Specific Comments To Authors:

This reviewer carefully reviewed the paper entitled, " Development and in vitro study of bi-specific MRI molecular probe for hepatocellular carcinoma". In this manuscript, the authors showed the possibility of usefulness of the bi-specific MRI probes in the early diagnosis of patients with hepatocellular carcinoma. This study was performed by appropriate methods, and the manuscript is written concisely with the adequate numbers of figures and tables. Major part of these results is interesting and a significant contribution to our knowledge on hepatocarcinogenesis. There is a major concern in the present experiment that this reviewer must point out. The internalization of bi-specific probe in the hepatocyte is proven in vitro experiment. However, there are many Kupffer cells in the liver of a patient with hepatocellular carcinoma, and bi-specific SPIO prove might be easily incorporated into Kupffer cells rather than hepatocytes. Non-specific incorporation of background Kupffer cells may interfere the visualization of small size HCC. How do the authors overwhelm this problem?

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Thank you very much for pointing out this important issue!

To answer the reviewer, we have listed several points and preliminary experimental data.

1. The non-specific uptake of SPIO by Kupffer cells in the liver should be avoided during *in vivo* HCC imaging.

We agree with the reviewer that the SPIO may be easily sequestered by the reticuloendothelial system (RES) cells in the liver or spleen. The cellular study of bi-specific probes in our present manuscript were preliminary work for pre-clinical *in vivo* HCC MR imaging study in the next step. Therefore, the non-specific uptake of such

nanoparticle-based probes by background Kupffer cells in the liver is a key point to be considered and weakened during our probes design.

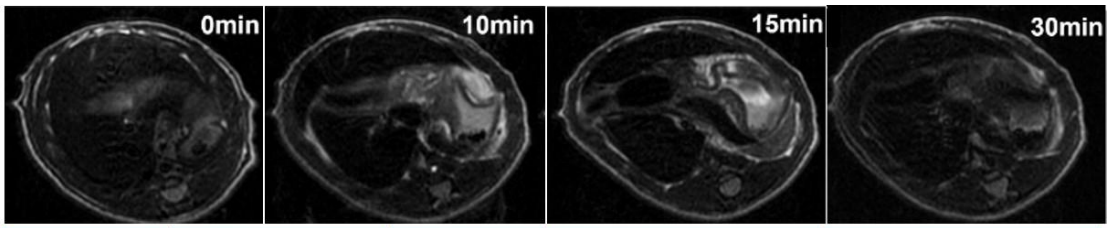
2. USPIO with smaller hydrodynamic size was synthesized in the study to enhance tumor penetration and avoid RES system recognition.

It has been reported by many review articles about the iron oxide nanoparticles for biomedical application that the biodistribution, pharmacokinetics and *in vivo* cellular uptake of iron oxide nanoparticles are related to their hydrodynamic size, charge, shape or surface modifications. [1-3]

The hydrodynamic size of the nanoparticle plays a crucial role in their concentration in blood, circulation time and tissue penetration.[1] As for solid tumor imaging, it has been suggested that the nanoparticles should be located within 10 nm-100 nm to penetrate capillary walls and realize effective tumor distribution, while to prevent rapid leakage into blood, or fast clearance and phagocytosis by macrophages rich in organs of RES systems, such as liver or spleen.[4-6] Thus, the development of the ultra-small SPIO (USPIO) with size around or less than 50 nm have been prioritized by companies and clinical aspect, as for their longer blood half-life and slow phagocytosis by macrophages. [7]

In our study, the USPIO with small core size (~5 nm) was adopted as the platform to further conjugate with targeting biomarkers such as AFP- and GPC3-antibodies. The final hydrodynamic size of non-targeted and bi-specific (double-targeted) USPIO probes were range from 40.4-59.6 nm, which meet the particle size requirement for the tumor imaging as mentioned above. In addition, our preliminary works (under review) about USPIO (~5 nm core size) absorption and clearance properties in normal rat liver studied by T2-weighted MRI, also confirmed that the USPIO with such small core size could be cleared by the normal liver of rat within ~30 min after tail vein administration, as shown in **Figure 1**.

Figure 1. The T2-weighted MR image of liver and liver-to-muscle signal ratio before and at different time point after 5 nm USPIO administration into normal rat.



	Pre-injection	10min	15min	35min
T2 L/M	0.45	0.30	0.34	0.44

(L/M: Liver-to-paraspinal muscle signal ratio)

Therefore, considering the appropriate hydrodynamic size of our targeted USPIO probes and the relative fast clearance of non-targeted USPIO (~5 nm core size) by normal liver within ~30 min, we hypothesize that the strategy of smaller USPIO may facilitate bi-specific probes binding to HCC tumors with low level background signal from normal liver.

The adoption of USPIO with 5 nm core size and discussion about hydrodynamic size of resulted bi-specific USPIO probes were described in the introduction part (*Page 8 Line 6-8*) and discussion part (*Page 24 Line 6-17*), colored in blue. The related references are also cited.

3. Surface modification of the USPIO will be optional strategy in the further *in vivo* experiment.

The surface coating is an equally important factor for *in vivo* fate of nanoparticle-based probes. Compared with hydrophobic coatings, hydrophilic surface may help nanoparticles to avoid plasma protein adsorption and accumulation which could lead to reticuloendothelial system (RES) recognition and uptake. [1] Therefore, if necessary, to further reduce non-specific uptake of USPIO by RES system, we may also consider adopting some surface modifications for the USPIO, such as introducing hydrophilic

PEG coatings in our *in vivo* experiments.

Reference

1. Laurent S, Saei A A, Behzadi S, et al. Superparamagnetic iron oxide nanoparticles for delivery of therapeutic agents: opportunities and challenges. *Expert Opinion on Drug Delivery*, 2014, **11**(9):1449-1470.

2. Jin R, Lin B, Li D, et al. Superparamagnetic iron oxide nanoparticles for MR imaging and therapy: design considerations and clinical applications. *Current Opinion in Pharmacology*, 2014, **18**:18-27.

3. Wang Y X J, Hussain S M, Krestin G P. Superparamagnetic iron oxide contrast agents: physicochemical characteristics and applications in MR imaging. *European radiology*, 2001; **11**(11): 2319-2331.

4. Cortajarena AL, Mestresortega D, Ocampo SM, García A, Couleaud P, Miranda R, Beldainiesta C, Ayusosacido A, Garcissastre A, Cortajarena A. Engineering Iron Oxide Nanoparticles for Clinical Settings. *Nanobiomedicine*, 2014, **1**(2): 1-20.

5. Feng QY, Liu YP, Huang J, Chen K, Huang JX, Xiao K. Uptake, distribution, clearance, and toxicity of iron oxide nanoparticles with different sizes and coatings. *Scientific Reports*, 2018, **8**(1): 2082-2093.

6. Shakir A M, Ahmad B S, Clayton Y, et al. Double-receptor-targeting multifunctional iron oxide nanoparticles drug delivery system for the treatment and imaging of prostate cancer. *International Journal of Nanomedicine*, 2017, **12**:6973-6984.

7. Daldrup-Link HE. Ten Things You Might Not Know about Iron Oxide Nanoparticles. *Radiology*, 2017, **284**(3): 616-629.