is a glycosylphosphatidylinositol (GPI)-anchored, high-affinity membrane folate binding protein which is over-expressed in a wide variety of human tumors. Since normal tissue distribution of folate receptor is highly restricted, it provides a useful marker for targeted drug delivery. According to previous studies, folic acid is potentially superior to

antibodies as a targeting ligand because of its small size, lack of immunogenicity, ready availability, and defined conjugation chemistry [22-24]. It must be mentioned that CD20 is expressed from late pro-B cells through memory cells, and it is found that CD20 is over-expressed in B-cell lymphomas, hairy cell leukemia, B-cell chronic lymphocytic leukemia, and melanoma cancer stem cells [25, 26].

For the first time, this study presented a new radioactive nanoparticle for detection and simultaneous treatment purposes. Previously, other RNPs have been used by other researchers. For example, Chanda et al. worked on gum Arabic glycoprotein-functionalized radioactive gold nanoparticles for targeting and destroying tumor cells [18]. They showed that these nanoparticles had significant therapeutic efficacy. In another study, Bouchat et al. demonstrated that $^{90}Y_2O_3$ RNPs can be applied for treatment of vascularized tumors. They indicated that normal and tumoral cells are not equally sensitive to radiation [17]. At the University of Missouri, researchers showed that radioactive gold nanoparticles conjugated with epigallocatechin-gallate (EGCg) could treat prostate cancer cells [18]. Recently, some studies declared that the use of RNPs could improve brachy therapy efficacy [12-14]. According to these studies, RNPs are an ideal choice for detection and treatment of cancers in future. But their biocompatibility, tolerance, and toxicity must be evaluated in vivo and in clinical trials. In conclusion, this study shows that ThO_2 RNPs conjugated with folic acid and antibody deliver a higher toxicity on the CLL cancer cells than normal cells, and may be used for treatment of cancer cells in future.

Conflict of Interest

The authors declare no competing financial interest.

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