

Synthesis, Radiolabeling, and Biological Evaluation of an RGD-Conjugated ^{99m}Tc Dendrimer for Breast Cancer SPECT Imaging

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Abstract

Introduction:

Breast cancer remains one of the leading causes of cancer-related mortality worldwide. Early and accurate molecular imaging is essential for improving diagnosis and patient outcomes. Nanotechnology-based imaging probes offer promising opportunities to overcome limitations associated with tumour heterogeneity.

Materials and Methods

Third-generation polyethylene glycol (PEG)–citrate dendrimers were synthesised and functionalised with arginylglycylaspartic acid (RGD) peptides targeting $\alpha\beta3$ integrins. The nanoconjugates were radiolabeled with technetium-99m (^{99m}Tc) and characterised using nuclear magnetic resonance (NMR), liquid chromatography–mass spectrometry (LC–MS), Fourier-transform infrared (FTIR) spectroscopy, dynamic light scattering (DLS), and electron microscopy. In vitro cytotoxicity was assessed using the XTT assay. In vivo imaging and biodistribution studies were conducted in tumour-bearing nude mice using single-photon emission computed tomography/computed tomography (SPECT/CT).

Results

The synthesised dendrimers demonstrated favourable physicochemical properties and high radiochemical purity. In vitro assays confirmed good biocompatibility, with no significant cytotoxicity observed at concentrations up to 8 mg/mL. SPECT imaging revealed rapid and specific tumour accumulation within the first hour post-injection, with maximal uptake observed at approximately 90 minutes. Biodistribution studies indicated predominant renal clearance and minimal nonspecific uptake in non-target organs. The radiolabeled nanoconjugates remained stable in human serum for up to 24 hours.

Conclusion

RGD-functionalized, ^{99m}Tc -labeled PEG–citrate dendrimers exhibit strong potential as targeted SPECT imaging agents for breast cancer. Their favourable targeting specificity, imaging contrast, and pharmacokinetic profile support further development for molecular imaging and theranostic applications.

Introduction

Breast cancer (BC) continues to represent a major global health burden, ranking second among causes of cancer-related mortality after lung cancer, while remaining the primary cause of cancer-associated death in women worldwide (1). According to global estimates from 2022, breast cancer was the most commonly diagnosed malignancy, with approximately 2.3 million newly reported cases and nearly 670,000 associated deaths (2). According to GLOBOCAN 2022, BC accounts for over 11% of all global cancer cases, with mortality rates highest in low- and middle-income regions due to barriers in early detection and limited access to treatment. As the global population grows and ages, the burden of breast cancer is projected to increase substantially in the coming decades, intensifying this public health challenge (3). Early detection of malignant cells is essential for improving treatment outcomes and overall survival in cancer patients. Current detection methods utilise various imaging modalities, including ultrasound imaging (4), digital mammography (5, 6), computer-aided detection systems (7, 8) and magnetic resonance imaging (MRI) (9, 10). However, the phenotypic and genotypic heterogeneity within and between tumours presents significant obstacles to accurate diagnosis and effective therapeutic decision-making. Histopathological biomarkers, such as estrogen, progesterone, and HER2 receptor expression, are critical for stratifying breast cancer subtypes and guiding clinical management (11, 12). Ongoing research efforts are focused on identifying diagnostic approaches capable of detecting specific molecular signatures associated with pre-malignant and malignant breast tumors at early stages. These efforts include optimising existing detection platforms and developing novel imaging strategies for early cancer diagnosis (13, 14).

Recent advancements in molecular imaging technologies, such as Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET), have significantly enhanced diagnostic capabilities (15) have improved the detection of tumour-specific biomarkers, such as HER2, across various tissues. These technologies enable non-invasive differentiation of pathological changes and provide critical insights into tumour biology (16). Nanoparticles (NPs) demonstrate significant potential for selective tumour targeting due to their precisely controlled nanoscale dimensions, typically ranging from 10 to 100 nm (17). Nanoparticles engineered for radionuclide imaging have attracted considerable interest in both preclinical research and emerging clinical applications (18). In nuclear medicine, dendrimers are increasingly investigated to improve tumour diagnosis and therapeutic management. The established strategy of conjugating nanodrugs to water-soluble, nontoxic, and biocompatible polymeric carriers offers benefits such as prolonged circulation time and reduced systemic toxicity (19–22). Dendrimers possess several advantages over linear polymers, including precise control of surface functionalities and the capacity to conjugate with diverse therapeutic agents, targeting molecules, and imaging agents. These features make dendrimers promising theranostic agents for cancer imaging and therapy. However, their high peripheral cationic charge density can cause cytotoxicity by disrupting cell membrane integrity through interactions with negatively charged phospholipids (23).

The generation of dendrimers is crucial in defining their physicochemical and biological characteristics. Higher-generation dendrimers, such as third-generation (G3) dendrimers, contain a greater number of peripheral functional groups, which facilitate the attachment of larger quantities of radionuclides and targeting agents. G3 dendrimers offer greater structural strength and more precise imaging signals than

second-generation (G2) dendrimers. Dendrimers functionalized with cancer-selective molecular markers can selectively target cancer cells, reducing nonspecific toxicity and improving therapeutic efficacy.

This study developed a novel breast cancer imaging agent using PEG-citrate dendrimers, functionalized with technetium-99m (^{99}mTc) for imaging and arginyglycylaspartic acid (RGD) for targeting. This study introduces the first third-generation citric acid-based dendrimers for biomedical use, focusing on nuclear medicine.

Materials and Methods

Chemicals

Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), Trypsin/EDTA, streptomycin, fetal bovine serum (FBS), and penicillin were sourced from Gibco BRL (Eggenstein, Germany). HEK-293 cell lines and Female nude mice were supplied by the Pasteur Institute of Iran. Xylazine and Ketamine were purchased from Alfasan (Woerden, The Netherlands).

Dimethyl sulfoxide (DMSO), folic acid, hydrochloric acid (HCl), citric acid, adipic acid dihydrazide (ADH), and trichloroacetic acid were procured from Merck (Darmstadt, Germany). Polyethylene glycol (PEG) diacid 600, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), and N, N-dicyclohexylcarbodiimide (DCC) were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Dendrimer Fabrication and Conjugation

G2 dendrimers were synthesized according to previously described protocols. Polyethylene glycol diacid 600 was dissolved in DMSO. Dicyclohexylcarbodiimide in DMSO-d₆ was then added as the activating agent.

The mixture was continuously stirred at room temperature. Citric acid monomers were introduced and allowed to react for 24 hours while maintaining continuous stirring. Unreacted DCC was removed by precipitation in distilled water, followed by purification of the product through dialysis. The G1 dendrimer was then activated with DCC in DMSO-d₆ and reacted with citric acid under continuous stirring for 24 hours at room temperature to form the G2 dendrimer. The same purification procedure, involving the precipitation of residual DCC followed by dialysis, was applied. This synthetic cycle was repeated to obtain the third-generation dendrimer (G3) (Fig. 1).

Terminal amine (NH_2) functional groups were introduced by modifying the surface of the synthesized G3 dendrimer with adipic acid dihydrazide (ADH). A DMSO solution (5 mL) containing 150 mg of ADH and 1 g of DCC was introduced into the G3 dendrimer solution. The reaction mixture was continuously stirred for four days. Purification was carried out following established protocols. RGD was then conjugated to the synthesized G3 dendrimer, as shown in **Figure 2**. A total of 0.05 g of dendrimer-G3 was activated with 0.02 g of DCC in 10 mL of DMSO, after which the solution was stirred for 30 minutes. The activated dendrimer solution was then mixed with 0.2 g of RGD and stirred for 48 hours at room temperature to

facilitate complete coupling to the dendrimer's carboxylate termini. Deprotection of the reaction products was performed with 0.1% HCl, after which distilled water was added to the mixture to promote DCU precipitation, resulting in the final products.

2.3 ^{99}Tc radiolabelling of dendrimer–RGD conjugate

Radiolabeling was carried out in accordance with an established protocol. Briefly, **10 mg of the freeze-dried nanocomplex** was reconstituted in **2 mL of double-distilled water (DD water)**. Subsequently, **0.4 mL of an antioxidant preparation containing ascorbic acid (5 mg/mL)** and **0.3 mL of a tin chloride solution (1 mg/mL)** were introduced. The **pH of the reaction medium** was then adjusted to **8**. The resulting formulation was subjected to **lyophilization using a freeze-drying system** (Telstar, Terrassa, Spain) for **24 h at $-54\text{ }^{\circ}\text{C}$** . In the final step, **370 MBq of freshly eluted $^{99}\text{TcO}_4^-$ obtained from a $^{99}\text{Mo}/^{99}\text{Tc}$ generator** was allowed to react with the dendrimer formulation at **ambient temperature for 10 min**.

2.4 Characterisation

The infrared profiles of all formulations were acquired using a PerkinElmer Spectrum BX-II Fourier-transform infrared spectrometer (PerkinElmer Inc., Waltham, MA, USA). Structural verification of both the native and conjugated dendrimers was performed by ^1H nuclear magnetic resonance spectroscopy, recorded on a Bruker 500 MHz spectrometer (Billerica, MA, USA), with $\text{DMSO-}d_6$ employed as the deuterated medium.

The hydrodynamic diameter of the nanoparticles was determined via dynamic light scattering (DLS) using a Malvern Nano-ZS analyzer. In addition, surface topology and morphological features of the dendrimers were examined by atomic force microscopy (AFM; JPK Nanowizard II) and transmission electron microscopy (TEM; JEOL JEM-2100).

2.5 Measurement of radiochemical purity

The dendrimers' radiochemical purity (RCP) was evaluated with Whatman paper as the solid phase and either saline or a 1:1 acetone-methanol mixture as the mobile phase. This method relies on the different migration rates of radiolabeled dendrimer, free pertechnetate, and $^{99}\text{TcO}_2$ on paper during solvent flow. A 5 μL sample was applied to one end of the paper. Following separation, a γ well-type counter was used to quantify the radioactivity of each segment. When saline served as the solvent, both the radiolabeled dendrimer and free pertechnetate and the migrated with the solvent front, whereas $^{99}\text{TcO}_2$ remained at the origin. In contrast, when acetone/methanol was used, free pertechnetate migrated with the solvent, but both $^{99}\text{TcO}_2$ and the radiolabeled dendrimer were retained at the origin. The RCP value was calculated using the following equation:

$$\text{Radiochemical Purity} = 100 - (\text{free pertechnetate} - ^{99}\text{TcO}_2)$$

2.6 Stability test

The **in vitro stability** of the nanoconjugate was examined under **physiological conditions in human serum (37 °C)** as well as in **phosphate-buffered saline maintained at ambient temperature**. For this purpose, **300 µL of the nanocomplex dispersion** was combined with **600 µL of either human serum or PBS**. At predetermined intervals, **100 µL of trichloroacetic acid solution (10%)** was introduced to terminate the reaction and precipitate serum components. Following **centrifugation**, the supernatant was collected, and **conjugate stability was subsequently analyzed using chromatographic methods**.

2.7 Assessment of cellular toxicity

Cellular toxicity of the synthesized dendrimers was evaluated using the XTT cell proliferation assay in HEK-293 cells. This assay relies on a colorimetric readout reflecting mitochondrial metabolic activity, which serves as an indicator of cell viability. Cells were cultured in DMEM/F12 growth medium supplemented with 10% fetal bovine serum, along with penicillin (100 U/mL) and streptomycin (100 µg/mL).

For the experiment, 1×10^4 cells were dispensed into each well of a 96-well plate in 100 µL of culture medium and allowed to adhere for 24 h. The cells were then exposed to nanoconjugate formulations at concentrations of 0.25, 0.5, 1, 2, 4, and 8 mg/mL, followed by incubation at 37 °C under a humidified atmosphere containing 5% CO₂ for 24 or 48 h.

After each exposure period, 25 µL of XTT reagent mixed with N-methylphenazonium methyl sulfate (PMS) in a 50:1 ratio was added to each well, and the plates were further incubated for 2 h. Absorbance values were subsequently recorded at 450 nm using a microplate spectrophotometer, and cell viability was quantified relative to untreated control cells.

2.8 In vivo study

Female **athymic nude mice** weighing **20–28 g** were employed for the in vivo experiments. All animal procedures were conducted in strict compliance with the guidelines approved by the **Ethics Committee of Tehran University of Medical Sciences** (approval code: **IR.TUMS.REC.1394.1509**). Tumor establishment was performed according to previously reported methodologies (24, 25). Briefly, a suspension containing **1×10^6 HEK-293 cells** prepared in **100 µL of phosphate-buffered saline (PBS)** was administered **subcutaneously into the right flank** of each mouse.

2.8.1 Radioactive imaging results

To achieve anesthesia, mice were administered an intraperitoneal injection of ketamine (100 mg/kg) in combination with xylazine (10 mg/kg), with dosages adjusted according to individual body mass. Following anesthesia, tumor-bearing nude mice were intravenously injected via the tail vein with 3.7 MBq

of ^{99}Tc -labeled dendrimer. SPECT/CT acquisition was performed 3 h post-administration ($n = 3$) using a Symbia T2 system (Siemens) equipped with low-energy high-resolution collimators.

During image acquisition, animals were positioned supine on the imaging bed, and data were recorded using a 256×256 pixel matrix with a 20% energy window centered at 140 keV.

2.8.2 Biodistribution assessment

The ^{99}Tc -labeled dendrimer (3.7 MBq) was delivered **systemically through tail vein injection** under anesthesia. Animals were sacrificed at **predetermined time points** (30, 60, 90, and 120 min), after which selected organs and tissues, including the **kidneys, liver, spleen, and cortical bone**, were carefully harvested and weighed. The **radioactivity content** of each sample was determined using a **properly calibrated gamma counting system**. Biodistribution data were subsequently expressed as the **percentage of injected dose per gram of tissue (%ID/g)**, normalized to the **total administered radioactivity**.

2.9 Quantitative analysis

All data analyses were performed using **GraphPad Prism version 5** in combination with **Microsoft Excel (Office 2013)**. Group comparisons were carried out by **one-way analysis of variance (ANOVA)** followed by **Tukey's multiple-comparison procedure**. Differences were considered **statistically meaningful when the probability value was below 0.05**.

Results

3.1 Conjugate size, surface properties, and zeta potential

The processes of **synthesis, conjugation, and radiolabeling** of the dendrimers were performed following the procedures described in **Figures 1 and 2**. Structural integrity and chemical composition of the resulting dendrimers were confirmed by ^1H NMR spectroscopy in DMSO-d_6 , as presented in **Figure 3**. Resonance signals detected at **2.7 ppm (2H, $J = 15.5$ Hz)**, **3.5 ppm (3H, $J = 5$ Hz)**, and **5.02 ppm (2H)** were attributed to the **$-\text{CH}_2\text{COOH}$ moiety of citric acid**, as well as the **$-\text{OCH}_2\text{CH}_2-$ and $-\text{OCH}_2\text{CO}-$ segments of PEG**, respectively. These spectral features verify the effective incorporation of **citric acid and PEG into the G2 dendrimer framework**.

As illustrated in **Figure 3**, the formation of **dendrimer-G3** was similarly confirmed. The experimentally determined and theoretical **citric acid-H to PEG-H ratios** were calculated as **0.77 and 0.75**, respectively. Signals appearing in the **2.3–2.8 ppm range** were assigned to **citric acid-H protons**, whereas resonances observed above **3.5 ppm** corresponded to **PEG-CH₂ protons**. In addition, a minor resonance at **4.17 ppm** was associated with a **single PEG-H unit**. The presence of **sharper and more resolved peaks** for citric

acid and PEG in **dendrimer-G3**, relative to **dendrimer-G2**, further substantiates the **successful progression from generation G2 to G3**.

Liquid chromatography–mass spectrometry (LC–MS) analysis provided additional evidence for both dendrimer formation and successful conjugation with RGD, as depicted in Figure 4. A signal observed at 306 m/z was assigned to a fragment consisting of a citric acid moiety linked to a second citric acid unit along with a recurring PEG segment. The ion detected at approximately 512 m/z was attributed to a structure containing five PEG units conjugated to citric acid residues, whereas the peak at 696 m/z corresponded to seven PEG chains associated with two citric acid molecules.

Collectively, these spectral features confirm that citric acid units are coupled to PEG via their carboxyl functional groups, consistent with the anticipated citrate–PEG dendrimer architecture. Moreover, the increased signal intensities observed in Figure 4a further substantiate the structural progression from G2 to G3 dendrimers. As shown in Figure 4b, LC–MS data also verify the efficient attachment of RGD moieties to the dendrimer scaffold.

Fourier-transform infrared (FTIR) spectroscopy was utilized to confirm both the successful synthesis of the dendrimers and their subsequent conjugation, as shown in Figure 5. The absorption band detected at 1232 cm^{-1} was attributed to C–O stretching vibrations of ester linkages formed between the terminal citric acid groups of dendrimer-G2 and citric acid monomers, indicating the generation of dendrimer-G3. In addition, the characteristic peak observed near 1726 cm^{-1} corresponds to carbonyl (C=O) stretching of terminal citric acid residues, providing further evidence for ester bond formation. Signals associated with PEG C–O functional groups were identified at 1084 cm^{-1} , while the broad absorption band around 3500 cm^{-1} was assigned to O–H stretching vibrations of citric acid moieties. Moreover, a distinct C–O stretching vibration was observed at approximately 2925 cm^{-1} . Following the synthesis and surface optimization of the third-generation dendrimer, the terminal functional groups were conjugated with the RGD peptide, after which comprehensive characterization was performed. Post-conjugation analyses included LC–MS, FTIR, transmission electron microscopy (TEM), dynamic light scattering (DLS), and ZetaSizer measurements.

Figure 5 presents the LC-MS spectrum of the third-generation dendrimer conjugated with the RGD peptide. Binding units are attached to a portion of a citric acid group and to a complete citric acid molecule. The peak at m/z 530 confirms the conjugation of the RGD peptide to the dendrimer. Next, FTIR spectroscopy was used to investigate the conjugation of the RGD peptide to the third-generation dendrimer. In Figure 6, the peaks corresponding to citric acid (hydroxyl group: $3400\text{ to }3500\text{ cm}^{-1}$), PEG (C-O PEG groups in the region of 1106 cm^{-1}), and the formation of an ester bond between these two compounds in the third-generation dendrimer conjugated with RGD are visible.

The infrared band observed at 1633 cm^{-1} corresponds to the **stretching mode of carbonyl (C=O) functionalities**, characteristic of **carboxylic acid groups**. Furthermore, the detection of **Amide II ($\sim 1580\text{ cm}^{-1}$)** and **Amide III (1287 cm^{-1})** bands in the **third-generation dendrimer** provides clear confirmation of the **successful attachment of the RGD peptide** to the dendrimer framework.

3.2 Stability test results

Maintaining the **structural integrity of nanocomplexes during systemic circulation** is essential for achieving effective **molecular imaging at the target site**. Following administration, **serum proteins rapidly associate with nanocomplex surfaces**, leading to the formation of a protein corona that can markedly influence their biological performance. The development of an unfavorable corona may accelerate clearance from circulation and compromise imaging efficiency. To assess this aspect, the **stability of ^{99}Tc -labeled dendrimers** was investigated in **human serum and phosphate-buffered saline (PBS)** over a **24-h period**. As shown in **Figure 7**, the nanoconjugates exhibited **good stability in both environments**, with **radiochemical loss remaining below 15%**. These findings demonstrate that the **^{99}Tc -radiolabeled dendrimers** possess adequate robustness for imaging applications in **biologically complex media such as human serum**.

3.3 Toxicity assessment results

The **cytotoxic potential** of the synthesized dendrimers was examined in **HEK-293 cells** across a concentration range of **0.25–8 mg/ μL** (Figure 8). The **^{99}Tc -labeled nanoconjugates** showed **no notable cytotoxic effects**, even at the highest tested concentration of **8 mg/ μL after 24 h of incubation**.

3.4 Scintigraphic imaging and biodistribution analysis

SPECT imaging was performed in **tumor-bearing mice** to examine the **targeting performance** of the developed system and to investigate the **in vivo distribution** of the nanoconjugates. As illustrated in **Figure 9**, a clear accumulation of the **radiolabeled dendrimer conjugates** was observed at the tumor site **60 min after intravenous administration of 3.7 MBq**. Tumor localization became evident within the first hour post-injection, confirming effective in vivo targeting. These findings demonstrate that **RGD-functionalized ^{99}Tc -labeled dendrimers** generate a strong and detectable **SPECT signal** in living subjects. To further characterize tissue-specific uptake, **complementary biodistribution experiments** were subsequently carried out across major organs.

3.5 Biodistribution results

Following injection of the RGD peptide-conjugated dendrimers, the highest accumulation was detected in kidney tissue, followed by the liver, as shown in Figure 10. These findings highlight the involvement of renal clearance pathways and the reticuloendothelial system in nanoparticle elimination. The kidney exhibited substantial uptake of nanoconjugates, with a notable decrease observed between 30 and 120 minutes post-injection. At 30 minutes post-injection, the kidney displayed an ID/g (%) value of $3.4 \pm 0.4\%$. This value decreased significantly to $1.0 \pm 0.2\%$ by 120 minutes, indicating a reduction in nanoconjugate concentration over time, likely due to renal clearance.

Discussion

This study presents the synthesis, radiolabeling, and biological evaluation of RGD-conjugated PEG–citrate dendrimers for $\alpha\beta 3$ -integrin–targeted tumor imaging using SPECT. Cyclic RGD peptides are established ligands for $\alpha\beta 3$ integrins, which are overexpressed in various solid tumors, including breast cancer (26, 27). Their specific affinity for integrin-rich tumor vasculature enhances the selectivity and targeting precision of radiotracers for molecular imaging. The results align with previous investigations demonstrating that radiolabeled RGD peptides efficiently target $\alpha\beta 3$ -positive tumors, including breast cancer models. Multivalent RGD constructs, such as dimers and tetramers, have been shown to enhance tumor uptake by increasing integrin-binding avidity (28, 29). Comparable outcomes were reported in clinical studies, such as Kang et al. (2017) (30) where ^{68}Ga -RGD2 PET imaging demonstrated strong tumor uptake in lung cancer patients, confirming the translational value of RGD-based targeting.

The biodistribution of ^{99}mTc -labeled RGD-conjugated dendrimers demonstrated optimal tumor uptake at 90 minutes post-injection, with clear contrast between tumor and surrounding healthy tissues. The tracer was predominantly localized within the tumor, while a substantial portion was rapidly eliminated via the renal system. This pattern is consistent with the findings of Liu et al. (2014) (31) who reported strong tumor uptake of ^{99}mTc -3PRGD2 in malignant lesions followed by renal clearance, a behavior characteristic of small-molecule and peptide-based radiotracers. Axelsson et al. (2010) (32) also demonstrated that renal excretion was the principal route of elimination for ^{99}mTc -NC100692, which exhibited high uptake in both primary tumors and metastatic lesions while maintaining minimal nonspecific retention in healthy organs.

The biodistribution results also revealed detectable intestinal activity at approximately 2 hours post-injection, indicating relatively rapid intestinal washout in addition to renal elimination. This observation is consistent with previous work and supports the conclusion that these dendrimers follow dual renal–intestinal clearance pathways. Furthermore, the formulation demonstrated minimal accumulation in the lungs, heart, and blood, reinforcing the high specificity of the RGD-modified dendrimers for tumor tissue and reducing concerns regarding off-target biodistribution. Comparable organ-distribution behavior was observed by Peng et al. (2016) (33) in their evaluation of PEGylated dendrimer-entrapped gold nanoparticles, although in this study, tumor accumulation remained the most prominent finding. Compared with other imaging systems, such as ^{18}F -glutamine dendrimers, the RGD-conjugated dendrimers in this study exhibited a more favorable biodistribution profile, demonstrating improved tumor targeting and greater specificity. These characteristics underscore the potential of RGD-functionalized dendrimers as promising agents for early detection of breast cancer and other $\alpha\beta 3$ -expressing malignancies, supporting both effective targeting and efficient systemic clearance suitable for clinical translation.

SPECT imaging confirmed the targeting efficiency of ^{99}mTc -RGD-dendrimers, yielding bright, well-defined tumor signals. The optimal tumor uptake observed between 90 and 120 minutes is consistent with previous reports on RGD-modified dendrimer systems. Tsiapa et al. (2013) (34) similarly demonstrated

high tumor uptake values using ^{99}mTc -labeled RGD peptides for angiogenesis imaging, with renal clearance as the dominant excretion route and minimal off-target uptake, patterns closely paralleling these findings.

RGDyC/PEG-PAMAM dendrimers have previously demonstrated enhanced pharmacokinetics, increased half-life, and elevated tumor cell uptake, further supporting $\alpha\text{v}\beta 3$ -targeted nanoplateforms as effective imaging agents (35). Song et al. (2017) (36) demonstrated comparable tumor-specific enhancement with cRGD-modified liposomes, with significantly higher tumor uptake compared with non-targeted systems, and confirmed renal clearance as the dominant pathway. While their study reported negligible intestinal involvement, our formulation displayed modest intestinal accumulation, suggesting that structural or size variations across systems may influence clearance mechanisms. Additional parallels were observed with the findings of Xing et al. (2018) (37) who used ^{99}mTc -labeled dendrimer-entrapped gold nanoparticles for SPECT/CT imaging, reporting tumor-specific uptake and renal excretion, findings similar to those of the current system. These concordant results reinforce the robustness of dendrimer-based architectures for targeted tumor imaging.

The RGD-conjugated dendrimers demonstrated high radiochemical stability in biological media for up to 24 hours. This observation is consistent with the reports of Swidan et al. (2019) (38) who showed high radiochemical stability of ^{99}mTc -labeled iron oxide nanoparticles with minimal radionuclide leaching after 8 hours. Although their systems used different nanoparticle structures, both studies highlight the capacity of well-engineered nanoplateforms to maintain radiochemical integrity, which is a crucial factor for reliable imaging performance.

Collectively, these findings demonstrate that PEG–citrate dendrimers conjugated with RGD peptides and radiolabeled with ^{99}mTc possess key attributes for SPECT imaging, such as straightforward synthesis, high biocompatibility, effective receptor-mediated tumor targeting, robust in vitro and in vivo stability, and strong imaging contrast at early time points. These characteristics establish the RGD-dendrimer nanoplateform as a promising contrast agent for breast cancer imaging and potentially for other $\alpha\text{v}\beta 3$ -integrin–positive malignancies. Future research should evaluate long-term stability, extended pharmacokinetics, and the biodistribution profile of the nanocomplex. The modular nature of the dendrimer platform also warrants investigation into theranostic adaptations that combine imaging and targeted therapy, which may facilitate translational progress.

Conclusion

The present study demonstrates that third-generation PEG–citrate dendrimers, functionalized with RGD peptides and radiolabeled with ^{99}mTc , serve as a stable, biocompatible, and highly selective platform for breast cancer imaging. The resulting nanoconjugates exhibited pronounced $\alpha\text{v}\beta 3$ -mediated tumour uptake, clear SPECT contrast, and rapid renal clearance with minimal off-target accumulation. These results highlight the potential of RGD-modified dendrimers as molecular imaging agents and represent the first application of citric acid–based G3 dendrimers in nuclear medicine. Further research into long-

term pharmacokinetics and integration with therapeutic payloads may facilitate the advancement of this platform for future diagnostic applications.

Declarations

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Authors' contributions: Saeedeh Zamani: Conceptualisation, Methodology, Investigation, Data curation, Writing – original draft. Amir Khanmirzaei: Conceptualisation, Methodology, Investigation, Writing – original draft. Mehdi Shafiee Ardestani: Supervision, Resources, Validation, Writing – review & editing. Sepehr Miran: Formal analysis, Writing – review & editing. Bahar Amiri: Data curation, Visualization, Writing – review & editing. Arya Behzadi: Formal analysis, Writing – review & editing. Arian Karimi Rouzbahani: Conceptualisation, Supervision, Project administration, Writing – review & editing

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Figures

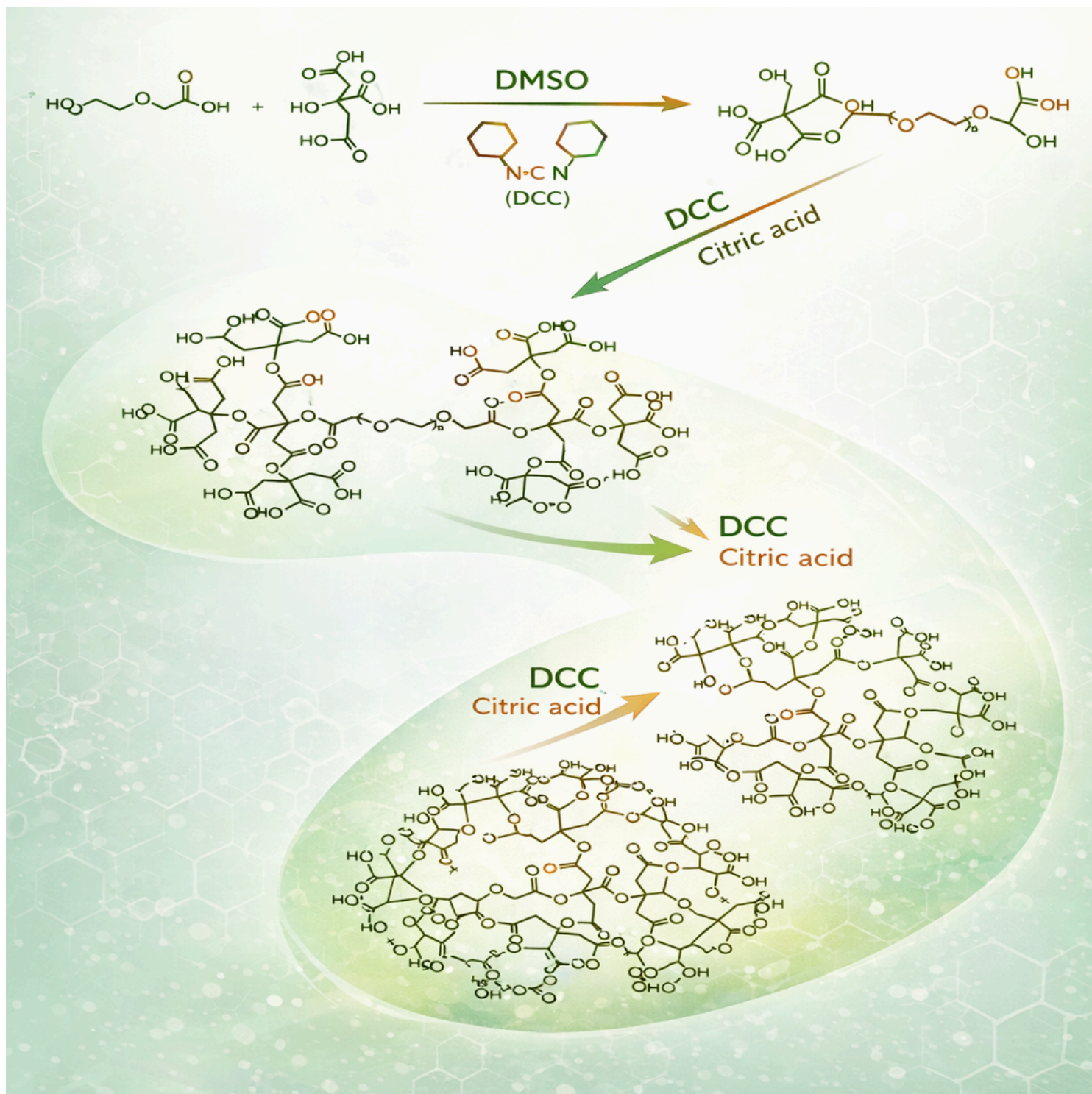


Figure 1

PEG-citrate dendrimers fabrication process. PEG diacid 600 was activated by DCC in DMSO-d₆, then the monomers (citric acid) were added to the solution to generate G1 dendrimer. These processes were repeated to synthesize G2 and G3 dendrimers.

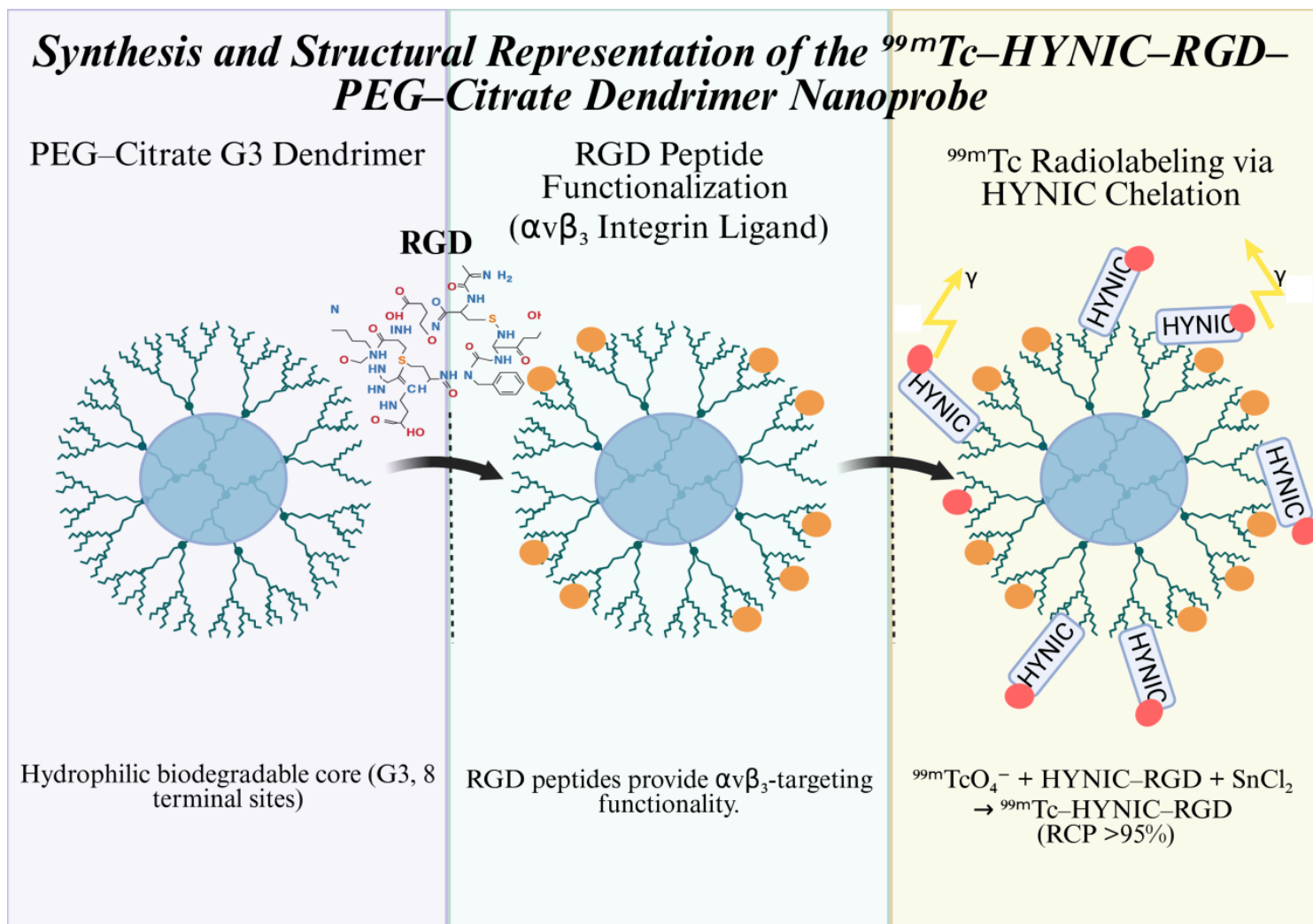


Figure 2

Schematic illustration of the synthesis and structural design of the ^{99m}Tc -HYNIC-RGD-PEG-citrate dendrimer nanoprobe. The PEG-citrate generation-3 (G3) dendrimer, which possesses multiple terminal functional groups, serves as a hydrophilic and biodegradable nanocarrier. Surface conjugation with RGD peptides confers specific targeting ability toward $\alpha_v\beta_3$ integrin receptors. Radiolabeling is achieved via HYNIC chelation, enabling efficient coordination of technetium-99m (^{99m}Tc) with SnCl_2 as the reducing agent. The resulting nanoprobe demonstrates radiochemical stability and a radiochemical purity greater than 95%.

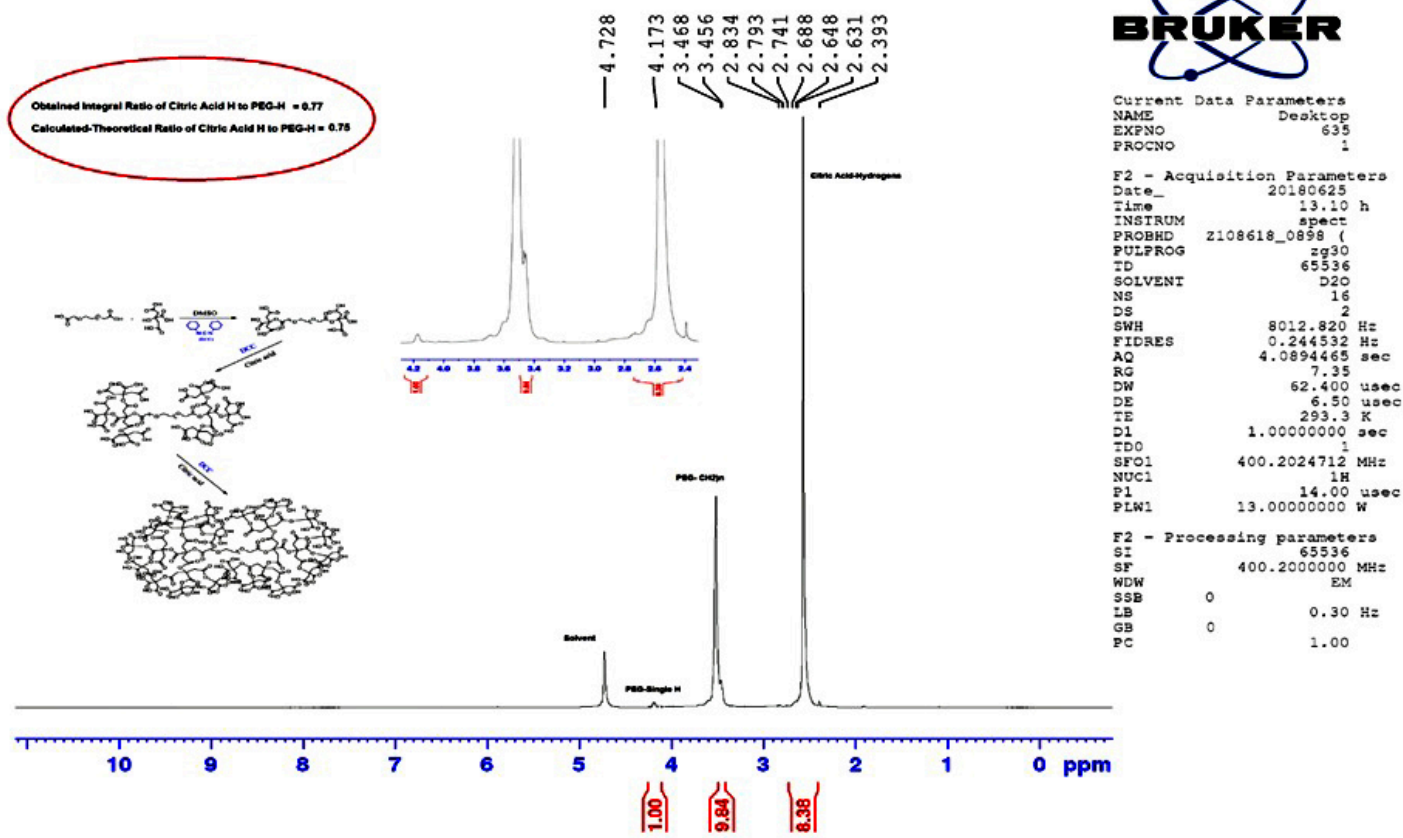


Figure 3

^1H NMR (500 MHz) spectra of Dendrimer-G3. Peaks around 2.7 ($2J = 15.5$ Hz, 2 h), 3.5 (t, $3J = 5$ Hz, 2 h), and 5.02 (s, 2 h) in Fig. 3a are related to the CH_2COOH functional group of citric acid, OCH_2CH_2 , and OCH_2CO functional groups of PEG, respectively.

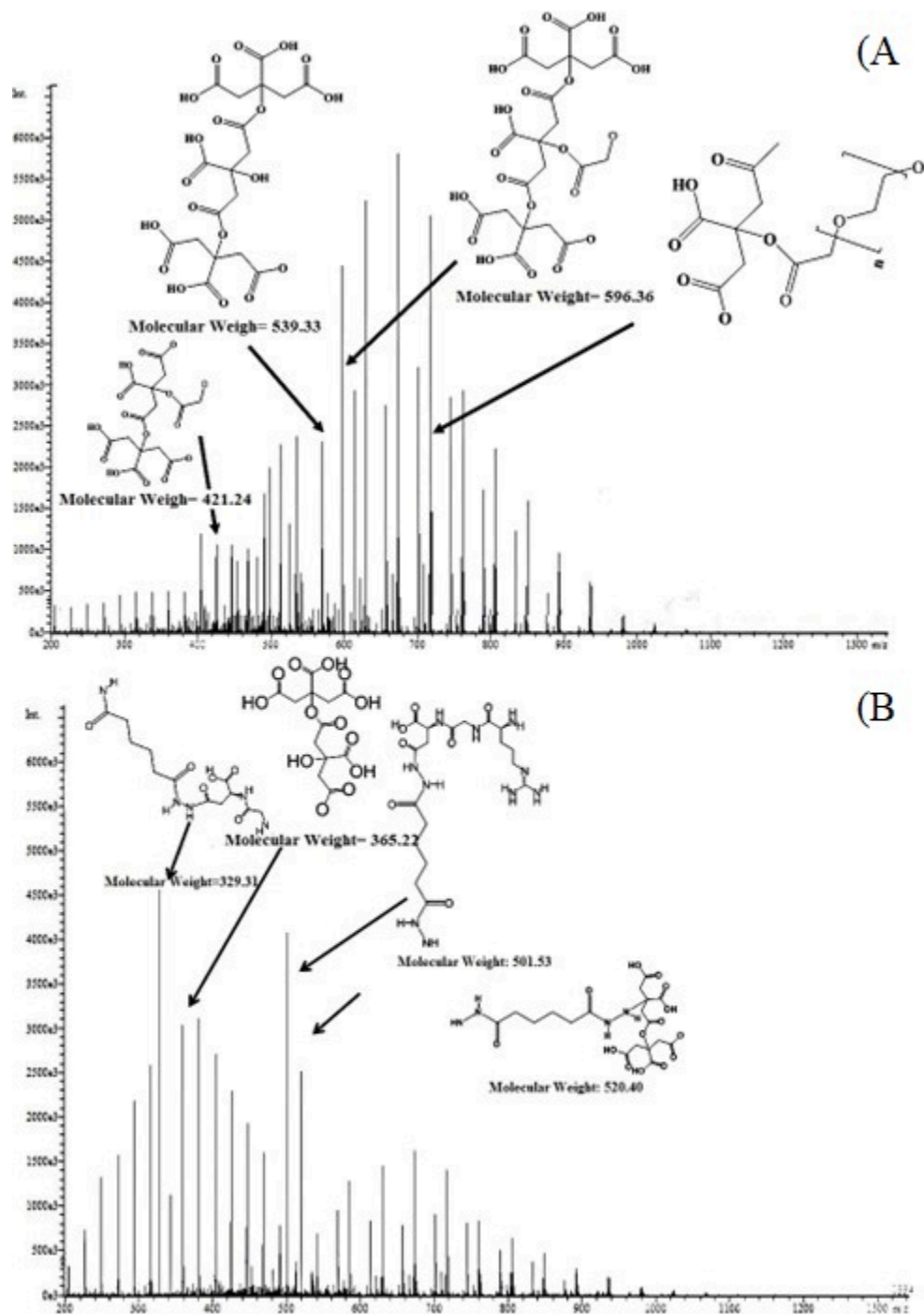


Figure 4

A) Dendrimer-G3, B) Dendrimer-G3-RGD. The peak at approximately 306 m/z corresponds to the fragmentation of a citric acid unit attached to a second citric acid molecule, along with a repetitive unit of PEG. The peak around 512 m/z represents five repetitive units of PEG conjugated to citric acid molecules, while the peak at around 696 m/z indicates seven repetitive units of PEG along with two citric acid molecules. The peak at m/z 500 corresponds to PEG repeating units attached to a part of a citric acid group and a complete citric acid molecule. The peak at m/z 530 confirms the conjugation of the RGD peptide to the dendrimer. Next, FTIR spectroscopy was used to investigate the conjugation of the RGD peptide to the third-generation dendrimer.

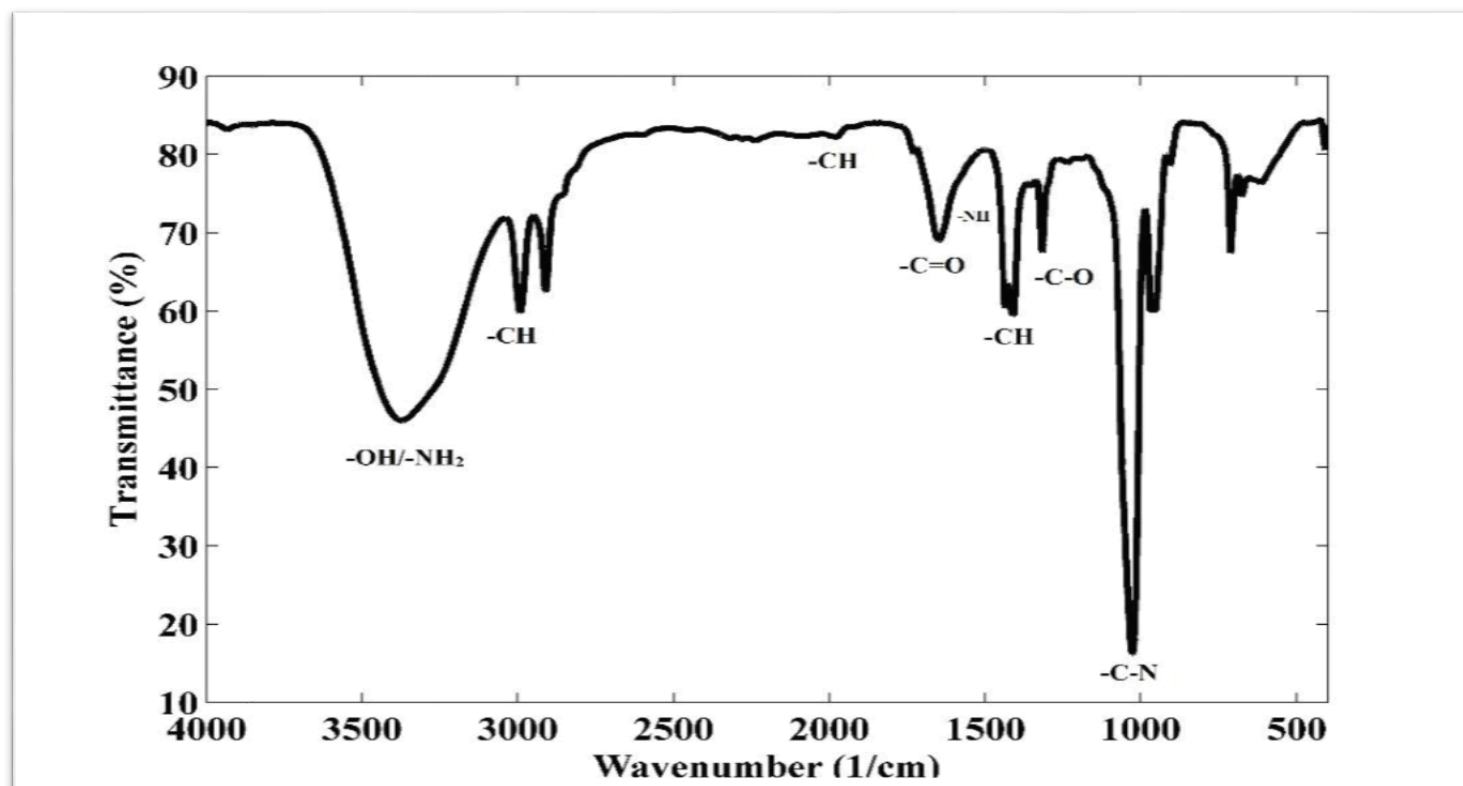


Figure 5

FTIR Spectrum of Third-Generation Dendrimer Conjugated with RGD. The spectrum shows characteristic peaks for citric acid (hydroxyl group: $3400\text{--}3500\text{ cm}^{-1}$), PEG (C-O PEG groups at 1106 cm^{-1}), and the formation of an ester bond between citric acid and PEG. The peak at 1633 cm^{-1} corresponds to the stretching vibration of the -C=O group, indicative of carboxyl groups. Additionally, the presence of Amide II ($\sim 1580\text{ cm}^{-1}$) and Amide III (1287 cm^{-1}) bands confirms the conjugation of the RGD peptide to the third-generation dendrimer.

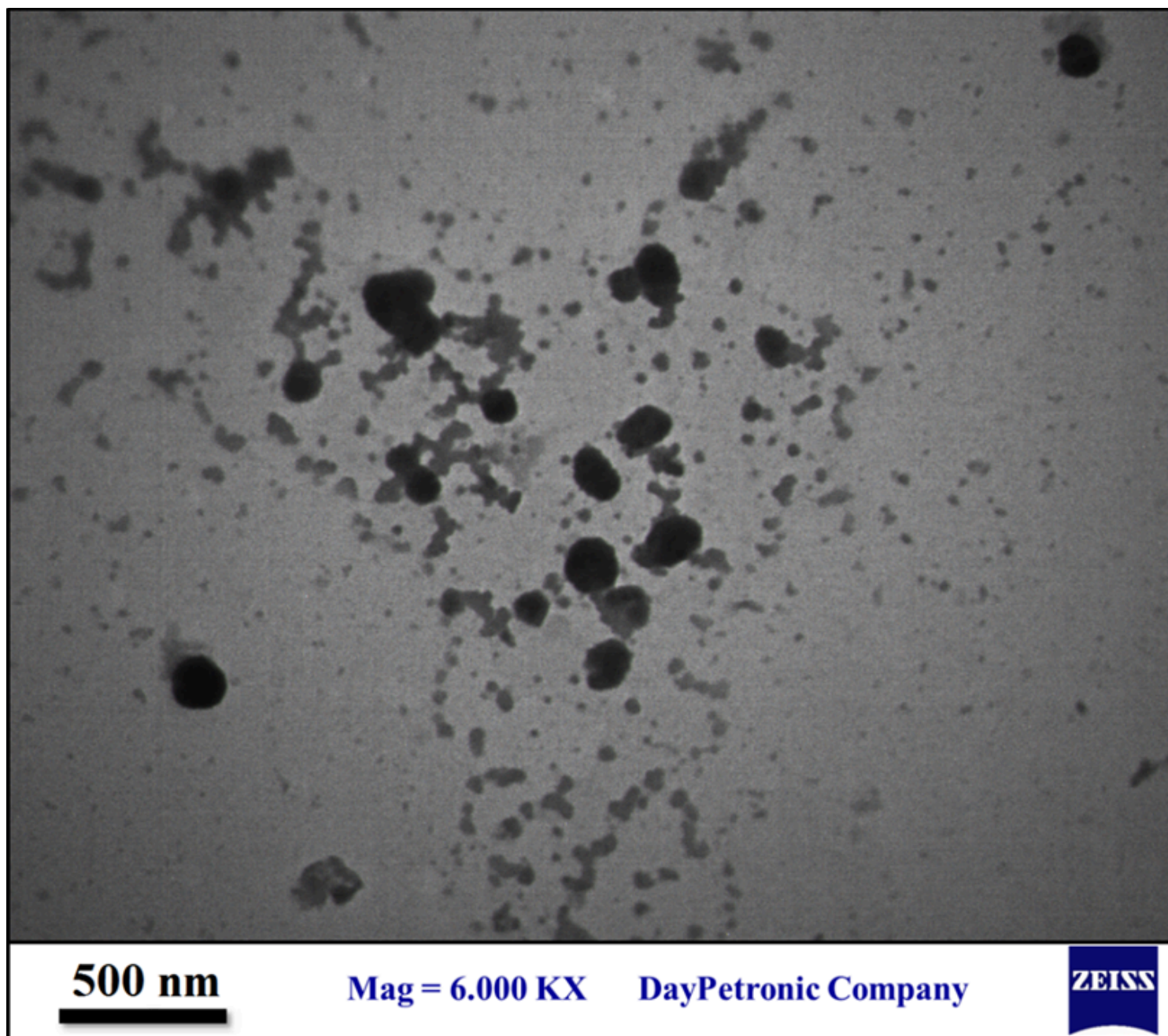


Figure 6

The peaks corresponding to citric acid (hydroxyl group: 3400 to 3500 cm^{-1}), PEG (C-O PEG groups in the region of 1106 cm^{-1}), and the formation of an ester bond between these two compounds in the third-generation dendrimer conjugated with RGD are visible. The peak observed at a wavenumber of 1633 cm^{-1} indicates the stretching vibration of -C=O , corresponding to carboxyl groups. Additionally, the presence of Amide II ($\sim 1580\text{ cm}^{-1}$) and Amide III (1287 cm^{-1}) in the third-generation dendrimer confirms the conjugation of the RGD peptide to the dendrimer.

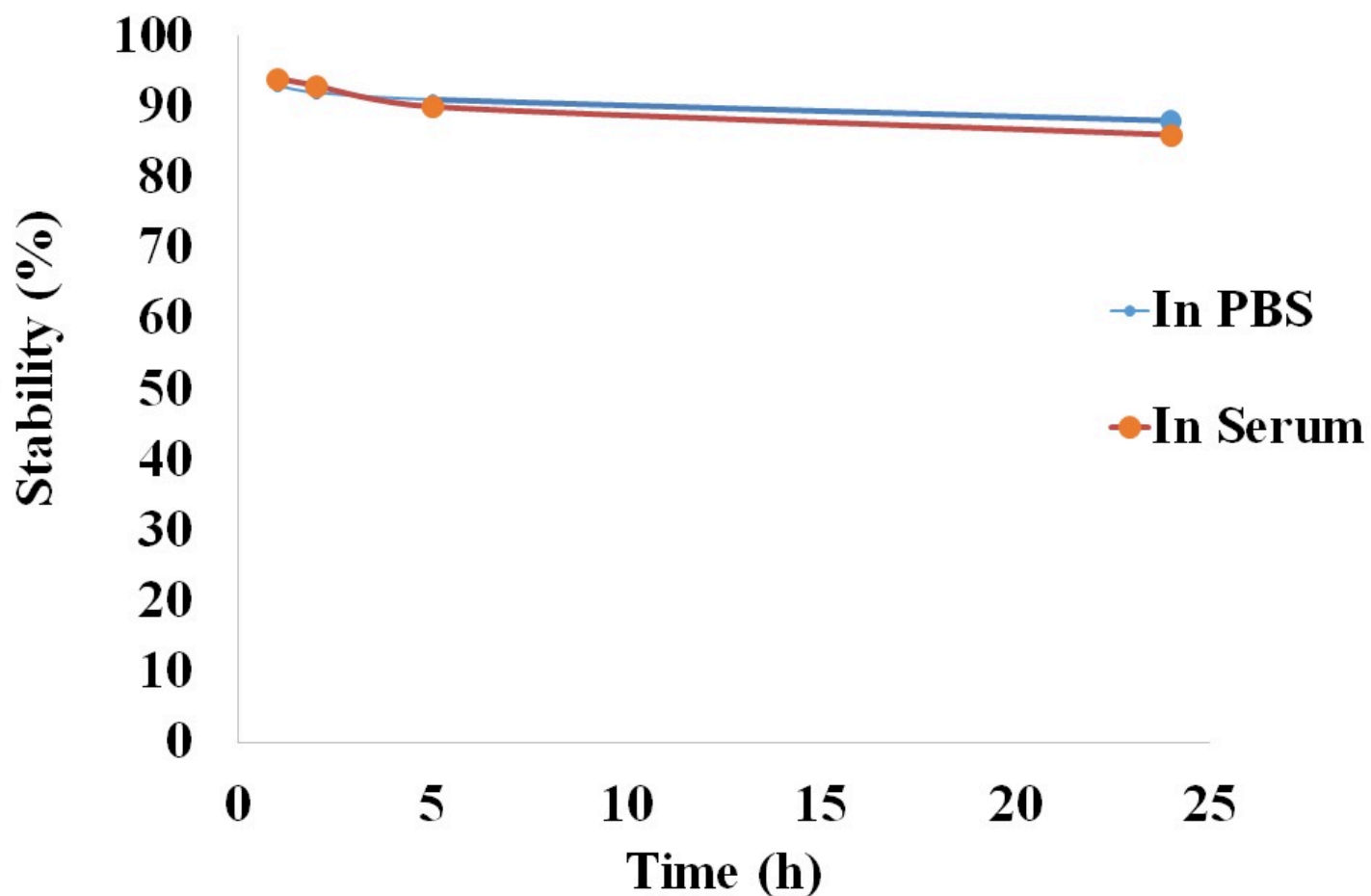


Figure 7

In vitro stability of ^{99}mTc nanoconjugate complex in human serum at 37°C and PBS at room temperature for 24 h. PBS, phosphate-buffered saline.

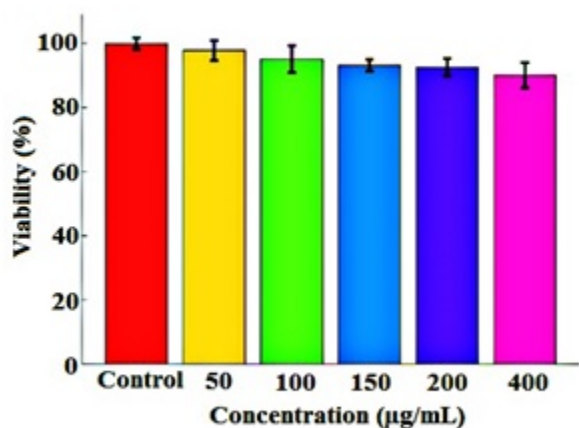


Figure 8

Cell viability of HEK-293 cells under incubation with the

synthesised nanocomplex at 24 h, measured by the XTT assay kit. There is an inverse relationship between the concentration of the nanocomplex and cell viability.

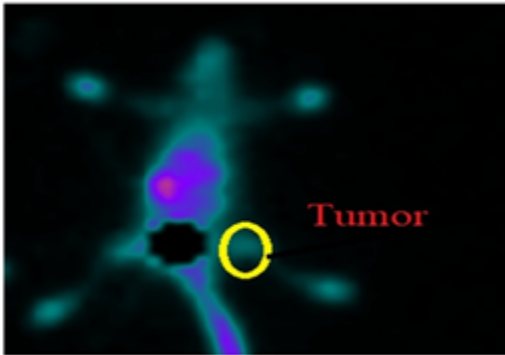


Figure 9

Planar images of mice with ^{99m}Tc-radiolabeled dendrimer acquired 60 minutes post-injection, shown in the posterior position. The circle highlights the tumor site. The accumulation of the nanocomplex at the tumor site is clearly visible in the image.

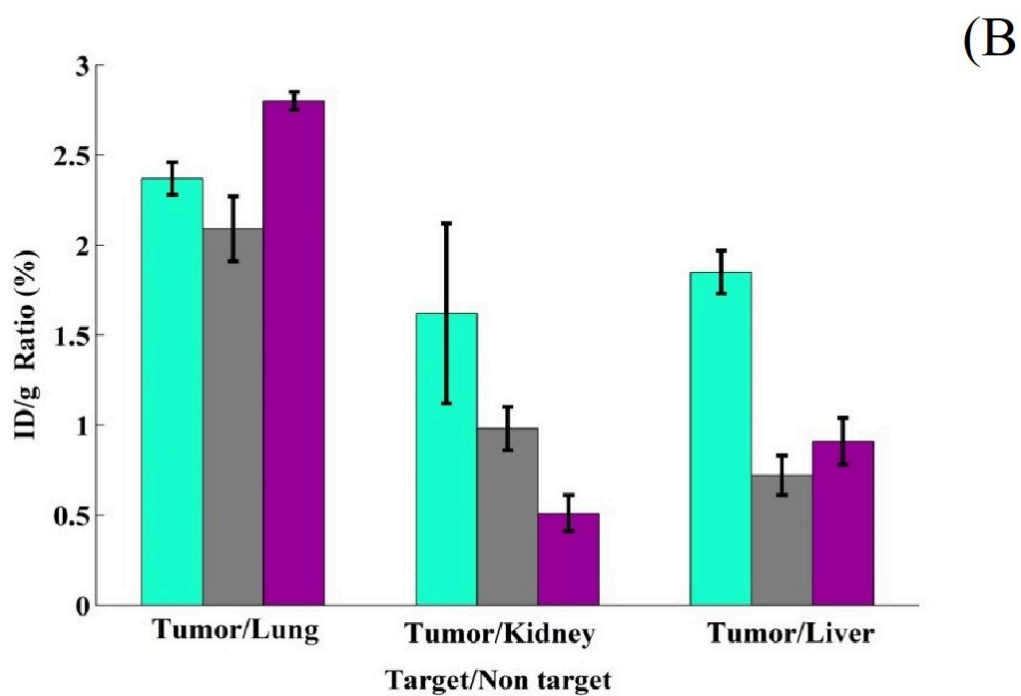
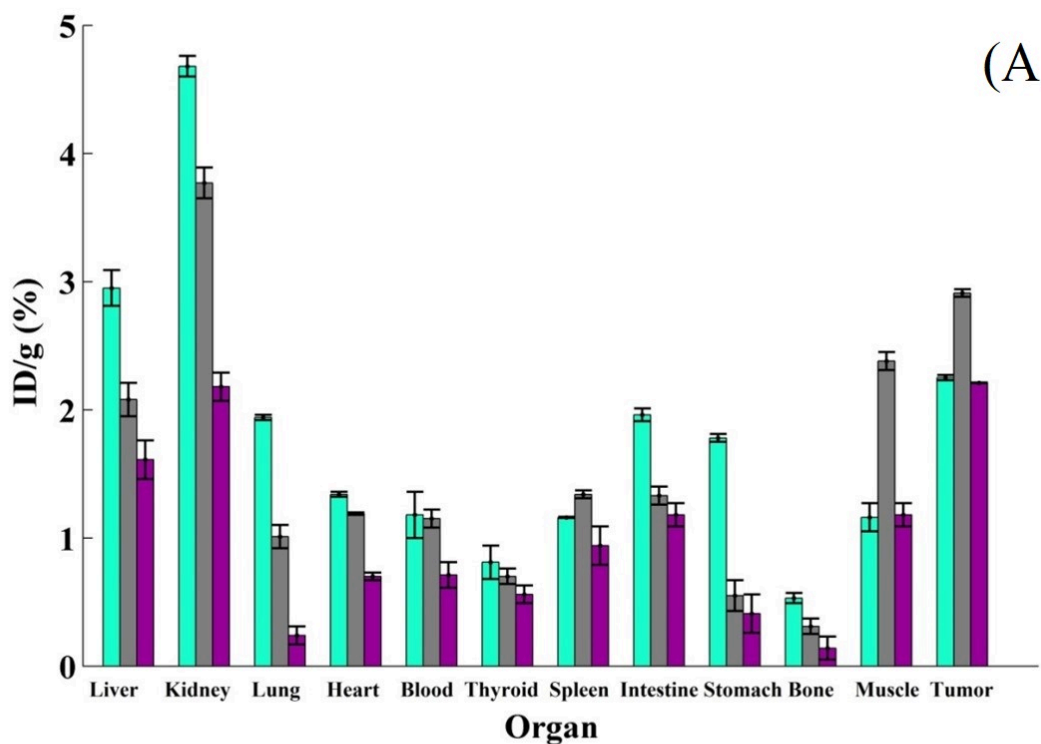


Figure 10

A) The biodistribution profile of the nanoconjugate at 1, 2, and 3-hour intervals (dose per gram of tissue) showed the highest accumulation in the kidney tissue, followed by the liver, which contained the highest amount of nanoparticles. B) It shows the ratio of nanoparticle accumulation in the tumor compared to other tissues. The highest ratio in all three time intervals corresponds to the tumor-to-lung tissue ratio.