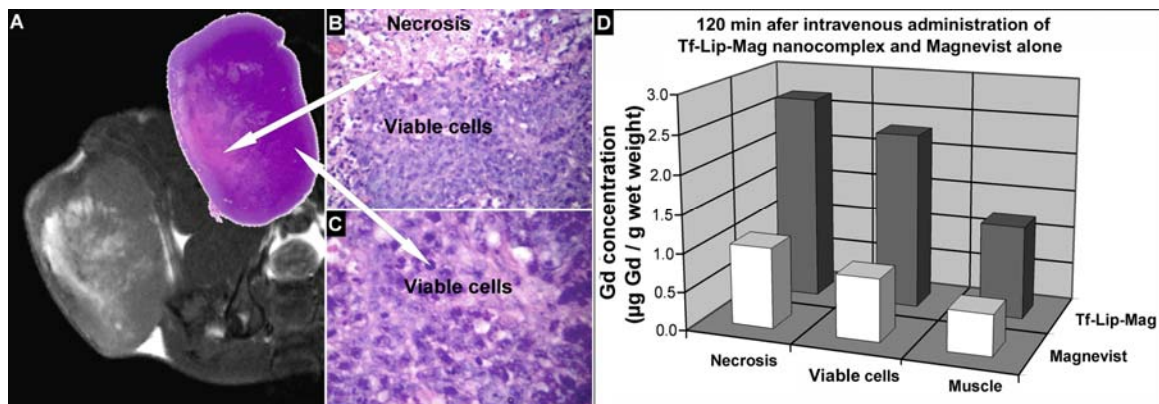


A Nanocomplex System as Targeted Contrast Agent Delivery Vehicle for MRI Dynamic Contrast Enhancement Study

Alexandru Korotcov, Liang Shan, Huan Meng, Tongxin Wang, Rajagopalan Sridhar, Yuliang Zhao, Xing-Jie Liang, and Paul C. Wang*



We constructed a nanocomplex system containing Gd-DTPA as an encapsulated payload inside transferrin coated cationic liposomes. This nanocomplex was evaluated as a tumor targeted magnetic resonance (MR) contrast agent in prostate tumor xenografts grown in nude mice. The probe greatly enhanced the MR imaging (MRI) signals, and the MR contrast enhancement revealed a distinct heterogeneous pattern in tumors which correlated well with histological findings. The results indicate the utility of the probe for tumor targeted dynamic contrast enhanced MRI study. This targeted MR contrast agent delivery system can be used for cancer prognosis and non-invasive monitoring the response to therapy.

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Alexandru Korotcov,¹ Liang Shan,¹ Huan Meng,² Tongxin Wang,¹ Rajagopalan Sridhar,⁴ Yuliang Zhao,² Xing-Jie Liang,³ Paul C. Wang^{1,*}

¹Department of Radiology, Howard University, Washington DC, 20060, USA

²CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, Institute of High Energy Physics, Chinese Academy of Sciences, Beijing, 100049, PRC

³CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology of China, Beijing 100190, PRC

⁴Department of Radiation Oncology, Howard University, Washington DC, 20060, USA

* pwang@howard.edu

Abstract: We have developed and tested a liposomal nanocomplex system, which contains Gd-DTPA as a payload and transferrin on the surface, as a tumor specific targeting MRI contrast agent for studying prostate cancer tumors in mice. *In vivo*, the probe significantly enhanced the MRI signal. The image contrast between the peripheral region of the tumor and the non-involved muscle was nearly 50% higher two hours after administration of the nanocomplex. The liposomal nanocomplex increased the amount of Gd accumulated in tumors by factor 2.8 compared to that accumulated by using Magnevist alone. Moreover, the heterogeneous MRI image features correlate well with the tumor pathology. The image enhancement patterns can be used for cancer prognosis and non-invasive monitoring of the response to therapy.

Keywords: targeted drug delivery, dynamic contrast enhancement (DCE)-MRI, cancer, liposome, transferrin, nanoparticle

1. INTRODUCTION

The advances in magnetic resonance imaging (MRI) techniques have led to increasing use of parametric images, which are designed to display physiological, pathological and morphological features of tissue along with anatomical details. Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) using Gd-DTPA as a contrast agent (CA) has been widely used for the assessment of the malignancy of tumors.¹ The DCE-MRI involves acquisition of a series of MR images before, during and after an intravenous (i.v.) injection of a CA.² The dynamic changes in the MR image intensity reveals details of microenvironment inside the tumor.^{3,4} It has been used successfully in many types of cancers for tumor detection and staging capabilities.¹⁻⁷ One of the attractive strategies to further enhance the DCE-MRI is to utilize a specific targeted delivery of the CA to the cancer cells, thereby, improving the specificity of tumor detection.^{8,9} Transferrin receptor (TfR) is found to be overexpressed on the surface of many cancer cells.^{10,11} The elevated expression of TfR correlates with the malignancy of tumors.¹² It has been widely used to explore for receptor-mediated delivery of anticancer agents.^{13,14} In our previous study we constructed a TfR targeted liposomal nanocomplex with both optical and MRI reporters.¹⁵ There was higher uptake of these dual labeled nanoprobe by MDA-MB-231-luc breast cancer cells *in vitro* and an increased image enhancement in MRI of tumor xenografts *in vivo*. Encapsulating MRI CA within Tf based tumor-targeted liposomal complex offers potential advantages for enhanced sensitivity, detection of metastases, and diagnosis of cancer.

In this study, we have used a cationic liposome (Lip) complex modified with Tf on the surface as a ligand for specific targeting and CA (Magnevist) inside to be the payload (Tf-Lip-Mag) for DCE-MRI. The goals of this study are: (1) to test this Tf-liposome nanocomplex as a targeted MRI probe for systemic delivery of a gadolinium based CA to PC-3M-luc prostate cancer tumor xenografts and (2) to study the relationship of MRI dynamic contrast enhancement patterns with tumor pathology and tumor vasculature after i.v. administration of the nanocomplex. We found that the nanocomplex exhibits significantly enhanced MRI signals in solid tumor xenografts of prostate cancer in nude mice. It was superior to the use of contrast agent alone without specific targeting. The increase in MR image intensity can be used for the early detection of small tumors or to reduce CA dose for patients with impaired renal function. The characteristic image enhancement pattern can be used in the clinic as a noninvasive method for studying tumor pathology.

2. EXPERIMENTAL DETAILS

Cell Line and Animal Model: Human prostate cancer cells PC-3M-Luc (Caliper Life Sciences, Hopkinton, MA) were grown to 70% confluence. 1×10^7 PC-3M-Luc cells in 100 μL saline buffer were inoculated in the flank of ten 6-8 weeks athymic nude mice (Harlan, Indianapolis, IN).

Nanocomplex Preparation: Cationic liposomes were prepared by premixing 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) (Avanti Polar Lipids, Alabaster, AL) with 1:1 weight ratio as described by Shan et al.¹⁵ with modification. The mixture of phospholipids (0.25 μmol in 7.2 μL) was dried to a thin film using dry nitrogen gas and then hydrated by adding 70 μL of 0.9% saline and 30 μL of MRI contrast agent, Magnevist (469.01 mg/mL Gd-DTPA, Berlex, Inc., Montville, NJ). The total volume of mixture was adjusted to 347.5 μL by adding 0.9% saline. The suspension was incubated for 10-15 minutes at room temperature then was placed in a 90W sonicator for downsizing for 10 min. Conjugation of transferrin to the liposomal surface was achieved by adding human transferrin (5 mg/mL, Sigma-Aldrich Co., St. Louis, MO) to the liposome solution and incubating the mixture for 15 minutes. Transferrin was adsorbed to the surface of liposome (Lip) via electrostatic interactions. The Tf:Lip ratio was 12.5 μg :10 nmol, which was similar to the optimized ratio derived from systematic gene delivery study using transferrin-liposome-DNA complex.¹⁶ The size of the Lip-Mag and Tf-Lip-Mag complexes were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK).

MRI and Tumor Histology: DCE-MRI was performed using a 9.4 T, 89 mm vertical bore NMR spectrometer (Bruker Biospin MRI, Billerica, MA) with a 25 mm quadrature birdcage transmit/receive radiofrequency coil. The *in vivo* studies were performed when the tumor size reached about 1 cm in diameter. The imaging sequence used in DCE-MRI was a multi slice multi echo (MSME) sequence: TE = 11.4 msec; TR = 605 msec; $\alpha = 90^\circ$; 2 averages, field of view 25.6 \times 25.6 mm; the matrix size 256 \times 128, slice thickness 1 mm. Free Magnevist or Tf-Lip-Magnevist nanocomplex (0.3 mmol/kg) was systematically administrated by i.v. injection into the tail vein and images were acquired every 2.5 minutes for the 120 minutes. First, the animal was given an injection of Magnevist alone for obtaining a control DCE-MRI. Forty eight hours later the same animal was used in the targeted DCE-MRI study with Tf-Lip-Magnevist nanocomplex. Excised tumors were Hematoxylin and Eosin stained. The DCE-MRI signal enhancement patterns and pathologic findings were carefully compared.

Distribution of Gd in the tumors: The muscle samples were taken from the leg, and the tumor tissues were collected from necrotic and non-necrotic regions of the tumors. The collected tissues were weighed and freeze-dried. Inductively Coupled Plasma mass spectrometry (ICP-MS) (Elemental X7 ICP-MS, Thermo Fisher Scientific, Waltham, MA) measurements were used for the Gd distribution analysis.

3. RESULTS AND DISCUSSION

Although Magnevist does not cross the cell membranes *in vivo*, cellular uptake of Magnevist has been observed *in vitro* using Tf-conjugated liposomes loaded with Gd-DTPA.¹⁵ The efficiency of the Tf-mediated cellular uptake of Gd-DTPA *in vitro* was confirmed by confocal microscopic observation and by measuring the NMR T₁ relaxation time of the MDA-MB-231-luc cell pellets following one hour incubation of the cells with Tf-Lip-Mag nanocomplex. Tf has demonstrated its ability to direct cationic liposomes containing MRI contrast agent to receptor-bearing breast cancer tumor cells *in vitro*. The results indicate the importance and specificity of Tf moiety for targeting and illustrate the Tf-Lip-Mag nanoprobe internalization by tumor cells *in vitro*.¹⁵

Similar to our previous study using the Tf-Lip-Mag nanocomplex as a dual labeled probe for studying breast cancer xenografts in nude mice,⁷ we tested the Tf-Lip-Mag nanocomplex in a prostate cancer tumor model using PC-3M-luc cancer cells. The sizes of the freshly prepared Lip-Mag and Tf-Lip-Mag were 123.1 \pm 6.9 nm and 134.8 \pm 6.3 nm respectively. Since the dimension of transferrin is approximately 8 \times 10 nm^{2,11,17} the size measurements of Lip-Mag and Tf-Lip-Mag confirm the binding of Tf to the surface of liposome.

Representative DCE-MRI images of PC-3M-luc mouse xenografts are shown in Figure 1. Images obtained 60 minutes after CA and nanoprobe administration (second column) show significant differences in enhancement patterns. The observed increasing signal intensity and greater contrast induced by nanocomplex compared to the CA alone are very promising for future clinical applications. The underlying mechanism of this heterogeneous and prolonged image enhancement still needs further investigations. Nonetheless, these unique image enhancement pattern and correlation to the pathology indicate potential for future clinical use.

Figure 2A displays the probe-mediated dynamic contrast enhancement in different parts of the tumor which is well correlated with the pathological findings in Figure 2B, and 2C. We have assessed the images of tumors in three different regions: peripheral region with abundant of blood vessels, deep-seated non-necrotic region away from blood supply, and the necrotic region. In the peripheral region, because of the abundant blood vessels the nanocomplexes were at first leaking out into this region. The image intensity increases in this region first, and then the CA gradually diffuses into the second deeper region and image intensity gradually increases with time. The third region, necrotic area, serves as a sink for CA accumulation due to lack of washout mechanisms.

Figure 2D shows the dynamic image intensity ratio of tumor to muscle from these three regions after Tf-Lip-Mag or Magnevist i.v. administration. There is a significant signal intensity increase in the peripheral region during the first 10 minutes after injection (Figure 2D, curve 1). This is the initial stage of the CA distribution in tumor. During this time the Tf-Lip-Mag starts leaking out from the leaky blood vessels into the extravascular extracellular space (EES). Then, the nanocomplexes enter into cancer cells through endocytosis and release the CA in the cytoplasm. The CA or nanocomplexes may also be pumped out from the cytoplasm into the EES. For a well perfused area such as in the peripheral region of the tumor, there will be a higher concentration of nanocomplexes. The contrast level in the deeper tumor tissues containing viable cells gradually increases with time (Figure 2D, curve 2), and the rate of the contrast enhancing observed in the necrotic area (Figure 2D, curve 3) is higher than in the viable deep tissues. The untrapped nanoprobe and/or already released free Magnevist in EES gradually diffuse into the necrotic area from the viable tumor cells. The necrotic area becomes a depot for CA. There are a few basic competitive processes: (1) a flow of CA into the tumor, probes being trapped by viable cells and consequently being released from cells, followed by diffusion in and out of EES (2) CA washing out from EES back to the vascular system by diffusion and due to the CA concentration gradient. An equilibrium stage was achieved between 40-70 minutes after administration of the nanocomplex and lasted for more than 60 minutes. At the equilibrium, the contrast in viable cell areas, which includes the peripheral and deep tissue regions, are similar regardless of the blood supply (Figure 2D, curves 1 and 2). In comparison with using CA alone, at a late stage, 120 min after nanocomplex administration, the tumor-muscle contrast is still elevated and approximately 1.5 times higher in the area of viable cells regions and 2.7 times higher in the necrotic area. The contrast at later stages in the deeper viable tissue is slightly higher than in peripheral area which could be due to the slow out flow of CA from necrotic region. The overall process of the washing out of CA from the tumor in the targeted DCE-MRI is slow and lasts for a few hours.

Comparison of the targeted DCE-MRI (Figure 2D, curve 1), the DCE-MRI of free Magnevist illustrated in Figure 2D (curve 4) showed less contrast and the image intensity rapidly decreased in the peripheral area starting 5 minutes after Magnevist administration. After 2-3 hours, the Magnevist washed out from the body completely. These differences in contrast enhancement in tumors suggest that the elevated and prolonged image intensity is due to the receptor mediated endocytosis of nanocomplex similar to that observed *in vitro* studies. However, the internalization of the nanocomplex into the tumor cells *in vivo* still needs further direct confirmation. The other possible causes for the elevated level of the contrast could be due to the entrapment of the nanocomplexes in the EES of the peripheral region and the slow process of being washed out. In addition, the size of the nanocomplex also will influence the circulation time of the probe in the blood system and the retention in tumor. The uptake and retention of the Tf-Lip-Mag liposomal nanocomplex in tumor was significantly increased in comparison to Magnevist alone. Moreover, the observed enhancement patterns correlated well with tumor histology and it may be used to provide pathological information noninvasively.

Quantitative analysis of Gd concentration from muscle, necrotic areas and area of viable cells of the tumor reveals that after 120 min of i.v. administration the Gd concentrations are about 2.5 times higher in the samples collected in the mice injected with the Tf-Lip-Mag nanocomplex than with the mice injected with Magnevist only (Figure 3). The highest Gd concentration ratio of 2.8 was observed in the samples collected in the region of viable cells in the tumor. These results are consistent with DCE-MRI contrast enhancement patterns. This implies that a lower dose of Gd in the form of liposomal nanocomplex can be used to achieve the MRI enhancement that is achieved with nearly 2.5 times higher concentration of Gd in the form of free Magnevist.

4. CONCLUSIONS

In conclusion, we have shown that the targeted nanocomplex, Tf-Lip-Mag (~130 nm) significantly enhanced the MRI signals in PC-3M-luc prostate cancer xenograft tumors in mice. The image enhancement was superior to that obtained using contrast agent alone. This superior enhancement capability of the nanocomplex can be used to increase the sensitivity of detecting small tumors. A much higher contrast in viable cells in the periphery of tumor was observed at the initial stage of the targeted DCE-MRI. The image intensity was persistently higher compared to the DCE-MRI of free CA and lasts for several hours. The nature of the elevated contrast level at the initial stage of targeted DCE-MRI *in vivo* may be due to receptor mediated endocytosis. The tumor enhancement patterns are well correlated with histology, and could be used to evaluate tumor pathology *in vivo* and provide very useful timely information for the clinicians. In future specially formulated nanocomplex CA can be used for quantifying the specific biomarkers expressed in tumors, which will be helpful to determine the patient's prognosis and response to treatment.

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Figure 1, A. Korotcov et al. A Nanocomplex System as Targeted Contrast Agent Delivery Vehicle for MRI Dynamic Contrast Enhancement Study.

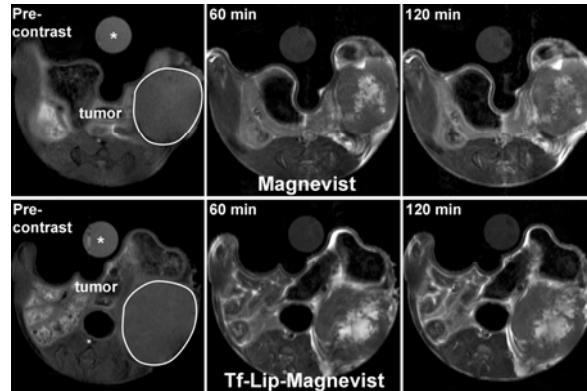


Figure 1. DCE-MRI images of the same mouse (PC-3M-luc solid tumor xenograft) studied with Magnevist alone (upper row) and with the Tf-Lip-Mag nanocomplex (lower row). There is 48 hours interval between the studies. * 0.9% saline was used as a reference.

Figure 2, A. Korotcov et al., A Nanocomplex System as Targeted Contrast Agent Delivery Vehicle for MRI Dynamic Contrast Enhancement Study (color figure).

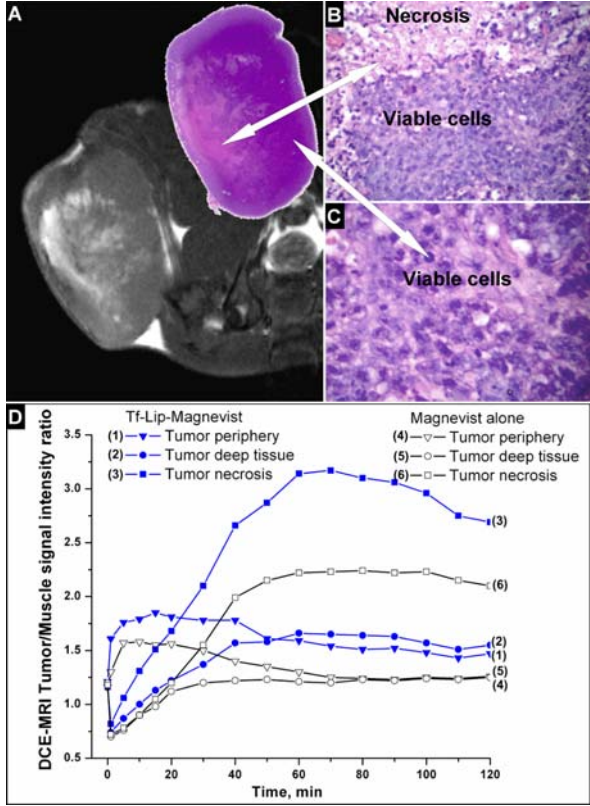


Figure 2. The probe-mediated MRI Image enhancement pattern (A) is well correlated with the pathological findings (B, C). Figure 2.A is a representative MR image showing heterogeneous enhancement pattern. Figure 2.B shows a region with high concentration of viable cells and necrosis (magnification $\times 250$). Figure 2.C shows the viable cells with high mitotic activity (magnification $\times 400$). In Figure 2.D, typical DCE-MRI curves show the MRI image intensities increase initially and later decrease at various rates for different regions in the tumor using either Tf-Lip-Mag liposomal nanocomplex or Magnevist. These distinct differences are due to the specific targeting and prolong retention of the Tf-Lip-Mag liposomal nanocomplex in the tumor.

Figure 3, A. Korotcov et al., A Nanocomplex System as Targeted Contrast Agent Delivery Vehicle for MRI Dynamic Contrast Enhancement Study.

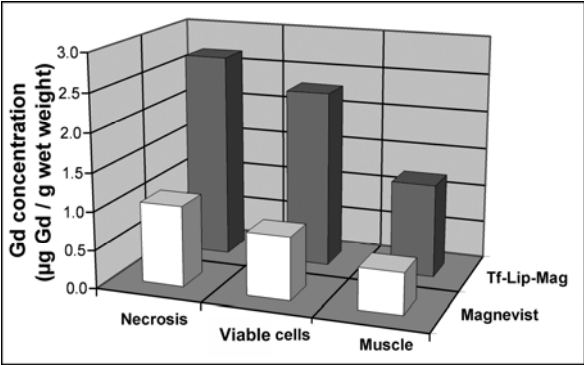


Figure 3. Average biodistribution of Gd in tumor-bearing mice (expressed in Gd concentrations in tissues, $\mu\text{g Gd} / \text{g wet weight}$). The samples were taken from muscle, necrotic area and viable cells area in the tumor after 120 min of i.v. administration of Magnevist or Tf-Lip-Mag liposomal nanocomplex.

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Address of the corresponding author:

Author: Paul C. Wang
Institute: Howard University, Radiology Department
Street: 2041 Georgia Ave., N.W.
City: Washington, DC 20060
Country: USA
Telephone: + 202-865-3711
Fax: + 202-865-3722
Email: pwang@howard.edu