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RESEARCH ARTICLE

Site-Specific Labeling of F-18 Proteins Using a Supplemented Cell-Free Protein Synthesis System and *O*-2-[^{18}F]Fluoroethyl-L-Tyrosine: [^{18}F]FET-HER2 Affibody Molecule

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Abstract

Purpose: Although a preparation method for F-18-labeled proteins that used a cell-free translation system and 4-[^{18}F]fluoro-L-proline instead of L-proline has been reported, its introduction depends on amino acid sequences of target proteins. The purpose of the study was to propose site-specific labeling method of F-18 by using cell-free translation systems supplemented with an engineered orthogonal aminoacyl-tRNA synthetase derived from *Methanocaldococcus jannaschii* (*p*CNF-RS)/suppressor tRNA ($tRNA_{CUA}^{opt}$) pair, *O*-2-[^{18}F]fluoroethyl-L-tyrosine ([^{18}F]FET), and template DNA inserted with an amber codon.

Procedures: [^{18}F]FET was prepared from the corresponding precursor and determined whether [^{18}F]FET could be incorporated into an affibody molecule for human epidermal growth factor receptor type 2 (HER2; $Z_{HER2:342}$) as the 21st amino acid used with the *p*CNF-RS-$tRNA_{CUA}^{opt}$ pair and template DNA inserted with an amber codon in a cell-free translation system. Using SKOV-3 cells, we performed an *in vitro* binding assay of [^{18}F]FET-$Z_{HER2:342}$. Furthermore, *in vivo* positron emission tomography (PET) imaging in SKOV-3 xenograft-bearing mice was performed after the intravenous administration of [^{18}F]FET-$Z_{HER2:342}$.

Results: [^{18}F]FET was successfully incorporated into proteins by using commercially available cell-free protein synthesis reagents with a *p*CNF-RS-$tRNA_{CUA}^{opt}$ pair and template DNA of the desired proteins inserted with an amber codon. The mean radiochemical yield (non-decay-corrected) of [^{18}F]FET-$Z_{HER2:342}$ was 6.5 ± 4.1 %. An *in vitro* cell binding assay revealed that SKOV-3 cells–bound [^{18}F]FET-$Z_{HER2:342}$ expressed HER2. The *in vivo* PET imaging in SKOV-3 xenograft-bearing mice revealed that [^{18}F]FET-$Z_{HER2:342}$ accumulated in SKOV-3 xenografts.

Conclusion: The method proposed in this study might be useful for preparing proteins with F-18 and molecular imaging in the preclinical development.

Key words: Cell-free protein synthesis, Engineered orthogonal aminoacyl-tRNA synthetase, [^{18}F]FET, Affibody

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Introduction

Positron emission tomography (PET) imaging provides physiological and pathological information in living subjects from small animals to humans by detecting annihilation gamma-rays from PET radiopharmaceuticals, which are widely used in research and development. Although 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG) is broadly used for imaging tumors in clinical practice, the development of radiotracers based on macromolecules, such as proteins, is an attractive but big challenge. Unlike small molecules, macromolecules have unique recognition mechanisms for binding to target molecules, such as post-translational modification sites [1], which exhibit high affinity and high specificity for their targets. Thus, protein ligands are attractive molecules for the labeling of positron-emitting radionuclides [2]. The most promising candidates for PET radiotracers are monoclonal antibodies. Previously, clinical studies have developed and have tested several antibody-based PET tracers labeled with Cu-64, Ga-68, Zr-89, and I-124 [3]. However, they tended to exhibit slow clearance from the body, which led to long waiting times to acquire PET images and limited the tumor penetration because of their molecular size. Recently, a promising alternative protein ligand of approximately 6.5 kDa, an affibody molecule, was developed, which is much smaller than antibodies and has a high binding affinity to its targets and fast pharmacokinetics [4, 5]. Affibody molecules exhibit rapid clearance from the body, facilitating labeling with short half-life radionuclides, such as C-11 and F-18, to obtain high-contrast images [6, 7].

We previously proposed a unique approach to the preparation of positron-emitting radionuclide-labeled proteins that uses cell-free translation systems with C-11 and F-18-labeled amino acids. In addition, we successfully produced C-11 and F-18-labeled interleukin-8 (IL-8) by using [^{11}C]-L-methionine and 4-[^{18}F]fluoro-L-proline ([^{18}F]FPro), respectively [8, 9]. Reportedly, cell-free translation systems are reagents for protein synthesis *in vitro* comprising essential elements, such as the ribosome, amino acids, nucleoside triphosphates, and aminoacyl-tRNA synthetase-tRNA pairs, and can synthesize interesting proteins by just adding their template DNA in a short reaction time [10]. Our previous strategy used a radiolabeled amino acid instead of a natural amino acid; in other words, one of the canonical 20 amino acids was removed from the reaction and was replaced with the corresponding radiolabeled amino acid. Since F-18 has a longer half-life (109.8 min) than that of C-11 (20.4 min), which facilitates longer scan times for slow pharmacokinetic tracers, we extended our approach from C-11 to F-18 [8]. However, [^{11}C]-L-methionine exhibited relatively more efficient incorporation into IL-8 in this system because of the natural amino acid, whereas [^{18}F]FPro is an unnatural amino acid that exhibited less efficient incorporation and a slow incorporation rate despite the successful incorporation into IL-8. Another limitation of this approach was that it depended on amino acid sequences of interesting proteins for the incorporation of radiolabeled amino acids.

Recent progress in the biotechnology field regarding the development of new orthogonal aminoacyl-tRNA synthetase-suppressor-tRNA ($tRNA_{CUA}$) pairs resulted in the experimental addition of unnatural amino acid to the genetic codes of various organisms [11]. Initially, orthogonal $tRNA_{CUA}$ pairs evoked from *Methanocaldococcus jannaschii* were developed in *Escherichia* coli and have been used in cell-free translation systems for protein synthesis [12–14]. Remarkably, engineered *p*-cyanophenylalanine aminoacyl-tRNA synthetase (*p*CNF-RS) exhibits high substrate permissivity for unnatural amino acids while upholding its ability to differentiate against canonical 20 natural amino acids [15].

This study reports the successful preparation of an *O*-2-[^{18}F]fluoroethyl-L-tyrosine ([^{18}F]FET)-labeled affibody molecule for human epidermal growth factor receptor type 2 (HER2) receptor ([^{18}F]FET-$Z_{HER2:342}$) that improved our approach for preparing F-18-labeled proteins for use with commercially available cell-free translation systems supplemented with orthogonal *p*CNF-RS-$tRNA_{CUA}^{opt}$ pairs, [^{18}F]FET, and template DNA inserted with an amber codon without requiring replacement of canonical 20 amino acids (Fig. 1a). In addition, this study aims to investigate the *in vitro* and *in vivo* properties of [^{18}F]FET-$Z_{HER2:342}$ to assess its efficacy in our improved strategy.

Methods

Materials

We obtained the RTS 100 *E. coli* HY Kit from Biotechrabbit GmbH (Hennigsdorf, Germany) for cell-free protein synthesis, and the RNase inhibitor was obtained from New England Bio Labs (Ipswich, MA). Isopropyl β-D-1-thiogalactopyranoside was obtained from Wako Chemicals (Osaka, Japan). In addition, the $Z_{HER2:342}$ and *p*CNF-RS genes were custom-synthesized by GeneScript (Piscataway, NJ) and subcloned into the pET-21a and pET-28a expression vectors, respectively. $Z_{HER2:342}$ affibody with an N-terminal histidine-glutamate $(HE)_3$ tag and *p*CNF-RS with an N-terminal histidine tag were prepared as described previously [14, 16]. IL-8 and $Z_{HER2:342}$ inserted with amber codon genes were prepared by site-directed mutagenesis PCR. We purchased *O*-2-fluoroethyl-L-tyrosine and its precursor (TET, (2*S*)-*O*-(2′-tosyloxyethyl)-N-trityl-tyrosine-tert-butyl ester) from ABX GmbH (Radeberg, Germany). Furthermore, reported optimized $tRNA_{CUA}^{opt}$ was custom-synthesized by GeneDesign, Inc. [17]. SKOV-3 and HEK293 cells were obtained from the ATCC (Manassas, VA).

Radiosynthesis of O-2-[^{18}F]Fluoroethyl Tyrosine

[^{18}F]Fluoride was produced by the ^{18}O(p,n)^{18}F reaction on enriched ^{18}O-H_2O (Taiyo-Nippon Sanso) with a Cypris HM-12 cyclotron (Sumitomo Heavy Industries) at the Cyclotron and Radioisotope Center of Tohoku University. [^{18}F]FET

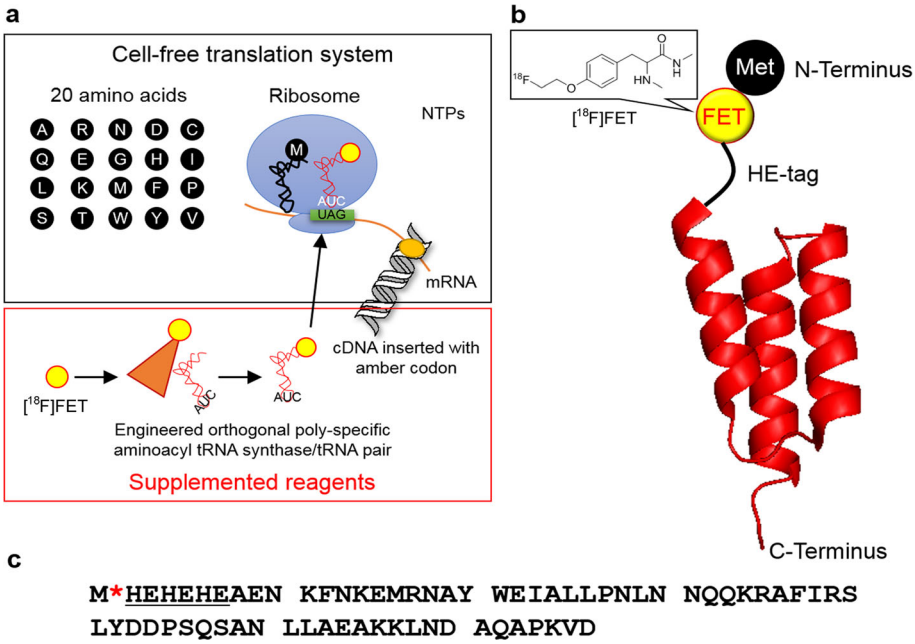

Fig. 1. **a** Schematic illustration of the proposed F-18-labeled preparation system using a commercially available cell-free synthesis and supplemented reagents, such as engineered orthogonal aminoacyl-tRNA synthetase-tRNA pair, cDNA inserted with an amber codon and [^{18}F]fluoroethyl-L-tyrosine ([^{18}F]FET). **b** The schematic illustration of the [^{18}F]FET-labeled $Z_{HER2:342}$ affibody (PDB ID: 3MZW). **c** Amino acid sequences of the $(HE)_3$-tagged $Z_{HER2:342}$ affibody with FET. *, the introduction site of FET.

was prepared using microscale radiosynthesis as described previously [18]. Briefly, reactive [^{18}F]fluoride, as a complex form of Kryptofix 222/KF dissolved in DMSO (20–30 µl), was prepared from no-carrier-added [^{18}F]fluoride by separation on an Oasis MAX cartridge and reacted with a precursor (TET, ABX) at 120 °C for 5 min followed by deprotection with HCl (2 M, 30 µl) at the same temperature for 10 min. The reaction was quenched by adding 30–50 µl of phosphate-buffered saline (PBS), then the whole solution was injected onto a small analytical C18 column (InertSustain 150 × 4.6 mm with a solvent system of EtOH/50 mM AcOH, 10:90). A fraction containing [^{18}F]FET was collected and evaporated to dryness on a rotary evaporator. The overall synthesis time was approximately 60 min. The radiochemical purity exceeded 95 %. The decay-corrected radiochemical yields of [^{18}F]FET were 55.6 ± 9.4 % (n = 9), which was comparable to that reported in literature [19]. The molar radioactivity was 566 ± 244 GBq/µmol at the end of synthesis (n = 9). Dry [^{18}F]FET was dissolved in Reconstitution Buffer supplied from the RTS 100 *E. coli* HY Kit.

Radiosynthesis of IL-8 and Anti-HER2 Affibody Containing [^{18}F]FET by Using a Supplemented RTS 100 E. coli HY Kit

The reaction mixture (375 µl) comprised a commercially available RTS 100 *E. coli* HY Kit (*E. coli* Lysate, Amino acids (Final concentration; 2 mM), and Reaction Mix) with supplements, including isopropyl β-D-1-thiogalactopyranoside and RNase inhibitor, and additionally [^{18}F]FET (359–984 MBq; molar radioactivity, 566 ± 244 GBq/µmol; n = 9), tRNA $_{CUA}^{opt}$ (5 µM), *p*CNF-RS (22 µM), and template DNA inserted with an amber codon (5 µg). Lyophilized reagents (*E. coli* Lysate, Amino acids, and Reaction Mix) from the RTS 100 *E. coli* HY Kit were dissolved in [^{18}F]FET solution. To the solution containing [^{18}F]FET, *E.* coli lysate, amino acids, and reaction mix in one tube, other reagents were added, followed by incubating the reaction mixture at 30 °C for 30, 60, 90, and 120 min. Protein production was analyzed by gel autoradiography using NuPAGE™ gel (12 % Bis-Tris gel with MES-SDS Running buffer). Gels were exposed to a BAS-MS2025 imaging plate (GE Healthcare) overnight. Autoradiographic images were obtained by FLA-9500 phosphor imaging instrument (GE Healthcare). For purification of [^{18}F]FET-$Z_{HER2:342}$, the incubated reaction mixture was heated at 60 °C for 5 min and centrifuged at 15,000*g* for 5 min. The collected supernatant was diluted and loaded to His Spin Trap (GE Healthcare). After washing with PBS, [^{18}F]FET-$Z_{HER2:342}$ was eluted with phosphate-buffered saline containing 50 mM imidazole to separate $(HE)_3$-tagged $Z_{HER2:342}$ from His-tagged *p*CNF-RS. The eluted fraction was transferred to a NAP-5 column (GE Healthcare) to obtain injectable [^{18}F]FET-$Z_{HER2:342}$. We used unlabeled FET in the reaction instead of [^{18}F]FET to produce unlabeled FET-$Z_{HER2:342}$. After the Zip TipC18 (Millipore) treatment, mass spectrometry was performed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF-MS; AXIMA Performance; Shimazu, Kyoto, Japan) to confirm the specific incorporation of FET. Protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, MA).

In Vitro Cell Binding Assay of [^{18}F]FET-$Z_{HER2:342}$

In vitro binding of [^{18}F]FET-$Z_{HER2:342}$ was examined by using SKOV-3 cells with high HER2 expression than that of HEK293 cells with low HER2 expression (1.0×10^5 cells/well) in 24-well dishes. The cells were incubated with [^{18}F]FET-$Z_{HER2:342}$ (30 kBq/ml) at 37 °C for 1 h. The reactions were performed in the presence of unlabeled $Z_{HER2:342}$ (15 μg/ml) to account for non-specific binding. Then, the culture medium was removed, was washed two times, and was harvested with 0.1 M NaOH. The radioactivity was measured on an γ-counter (AccFLEX γ7000, ALOKA). *In vitro* saturation binding of [^{18}F]FET-$Z_{HER2:342}$ was performed against SKOV-3 cells in 48-well dishes. The concentration of [^{18}F]FET-$Z_{HER2:342}$ was determined based on the molar radioactivity of [^{18}F]FET. Furthermore, we performed the competitive binding assay at the different concentrations of unlabeled $Z_{HER2:342}$ and FET-$Z_{HER2:342}$ (0.001–1000 nM). All experiments were performed in triplicate.

Small Animal PET Studies

The Laboratory Animal Care Committee of Tohoku University approved all the animal experiment protocols described in this study. *In vivo* PET studies were performed by using female immunodeficient mice (BALB/c-ν:CAnN.Cg-*Foxn1*^{nu}/CrlCrlj) with SKOV-3 xenografts. The mice were inoculated under the arm by subcutaneous injection of SKOV-3 cell (10^6 cells). SKOV-3 xenografts were allowed to grow for 3 to 4 weeks. PET images were acquired by using a Clairvivo-PET (Shimazu) under 1.5 % (*v*/*v*) isoflurane after the intravenous administration of [^{18}F]FET-$Z_{HER2:342}$ (3.3 ± 0.2 MBq, 0.2 ml in PBS) ($n = 5$). PET list-mode data were collected for 120 min. In addition, the regions of interest were drawn by using AMIDE software [20]. Furthermore, the mice ($n = 3$) were intravenously injected with unlabeled $Z_{HER2:342}$ (250 μg) 30 min before administering [^{18}F]FET-$Z_{HER2:342}$ to determine the specificity of tumor uptake. For statistical analysis, the unpaired *t* test was used. The HER2 expression in SKOV-3 xenografts was confirmed by the HERcep Test (Agilent, Santa Clara, CA).

Results

Incorporation of [^{18}F]FET into IL-8 by Using Engineered Orthogonal Aminoacyl-tRNA Synthetase-tRNA Pairs

Using a commercially available cell-free protein synthesis kit, we previously illustrated that FET could not have been incorporated into IL-8 in place of tyrosine because natural tyrosyl tRNA synthase does not recognize FET [8]. Although engineered *p*CNF-RS has been reported to recognize 18 unnatural amino acids, including *p*-fluorophenyl alanine [15], other fluorinated amino acids, such as FET, remain unknown. Therefore, we first ascertained whether [^{18}F]FET could be incorporated into IL-8 by using a commercially available cell-free protein synthesis kit supplemented with *p*CNF-RS-$tRNA_{CUA}^{opt}$ pairs and template DNA containing the IL-8 gene inserted with a single amber codon after the start codon. Gel autoradiography suggested the successful production of [^{18}F]FET-IL-8 incorporated with [^{18}F]FET. In addition, the production of [^{18}F]FET-IL-8 gradually increased over 120 min (Fig. 2a). We assessed the optimal concentrations of *p*CNF-RS-$tRNA_{CUA}^{opt}$ pairs and found that the optimal concentrations of *p*CNF-RS and tRNA were 22 and 5 μM, respectively (Supplemental Material, Fig. S1). Furthermore, the higher concentration of [^{18}F]FET was much better for producing F-18-labeled protein (Supplemental Material, Fig. S2).

Radiosynthesis of ^{18}F-FET-Labeled $Z_{HER2:342}$ Affibody

Next, we applied this technique to a protein of interest, anti-HER2 affibody ($Z_{HER2:342}$), for imaging HER2-positive breast cancer. We designed DNA sequences that contained a unique amber codon for the single introduction of FET in the $Z_{HER2:342}$ molecule (Fig. 1). MALDI-TOF MS revealed that the molecular weight of FET-$Z_{HER2:342}$ was 208 Da heavier than $Z_{HER2:342}$, which is equivalent to single FET (Fig. 2b). Moreover, gel autoradiography suggested that the dominant single radioactive band between 3.5 and 10 kDa was increased in a time-dependent manner, suggesting the successful production of [^{18}F]FET-$Z_{HER2:342}$ (Supplemental Material, Fig. S3). Using relatively high radioactivity of [^{18}F]FET, the production of [^{18}F]FET-$Z_{HER2:342}$ reached a plateau at 30 min, considering the decay-corrected time (Supplemental Material, Fig. S3). Reportedly, the $(HE)_3$-tagged $Z_{HER2:342}$ affibody can be purified by immobilized metal ion-affinity chromatography and exhibited less hepatic radioactivity accumulation than the His-tagged $Z_{HER2:342}$ affibody [16]. In this study, we used $(HE)_3$-tagged anti-HER2 affibody [$(HE)_3$-tagged $Z_{HER2:342}$] for the introduction of [^{18}F]FET. Using a His Spin column, [^{18}F]FET-$Z_{HER2:342}$ was successfully purified (Fig. 3). As determined by gel autoradiography, the radiochemical purity was > 99 %. The mean radiochemical yield of [^{18}F]FET-$Z_{HER2:342}$ was evaluated as 6.5 ± 4.1 % (non-decay-corrected) based on [^{18}F]FET at the total synthesis time of 60 min (3.3 ± 2.1 % for the non-decay-corrected radiochemical yield based on starting [^{18}F]fluoride at the total synthesis time of 120 min). Of note, protein amounts of [^{18}F]FET-$Z_{HER2:342}$ could not be determined by the BCA assay, but the the working range of the assay (20–2000 μg/ml) suggests 20 GBq/μmol as lower limit on the product's molar radioactivity. The molar radioactivity of

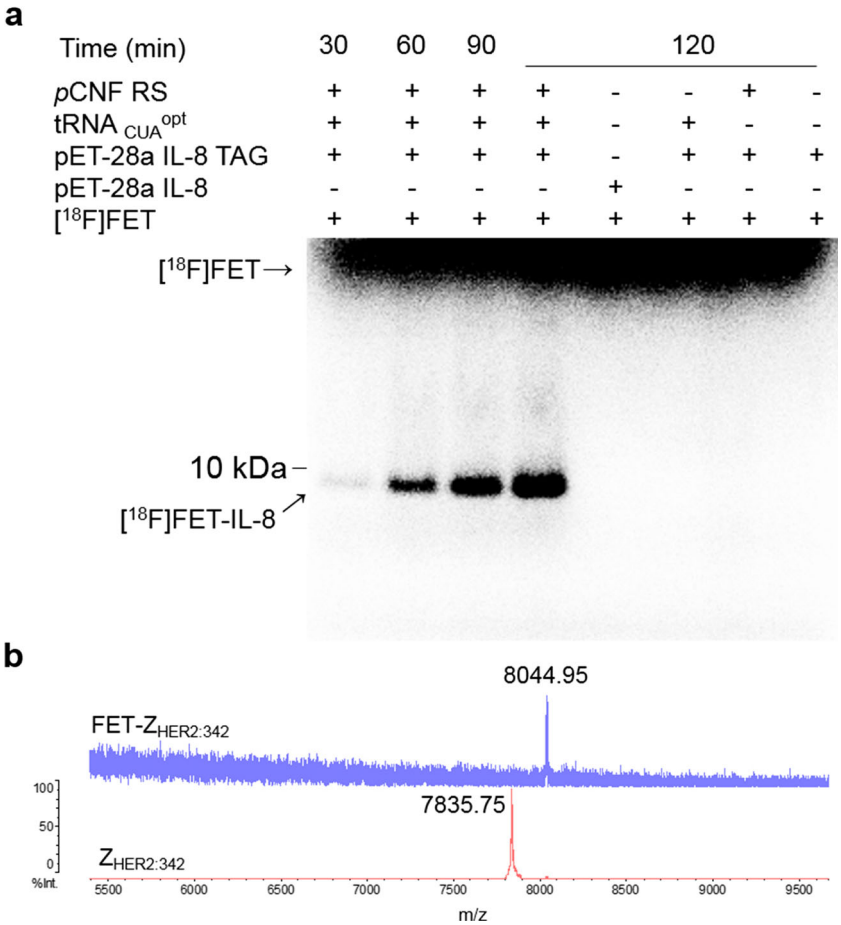

Fig. 2. **a** Gel autoradiography analysis of radiosynthesis of [^{18}F]FET-IL-8 using an RTS 100 *E. coli* HY kit with orthogonal aminoacyl-tRNA synthetase/suppressor tRNA pairs and template DNA inserted with an amber codon. **b** MALDI-TOF MS analysis of FET-$Z_{HER2:342}$ and $Z_{HER2:342}$.

[^{18}F]FET-$Z_{HER2:342}$ calculated based on the molar radioactivity of [^{18}F]FET, because single [^{18}F]FET can be incorporated into proteins in a site-specific manner by using this method, was 468 ± 336 GBq/μmol at the end of synthesis ($n = 8$).

In Vitro Cell Binding Assay of [^{18}F]FET-$Z_{HER2:342}$

The *in vitro* cell binding assay of [^{18}F]FET-$Z_{HER2:342}$ exhibited much higher binding to HER2-expressing SKOV-3 cells at high levels than binding to HEK293 cells as controls (Fig. 4a). The binding was completely blocked in the presence of an unlabeled excess of the $Z_{HER2:342}$ affibody, suggesting that the binding was specific. These findings suggested that [^{18}F]FET-$Z_{HER2:342}$ was biologically active, implying proper folding. In addition, the *in vitro* saturation binding assay exhibited the high binding affinity ($K_D = 1.0 \pm 0.3$ nM) of [^{18}F]FET-$Z_{HER2:342}$ against SKOV-3 cells (Fig. 4b). Furthermore, the competitive binding assay of [^{18}F]FET-$Z_{HER2:342}$ exhibited a small reduction of FET-$Z_{HER2:34}$ ($K_i = 8.2 \pm 0.7$ nM) compared with unlabeled parent $Z_{HER2:342}$ ($K_i = 3.0 \pm 1.3$ nM; Fig. 4c).

[^{18}F]FET-$Z_{HER2:342}$ PET Imaging in Xenograft-Bearing Mice

Small animal PET studies demonstrated that [^{18}F]FET-$Z_{HER2:342}$ was rapidly excreted to the kidneys and urinary tract, with low uptake observed in the liver and bone. Fig. 5a presents the PET images summed from 75 to 120 min after the intravenous injection of [^{18}F]FET-$Z_{HER2:342}$. [^{18}F]FET-$Z_{HER2:342}$ exhibited the high uptake in SKOV-3 xenografts. Fig. 5b presents the time–activity curve of [^{18}F]FET-$Z_{HER2:342}$ in the SKOV-3 xenografts. The uptake of [^{18}F]FET-$Z_{HER2:342}$ in the SKOV-3 xenografts reached a plateau at 60 min post-injection. After blocking with unlabeled $Z_{HER2:342}$, the uptake of [^{18}F]FET-$Z_{HER2:342}$ in mice decreased significantly ($P = 0.0023$) (Fig. 6), suggesting that the binding to SKOV-3 xenografts was specific. Furthermore, the HER2 expression in the SKOV-3 xenografts was confirmed by HER2 immunohistochemistry.

Discussion

We previously reported a technique for labeling proteins with F-18 using a cell-free translation system and [^{18}F]FPro

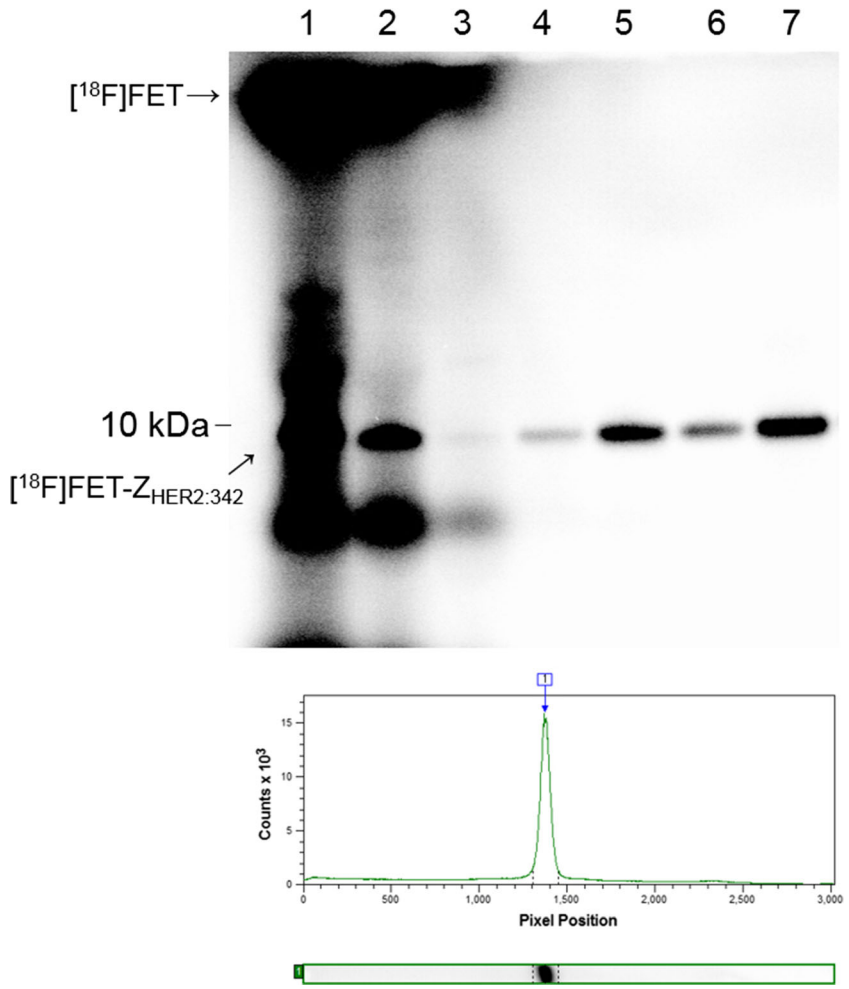

Fig. 3. Purification of [^{18}F]FET-$Z_{HER2:342}$ on a HisSpin column. Lane 1: crude reaction solution (30 min), lane 2: flowthrough, lane 3: washing solution with PBS (0.6 ml), lane 4: eluted solution with PBS (0.25 ml), 50 mM imidazole, lane 5: eluted solution with PBS and 50 mM imidazole (0.25 ml), lane 6: eluted solution with PBS, 50 mM imidazole (0.25 ml), lane 7: after desalting (1.0 ml).

[8]. However, there were some limitations to the production of F-18-labeled proteins. First, incorporation sites of [^{18}F]FPro depended on amino acid sequences of the desired proteins. Second, [^{18}F]FPro was not proficiently incorporated into the desired protein because of the presence of unnatural amino acids. To overcome these limitations, we proposed a better strategy to use with the supplemented cell-free protein synthesis system comprising the engineered

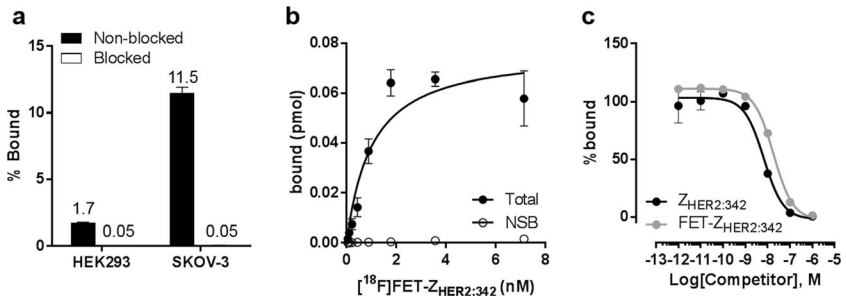

Fig. 4. **a** *In vitro* cell binding assay of [^{18}F]FET-$Z_{HER2:342}$ to HEK293 and SKOV-3 cells. HEK293 is a cell with low HER2 expression, and SKOV-3 has low HER2 expression. The percentage bound (%bound) was obtained by considering radioactivity bound to cells divided by that added to wells. **b** *In vitro* saturation binding of [^{18}F]FET-$Z_{HER2:342}$ against SKOV-3 cells. **c** Competitive binding of unlabeled $Z_{HER2:342}$ and FET-$Z_{HER2:342}$ to SKOV-3 cells. [^{18}F]FET-$Z_{HER2:342}$ was used as a radioactive tracer. Error bars depict mean ± SEM.

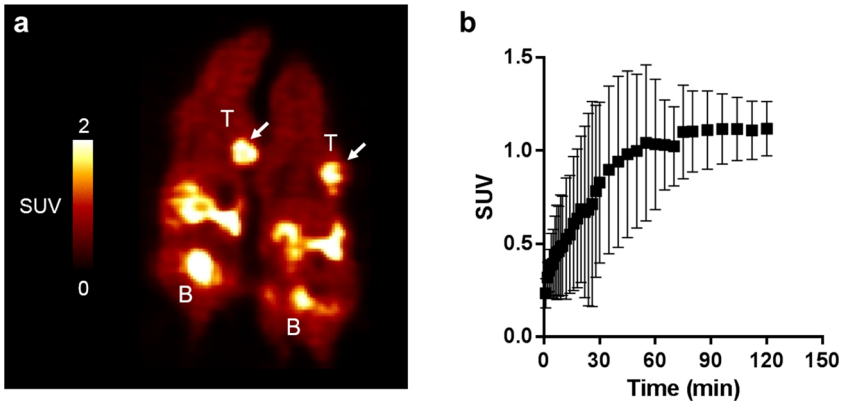

Fig. 5. Small animal PET imaging of [^{18}F]FET-$Z_{HER2:342}$ in a SKOV-3 tumor xenograft mouse model. **a** Representative PET images of [^{18}F]FET-$Z_{HER2:342}$ from 80 to 120 min in SKOV-3 xenograft-bearing mice after the intravenous administration of radiotracers. B, bladder; T, tumor. **b** Time–activity curves of ^{18}F-FET-$Z_{HER2:342}$ in tumors of HER2 receptor-expressing xenografts ($n = 3$). Error bars, mean ± SD.

orthogonal aminoacyl-tRNA synthetase-suppressor-tRNA pair so that the F-18-labeled amino acid could be introduced into the desired proteins as the 21st amino acid without the replacement of the canonical 20 amino acids.

Principally, radiolabeled amino acid derivatives are transported into cells through amino acid transporters on the cell membrane, and PET images reflect amino acid metabolism. Recently, several F-18 fluorinated amino acids have been developed as radiopharmaceuticals for imaging tumor metabolism [21]. Of these, [^{18}F]FET is used for imaging tumors, such as gliomas, and has been attained with a high radiochemical yield [19, 22, 23]. A recent study using our microscale one-pot radiosynthesis reported the production of [^{18}F]FET with a comparable radiochemical yield and rapid purification by an analytical HPLC column [18]. In addition, FET was not recognized by natural tyrosyl tRNA synthetase derived from *E. coli*, as described previously [8]. Hence, in this sudy, we selected [^{18}F]FET as the amino acid source in the cell-free translation system.

Typically, unnatural amino acids are not incorporated into proteins because aminoacyl-tRNA synthetases exhibit high specificity for each amino acid. However, rigorous efforts to develop new orthogonal aminoacyl-tRNA synthetase-suppressor-tRNA pairs have resulted in the experimental additions of unnatural amino acids to the genetic codes of various organisms [11]. Young, et al. reported that *p*CNF-RS, which is designed to precisely recognize *p*-cyanophenylalanine, exhibits high substrate permissivity for unnatural amino acids while upholding its ability to distinguish against canonical 20 natural amino acids [15]. Previously, we focused on the permissivity of natural aminoacyl-tRNA synthetase for F-18-labeled amino acid and found that a natural prolyl aminoacyl-tRNA synthetase-tRNA pair derived from *E. coli* could recognize [^{18}F]FPro. Conversely, this study focused on the selectivity of natural aminoacyl-tRNA synthetase for F-18-labeled amino acid (*i.e.*, F-18-labeled amino acid fails to be incorporated into protein instead of natural amino acid) and permissivity of

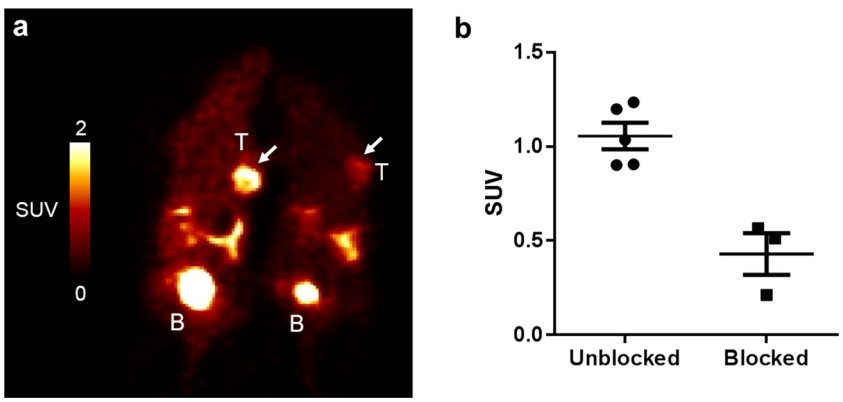

Fig. 6. *In vivo* binding specificity of [^{18}F]FET-$Z_{HER2:342}$ in a SKOV-3 tumor xenograft mouse model. **a** Representative PET images of [^{18}F]FET-$Z_{HER2:342}$ without blocking (left) and after blocking with excess of cold $Z_{HER2:342}$ (right) 130 min post-injection. B, bladder; T, tumor. **b** The tumor uptake of [^{18}F]FET-$Z_{HER2:342}$ in SKOV-3 xenografts was significantly reduced ($P = 0.0023$) after blocking of HER2 receptors with cold $Z_{HER2:342}$ (right) ($n = 3$~5). Significant reductions are marked with asterisks in the figure. *$P < 0.05$.

engineered aminoacyl-tRNA synthetase for F-18-labeled amino acid. We found that *p*CNF-RS with $tRNA_{CUA}^{opt}$ recognized [^{18}F]FET and could incorporate [^{18}F]FET into the desired protein by using a commercially available cell-free translation system. Of note, using orthogonal *p*CNF-RS along with $tRNA_{CUA}^{opt}$ in a commercially available cell-free translation system improved one of the limitations of our previous study and provided the desired site-specific incorporation of [^{18}F]FET into desired proteins. Another limitation of our previous study was the low radiochemical yield and the slow synthesis rate. Compared with the previous mean radiochemical yield (0.29 ± 0.2 %, non-decay-corrected), the reaction in this study successfully improved the radiochemical yield to 6.5 ± 4.1 % (non-decay-corrected). Interestingly, the use of higher radioactivity of [^{18}F]FET led to a faster reaction rate to reach a plateau at 30 min. However, the radiochemical yield was still lower than that of other affibody tracers labeled with F-18 reported previously [7, 24]. Thus, further studies are warranted to enhance the radiochemical yield of F-18-labeled proteins.

Previously, several number of diverse approaches for site-specific radiolabeling of proteins with F-18 have been reported [25]. Single C-terminal cysteine modification approaches can site-specifically label proteins by the maleimide-linking strategy. Glaser, et al. directly compared three different F-18 radiolabeling approaches of HER2-specific affibody $Z_{HER2:2891}$ comprising a C-terminal cysteine using [^{18}F]fluorobenzaldehyde ([^{18}F]FBA)/aminooxy coupling, silicon-fluoride acceptor approach ([^{18}F]SiFA) wtith an isotopic ^{19}F/^{18}F exchange reaction, and $([^{18}F]AlF)^{2+}$ with 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) coupling [7]. The molar radioactivity of these methods was 0.6–4.3 GBq/μmol. Of these, the [^{18}F]SiFA protocol exhibited the best radiochemical yield (38 ± 2 %, non-decay-corrected). However, [^{18}F]SiFA-labeled $Z_{HER2:2891}$ displayed worse pharmacokinetics such as defluorination, higher blood, liver, and lung uptake, but lower tumor uptake despite the binding affinity to HER2 similar to other F-18-labeled $Z_{HER2:2891}$ affibodies.

One of the characteristics of our approach for site-specific F-18 radiolabelling was minimal additions of the functional group for F-18 radiolabeling. When single tyrosine residue replaces [^{18}F]FET in proteins, the addition of molecular weight is 63 Da (only fluoroethyl side-chain), permitting the use of almost the same scaffold of original proteins. Nevertheless, tyrosine residues in proteins are occasionally critical for binding to their targets. Reportedly, two tyrosine at residues 13 and 35 in the $Z_{HER2:342}$ affibody interacted with HER2 receptor determined by the crystal structure of the complex between HER2 and $Z_{HER2:342}$ [26]. Therefore, we designed the single addition of [^{18}F]FET (the addition of molecular weight was 208 Da) in the N-terminal region of the $Z_{HER2:342}$ affibody rather than the replacement of tyrosine residues to avoid a reduction of the binding affinity. Indeed, [^{18}F]FET-$Z_{HER2:342}$ exhibited the high binding affinity and was comparable to parent unlabeled $Z_{HER2:342}$. Another characteristic of our approach was the stability against defluorination. Although we assessed only the $Z_{HER2:342}$ affibody as a model protein in this study, the [^{18}F]FET-labeled $Z_{HER2:342}$ affibody demonstrated preferable pharmacokinetics such as little defluorination *in vivo* due to the high stability of [^{18}F]FET itself [27]. A single [^{18}F]FET can be incorporated into proteins in a site-specific manner by using this method. Therefore, the molar radioactivity of [^{18}F]FET-labeled protein should be theoretically the same as [^{18}F]FET, although the molar radioactivity of [^{18}F]FET-labeled protein was not determined in this study. Since we used [^{18}F]FET with high molar radioactivity (566 ± 244 GBq/μmol) in this study, the molar radioactivity of the [^{18}F]FET-labeled protein should be high. Relative to the amounts needed in conventional labeling methods for proteins, smaller amounts of labeled proteins were prepared in this reaction. The protein amount is too small to be determined by conventional protein assays and absorbance at ultraviolet wavelength. Although enzyme-linked immunosorbent assays are highly sensitive to protein amounts, it takes several hours to obtain the results and requires specific antibodies. Further studies are required to measure small amounts of radiolabeled proteins for the calculation of molar radioactivity. Our approach for site-specific F-18 radiolabeling of proteins is based on biological enzyme reactions; the method can be easily performed at physiological reaction conditions by just adding the template DNA to the reaction for preparing the desired F-18-labeled proteins as biologists usually do. Hence, multiple radiolabeled proteins can be prepared by this method and applied to high-throughput screening of candidates in the preclinical setting. Furthermore, this radiosynthesis is a feasible one-pot method that can be easily automated using a robotic device.

Affibody molecules, which are small protein ligands of approximately 6.5 kDa that modify the 3-helical B domain of the IgG-binding region of staphylococcal protein A, have high binding affinity to their targets and fast pharmacokinetics [28]. Therefore, affibody molecules are expected to be excellent alternative radiotracers to antibodies for molecular imaging, as previously demonstrated [4]. The $Z_{HER2:342}$ affibody, which is a first-generation affibody molecule with high affinity to HER2, has been produced by *E. coli* and characterized well [5, 29]. $Z_{HER2:342}$ binds to the HER2 receptor expressed in breast cancer with high affinity (K_D = 22 pM), but its binding does not compete with traszumab [5, 26, 30]. Although full characterization of [^{18}F]FET-$Z_{HER2:342}$ by direct comparison with reported F-18-labeled HER affibodies was required, it exhibited good pharmacokinetics with fast clearance from the body in mice *via* urinary excretion with high binding to HER2-expressed cells, which was consistent with promising F-18-labeled $Z_{HER2:342}$ affibody molecules reported previously [7].

Conclusions

This study demonstrates that a commercially available cell-free protein synthesis system combined with an engineered orthogonal aminoacyl-tRNA synthetase-suppressor tRNA pair, cDNA inserted with an amber codon, and [^{18}F]FET with high molar radioactivity efficiently produces F-18-labeled affibody molecules. This method might be useful for the preparation of proteins with F-18 and in molecular imaging during the preclinical development.

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Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

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