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Rapid biochemical synthesis of ^{11}C-labeled single chain variable fragment antibody for immuno-PET by cell-free protein synthesis

Takayoshi Matsuda [a,b], Shozo Furumoto [c,d], Kae Higuchi [a,b], Jun Yokoyama [a,b,e], Ming-Rong Zhang [f], Kazuhiko Yanai [c], Ren Iwata [d], Takanori Kigawa [a,b,g,*]

[a] NMR Pipeline Methodology Research Team, RIKEN Systems and Structural Biology Center, 1-7-22 Suehiro-cho, Tsurumi, Yokohama 230-0045, Japan
[b] Cell-Free Technology Application Laboratory, RIKEN Innovation Center, 1-7-22 Suehiro-cho, Tsurumi, Yokohama 230-0045, Japan
[c] Department of Pharmacology, Graduate School of Medicine, Tohoku University, Sendai 980-8575, Japan
[d] Division of Radiopharmaceutical Chemistry, Cyclotron and Radioisotope Centre, Tohoku University, Sendai 980-8578, Japan
[e] Tsukuba Laboratory, Taiyo Nippon Sanso, Tsukuba, Ibaraki 300-2611, Japan
[f] Molecular Imaging Center, National Institute of Radiological Sciences, Chiba 263-8555, Japan
[g] Department of Computational Intelligence and Systems Science, Interdisciplinary Graduate School of Science and Engineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8502, Japan

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ABSTRACT

Immuno-PET is a promising approach for improved cancer diagnosis, by taking advantage of the high specificity of antibodies. Here, we present a novel cell-free protein synthesis method for preparing a positron emitter labeled-antibody. Functional anti-human EGFRvIII single chain Fv, MR1-1, was successfully labeled with carbon-11 (half-life = 20.4 min) in 5 min (36% yield) by the direct incorporation of the clinical PET tracer, L-[^{11}C]methionine. The product [^{11}C]MR1-1 was easily and rapidly isolated with high radiochemical purity (>95%) from the reaction solution, by affinity purification. This method would be widely applicable to the preparation of radiolabeled antibodies for PET imaging.

1. Introduction

Positron emission tomography (PET) is a key molecular imaging modality in clinical and biomedical research. Noninvasive and quantitative imaging by PET provides valuable information on the functions of biomolecules, such as enzymes, receptors, and transporters, in the living body. Their substrates or ligands, labeled with a positron emitter, are generally used as imaging probes for PET. 2-[^{18}F]-fluoro-2-deoxy-D-glucose ([^{18}F]FDG), a substrate for glucose metabolism, is the most widely used PET tracer for clinical diagnoses of tumors and searches for metastasis by whole body scans. [^{11}C]Methionine is also a useful PET tracer, which is most commonly and widely used for imaging brain and peripheral tumors in clinical diagnosis and investigation.[1] In addition to these conventional imaging techniques using small compounds, immuno-PET, a novel tumor imaging approach using a radiolabeled monoclonal antibody or an antibody fragment, has been drawing much attention. Since immuno-PET is expected to improve the characterization, diagnosis, and immunotherapy of tumors, many studies have been performed to prepare and evaluate positron-emitter labeled antibodies.[2]

The cell-free (or *in vitro*) protein synthesis system, which produces proteins using lysates from bacterial or eukaryotic origin, has become an important tool for molecular biology. The produced protein can be easily purified after a brief protein synthesis reaction, without time-consuming cell disruption. The openness of the system enables the direct redox control of the system and the addition of a disulfide-reshuffling enzyme, so that disulfide-containing proteins, such as single chain Fv (scFv), can be produced within an hour.[3] Additionally, labeled proteins can be easily synthesized by simply replacing the target amino acid(s) with the labeled one(s).[4] These features indicate that the cell-free system is potentially applicable to the preparation of radiolabeled scFv, using a positron-emitter labeled amino acid. For this purpose, [^{11}C]methionine is a suitable candidate, because its production method is easy and well established.

Abbreviations: PET, positron emission tomography; EGFRvIII, epidermal growth factor receptor variant III; scFv, single chain Fv; L-[methyl-^{11}C]methionine, [^{11}C]Met; CT, computed tomography; [^{18}F]FDG, 2-[^{18}F]-fluoro-2-deoxy-D-glucose; MWCO, molecular weight cut-off.

* Corresponding author. Tel.: +81 45 503 9203; fax: +81 45 503 9643.
E-mail address: kigawa@riken.jp (T. Kigawa).

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In the present study, we first introduced a positron-emitter labeled amino acid into the cell-free synthesis reaction, to produce a radiolabeled antibody fragment for immuno-PET applications. As an imaging target, epidermal growth factor receptor variant III (EGFRvIII) is attractive, because it is overexpressed in a substantial proportion of malignant gliomas and other human cancers,[5] and thus is considered to be a potential target for immunotherapy and immno-PET. We have successfully utilized cell-free synthesis to produce a carbon-11 labeled, functional MR1-1, which is an scFv that recognizes the specific linear epitope LEEKKGNYVVTDH of EGFRvIII.[6]

2. Materials and methods

2.1. Template DNA for cell-free synthesis

The DNA encoding MR1-1[7] was subcloned into the pCR2.1 TOPO vector by the two-step PCR method,[8] from the pUC MR1-1 vector synthesized by a gene synthesis service (Invitrogen, USA). By the two-step PCR method, the T7 promoter and the ribosome binding site were attached to the 5′ end, while the T7 terminator was attached to the 3′ end of the coding sequence.

2.2. Cell-free synthesis of MR1-1

The *Escherichia coli* extract was prepared according to our standard protocol,[9] with minor modifications. The S30 extract was dialyzed against S30 buffer without DTT, and then was concentrated twofold, using an Amicon ultra MWCO 10 kDa device (Millipore, USA). The reaction solution, based on the D-glutamate system,[10] consisted of 60 mM HEPES-KOH (pH 7.5), 230 mM potassium D-glutamate, 1.3 mM ATP, 0.9 mM each of GTP, CTP, UTP, 20 mM magnesium acetate, 36 μg/ml folinic acid, 80 mM creatine phosphate, 150 μg/ml *E. coli* tRNA, 5 mM oxidized glutathione, 4.5 mM each of cysteine, serine, arginine, glutamine, tryptophan, 1.5 mM each of the other amino acids, 50 μg/ml creatine kinase, 100 μg/ml T7 RNA polymerase, 400 μg/ml DsbC, 24% volume of concentrated *E. coli* S30 extract, and 4 μg/ml template DNA. The reaction was performed at 37 °C for 60 min, unless otherwise noted. The reaction solution used for radiosynthesis included the same components, but lacked methionine.

2.3. Affinity purification of MR1-1 using an epitope peptide

An affinity column was prepared with a Tricone column (GE Healthcare, USA), biotin labeled EGFRvIII peptide (LEEKKGNYVVTDHSGGK-biotin)[6] and streptavidin agarose (GE Healthcare). After protein synthesis, 2.5 ml of the reaction solution were mixed with 2.5 ml of buffer A [50 mM Tris–HCl (pH 7.5), 150 mM NaCl], followed by centrifugation at 10,000 rpm for 5 min. The supernatant containing MR1-1 was loaded onto the column, which was washed with 10 column volumes of buffer A. The MR1-1 was then eluted with buffer B [100 mM glycine-HCl (pH 2.9)]. The acidic pH of the eluted fraction was immediately neutralized by adding 1 M Tris–HCl (pH 10.8).

2.4. Kinetic analysis

The binding kinetics of MR1-1 to the EGFRvIII peptide was measured by a BIAcore 3000 (GE Healthcare, USA). The biotinylated peptide was immobilized on the sensor chip SA. MR1-1 (1.25, 2.5, 5, 10, 20 μg/ml) in HBS-EP buffer was injected. Regeneration was accomplished with 10 mM glycine-HCl (pH 1.5). The binding specificity of MR1-1 was examined with two kinds of mutant peptides (EGFRvIII-A: Val10Ala; EGFRvIII-3A: Tyr8Ala, Val10Ala, Asp12Ala).

2.5. Cell binding assay

MR1-1 was fluorescently labeled with Alexa Fluor 488 succinimidyl esters, by a standard amine-coupling method (Invitrogen, USA). The F98 and $F98_{EGFRvIII}$ rat glioma cells were obtained from ATCC and cultured in Lab-tek chamber slides (Thermo Scientific, USA). $F98_{EGFRvIII}$ cells over-express human EGFRvIII.[11] Assays were performed by incubating the cells with Alexa Fluor 488 labeled MR1-1 in PBS buffer for 30 min. To remove the unbound MR1-1, the cells were washed twice with PBS buffer. The MR1-1 bound cells were visualized by confocal fluorescence microscopy, using an LSM510 Confocal Imaging System (Carl Zeiss, Germany).

2.6. Cell-free synthesis of ^{11}C-labeled MR1-1

L-[^{11}C]Methionine ([^{11}C]Met) was prepared from L-homocysteine thiolactone hydrochloride (Sigma), according to the on-column methylation method reported previously.[12] Briefly, L-homocysteine thiolactone hydrochloride (6 mg) was reacted with [^{11}C]methyl iodide on a Sep-Pak Plus tC18 cartridge (Waters). The product was eluted with 0.5% acetic acid, and evaporated. The radioactive residue was dissolved in saline.[12] An 80 μl portion of the [^{11}C]Met solution (2.3–3.6 mCi/100 μl) per 200 μl scale cell-free reaction was used for [^{11}C]MR1-1 synthesis. The [^{11}C]Met was added to the cell-free reaction solution lacking methionine, and incubated at 37 °C for an appropriate period of time. The radiochemical yields of [^{11}C]MR1-1 during a 30 min reaction were determined by non-reducing SDS–PAGE autoradiography (ARG), using a BAS5000 phosphorimager (FUJIFILM, Japan). The synthesized [^{11}C]MR1-1 was isolated by the affinity purification method with the epitope peptide-conjugated streptavidin spin column (GE Healthcare), according to described above (see 2.3.). The total duration of this purification process was 20 min.

3. Results and discussion

3.1. Cell-free synthesis of MR1-1

Considering the short half-life of carbon-11, the protein synthesis rate was accelerated by using a condensed cell extract.[13] MR1-1 contains two pairs of disulfide bonds (one pair in each of the V_H and V_L domains). The cell-free system provides the ability to control the redox conditions of the reaction. To produce functional MR1-1, we introduced glutathione buffer and disulfide isomerase, DsbC, to the cell-free reaction, to facilitate disulfide-bond formation and the re-shuffling of incorrectly paired disulfide bonds, respectively. Soluble MR1-1 was synthesized in the presence of 5 mM GSSG and 400 μg/ml DsbC (Fig. 1), and after purification on the epitope column, about 110 μg of functional MR1-1 were obtained from 1 ml of reaction solution. When MR1-1 was synthesized under relatively reductive conditions or in the absence of DsbC, most of the MR1-1 precipitated (data not shown). These results indicate that the formation of properly paired disulfide bonds is essential in the folding process of MR1-1. The two pairs of disulfide bonds within MR1-1 were confirmed by MALDI-TOF MS, using a thiol-alkylating reagent (data not shown).

3.2. Affinity and specificity

Kinetic analyses of MR1-1 were performed by surface plasmon resonance, using a BIAcore 3000. The biotin labeled EGFRvIII peptide was linked to the SA chip. MR1-1 showed high affinity for the wild type peptide, with a K_D value of 1.06×10^{-8} M, while its affinity was significantly decreased for the mutant peptides (Fig. 2). These results demonstrated that the cell-free synthesized MR1-1 was able to strictly discriminate the antigen peptide.

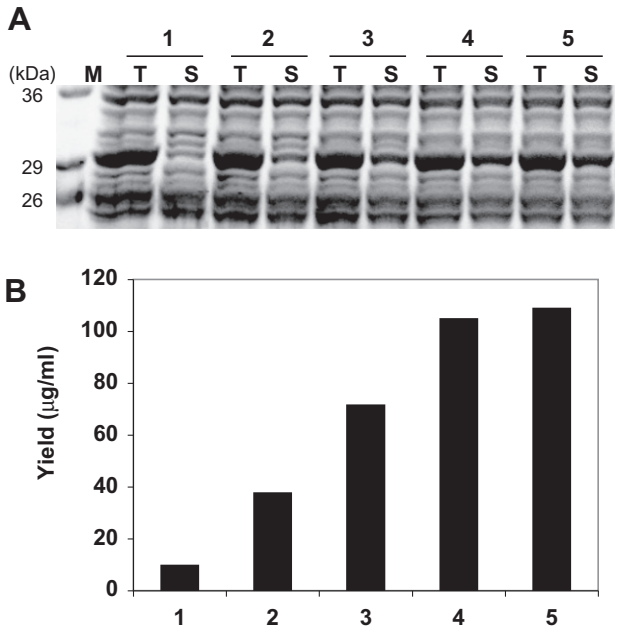

Figure 1. Cell-free synthesis of non-labeled MR1-1. (A) SDS–PAGE analysis. MR1-1 was synthesized in the presence of 5 mM GSH (1), 4 mM GSH and 1 mM GSSG (2), 2.5 mM GSH and 2.5 mM GSSG (3), 1 mM GSH and 4 mM GSSG (4), and 5 mM GSSG (5) Total (*T*) and supernatant (S) fractions of the reaction solution were loaded on the gel. The gel was stained with CBB. (B) Yield of MR1-1. MR1-1 was purified using an epitope column, and the yields of MR1-1 synthesized under different redox conditions were determined by the Bradford method[17], using BSA as a standard.

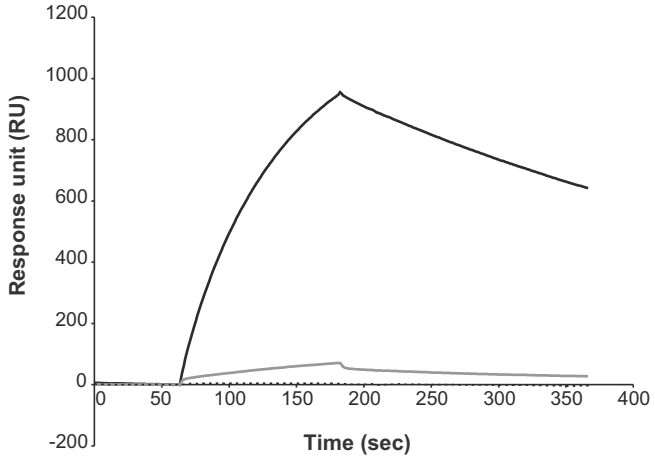

Figure 2. Surface plasmon resonance sensorgram of MR1-1. The binding of MR1-1 to epitope peptides, EGFRvIII (black line), EGFRvIII-A (gray line), and EGFRvIII-3A (dashed line), was analyzed.

3.3. Cell binding assay

The specific binding of MR1-1 to human EGFRvIII expressed on rat glioma cells, $F98_{EGFRVIII}$, was investigated. The binding of Alexa 488-labeled MR1-1 to the $F98_{EGFRVIII}$ cells was clearly observed (Fig. 3). On the other hand, MR1-1 did not bind to F98 cells, which do not express human EGFRvIII, indicating that the cell-free synthesized MR1-1 specifically binds to the intact target receptor expressed on cells.

3.4. Carbon-11 labeling and affinity purification of MR1-1

The major challenge of using [^{11}C]Met for immuno-PET is overcoming the short half-life (20.4 min) of carbon-11. The protein synthesis and purification must be completed quickly, in order to retain sufficient radioactivity for PET imaging. For this purpose, it is important to remove methionine from the components of the reaction solution. The radiosynthesis of [^{11}C]MR1-1 was initiated by adding a [^{11}C]Met solution, prepared for clinical use, to the reaction solution lacking methionine. As compared with the methionine-containing solution, the radiosynthesis in the methionine-lacking solution resulted in a better yield of [^{11}C]MR1-1 (data not shown).

The rate of [^{11}C]MR1-1 production under these conditions was very fast. The decay-corrected yield of [^{11}C]MR1-1 at 5 min was 36.2%, and the value was nearly constant (38%) after 10 min (Fig. 4A). However, the yield of [^{11}C]MR1-1 isolated after affinity purification was about 8.1% after a 20 min reaction. The difference between the calculated yield of [^{11}C]MR1-1 and the isolated one was mainly due to its elution into the flow-through fraction, suggesting that the binding of [^{11}C]MR1-1 to the epitope is insufficient. Although this low isolation efficiency should be improved, the current yield of the isolated [^{11}C]MR1-1 is sufficient for an animal PET study.

Through the purification step using epitope conjugated spin column, we successfully obtained [^{11}C]MR1-1 which certainly maintained the epitope binding activity. Functional [^{11}C]MR1-1 with 95.5% radiochemical purity was obtained in the elution fraction (Fig. 4B), indicating that it is sufficient for use as a PET tracer in animals. In the flow-through fraction, two bands of [^{11}C]MR1-1 were observed. Since these bands became a single band upon reducing SDS–PAGE (data not shown), the lower band is not truncated MR1-1. The lower band is probably the MR1-1 with incomplete or improper disulfide-bonds, which would lead to lower binding activity, due to the inability to form the antigen-binding site. Non-labeled MR1-1 was also prepared by using cell-free synthesis and subsequent epitope column purification under the same condition as [^{11}C]MR1-1 preparation except that non-labeled methionine was used in the cell-free synthesis reaction in place of [^{11}C]Met. An SDS–PAGE analysis clearly indicated that the addition of ^{11}C-Met does not affect the production and S–S formation of the MR1-1 scFv (Supplementary Fig. S1). In general, disulfide-bond formation is slow and the rate-limiting step in the folding pathway.[14] Further facilitation of proper folding with disulfide bond formation will improve the yield of functional MR1-1.

The conventional method for labeling an antibody requires the chemical modification of the amino acid side chains to be connected with a positron nuclide or a chelator for a metal positron nuclide.[15] Such chemical modifications sometimes reduce the binding activity of the antibody. In contrast, the activity of the carbon-11 labeled antibody produced in the present study should be intact, because the [^{11}C]Met used for labeling has the same chemical properties as the natural methionine.

Recently, the carbon-11 labeling of IL-8 was performed using the reconstituted cell-free system (PUREsystem). The IL-8 was successfully labeled with [^{11}C]Met and utilized for whole-body imaging in a mouse.[16] However, the [^{11}C]IL-8 had been synthesized under reducing conditions, so it was unable to form disulfide-bonds and was presumably inactive. Additionally, [^{11}C]Met dissolved in saline cannot be used in the reconstituted cell-free system, because a high concentration of NaCl inhibits the protein synthesis. In contrast, our approach allows us to synthesize functional scFv by facilitating proper disulfide-bond formation, and furthermore, clinical [^{11}C]Met in saline is usable without any modifications.

In conclusion, we have succeeded in the biochemical preparation of a carbon-11 labeled scFv, [^{11}C]MR1-1, by the cell-free protein synthesis method. The biochemical reaction using [^{11}C]Met in saline proceeded efficiently. The radiolabeled antibody could be easily purified on an affinity spin column with high chemical purity. A preclinical PET study using [^{11}C]MR1-1 is in progress.

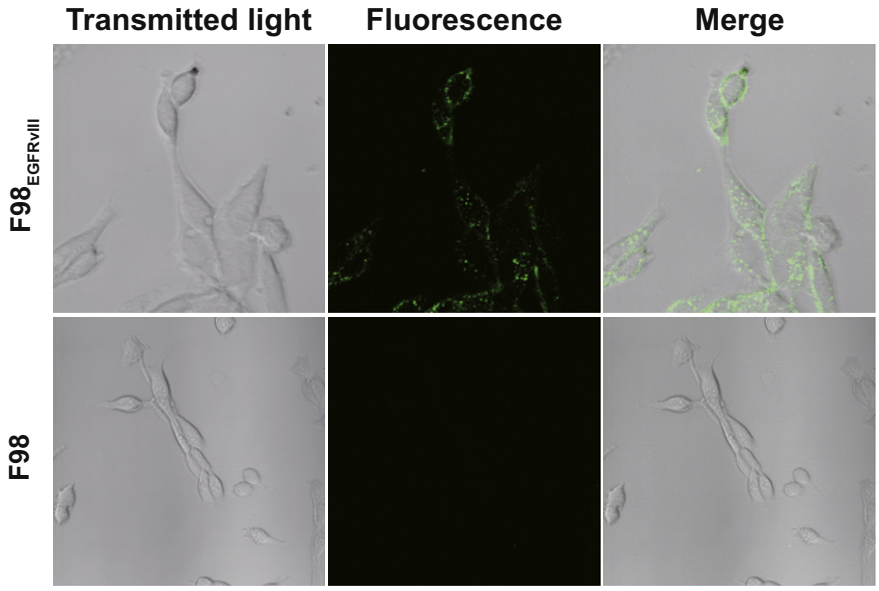

Figure 3. Specific interaction between MR1-1 and $F98_{EGFRvIII}$ cells. Alexa-488 labeled MR1-1 was incubated with F98 or $F98_{EGFRvIII}$ cells. After serial washes with PBS, the bound MR1-1 was visualized by confocal laser microscopy (Ex = 488 nm, Em = 519 nm).

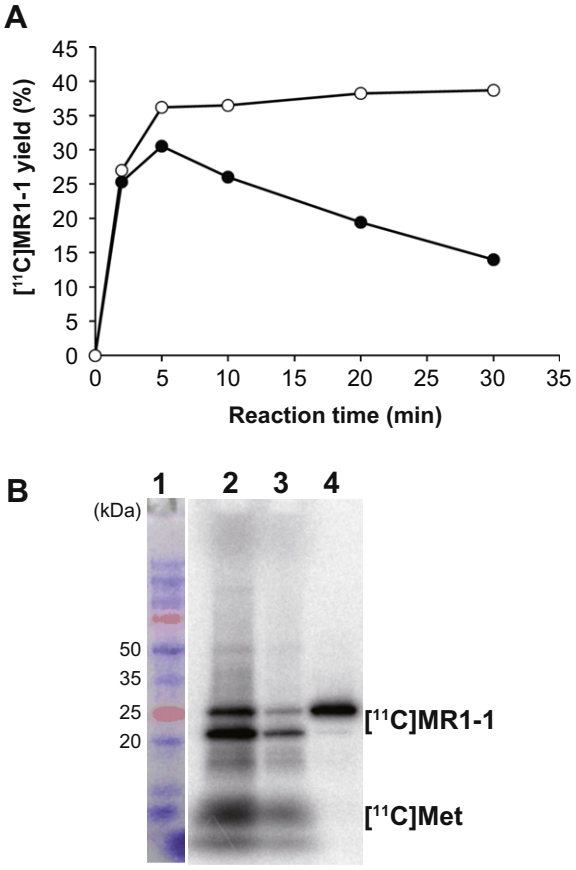

Figure 4. Cell-free synthesis of [^{11}C]MR1-1. (A) Time course of [^{11}C]MR1-1 synthesis. Non-decay-corrected (closed circles) and decay-corrected (open circles) yields of [^{11}C]MR1-1 at each reaction time were determined by SDS–PAGE of crude reaction samples, followed by autoradiography. (B) Purification of [^{11}C]MR1-1. Cell-free synthesized [^{11}C]MR1-1 was purified with an epitope peptide-conjugated spin column. Samples at each purification step were analyzed by non-reducing SDS–PAGE and detected by autoradiography. Lane 1: markers stained with CBB. Lane 2: flow-through fraction. Lane 3: wash fraction. Lane 4: elute fraction.

Although further improvement of the purification process is necessary, this novel approach would be a potential option for the preparation of immuno-PET radiotracers.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.09.038.

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