Bioorganic & Medicinal Chemistry 20 (2012) 96–100

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Heavy metal-free ^{19}F NMR probes for quantitative measurements of glutathione reductase activity using silica nanoparticles as a signal quencher

Kazuo Tanaka, Narufumi Kitamura, Yoshiki Chujo *

Department of Polymer Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan

ARTICLE INFO

Article history:
Received 26 September 2011
Revised 14 November 2011
Accepted 15 November 2011
Available online 23 November 2011

Keywords:
Glutathione reductase
^{19}F NMR
Silica nanoparticle
Molecular probe
Quantitative assay

ABSTRACT

For the quantitative assessment of the glutathione reductase (GR) activity with a ^{19}F NMR spectroscopy, we developed the heavy metal-free probes based on silica nanoparticles modified with water-soluble perfluorinated dendrimers via the disulfide linkers. Before enzymatic reaction, the molecular rotation of the perfluorinated dendrimers is highly restricted, and the magnitude of ^{19}F NMR signals from the perfluorinated dendrimers can be suppressed. By the reductive cleavage of the disulfide linkers with the reduced glutathione-mediated enzymatic reaction of GR, perfluorinated dendrimers can be released from the surfaces of the nanoparticles. Consequently, the ^{19}F NMR signals of perfluorinated dendrimers were recovered. The enzymatic activity of GR was determined from the increase of the magnitude of ^{19}F NMR signals. Finally, to demonstrate the feasibility of the probe in the presence of miscellaneous molecules under bio-mimetic conditions, the comparison study was executed with the cancer cell lysate. The value determined from our method showed a good agreement with that from the conventional method.

1. Introduction

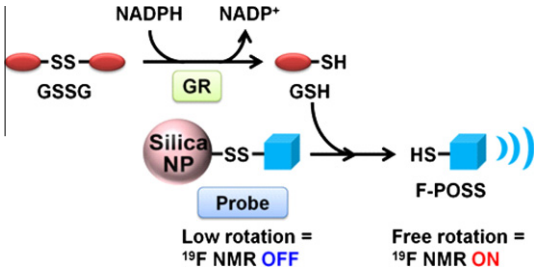
Glutathione reductase (GR) is the NADPH-dependent enzyme which catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). The GSH production plays a central role in buffering an intercellular redox condition and in activating the anti-oxidation system.[1] In addition, it has been reported that a significant enhancement of the GR activity was observed in malignant tumor cells.[2] Therefore, the quantification of the GR activity at the deep spot inside vital organs is of great significance for further understanding of the stress responses and carcinogenesis. On the other hand, the standard assay for measuring the GR activity is to follow the decrease in absorbance of NADPH at 340 nm.[3] Because of the low light permeability through a body, it is far difficult to apply the conventional methods for the in vivo assay. Hence, the new methodology is desired for the quantitative analysis of the GR activity.

Magnetic resonance imaging (MRI) contrast agents provide us the information from the deep spots inside vital organisms. In the recent years, a variety of fluorinated compounds have been developed as a versatile probe with a ^{19}F MRI or a ^{19}F NMR spectroscopy for monitoring the biological molecules and events such as gene repression,[4] protein existence,[5,6] enzymatic activity,[7–10] environmental alteration,[11–16] and biological reactions.[17] In advance, the quantitative evaluation of the enzymatic activity has been reported.[8] The stimuli-responsive MR probes can be classified in two groups. One is based on the metal complexes in which the center metal ion such as a gadolinium or a manganese ion can show the different contrast ability by recognizing the target.[18] However, GR itself would be activated in the presence of these heavy metal species in the cells.[1] Therefore, it should be difficult to eliminate errors on the detection using the metal complex-based MR probes. Another group is categorized into the surface-modified nanoparticles (NPs) such as superparamagnetic iron oxide.[19–21] By changing the aggregation/dispersion states of the NPs, the contrast ability can be greatly controlled.[21] However, because of difficulties to maintain the uniform morphology and particularly assemble the NPs in the cells, there are many problems to be overcome for realizing the quantitative assay with the NP-based probes. We have previously reported the silica NP can work as a quencher for the ^{19}F NMR signals.[7,8] Although the probe is composed of NPs, the interparticle interaction was not necessary for the target detection. Consequently, the quantitative evaluation of enzymatic reactions can be accomplished with this system.[8] In addition, silica is known to be a biocompatible material.[22] It can be expected to avoid the perturbation originated from the antioxidant activity. Because of these advantages, we aimed to construct the assay system for the GR activity using the modified silica NPs.

Herein, we report the quantitative assay in ^{19}F NMR for the GR activity. Perfluorinated dendrimers were anchored on silica NPs via the disulfide linkers (Scheme 1). On silica NPs, the molecular rotation of the perfluorinated dendrimers should be highly restricted,

* Corresponding author. Tel.: +81 75 383 2604; fax: +81 75 383 2605.
E-mail address: chujo@chujo.synchem.kyoto-u.ac.jp (Y. Chujo).

0968-0896/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2011.11.026



Scheme 1. Schematic mechanism for GR activity using the NP probe.

and the NMR signals from the perfluorinated dendrimers can be suppressed. Corresponded to progress the enzymatic reaction with GR in the presence of GSH and β-NADPH as a cofactor, the perfluorinated dendrimers were released from the surfaces, and the NMR signals from perfluorinated dendrimers were enhanced due to the recovery of the molecular rotation. Consequently, the enzymatic activity of GR can be evaluated as the increases of the magnitude of ^{19}F NMR signals. Furthermore, the values of the GR activities determined from the conventional and our method were compared using the cell lysate to demonstrate the feasibility of the probe in the presence of miscellaneous molecules under bio-mimetic conditions.

2. Experimental section

2.1. General

^{1}H and ^{13}C NMR spectra were measured with a JEOL EX-400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. ^{19}F and ^{29}Si NMR spectra were measured with a JEOL JNM-A400 spectrometer operating at 370 MHz for ^{19}F and 80 MHz for ^{29}Si. Coupling constants (J value) are reported in Hertz. The chemical shifts in ^{19}F NMR are expressed in ppm downfield from trifluoroacetic acid as an external reference. Masses were determined with a MALDI-TOF mass spectroscopy (acceleration voltage 21 kV, negative mode) with 2,5-dihydroxybenzoic acid (DHB) as a matrix. Transmission electron microscopy (TEM) was performed using a JEOL JEM-100SX microscope operating at 100 kV. The sizes of the particles were determined as an average of 100 particles in the TEM images. Emission spectra of the samples were monitored by a Perkin Elmer LS50B fluorometer at 25 °C using a 1 cm path length cell. The excitation bandwidth was 0.1 nm. The emission bandwidth was 0.1 nm. GR, GSH, GSSG, and β-NADPH were purchased from Wako Pure Chemicals Industries, Ltd (Osaka, Japan).

2.2. Perfluorinated dendrimer, F-POSS, 1[7]

To a suspension of octaammonium polyhedral oligomeric silsesquioxane (POSS)[23,24] (1 g, 0.852 mmol) and ethyl trifluoroacetate (406 μL, 3.41 mmol) in methanol (20 mL), triethylamine (2 mL, 14.4 mmol) was added, and the reaction mixture was stirred at room temperature for 3 h. The resulting mixture was evaporated, and the crude **1** was directly used in the next step. The analyzed sample as a clear oil was obtained after dialysis (895 mg, 83%). ^{1}H NMR (D_2O, 400 MHz) δ 3.26 (t, 8H, J = 7.0 Hz), 2.94 (t, 8H, J = 7.0 Hz), 1.70 (br s, 8H), 1.61 (br s, 8H), 0.64 (m, 16H). ^{13}C NMR (D_2O, 100 MHz) δ 155.9, 117.4, 41.52, 40.92, 21.76, 20.05, 8.77, 8.72. ^{29}Si NMR (D_2O, 80 MHz) δ −68.7, −68.4. ^{19}F NMR (D_2O, 373 MHz) δ −75.4. MALDI-TOF [$(M+H)^+$], [POSS-TFA_2] calcd 1074.52, found 1073.29, [POSS-TFA_3] calcd 1170.53, found 1170.00, [POSS-TFA_4] calcd 1266.53, found 1266.16, [POSS-TFA_5] calcd 1362.54, found 1361.76.

2.3. Compound 2[25]

To the solution of 3,3′-dithioldipropionic acid (1 g, 4.75 mmol) in dichloromethane (20 mL), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC) (2.16 g, 10.5 mmol) and *N*-hydroxysuccinimide (1.21 g, 10.5 mmol) were added. The mixture was stirred for 2 h at room temperature. The solution was concentrated in vacuo, and the obtained residue was subjected to silica column chromatography (ethyl acetate/hexane = 1:5) to give the NHS ester **2** (1.63 g, 85%). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.16 (m, 8H), 2.82 (s, 8H). FAB (DMSO-d_6) [$(M+H)^+$] calcd 405, found 405.

2.4. Preparation of the amino-presenting silica NPs[7,26]

A solution containing 2 mL of tetraethoxysilane, 1 mL of 3-aminopropyltriethoxysilane, 7 mL of water, and 2 mL of ammonium hydroxide in 50 mL of ethanol was stirred at ambient temperature for 16 h, and then the white precipitate was separated by centrifugation. After washing with ethanol three times, the particles (152 ± 11 nm diameter) were obtained as a white powder. To determine the amounts of the reactive amino groups at the surface of the particles, we treated the silica nanoparticles with ethyl trifluoroacetate. After washing with methanol, the particles were dissolved in 1 N aqueous sodium hydroxide, and the peak height was compared to the standard samples. Consequently, it was found that 960 nmol of trifluoroacetyl groups were attached to the particles.

2.5. Preparation of the NP probes

To the solution of the NHS ester **2** (404 mg. 1 mmol) and triethylamine (1 mL) in dichloromethane, 100 mg of amino-presented silica NPs were added. After 1 h stirring at room temperature, NPs were centrifuged and washed with dichloromethane. After drying in vacuo, the solution of **1** and triethylamine in methanol was added. The mixture was stirred at room temperature under ultrasound irradiation. After 1 h stirring, the NPs were centrifuged and washed with methanol and dichloromethane. The white powder was obtained after drying (167 ± 10 nm diameter from TEM). To estimate the amount of **1** on the NP (18 nmol/mg, F atom: 216 nmol/mg), the ^{19}F NMR spectrum of the solution containing the NP probe dissolved in 1 N aqueous sodium hydroxide was compared to the spectra of the standard samples with various concentrations of trifluoroacetic acid.

2.6. Reaction conditions with the NP probe

The 500 μL of samples containing the NP probe (2.5 mg, 1.08 mM F atom) with 500 mM $NaBH_4$, 500 mM DTT, 1 mM β-NADPH, 1 mM GSH, or 1 mM GSSG in PBS were incubated at 37 °C for 30 min. In the case of enzymatic reactions with GR, the NP probe (2.5 mg) was incubated in 500 μL of the reaction solutions containing 1 mM β-NADPH and 0.5 mM GSSG with various concentrations of GR in PBS at 37 °C. The reaction was terminated by centrifuging the reaction mixture, and then the supernatants were analyzed by a ^{19}F NMR spectroscopy.

2.7. Preparation for HeLa cell lysate

HeLa cells (1.0×10^6 cells) were cultured in a dish (90% confluent in φ 100 mm dishes) and washed twice with ice-cold PBS(−). The cell lysate was then harvested with 2 mL of ice-cold CelLytic M Cell Lysis Reagent (SIGMA-ALDRICH, Inc., St. Louis, MO), kept at ambient temperature for 15 min, and centrifuged at 12,000 rpm for 5 min to remove the cell debris. The enzymatic volume activity (U/mL) of the supernatant was measured from the conversion rate

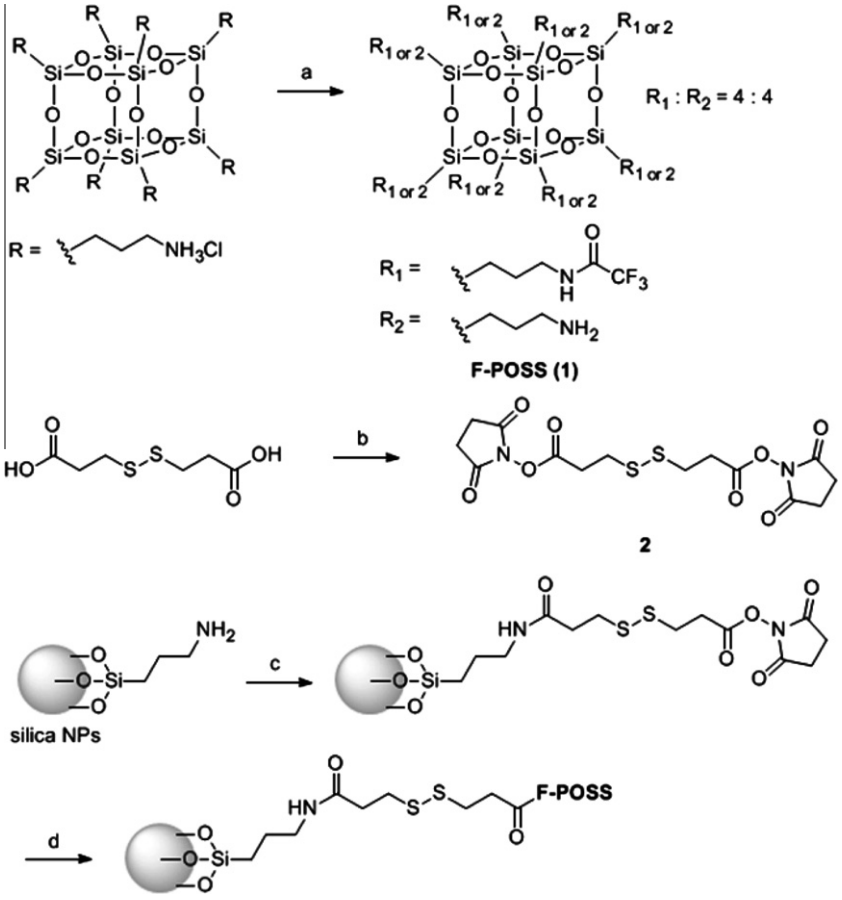
of NADPH. The solutions (100 μL) containing 1 μL of the cell lysate and 1 mM NADPH in PBS were incubated at 37 °C, and the absorption changes at 340 nm were monitored. The volume activity was estimated from fitting of the standard curve with GR.

2.8. ^{19}F NMR measurements for determining reaction yields and velocities in enzymatic reactions

Relaxation times of **1** and trifluoroacetic acid in ^{19}F NMR were taken with the following parameter sets; relaxation delay, 15 s; pulse width (90°), 51 μs; acquisition time, 88 ms; scan time, 4 times. ^{19}F longitudinal (T_1) relaxation data were collected with 10 delay times (1, 5, 10, 20, 50, 100, 250, 500, 1000, and 3000 ms) using a standard 1D inverse recovery pulse sequence. ^{19}F transverse (T_2) relaxation data were collected with nine delay times (400, 500, 600, 700, 800, 900, 1000, 2000, and 8000 ms) using a standard 1D Carr–Purcel–Meiboom–Gill pulse sequence. Resonance intensities in relaxation experiments were measured and fit to an exponential function. The reaction yields were determined as below: ^{19}F NMR spectra for the analysis of synthetic compounds were taken at 25 °C with the following parameter sets; relaxation delay, 6 s; pulse width (45°), 12 μs; acquisition time, 88 ms; scan time, 8 times; receiver gain 30. Totally 1 min was required for 1D NMR acquisition. Standard solutions containing **1** with the concentration region 100 μM to 1 mM were prepared, and the peak height was determined. The linear relationship was observed between the concentration and the peak height from trifluoromethyl groups in **1** in the ^{19}F NMR spectra. The reaction yields were determined by fitting the observed peak heights to the standard line. Initial velocities (v_i) were determined from the slopes of the increases of the ^{19}F NMR signal intensities from 0 to 15 min in Figure 3. Figure 4 represents the plots of these v_i values to the enzymatic concentrations and the approximate line with the plots in lower concentration region. The v_i value determined from the lysate sample was fitted on the approximate line, and the enzymatic activity was estimated as an x value.

3. Results and discussion

Scheme 1 illustrates the detection mechanism using the NP-based probe for enzymatic activity in this study. The probes consist of three significant components, the signal unit, the silica NP as a quencher for NMR signals, and the linker to release the signal unit after the target recognition. As a ^{19}F NMR signal unit, we used water-soluble perfluorinated POSS (F-POSS) which can be a good scaffold to construct functional ^{19}F MR probes by the modification with the signal regulation modulus because of high water-solubility and stability under biological conditions.[7,16,17] The silica NPs work as a quencher for ^{19}F NMR signals of the surface-tethered F-POSS molecules.[7,8] On the surface of the NP, the molecular rotation of F-POSS should be restricted like in the solid phase, which suppresses the NMR signals. Indeed, the T_1 and T_2 relaxation times of fluorine atoms were determined to be 2.90 and 1.94 s in trifluoroacetic acid and 1.067 and 0.992 s in F-POSS, respectively. On the other hand, those values in the NP probes were not determined because of shorter transverse relaxation times and the low signal intensity of the ^{19}F resonance. These results clearly indicate that the silica particles can accelerate the relaxation for ^{19}F NMR signals



Scheme 2. Synthetic scheme of the NP probe. Reagents and conditions: (a) Ethyl trifluoroacetate, triethylamine, methanol, rt, 3 h, 83%; (b) *N*-hydroxysuccinimide, WSC, dichloromethane, rt, 24 h, 81%; (c) amino-modified silica NPs, **2**, DMF, rt, 2 h; (d) F-POSS, triethylamine, methanol, rt, 2 h.

and work as a quencher for NMR signals. By the cleavage of the linker, F-POSS can be released, and the ^{19}F NMR signals of F-POSS are promised to be enhanced. The disulfide-containing compound was used as the linker between F-POSS and the surface of NP. Hence, the enzymatic reaction can be monitored by the increase of the signal molecule, F-POSS in ^{19}F NMR measurements.

F-POSS was synthesized using octaamino-POSS as a starting material by the introduction of trifluoroacetyl groups according to the previous reports.[7,16,17] We prepared amino-presenting silica NPs averaging 160 nm in diameter with the Stöber method,[7,26] and then the amino groups were modified with the disulfide linker (Scheme 2). Subsequently, F-POSS was reacted with another end of the linker on the surface of NPs. After washing with water and methanol thoroughly, the monodispersed modified NPs were obtained (Fig. 1). From the measurements of the particle radii with TEM, the slight increases were observed. In addition, less significant increases were observed from DLS measurements (287.5 ± 45.6 nm). These data indicate that the inter-particle cross-linking should hardly occur in the product. Precipitation or aggregation hardly occurred during all reactions.

To estimate the amount of F-POSS on the NP, the ^{19}F NMR signal intensities of the solution containing the NP probe dissolved in 1 N aqueous sodium hydroxide were compared to those of the standard samples with various concentrations of trifluoroacetic acid. Consequently, the amount of F-POSS was determined as 18 nmol on 1 mg of the NPs (F atom, 216 nmol/mg). The signals were not observed after 24 h incubation at 37 °C at pH 7.0 in the presence of proteinase K. In addition, the significant degradation following the undesired signal output or the aggregation was not observed by adding to BSA (1 mg/mL) which is known as an extracellular reducing material, the serum or the pH alteration between pH 5 and 9. These results suggest that the probe could provide clear signals without loss of sensitivity caused by unexpected interactions.

Initially, to examine the reactivity of the probes, we carried out the reactions with the conventional reducing agents, GSH, GSSG, and NADPH. The sample mixtures (0.5 mL) containing 5 mg/mL of the modified NPs (1.08 mM fluorine atoms) and the series of the reagents described on each spectrum in PBS were incubated at 37 °C for 30 min, and ^{19}F NMR signals from the supernatants after the centrifugation with the samples were monitored. The signals increased after incubation in the presence of $NaBH_4$, dithiothreitol (DTT), and GSH (Fig. 2). These results indicate that F-POSS can be released by reductive cleavage of disulfide bonds at the linker. On the other hand, both signals hardly increased after the incubation in the presence of GSSG and NADPH. This means that coexisting molecules should less affect the linker cleavage, and therefore only the GR activity can be evaluated using our probe as the increase of ^{19}F NMR signals.

We carried out the reactions with various concentrations of GR, and the signal intensities from the reaction mixtures were monitored with ^{19}F NMR measurements (Fig. 3). The signal intensities were compared with the standards and converted as the reaction yields. The initial velocity (v_i) was estimated from the slope of the increases of ^{19}F NMR signals. In the presence of 5 U/mL of GR, the reactions reached a plateau after the 20 min incubation. In contrast, less significant increase of the NMR signal intensity was observed in the absence of GR. The detection limits were correspondingly 0.5 U/mL of GR. These data present that the NP probes can provide the ^{19}F NMR signal via the enzymatic reaction with GR. Moreover, the reaction rates are seemed to be enhanced corresponded to the increase of the amount of GR in the reaction samples. In particular, the linear relationship was obtained between the GR concentration and the v_i values in the lower concentration region (Fig. 4). These results satisfy the requirements for quantitatively evaluating the GR activity from the degree of the reaction rates.

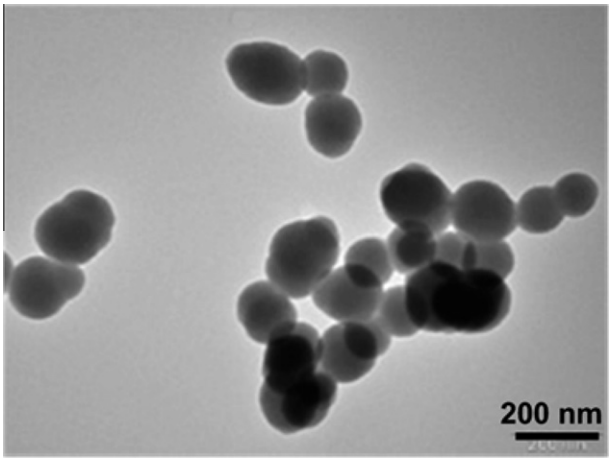


Figure 1. TEM image of the F-POSS-coated silica NPs. The scale bar represents 200 nm length.

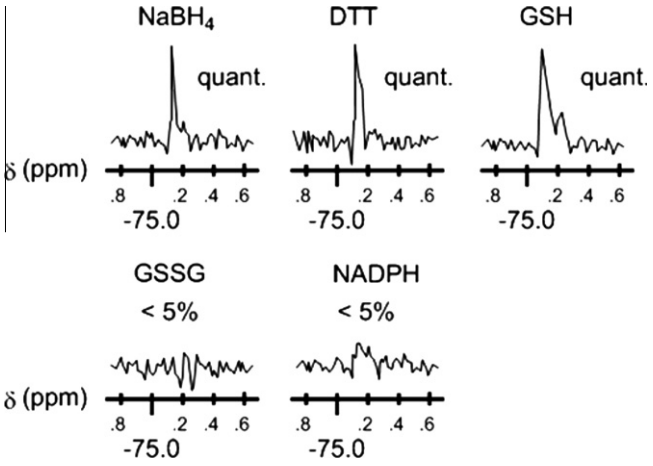


Figure 2. ^{19}F NMR spectra of the supernatants after the incubation. The reaction mixtures containing 5 mg/mL of the NPs were incubated in the presence of the reagents in PBS at 37 °C for 30 min.

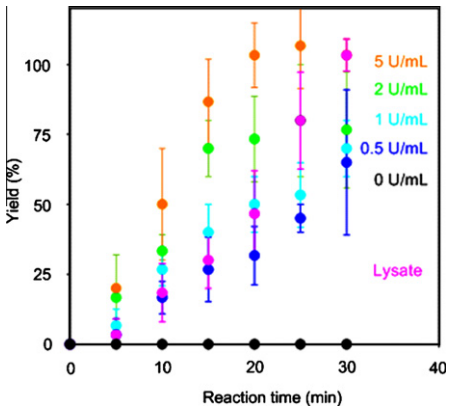


Figure 3. Time-courses of the intensity changes of ^{19}F NMR signals form the supernatants after enzymatic reactions. The NP probe (5 mg) was incubated in 500 μL of the reaction solutions containing various concentration of GR, 1 mM β-NADPH, 0.5 mM GSSG in PBS at 37 °C. The reaction yields were monitored with ^{19}F NMR and calculated by fitting on the standards. The errors represent the standard deviation calculated from the three data sets.

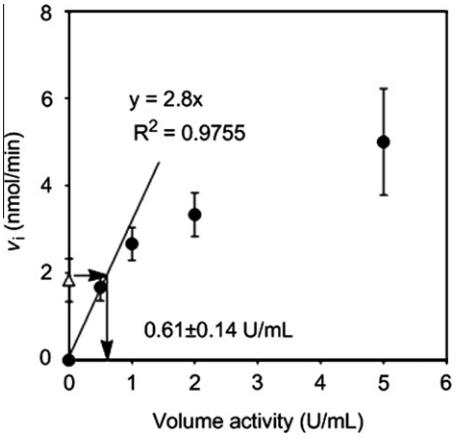


Figure 4. Determination of GR activity in the HeLa cell lysate. The vertical axis represents the initial velocities (v_i) determined from the increases of the ^{19}F NMR signal intensities of the samples. Fitting of the obtained value (clear triangular dot) from the ^{19}F NMR measurements to the standard line according to the results of Figure 3. The plots represent the averages calculated from the three data sets. The error bars represent the standard deviations.

To demonstrate feasibility of the analysis of the probe in the presence of the miscellaneous molecules under bio-mimetic conditions, the reactions were carried out with the HeLa cell lysate. Initially, the GR activity was determined from the evaluation of the absorption change of β-NADPH, and it was determined as 0.61 ± 0.05 U/mL. In contrast, the enzymatic activity of the cell lysate was evaluated to be 0.65 ± 0.14 U/mL by fitting the v_i value to the approximate line. This value determined with a ^{19}F NMR spectroscopy is in good agreement with the conventional method based on the light absorption measurement. These data suggest that our NP probes can be applied for imaging the GR activity under the reductive environment in the cells with MRI.

4. Conclusion

We present here the quantitative detection system in ^{19}F NMR for GR activity. Perfluorinated dendrimers tethered with silica NPs via disulfide bonds provided signal increase in the enzymatic reactions in the presence of GR. In addition, the quantitative detection was achieved by using our probe. Furthermore, the localization of the probes was regulated by modulating the diameter of the particles, for instance in the vessels and extracellular or intercellular matrices. Hence, this system promises to be a valid strategy for gathering the variety of quantitative information with MRI at various sites.

Acknowledgment

This research was partly supported by Magnetic Health Science Foundation (for K.T.).

References and notes

1. Nordberg, J.; Arnér, E. S. J. *Free Radical Biol. Med.* **2001**, *31*, 1287.
2. Malmgren, H.; Sylvén, B. *Cancer Res.* **1960**, *20*, 204.
3. Tietze, F. *Anal. Biochem.* **1969**, *27*, 502.
4. Cui, W.; Otten, P.; Li, Y.; Koeneman, K. S.; Yu, J.; Mason, R. P. *Magn. Reson. Med.* **2004**, *51*, 616.
5. Takaoka, Y.; Sakamoto, T.; Tsukiji, S.; Narazaki, M.; Matsuda, T.; Tochio, H.; Shirakawa, M.; Hamachi, I. *Nat. Chem.* **2009**, *1*, 557.
6. Higuchi, M.; Iwata, N.; Matsuba, Y.; Sato, K.; Sasamoto, K.; Saido, T. C. *Nat. Neurosci.* **2005**, *8*, 527.
7. Tanaka, K.; Kitamura, N.; Naka, K.; Chujo, Y. *Chem. Commun.* **2008**, 6176.
8. Tanaka, K.; Kitamura, N.; Chujo, Y. *Bioconjugate Chem.* **2011**, *22*, 1484.
9. Mizukami, S.; Takikawa, R.; Sugihara, F.; Hori, Y.; Tochio, H.; Wälchli, M.; Shirakawa, M.; Kikuchi, K. *J. Am. Chem. Soc.* **2008**, *130*, 794.
10. Mizukami, S.; Takikawa, R.; Sugihara, F.; Shirakawa, M.; Kikuchi, K. *Angew. Chem., Int. Ed.* **2009**, *48*, 3641.
11. Okada, S.; Mizukami, S.; Kikuchi, K. *ChemBioChem* **2010**, *11*, 785.
12. Tanabe, K.; Harada, H.; Narazaki, M.; Tanaka, K.; Inafuku, K.; Komatsu, H.; Ito, T.; Yamada, H.; Chujo, Y.; Matsuda, T.; Hiraoka, M.; Nishimoto, S. *J. Am. Chem. Soc.* **2009**, *131*, 15982.
13. Oishi, M.; Sumitani, S.; Nagasaki, Y. *Bioconjugate Chem.* **2007**, *18*, 1379.
14. Oishi, M.; Sumitani, S.; Bronich, T. K.; Kabanov, A. V.; Boska, M. D.; Nagasaki, Y. *Chem. Lett.* **2009**, *38*, 128.
15. Kenwright, A. M.; Kuprov, I.; Luca, E. D.; Parker, D.; Pandya, S. U.; Senanayake, P. K.; Smith, D. G. *Chem. Commun.* **2008**, 2514.
16. Tanaka, K.; Inafuku, K.; Chujo, Y. *Bioorg. Med. Chem.* **2008**, *16*, 10029.
17. Tanaka, K.; Kitamura, N.; Takahashi, Y.; Chujo, Y. *Bioorg. Med. Chem.* **2009**, *17*, 3818.
18. Tanaka, K.; Kitamura, N.; Naka, K.; Morita, M.; Inubushi, T.; Chujo, M.; Nagao, M.; Chujo, Y. *Polym. J.* **2009**, *41*, 287.
19. Tanaka, K.; Narita, A.; Kitamura, N.; Uchiyama, W.; Morita, M.; Inubushi, T.; Chujo, Y. *Langmuir* **2010**, *26*, 11759.
20. Hirata, N.; Tanabe, K.; Narita, A.; Tanaka, K.; Naka, K.; Chujo, Y.; Nishimoto, S. *Bioorg. Med. Chem.* **2009**, *17*, 3775.
21. Tanaka, K.; Kitamura, N.; Morita, M.; Inubushi, T.; Chujo, Y. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5463.
22. Jin, Y.; Kannan, S.; Wu, M.; Zhao, J. X. *Chem. Res. Toxicol.* **2007**, *20*, 1126.
23. Feher, F. J.; Wyndham, K. D. *Chem. Commun.* **1998**, 323.
24. Gravel, M.-C.; Zhang, C.; Dinderman, M.; Laine, R. M. *Appl. Organomet. Chem.* **1999**, *13*, 329.
25. Kim, E.; Kim, D.; Jung, H.; Lee, J.; Paul, S.; Selvapalam, N.; Yang, Y.; Lim, N.; Park, C. G.; Kim, K. *Angew. Chem., Int. Ed.* **2010**, *49*, 4405.
26. Stöber, W.; Fink, A.; Bohn, E. *J. Colloid Interface Sci.* **1968**, *26*, 62.