



Effects of serum matrix on molecular interactions between drugs and target proteins revealed by giant magneto-resistive bio-sensing techniques



Toshiro Saito ^{a,*}, Masayoshi Momiyama ^a, Chihiro Manri ^b, Heng Yu ^c, Kalidip Choudhury ^c, Sebastian J. Osterfeld ^c, Takashi Nakasawa ^a

^a Analytical & Medical Solution Business Group, Hitachi High-Tech Corporation, 1-17-1 Toranomon, Minato-ku, Tokyo, 105-6409, Japan

^b Center for Technology Innovation - Electronics and Instrumentation, Research & Development Group, Hitachi Ltd., 1-280 Higashi-koigakubo, Kokubunji, Tokyo, 185-8601, Japan

^c MagArray, Inc., 521 Cottonwood Drive, Suite 121, Milpitas, CA, 95035, USA

ARTICLE INFO

Article history:

Received 26 November 2020

Received in revised form 3 March 2021

Accepted 5 March 2021

Available online 8 March 2021

Keywords:

Matrix effect

Serum proteins

Molecular interaction

Giant magneto-resistive sensor

Superparamagnetic nanoparticle

ABSTRACT

We demonstrated that effects of serum matrix on molecular interactions between drugs and target proteins can be investigated in real time using magnetic bio-sensing techniques. A giant magneto-resistive (GMR) sensor was used on which target proteins were fixed and superparamagnetic nanoparticles (diameter: 50 nm) conjugated with drug were used in phosphate buffer, with and without serum. In this study, the following drug-protein pairs were investigated: quercetin and cAMP-dependent protein kinase A (PKA), Infliximab and tumor necrosis factor alpha (TNF α), and Bevacizumab and vascular endothelial growth factor (VEGF). For the quercetin and PKA pair, the time profile of the signal from the GMR sensor due to binding between quercetin and PKA clearly changed before and after the addition of serum. Moreover, it was revealed that not only the association process, but also the dissociation process was influenced by the addition of serum, suggesting that the quercetin and PKA complex may partially contain serum proteins, which affect the formation and stability of the complex. For antibody drugs, little effects of serum matrix were observed on both the association and dissociation processes. These clear differences may be attributed to the hydrophobic and electrostatic character of the drug molecule, target protein, and serum proteins. The real-time monitoring of molecular interactions in a biological matrix enabled by the GMR bio-sensing technique is a powerful tool to investigate such complicated molecular interactions. Understanding the molecular interactions that occur in a biological matrix is indispensable for determining the mechanism of action of the drugs and pharmacokinetics/pharmacodynamics inside the body. Additionally, this method can be applied for the analysis of the influence of any kind of third molecule that may have some interaction between two molecules, for example, an inhibitor drug against the interaction between two kinds of proteins.

© 2021 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Investigation of molecular interactions between drugs and biomolecules are indispensable for recognizing the mechanism of their action and their pharmacokinetics/pharmacodynamics. Analyses of protein interactions are typically performed in buffered salt solutions, which mimic natural conditions. However, these solutions lack many components that are present in the original biological liquid and can have significant effects on binding

behaviors. Most kinetics studies have been carried out in pure buffers because standard technology for such studies is based on surface plasmon resonance (SPR) measurements where non-specific binding due to plasma proteins distorts the kinetic data for highly concentrated (> 1%) serum samples [1]; thus, detailed kinetic studies in biological matrices are unavailable so far. Microscale thermophoresis [2] and high-performance affinity chromatography techniques [3] have been used for molecular interaction investigations between drugs and serum proteins, and have demonstrated their usefulness in obtaining equilibrium constants (for example, K_d : dissociation constant), although they cannot realize real-time interaction observations and cannot provide kinetic information, such as reaction rate constants.

* Corresponding author.

E-mail address: toshiro.saito.kf@hitachi-hightech.com (T. Saito).

It is well known that drugs interact with plasma proteins; especially, small molecule drugs, such as Warfarin, show strong affinity to plasma proteins, which can affect the distribution, activity, toxicity, and excretion rate of the drugs in the body [4]. For studies on such interactions, direct monitoring techniques of the molecular interaction in a biological matrix is required. Recently, a giant magneto-resistive (GMR) sensor and magnetic particle label combination have shown matrix-insensitivity in ligand binding assays and can quantify amounts of target proteins in serum [5]. In this method, one molecule (ligand) is fixed on the GMR sensor and another molecule (analyte) is labeled with magnetic particles. The magneto-resistance signal is obtained only when the magnetic particle label is located quite close (< 500 nm) to the GMR sensor because the signal induced in a GMR sensor by a properly oriented magnetic label would be approximately proportional to $1/d^3$ (where d is the sensor-to-label distance). For example, the signal attenuation with distance would be that of a simple dipole field, so that the binding event between the ligand and analyte molecules can be monitored in real-time and the association and dissociation processes rate constants can be determined. Analyte molecules are labeled with magnetic particles so that behaviors of only the analyte can be monitored and the matrix effect can be suppressed. This is quite an important advantage compared to SPR and bio-layer interferometry techniques where assignment of interacting molecules is very difficult because specific binding or nonspecific binding of serum proteins cannot be distinguished. Kinetic information, including the influence of the biological matrix, may allow more reliable conclusions to be made on protein functionality and may facilitate more efficient drug development.

In this study, we demonstrated the direct monitoring of molecular interactions between drugs and serum proteins by utilizing the advantage of the combination of the GMR sensor and magnetic particle techniques described above. We chose quercetin and cAMP-dependent protein kinase A (PKA) because they are known to be a typical system where serum proteins affect their interaction. The affinity between the small, uncharged inhibitor quercetin to the cAMP-dependent kinase PKA is distorted by 400-fold in human serum [2]. Quercetin is one of the typical flavonoids and is highly expressed in fruits, vegetables, red wine, and tea where consuming them may protect against the development of cardiovascular disease risks through their antioxidant and anti-inflammatory properties [6], although its low solubility in water has made it difficult to realize drug applications [7]. During the binding reaction we added tiny amounts of serum into a sample solution that included PKA fixed on the GMR sensor and quercetin-conjugated magnetic particles. We observed that the time profile due to the association reaction between quercetin and PKA changed significantly just after adding the droplet of serum into the sample solution, indicating that we observed the serum matrix effect on the molecular interaction directly in real time. We changed the amount of serum added to the sample and observed that complex formation was suppressed as the amount of serum added to the sample solution increased. Moreover, it was revealed that not only the association, but also the dissociation was influenced by the addition of serum. The dissociation rate constant increased with increasing amounts of added serum, indicating that the stability of the complex was reduced by the existence of serum proteins.

We also investigated the interactions of the drug-proteins pairs Infliximab and tumor necrosis factor alpha (TNF α), and Bevacizumab and vascular endothelial growth factor (VEGF), with and without serum proteins. In contrast to the quercetin and PKA, these drug-protein pairs showed little effects of serum matrix on both the association and dissociation processes. In addition, we investigated a spinal fluid matrix effect of these antibody drugs and observed little effects.

Using the GMR bio-sensing technique, it was revealed that the matrix effect of a biological liquid, such as serum, depends on the kind of drugs and target proteins. The real-time monitoring of molecular interactions in a biological matrix enabled by the GMR bio-sensing technique is a very powerful tool to investigate such complicated molecular interactions and is indispensable for determining the mechanism of action of the drug and the pharmacokinetics/pharmacodynamics inside the body. Specifically, we could directly observe the interaction change induced between two molecules by adding a third molecule into the sample solution. This is a huge advantage for studies on complicated interactions among more than two molecules; for example, an inhibitor drug against the interaction between two kinds of proteins. This is a very unique feature of this technique compared with other previous methods based on SPR, microscale thermophoresis, or bio-layer interferometry.

2. Materials and methods

2.1. Materials

Quercetin (Cayman Chemical, catalog #10,005,169) was used without further purification. cAMP-dependent protein kinase (Bio-Labs, catalog #P6000), Infliximab (BioVision, catalog #A1097–200), TNF α (RD Systems, catalog #210-TA), Bevacizumab (MedChem-Express, catalog #HY-P9906), and VEGF (PeproTech, catalog #100–20) were used. Lyphochek tumor marker plus control Lv.3 (BioRad, catalog #10,369) and Liquichek spinal fluid (BioRad, catalog #10,752) were used as serum and spinal fluid, respectively. Dextran-coated magnetic particles (Miltenyi Biotec, catalog #130–048-001) were used for the preparation of quercetin-conjugated magnetic particles. Quercetin-conjugated particles were prepared according to a previously reported procedure [8]. Briefly, 0.5 mL magnetic particle dispersed solution was added to 5 mL acetate buffer (pH 5.0) and gently stirred. The pre-determined amount of N-hydroxyl succinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was added to the magnetic particle solution and incubated at 25 °C for 15 min. Afterward, magnetic particles were separated with a column (micro column, Miltenyi Biotec, catalog #130–042-701) and washed twice with 400 μ L dimethyl sulfoxide (DMSO). Quercetin (5 mg) was dissolved in 5 mL DMSO and added to activated magnetic particles, which were then incubated overnight. The magnetic particles were separated with a column (micro column, Miltenyi Biotec), washed with 400 μ L DMSO, and twice with 400 μ L distilled water. To determine the concentration of magnetic particle dispersed solution, a 200 μ L droplet of 1~10-fold diluted magnetic particle solutions was spotted onto an amino-propyl silane-coated silicon oxide substrates (10 mm × 10 mm) and was gently dried. The spotted areas were observed by scanning electron beam microscope (Hitachi High-Tech, SE-8000) and the number of magnetic particles in a fixed area (2.95 μ m × 4.24 μ m) was counted to determine the concentration of magnetic particles. Finally, a 0.8 nM magnetic particle phosphate buffered saline (PBS) solution was prepared. For Bevacizumab- and Infliximab-conjugated magnetic particles, a bead labelling kit (Miltenyi Biotec, catalog #130–105–805) was used. Protein conjugation was performed according to the manufacturer's recommended protocol. Finally, pure PBS, PBS with 70 % serum, and PBS with 70 % spinal fluid solutions of the antibody drug-conjugated magnetic particles (1 nM) were prepared.

2.2. GMR sensor and instrument

The GMR sensor was an MR-813 chip provided by MagArray, Inc. (California). One chip had 80 sensors and the area of one sensor was

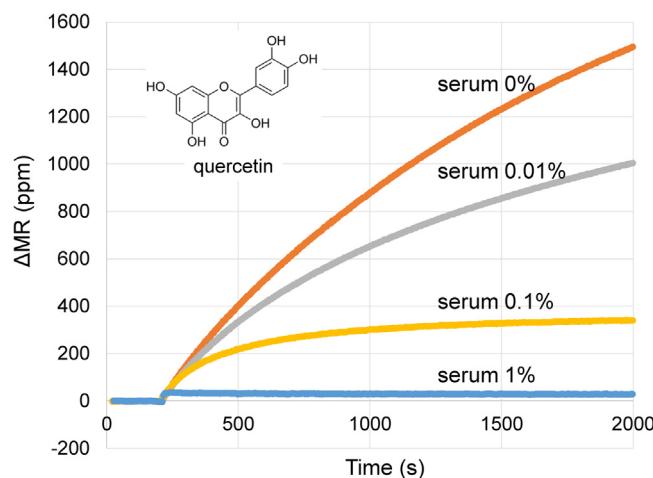


Fig. 1. Time profile of magnetic signal intensity (ΔMR) of the samples that contained several amounts of serum. The amount of serum is shown in the figure. Molecular structure of quercetin is inserted.

100 $\mu\text{m} \times 120\mu\text{m}$. Three–five droplets (300–360 pL/droplet) of PBS solution (pH 7.4) with PKA (50 $\mu\text{g}/\text{mL}$), TNF α (10 $\mu\text{g}/\text{mL}$) or VEGF (10 $\mu\text{g}/\text{mL}$) were spotted onto 20 sensors using a spotter (Scienion, S5 Spotter). For the reference sensor, 3–5 droplets of 0.1 % bovine serum albumin solution were spotted. After spotting, the blocking process was performed according to the MagArray's recommended protocol.

The magneto-resistance measurements were carried out with the MR-813 reader system provided by MagArray, Inc. The ratio of the resistance of the GMR sensor before and after dipping the GMR sensor into the magnetic particle dispersed solution was calculated as ΔMR . Sample and PBS solutions were stored in a 96-deep well plate contained within the instrument and the position of the GMR sensor was automatically controlled inside and outside of each deep well where sample or PBS buffer were kept. For the association reaction process, target proteins spotted on the GMR sensor chip were soaked in drug-conjugated magnetic particle solution for 30 min and the signal from the GMR sensor was monitored in real-time (one data point acquisition per 5 s). For the dissociation reaction process, the GMR sensor was soaked in pure PBS solution and was moved to the next well containing fresh PBS solution for 1 min intervals to suppress the increased concentration of free drug-conjugated magnetic particles. To stir the solutions, an up and down movement of the GMR sensor at 5 Hz in each solution in each deep well was performed. Coefficient of variation of rate constants evaluated by three repeated experiments was about 10 %.

3. Results & discussions

First, we prepared a mixture of quercetin-conjugated magnetic particle solution and serum, and then, the PKA-fixed GMR sensor was soaked in the mixed solution. Fig. 1 indicates the time profile of the magnetic signal intensity (ΔMR) of the samples that contained several amounts of serum. Signal intensity clearly depended on the amount of serum and decreased with increasing amounts of serum; the signal nearly disappeared when the amount of serum was more than 1%. These results could be interpreted as follows. Serum proteins, especially human albumin, attach to quercetin so well [9] that the attached proteins delay the association between quercetin and PKA. The initial mixing of quercetin with serum might cause a reduction of effective (free) quercetin concentration, so that the signal intensity showed saturation in a relatively early time frame. Therefore, these results indicated that initial mixing of quercetin-conjugated magnetic particles and serum before the

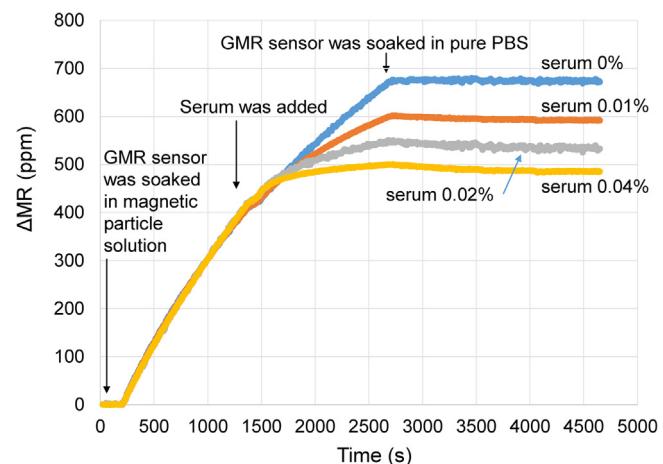


Fig. 2. Time profile of magnetic signal intensity (ΔMR) of the samples. Initially, the PKA-fixed GMR sensor was soaked in phosphate buffered saline (PBS) solution. At 3 min, it was placed into the sample solution containing quercetin-conjugated magnetic particles in each deep well. At 23 min, 4 μL of serum protein PBS solution at concentrations of 0.1 %, 0.2 % and 0.4 % were added to each sample solution (final concentrations were 0.01 %, 0.02 % and 0.04 %, respectively). The signal intensity at the time position of the addition of serum (23 min) was normalized to ensure the change due to the addition of serum was clear.

PKA-fixed GMR was soaked with them was not adequate to investigate the reaction kinetics of quercetin and PKA surrounded by serum matrix.

Next, we added a small amount of serum into the sample solution during the binding reaction to observe changes in the time profile of the GMR signal before and after adding the serum. First, the PKA-fixed GMR sensor was soaked in a PBS solution containing quercetin-conjugated magnetic particles kept in each deep well, and the signal measurement from the GMR sensor was started. The volume of sample solution was 400 μL . After 23 min, 4 μL PBS solution containing serum at concentrations of 1%, 2% and 4% were added to each sample solution (final concentrations were 0.01 %, 0.02 % and 0.04 %, respectively). The obtained result is shown in Fig. 2. The signal intensity at the time of adding serum (23 min after the measurement) was normalized to ensure that the change due to the addition of serum was clear. Obvious changes in the time profile of the ΔMR signal were observed before and after the addition of serum. Compared to the controls without serum, the curve was clearly suddenly bent just after adding serum, depending on the concentration of serum. Adding more serum suppressed increased ΔMR signal, which meant that serum proteins disturbed the association process between quercetin and PKA.

To interpret the serum matrix effect, we first analyzed the data based on reaction model (Fig. 3), where serum protein associated with quercetin-conjugated magnetic particles; such interactions may delay the association process between quercetin and PKA. In Fig. 3, serum protein is written as HSA (human serum albumin) because the majority (approximately 50–65 %) of serum protein is albumin and an interaction between quercetin and albumin has been reported [9]. Quercetin immobilized on the magnetic particles interacted with HSA to form a complex, which may dissociate to free quercetin and HSA. Quercetin also associated with PKA fixed on the GMR sensor. If the dissociation of the quercetin-HSA complex was slow, we could observe the competitive process between the formation of the quercetin-HSA complex and quercetin-PKA association, namely suppression of increased ΔMR signal intensity. Rate equations are shown in Fig. 3. The dissociation of the quercetin-PKA process was neglected for analysis of the association process because the signal decay without serum was slow compared to the signal increase (Fig. 2).

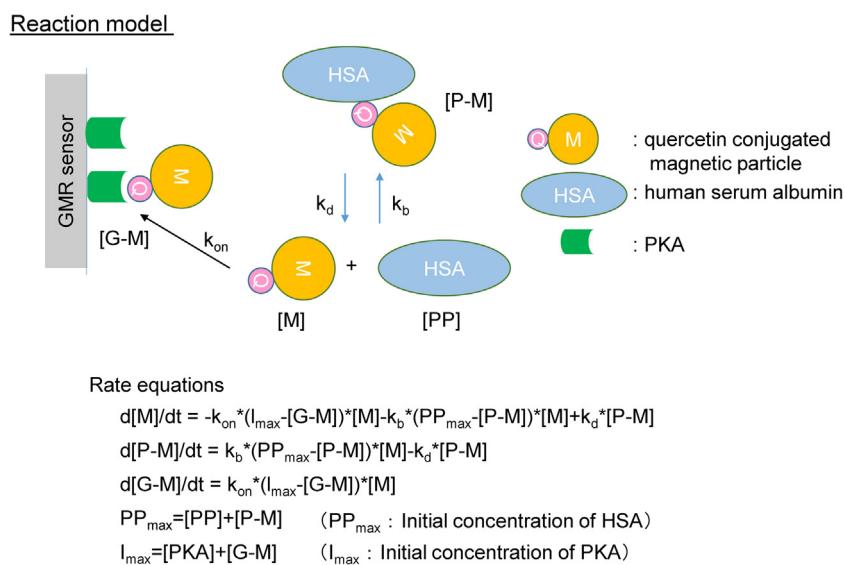


Fig. 3. Reaction model. Serum protein is written as HSA (human serum albumin). See the text for more details.

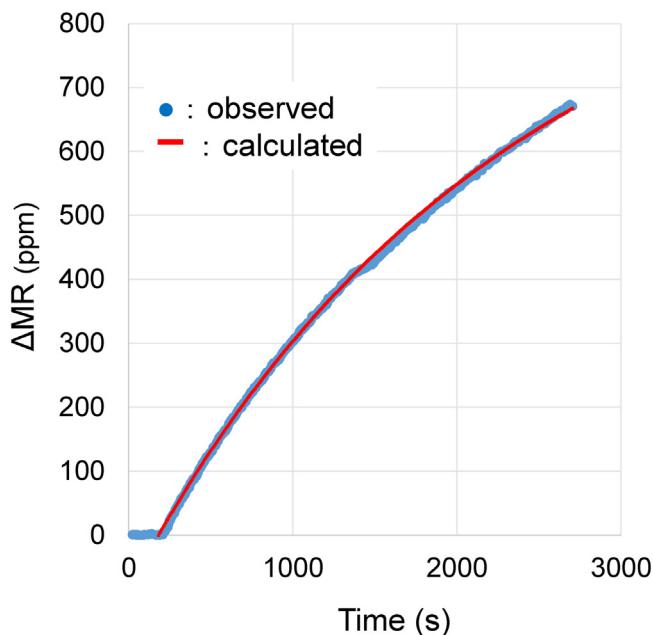


Fig. 4. Curve fitting to 0% serum data based on Langmuir's adsorption isotherm.

First we determined k_{on} by curve fitting the data without serum based on the Langmuir's adsorption isotherm equation, as shown below,

$$\text{signal}(t) = s_{\max} \times (1 - \exp(-k_{on} \times C_0 \times (t - t_0))),$$

where $\text{signal}(t)$: ΔMR signal intensity, s_{\max} : saturation value of signal intensity, k_{on} : association rate constant, C_0 : initial concentration of quercetin-conjugated magnetic particles, and t_0 : time interval between the starting point of the measurement and the real starting point of the binding process. C_0 was 0.8 nM, which we determined as described in section 2.1. The result of the curve fitting is shown Fig. 4, which shows that the observed data has been reproduced well by the calculated data. The determined k_{on} was $5.4 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. Next, curve fitting of the same data without serum using the least squares method was executed by numerical integration of rate equations to determine I_{max} (concentration of PKA on the GMR sensor) and a data conversion factor that converted the

ΔMR signal to mole concentration using the same k_{on} value determined above. The I_{max} and data conversion factor were $1.0 \times 10^{-10} \text{ M}$ and $1.0 \times 10^{13} \text{ ppm/M}$, respectively.

Next, we analyzed the data with 0.04 % serum, based on reaction model shown in Fig. 3. The reference range for albumin concentrations in serum is approximately 35–50 (average: 42.5) g/L [10], so that 0.04 % serum converted to HSA: $2.6 \times 10^{-7} \text{ M}$. k_b and k_d were determined by curve fitting using the same k_{on} , I_{max} and data conversion factor determined above. The observed data was adequately reproduced and k_b and k_d were determined as $1.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $2.0 \times 10^{-4} \text{ s}^{-1}$, respectively. The dissociation constant K_d ($= k_d/k_b$) was $1.5 \times 10^{-8} \text{ M}$. Previous studies using fluorescence measurements [11] and differential scanning calorimetry [12] report the dissociation constant K_d of quercetin and human serum albumin as $3-6 \times 10^{-6} \text{ M}$. The K_d value obtained in this study is very small compared with the ones reported in the previous study, suggesting that another process that strongly suppresses the association of quercetin and PKA, except quercetin-HSA binding, may exist. We considered that the association rate constant itself might be reduced by interaction between quercetin and serum proteins. However, a good reproduction of the observed time profile was not achieved.

The dissociation process of the complex of quercetin and PKA became faster with increasing amounts of serum, as shown in Fig. 2. We determined the dissociation rate constant by curve fitting based on the following equation;

$$\text{signal}(t) = \text{signal}(0) \times \exp(-k_{off} \times (t - t_0)),$$

where k_{off} : the dissociation rate constant, t_0 : time interval between start point of measurement and real start point of dissociation process. The obtained rate constants are plotted against serum concentration in Fig. 5. Obviously, the dissociation of the quercetin-PKA complex was faster with increasing serum concentration; ~3.7 times faster in 0.04 % serum than 0% serum (pure PBS). This indicated that the stability of the complex was decreased by the surrounding serum proteins. Although we tried the simulation considering the faster dissociation process, the observed data was not reproduced well. The results of the simulations indicated that much faster terms that suppress the association should be considered. The fast suppression of association and fast dissociation were induced by serum proteins, indicating that the interaction between quercetin and PKA in their complex depended on the amount of serum protein. This implies that the complex may partially include

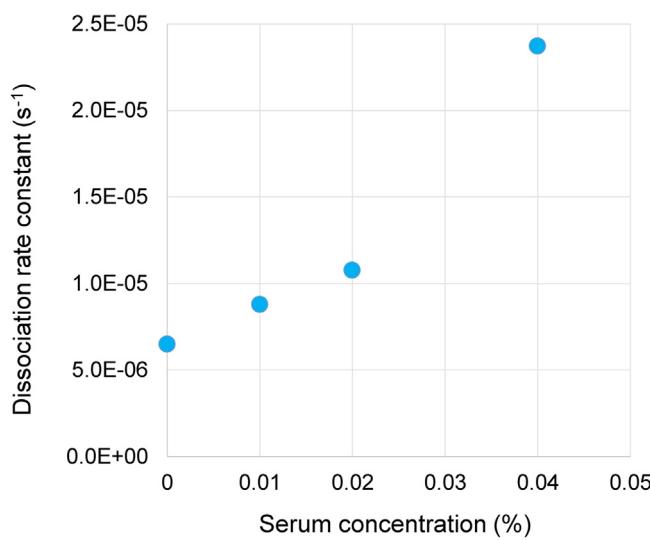


Fig. 5. Plot of rate constants of the dissociation process of the complex of quercetin and PKA versus the serum concentration.

serum albumin molecules or may attach to serum albumin, which has a few binding sites with different affinities [13], and that the albumin may also be involved in complex formation. It was likely that the association rate changed from true k_{on} (relatively fast) to a slower one over time due to binding of albumin to quercetin, depending on the concentration of albumin; also, the dissociation rate becomes faster over time. Such complicated mechanisms may govern the interaction among quercetin, PKA and serum proteins (mainly albumin).

Compared to the small drug quercetin (MW: 302 Da) and PKA pair mentioned above, we next investigated the serum matrix effect on large drug molecules, such as pairs of antibody drugs and their target proteins. We chose the pairs: Infliximab (MW: ~149,000 Da) and TNF α , and Bevacizumab (MW: ~149,000 Da) and VEGF.

Figs. 6 and 7 show the experimental results of the Infliximab and TNF α pair and the Bevacizumab and VEGF pair, respectively. The obtained rate constants are summarized in **Tables 1 and 2**, respectively. Contrary to the results of the quercetin and PKA pair, with and without serum, these two antibody drugs and target protein pairs showed little effects of serum matrix; the association and dissociation rate constants were almost the same among pure PBS, PBS with 70% serum and PBS with 70% spinal fluid. These obtained association and dissociation rate constants are very close to the reported values in the literature where the SPR method has been used to determine the rate constants in pure buffer solutions [14,15]. These results indicated that serum and also spinal fluid matrices did not have strong interactions with these antibody drugs and did not influence the interaction between the antibody drug and its target protein. It was also shown that these matrices did not have any strong interaction against the magnetic particles coated with dextran used in this study. Therefore, the matrix effects observed in the quercetin and PKA pair were attributed to the hydrophobic and electrostatic characteristics of the quercetin molecule, serum proteins (mainly human serum albumin), and PKA.

The advantages of GMR bio-sensing techniques over the conventional methods based on the photonics techniques like SPR and bio-layer interferometry are summarized as follows: 1) only specific interaction is measured even if the third molecule like serum protein coexists, 2) effect of the third molecule can be investigated. Moreover, compared with microscale thermophoresis [2] and high-performance affinity chromatography techniques [3] which are useful for molecular interaction investigations between drugs and serum proteins, the advantages of GMR bio-sensing techniques are: 1) association and dissociation processes are measured separately, 2) rate constant of each process can be determined. In previous study [1], we investigated the effect of adding serum proteins between VEGF and Bevacizumab by using SPR method and GMR-biosensing techniques and compared the results. Although the almost same rate constants were obtained even in 70% serum solution compared to those in pure buffer by using GMR-biosensing techniques as shown in **Table 2**, totally different rate constants were

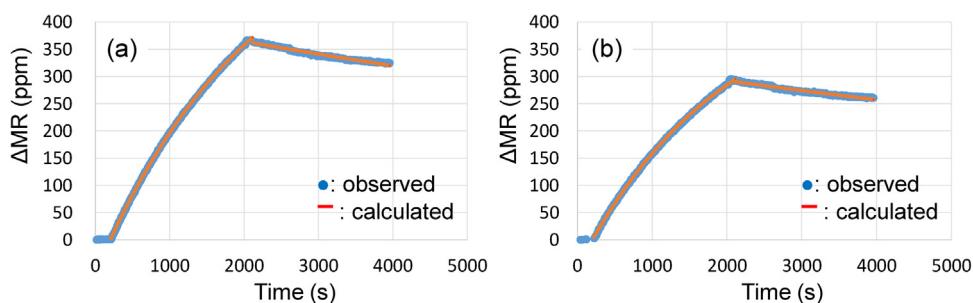


Fig. 6. Results of the binding assay of the Infliximab and tumor necrosis factor alpha (TNF α) pair and curve fitting in (a) pure phosphate buffered saline (PBS) buffer and (b) PBS buffer with 70% serum.

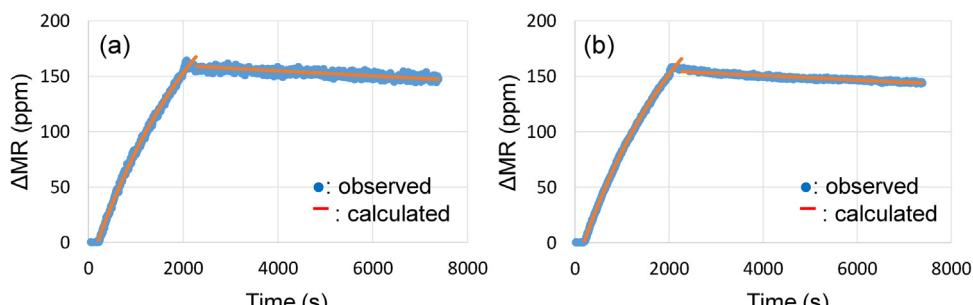


Fig. 7. Results of the binding assay of the Bevacizumab and vascular endothelial growth factor (VEGF) pair and curve fitting in (a) pure phosphate buffered saline (PBS) buffer and (b) PBS buffer with 70% serum.

Table 1Rate constants of the Infliximab/tumor necrosis factor α pair, with and without biological liquids.

	Pure buffer	70 % serum	70 % spinal fluid	Reported value in pure buffer [14]
k_{on} ($M^{-1}s^{-1}$)	4.7×10^5	5.1×10^5	6.0×10^5	4.8×10^5
k_{off} (s^{-1})	6.6×10^{-5}	6.4×10^{-5}	3.6×10^{-5}	5.5×10^{-5}
K_d ($= k_{off}/k_{on}$) (M)	1.4×10^{-10}	1.3×10^{-10}	5.9×10^{-11}	1.1×10^{-10}

Table 2

Rate constants of the Bevacizumab/ vascular endothelial growth factor pair, with and without biological liquids.

	Pure buffer	70 % serum	70 % spinal fluid	Reported value in pure buffer [15]
k_{on} ($M^{-1}s^{-1}$)	3.2×10^5	3.2×10^5	2.7×10^5	5.3×10^5
k_{off} (s^{-1})	1.2×10^{-5}	1.4×10^{-5}	1.4×10^{-5}	3.1×10^{-5}
K_d ($= k_{off}/k_{on}$) (M)	3.8×10^{-11}	4.2×10^{-11}	5.2×10^{-11}	5.8×10^{-11}

obtained by using SPR even in more than 1% serum solution which may be due to non-specific binding of serum protein to SPR chip surface. Duhr et al. reported that the affinity of quercetin to PKA was reduced by 400-fold in serum compared to that in buffer by using microscale thermophoresis [2]. However, they did not provide detailed information about which association or dissociation process was influenced and how they were changed by serum.

If we add the third molecule whose hydrophilicity and electrostatic character are well recognized into the sample solution that contains drugs and target molecules during their binding reaction, we can obtain important information on features of the molecular interactions between drugs and target molecules based on the analysis of the influence of the third molecule, which is not available in photonics techniques like SPR and interferometry because these non-labeling methods cannot distinguish the kinds of molecules binding to the surface of sensors.

Recently, intensive development of such inhibitor drugs for protein-protein interactions has shown that certain classes of protein-protein interactions are amenable to small molecule inhibition; typically these inhibitors disrupt the interaction between a globular protein and a single peptide chain on the partner protein and do so by binding to pockets on the surface of the globular protein [16]. The method described in this study is one of the powerful analysis tools to determine the action of such inhibitor drugs against the interaction between two kinds of proteins.

4. Conclusion

We have demonstrated that interactions between drugs and target molecules under the existence of a third molecule, such as serum and spinal fluid, can be monitored in real time using GMR bio-sensors. It was clearly shown that not only the association, but also the dissociation of the quercetin and PKA pair was affected by the addition of serum; the association was delayed and the dissociation was accelerated. This implied that the complex between quercetin and PKA may partially include serum proteins (mainly human serum albumin) or may attach to the serum proteins. In contrast, antibody drugs and their target protein pairs showed little matrix effects, even in 70 % spinal liquid. This discrepancy may be attributed to the hydrophobic and electrostatic properties of the drug molecules. Therefore, the technique based on a GMR bio-sensor with the addition of another third molecule into the sample solution during binding reaction has promising advantages for the analysis of molecular interactions between drugs and target molecules, including the biological matrix as the third molecule. This method can also be applied to the analysis of the influence of any kind of third molecule that may have some interaction between two molecules; for example, inhibitor drugs against the interaction between two kinds of proteins.

CRediT authorship contribution statement

Toshiro Saito: Conceptualization, Investigation, Data curation, Formal analysis, Writing - original draft. **Masayoshi Momiyama:** Methodology, Investigation, Data curation. **Chihiro Manri:** Methodology. **Heng Yu:** Methodology. **Kalidip Choudhury:** Conceptualization. **Sebastian J. Osterfeld:** Formal analysis. **Takashi Nakasawa:** Conceptualization.

Declaration of Competing Interest

TS, MM and TN are employees of Hitachi High-Tech Corporation and have received payments from the company. CM is employee of Hitachi, Ltd. and has received payment from the company. HY, KC and SJO are employees of MagArray Inc. and have received payments from the company.

Acknowledgments

The authors are grateful to Dr. Danfeng Yao of MagArray Inc. for helpful discussion on molecular interaction analysis using GMR sensors, and Dr. Naoshi Itabashi of Hitachi, Ltd. and Ms. Airi Kikuchi of Hitachi High-Tech Corporation for experimental supports on surface modification of GMR sensors and magnetic particles.

References

- [1] C. Manri, Y. Heng, N. Itabashi, T. Nakasawa, M. Momiyama, T. Saito, Development of molecular interaction assay using magneto-resistive sensor, Fukuoka, Japan, in: Poster Presented at: The 42nd Annual Meeting of the Molecular Biology Society of Japan, 5, 2019, December.
- [2] C.J. Wienken, P. Baaske, U. Rothbauer, D. Braun, S. Duhr, Protein-binding assays in biological liquids using microscale thermophoresis, *Nat. Commun.* 100 (2010) 1–7.
- [3] Z. Li, D.S. Hage, Analysis of stereoselective drug interactions with serum proteins by high-performance affinity chromatography: a historical perspective, *J. Pharm. Biomed. Anal.* 144 (2017) 12–24.
- [4] A. Vacobi, J.A. Udall, G.L. Pharm, Serum protein binding as a determinant of warfarin body clearance and anticoagulant effect, *Clin. Pharm. Therap.* 19 (1976) 552–558.
- [5] J.-R. Lee, D.J.B. Bechstein, C.C. Ooi, A. Patel, R.S. Gaster, E. Ng, L.C. Gonzalez, S.X. Wang, Magneto-nanosensor platform for probing low-affinity protein–protein interactions and identification of a low-affinity PD-L1/PD-L2 interaction, *Nat. Commun.* 7 (2016) 12220.
- [6] A. García-Lafuente, E. Guillamón, A. Villares, M.A. Rostagno, J.A. Martínez, Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease, *Inflamm. Res.* 58 (2009) 537–552.
- [7] R.E. Najafabadi, N. Kazemipour, A. Esmaeili, S. Beheshti, S. Nazifi, Using superparamagnetic iron oxide nanoparticles to enhance bioavailability of quercetin in the intact rat brain, *BMC Pharmacol. Toxicol.* 19 (2018) 59.
- [8] S.R. Kumar, S. Priyatharshni, V.N. Babu, D. Mangalaraj, C. Viswanathan, S. Kannan, N. Ponpandian, Quercetin conjugated superparamagnetic magnetite nanoparticles for *in-vitro* analysis of breast cancer cell lines for chemotherapy, *J. Colloid Interface Sci.* 436 (2014) 234–242.
- [9] O. Rolinski, A. Martin, D.J.S. Birch, Human serum albumin and quercetin interactions monitored by time-resolved fluorescence: evidence for enhanced discrete rotamer conformations, *J. Biochem. Optics* 12 (2007), 034013.

- [10] Pathology Harmony Group, Harmonisation of Reference Intervals, 2011 (accessed 4 February 2021) <https://web.archive.org/web/20130802082027/http://www.acb.org.uk/docs/Pathology%20Harmony%20for%20web.pdf/>.
- [11] B. Sengupta, P.K. Sengupta, The interaction of quercetin with human serum albumin: a fluorescence spectroscopic study, *Biochem. Biophys. Res. Commun.* 299 (2002) 400–403.
- [12] A. Precupas, R. Sandu, V.T. Popa, Quercetin influence on thermal denaturation of bovine serum albumin, *J. Phys. Chem. B* 120 (2016) 9362–9375.
- [13] A. Bolli, M. Marino, G. Rimbach, G. Fanali, M. Fasano, P. Ascenzi, Flavonoid binding to human albumin, *Biochem. Biophys. Res. Commun.* 398 (2010) 444–449.
- [14] M.-Y. Song, S.-K. Park, C.S. Kim, T.H. Yoo, B. Kim, M.-S. Kim, Y.-S. Kim, W.J. Kwag, B.-K. Lee, K. Baek, Characterization of a novel anti-human TNF- α murine monoclonal antibody with high binding affinity and neutralizing activity, *Exp. Mol. Med.* 40 (2008).
- [15] N. Papadopoulos, J. Martin, Q. Ruan, A. Rafique, M.P. Rosconi, E. Shi, E.A. Pyles, G.D. Yancopoulos, N. Stahl, S.J. Wiegand, Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF Trap, ranibizumab and bevacizumab, *Angiogenesis* 15 (2012) 171–185.
- [16] D.E. Scott, A.R. Bayly, C. Abell, J. Skidmore, Small molecules, big targets: drug discovery faces the protein-protein interaction challenge, *Nat. Rev. Drug Discov.* 15 (2016) 533–550.