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Radiolabeling and preliminary biodistribution study of ^{99m}Tc-labeled antibody-mimetic scaffold protein repebody for initial clearance properties

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ABSTRACT

Antibody-mimetic proteins are intensively being developed for biomedical applications including tumor imaging and therapy. Among them, repebody is a new class of protein that consists of highly diverse leucine-rich repeat (LRR) modules. Although all possible biomedical applications with repebody are ongoing, it's *in vivo* biodistribution and excretion pathway has not yet been explored. In this study, hexahistidine (His_6)-tag bearing repebody (rEgH9) was labeled with [^{99m}Tc]-tricarbonyl, and biodistribution was performed following intravenous (I.V.) or intraperitoneal (I.P.) injection. Repebody protein was radiolabeled with high radiolabeling efficiency (>90%) and radiolabeled compound was more than 99% pure after purification. Biodistribution data indicates radiotracer has a rapid clearance from blood and excreted through the kidneys for intravenous (I.V.) injection, but comparatively slow clearance for an intraperitoneal (I.P.) injection. SPECT-CT images were found to be in agreement with biodistribution data, high activity was found inside kidneys. The observed result for rapid blood clearance and renal excretion of repebody (rEgH9) provide useful information for the further development of therapeutic strategy.

Interleukin-6 (IL-6) is an active member of cytokine family that plays key role to regulate wide range of biological activities including hematopoiesis, immune response, tumorigenic process, and inflammation.[1] Usually it has little to do with body day-to-day function, however, like other cytokines it is front-line army against infections and diseases.[2] An abnormally high expression level of IL-6 could be associated with the pathology of many diseases such as rheumatoid arthritis, post-menopausal osteoporosis, psoriasis, castleman's disease, and multiple myeloma.[3–5] The understanding of biochemical mechanisms controlled by IL-6 and development of functional agonists and antagonists may offer therapeutic benefits.[6,7] The main therapeutic approaches against IL-6 signaling include the use of anti-receptor monoclonal antibodies (mAb), ligand-neutralizing mAb, and tyrosine kinase inhibitors.[8,9] Anti-IL6 therapeutics using monoclonal antibody (mAb) are able to neutralize IL-6 production and dramatically inhibit the proliferation of cancer cells. However, they have some drawbacks, such as limited clinical efficacy, high production cost, low tumor uptake, and slow clearance from plasma due to their large size.[10] Therefore, the construction of novel robust therapeutic antibodies with enhanced efficacy such as high tumor localization and sufficiently fast elimination from healthy tissues is an active area research. The development of alternative binding scaffold proteins may offer a small size, simple architecture, stable and highly soluble, and allow the specific tailoring of various molecular properties in addition to target affinity and specificity.[11–13]

Several radiolabeled antibody-mimetic protein such as DARPins[14], affibody molecules[15], anticalins[16], knottins[17], and fibronectin domains[18], have demonstrated their potential as tumor imaging agents in preclinical research. Among them, repebody is a new artificially designed binding scaffold that consists of highly diverse leucine-rich repeat (LRR) modules as their adaptive immune response to foreign antigens.[19] The repebody has a high level of soluble expression, easy library construction, and allows easy designing of the binding properties by modular

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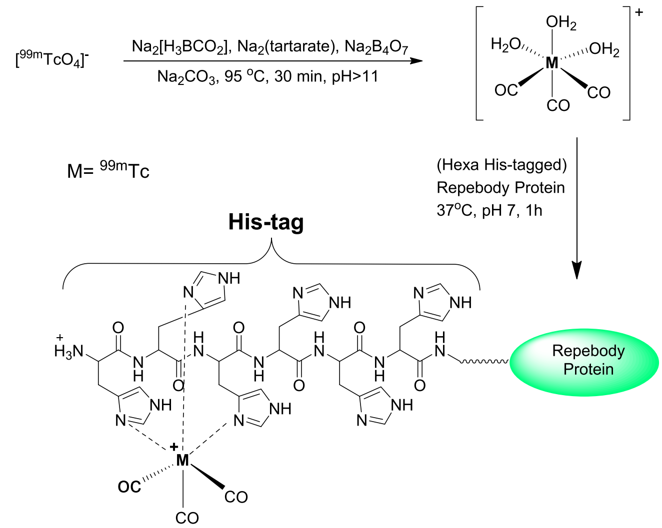
engineering.[19,20] Repebody libraries have been constructed for a phage display and binding affinity of selected repebodies for human IL-6 through isothermal titration calorimetry suggested that they can effectively inhibit the interaction between human IL-6 and its receptor.[20]

Pharmacokinetic (PK) study, particularly biodistribution, provides crucial quantitative and tracking information in all major tissues after administration of biological active molecule. It is also necessary for preclinical safety evaluation and development of improved therapy strategy of medicines.[21] In this study, we present the radiolabeling of repebody (rEgH9), extended at the N-terminus with the His6-tag by using $[^{99m}Tc(OH_2)_3(CO)_3]^+$ to study its biodistribution and localization in major organs of healthy mouse. Among various available radioisotopes, technetium-99m is the most commonly used radionuclide to evaluate biodistribution of molecules in the field of nuclear medicine. Its short half-life (6 h), favorable γ photon emission of 140 keV (nearly ideal for SPECT imaging), low radiation absorbed dose burden to patients, low price and excellent availability of $^{99}Mo/^{99m}Tc$ generators makes it the most suitable radioisotope for initial studies.[22] The pharmacokinetics data will not only help to develop therapeutic strategy but also provide important systematic information that might be useful in near future preclinical and clinical research using repebody proteins.

Several methods have been developed for ^{99m}Tc labeling of biologically active micro and macro molecules.[23,24] Among them, Waibel et al. have developed a site-specific direct labeling method employing a recombinant single-chain Fv antibody containing hexahistidine (His_6)-tag.[25] The His_6-tag allows facile and highly stable labeling with $[^{99m}Tc\ (OH_2)_3(CO)_3]^+$.[26,27] Moreover, radiolabeling of repebody (rEgH9) via His_6-tag, extended at the N-terminus may offer several advantages. Because, critical analysis of peptide-based chelating groups situated at the N-terminus of the biomolecule revealed that amino acid composition at the N-terminus played key role in biodistribution profile specifically the liver uptake and hepatobiliary excretion.[28–30] The use of amino acid groups with polar or charged hydrophilic side chains in N-terminal usually reduced the live uptake and caused low level of hepatobiliary excretion. However, the composition of C-terminus is usually independent of that effect.[31–33] The considerable low liver uptake and *in vivo* stability ^{99m}Tc-labeled protein are the main feature of chelator attached to N-terminus.[34]

The repebody molecules (PDB code: 4J4L, chain A), extended at the N-terminus with the His6-tag were synthesized, purified and characterized by immobilized metal affinity chromatography (IMAC) by using previously described procedure.[20] The synthesis of ^{99m}Tc-tricarbonyl and radiolabeling of repebody (rEgH9) was performed according to site-specific labeling method (Scheme 1). The ^{99m}Tc-tricarbonyl precursor was synthesized using an established procedure (Supporting information) to give more than 95% radiolabeling yield. The repebody (rEgH9) was labeled by incubating with ^{99m}Tc-tricarbonyl at 37 °C for 1 h. At the end of reaction, unconjugated ^{99m}Tc-tricarbonyl was removed by centrifuge using 10 kDa cut-off filter. The radiolabeling purity of labeled product was determined by radio-TLC (Fig. 1). The product was obtained in high radiochemical purity (>99%) with specific activity 925 MBq/mg.

To determine the stability of labeled compound, ^{99m}Tc-repebody (100 μL, 1.85 MBq) was incubated with 900 μL of mouse serum or saline at 37 °C. The stability of the ^{99m}Tc-repebody in saline or mouse serum was analyzed at different time points by radio-TLC. The radioactive conjugate of repebody keeps excellent *in vitro* stability in saline or mouse serum. (Fig. S1). To evaluate the bond strength of ^{99m}Tc-complex, a histidine challenge was performed. A known amount of ^{99m}Tc-repeboy was incubated with an excess amount of histidine (1000-fold molar excess) at 37 °C for 24 h.



Scheme 1. Synthesis of ^{99m}Tc-labeled repebody protein.

The radiochemical purity of ^{99m}Tc-repebody was more than 95% up to 24 h post incubation (Fig. S1). The lipophilicity of ^{9m}Tc-repebody was determined by using procedure reported elsewhere.[35,36] The partition coefficient of the ^{99m}Tc-repebody was determined by using measured radioactivity in octanol and water at pH 7.4. The partition coefficient (log $P_{o/w}$) was found to be −2.061, indicating that ^{99m}Tc-repebody was highly hydrophilic. It is preferentially distributed in the blood serum thus enhance the pharmacokinetic properties.

For biodistribution study, 6 weeks old female BALB/c mice were used. The animals were anesthetized with isoflurane and then received an intravenous (I.V.) or intraperitoneal (I.P.) dose of 1.85 MBq of ^{99m}Tc-repebody in 100 μL of saline per mouse. For the detail biodistribution study, mice were sacrificed under anesthesia. Radioactivity of blood and organs was measured in the gamma counter. Radioactivity in the samples was expressed as the percentage of injected radioactive dose per gram of tissue (% ID/g). The biodistribution data following intravenous injection (I. V) has been summarized in table 1. The radiotracer was rapidly cleared from the blood, 0.70 ± 0.14 %ID/g during the 1 h and at 6 h pi only 0.28 ± 0.02 %ID/g remained in the blood pool. Final activity in blood was 0.14 ± 0.01 %ID/g 24 h post injection (Fig. 2). It is considered that rapid clearance of radiotracer from blood in necessary for high tumor to blood ratio and hence better imaging. The distinguished feature of engineered scaffold protein (repebody) is it small size which not only provides fast extracellular diffusion but also rapid clearance of radiotracer from background organs. It has potential to provide high contrast images along with high affinity to target area. It has to be consider that the fast clearance of radiotracer from tissues and organs depends not only size or blood clearance but also on off-targeting interactions. There are several properties of engineered protein including lipophilicity, overall charge, specificity towards binding site, radioisotope and purification tag interactions which determine the *in vivo* behavior and off-target interactions. The predominant organ with high radioactivity concentration was kidneys. It can been seen that soon after administration, the labeled compound was rapidly cleared from the blood and body toward the kidneys. The kidneys were the main route of elimination with 53.38 ± 8.18 %ID/g 1 h and 29.16 ± 9.31 %ID/g 24 h post injection. The organ with the second highest radioactivity was the liver 7.15 ± 3.95 %ID/g, 1 h and there was little decrease during 3 h post injection. Overall little change was observed in its biodistribution