Effect of Time to Detection on the Measured Concentrations of Blood Proteins Associated with Alzheimer’s Disease

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Abstract
Background: For assays using immunomagnetic reduction, a reagent composed of antibody-functionalized magnetic nanoparticles is dispersed in phosphate-buffered saline solution. The real-time signals of alternating-current (ac) magnetic susceptibility, $\chi_{ac}$, of the reagent are subsequently recorded after mixing the reagent with a biofluid sample. After mixing the reagent and sample, the reduction in $\chi_{ac}$ of the mixture is calculated and used to quantify the concentration of the target biomarker in the sample. The reduction does not occur immediately but rather occurs at some time after mixing. This observation implies that the time elapsed before recording the real-time signals of $\chi_{ac}$ of a reagent-sample mixture needs to be investigated to ensure that the signals are fully recorded. In this work, the effect of time to detection on the measured concentrations of proteins in human plasma after mixing the reagent and sample is examined. Methods: The proteins analyzed are related to Alzheimer’s disease: amyloid $\beta$ 1–40, amyloid $\beta$ 1–42, and Tau protein. The investigated times to detection after mixing the reagent and sample are 0, 20, 30, 40, and 120 min. Results: The results show that the recording of real-time signals of $\chi_{ac}$ should be conducted within 20 min after mixing the reagent and sample.

Introduction
A trend to evaluate the risk of Alzheimer’s disease (AD) is the assay of biomarkers in blood [1–4]. The most recognized biomarkers related to AD are amyloid $\beta$ 1–40 (A$\beta_{1–40}$) and A$\beta_{1–42}$ peptides and total Tau protein (Tau) [5–9]. However, the concentrations of these biomarkers in human blood are very low, which is motivating the development of ultrasensitive assay technologies [10–15]. Superconducting-quantum-interference-device-based immunomagnetic reduction (SQUID-IMR) is one such technology [16]. According to reports [17, 18], the lower limits of detection of SQUID-IMR are at the level of pg/mL or lower, indicating that the method is sensitive enough to precisely quantify the levels of A$\beta_{1–40}$, A$\beta_{1–42}$ and Tau in human blood. Many papers have been published on the clinical validations of risk evaluations of AD by using SQUID-IMR to assay plasma biomarkers [19–21]. In SQUID-IMR, magnetic nanoparticles biofunctionalized with antibodies and dispersed in PBS (pH = 7.4)
solution are used as reagent [22]. The average diameter of the nanoparticles is approximately 55 nm. The application of alternating-current (ac) magnetic fields to the reagent causes the magnetic nanoparticles to oscillate with the fields. Signals due to the nanoparticle oscillation, so-called ac magnetic susceptibility (\(\chi_{ac}\)) signals, are generated by the reagent. To achieve the strongest signals, the frequency of the applied ac magnetic fields is adjusted to the resonant frequency of nanoparticle oscillation. After mixing the reagent with a plasma sample, the target biomarker molecules in plasma bind with the magnetic nanoparticles via antibody-antigen association. The physical sizes of the bound magnetic nanoparticles increase; thus, after biomarker-nanoparticle association, the resonant frequency of the bound nanoparticles no longer matches the frequency of the applied ac magnetic fields biomarker-nanoparticle association. Thus, the signals of ac magnetic susceptibility of the reagent are reduced. A typical real-time signal of ac magnetic susceptibility of the reagent after mixture is plotted in Figure 1a [23]. The concentration of the target biomarker is determined based on the reduction [24].

To determine the reduction, the real-time signals of ac magnetic susceptibility of the reagent are recorded after mixing the reagent with a plasma sample. After mixing the reagent with a sample, the signal of ac magnetic susceptibility initially remains unchanged (referred to as the initial period) before starting to decrease; it ultimately reaches a lower, stable level (referred to as the final period), as illustrated in Figure 1a. In our experience, both the initial and final periods span approximately 100 min. The signals during the initial period and final period are aver-

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**Fig. 1.** Schematic of real-time signals of ac magnetic susceptibility of reagent-sample mixture, i.e., the \(\chi_{ac}-t\) curve (a), and a comparison of the \(\chi_{ac}-t\) curves between the 1st vial and the last vial of the same biomarker concentration under shorter (b) or longer mixing periods (c).
aged, with the averages denoted as $\chi_{ac,0}$ and $\chi_{ac,\phi}$, respectively. The reduction in ac magnetic susceptibility, denoted as IMR ($\%$), of the reagent is calculated as follows:

$$\text{IMR ($\%$)} = \left(\frac{\chi_{ac,\phi}}{\chi_{ac,0}}\right) \times 100\%. \quad (1)$$

Samples with higher concentrations of a biomarker generate higher values of IMR ($\%$). The relationship between IMR ($\%$) and biomarker concentration has been established for the quantitative detection of several biomarkers.

In practice, not one sample but a batch of samples are assayed simultaneously. For example, 36 vials may be assayed in one batch of IMR measurements.[24] Usually, one reagent is mixed with one sample at a time; in this example, 36 vials would be mixed in sequence. This period is referred to as the mixing period and is shown in Figure 1b. After all 36 vials are prepared, the real-time signals of ac magnetic susceptibility of the 36 mixtures are recorded; this period is referred to as the recording period and is shown in Figure 1b. In this scenario, therecording period for each vial starts at a different point in the curve of real-time ac magnetic susceptibility, as plotted in Figure 1b. Once the mixing period becomes too long, the initial interval for the 1st vial may include data that lie in the descending interval, as plotted in Figure 1c. In such a case, even though the samples in the 1st and last (36th) vials have the same concentrations, the measured concentration for the 1st vial would be lower than that for the last (36th) vial, as shown in Figure 1c. Thus, the IMR ($\%$) calculated for the 1st vial would be inaccurate. Hence, it is necessary to clarify the maximum duration of the mixing period that enables accurate IMR measurement.

**Materials and Methods**

For each of Aβ$_{1-40}$, Aβ$_{1-42}$ and Tau, pure antigen was spiked in phosphate-buffered saline (PBS) solution. Three different concentrations, i.e., 10, 100, 1,000 pg/mL, of PBS samples were prepared for each biomarker. Biomarker concentrations from 10 to 1,000 pg/mL were prepared because the reported biomarker concentrations in human plasma are several tens of pg/mL [19–21]. Thus, the spiked concentrations 10–1,000 pg/mL cover the range of biomarker concentrations in real human samples.

For a given concentration of a biomarker, the biomarker PBS solution was aliquoted to five samples. Each aliquoted sample was subjected to one of several different durations of mixing period: 0, 20, 30, 40, and 120 min. Thus, the aliquoted five samples had the same concentration of biomarker but corresponded to different mixing period durations. The measured concentration for the 0-min mixing period, denoted as $\phi_0$, was used as a reference. The recovery rate corresponding to a given mixing period was calculated as follows:

$$\text{Recovery rate ($\%$)} = \frac{\phi_t}{\phi_0} \times 100\%. \quad (2)$$

where $\phi_t$ denotes the measured concentration for a t-min mixing period.

The concentrations of the PBS samples were measured with the aid of IMR reagents (MF-0060-AB0, MF-0060-AB2, MF-0060-TAU, MagQu) and an analyzer (XacPro-S, MagQu). For each IMR measurement, 60/40/60 μL reagent was mixed with 60/80/60 μL of PBS sample for the assay of Aβ$_{1-40}$/Aβ$_{1-42}$/Tau. Duplicate IMR measurements were performed for each sample.

**Results**

The measured concentrations of 10-/100-/1,000-pg/mL Aβ$_{1-40}$ PBS samples with various durations of mixing period from 0 to 120 min are listed in Table 1. The measured concentration of 10-/100-/1,000-pg/mL Aβ$_{1-40}$ samples with a zero-minute mixing period, wherein the recording of real-time ac magnetic signals was performed immediately after mixing the reagent and sample, was 10.22/97.66/1,034.3 pg/mL. The deviations between the measured concentrations of Aβ$_{1-40}$ from spiked concentrations were less than 5%, revealing the accuracy of IMR measurement. The measured concentration tended to decrease as the mixing-period duration increased from 0 to 120 min. For example, the measured concentration of 100-pg/mL Aβ$_{1-40}$ PBS samples with a 120-min mixing period was 47.48 pg/mL, which corresponded to a 48.6% recovery rate. These results suggest that when initiating the recording period at 120 min after the mixing of reagent and sample, the measured concentration was reduced approximately by 50% from that obtained immediately after mixing the reagent and sample. These findings confirmed that the duration of the mixing period affected the IMR-measured Aβ$_{1-40}$ concentration.

Reductions in the measured concentration with increasing mixing-period duration were similarly observed for the 10-/100-/1,000-pg/mL Aβ$_{1-42}$ PBS samples; the values are listed in Table 2. Recovery rates higher than 95% were found for the duration of 20 min. The recovery rates for the 100- and 1,000-pg/mL Aβ$_{1-42}$ PBS samples were higher than 90% for the 30-min mixing period. However, the recovery rate for the 10-pg/mL Aβ$_{1-42}$ PBS samples was lower than 90% for the 30-min period. For durations greater than 40 min, the 10-, 100-, and 1,000-pg/mL Aβ$_{1-42}$ PBS samples showed recovery rates lower than 90%. Conventionally, a recovery rate lower than 90% or higher than 110% is used to define a significant change in
Table 1. Replicate measurements of Aβ1-40 concentration for various mixing period durations

| Biomarker | Spiked concentration, pg/mL | Mixing period, min | Measured concentration | Recovery rate, % | SD, pg/mL | CV, % |
|-----------|-----------------------------|-------------------|------------------------|-----------------|-----------|-------|
| Aβ1-40    | 10                          | 0                 | 9.48 10.95 10.22 1.04 10.13 | 100             |           |       |
|           | 20                          | 10.10 9.82 9.96 0.20 1.99 | 97.5          |           |           |       |
|           | 30                          | 10.18 10.12 10.15 0.05 0.46 | 99.4          |           |           |       |
|           | 40                          | 8.85 9.17 9.01 0.22 2.47 | 88.2          |           |           |       |
|           | 120                         | 6.34 7.51 6.93 0.82 11.89 | 67.8          |           |           |       |
| 100       | 0                           | 94.15 101.17 97.66 4.97 5.08 | 100           |           |           |       |
|           | 20                          | 97.73 98.93 98.33 0.84 0.86 | 100.7         |           |           |       |
|           | 30                          | 95.70 97.64 96.67 1.37 1.42 | 99.0          |           |           |       |
|           | 40                          | 80.38 83.74 82.06 2.37 2.89 | 84.0          |           |           |       |
|           | 120                         | 46.25 48.71 47.48 1.74 3.66 | 48.6          |           |           |       |
| 1,000     | 0                           | 1,027.5 1,041.1 1,034.3 9.62 9.3 | 100           |           |           |       |
|           | 20                          | 934.4 1,055.3 994.8 85.46 8.59 | 96.2          |           |           |       |
|           | 30                          | 858.9 893.8 876.3 24.68 2.82 | 84.7          |           |           |       |
|           | 40                          | 729.4 666.6 698.0 44.43 6.37 | 67.5          |           |           |       |
|           | 120                         | 553.0 463.0 508.0 63.66 12.53 | 49.1          |           |           |       |

Mean = (1st measurement + 2nd measurement)/2. SD, standard deviation; CV, coefficient of variation.

Table 2. Replicate measurements of Aβ1-42 concentration for mixing period durations

| Biomarker | Spiked concentration, pg/mL | Mixing period, min | Measured concentration | Recovery rate, % | SD, pg/mL | CV, % |
|-----------|-----------------------------|-------------------|------------------------|-----------------|-----------|-------|
| Aβ1-42    | 10                          | 0                 | 10.87 11.63 11.25 0.54 4.77 | 100             |           |       |
|           | 20                          | 10.96 11.30 11.13 0.24 2.16 | 98.9          |           |           |       |
|           | 30                          | 9.41 8.88 9.15 0.38 4.12 | 81.3          |           |           |       |
|           | 40                          | 7.52 7.40 7.46 0.08 1.12 | 66.3          |           |           |       |
|           | 120                         | 5.87 6.11 5.99 0.17 2.76 | 53.2          |           |           |       |
| 100       | 0                           | 97.22 105.86 101.54 6.11 6.02 | 100           |           |           |       |
|           | 20                          | 90.73 102.11 96.42 8.05 8.35 | 95.0          |           |           |       |
|           | 30                          | 97.51 93.97 95.74 2.51 2.62 | 94.3          |           |           |       |
|           | 40                          | 83.19 88.60 85.89 3.82 4.45 | 84.6          |           |           |       |
|           | 120                         | 50.96 55.21 53.09 3.00 5.66 | 52.3          |           |           |       |
| 1,000     | 0                           | 1,018.8 1,000.2 1,009.5 13.19 1.31 | 100           |           |           |       |
|           | 20                          | 934.0 1,045.9 990.0 79.13 7.99 | 98.1          |           |           |       |
|           | 30                          | 1,004.4 859.6 932.0 102.39 10.99 | 92.3          |           |           |       |
|           | 40                          | 453.1 549.1 501.1 67.94 13.56 | 49.6          |           |           |       |
|           | 120                         | 374.5 301.6 338.1 51.54 15.25 | 33.5          |           |           |       |

Mean = (1st measurement + 2nd measurement)/2. SD, standard deviation; CV, coefficient of variation.

measured concentration. Accordingly, recovery rates between 90% and 110% indicate nonsignificant changes. The data in Table 2 seem to suggest that the mixing period should not exceed 20 min for assaying Aβ1-42 via IMR.

For Tau, similar results to those for Aβ1-40 and Aβ1-42 were found; the results are listed in Table 3. For the 10-pg/mL Tau PBS samples, nonsignificant changes in measured concentration were detected for the mixing time.
durations of 20 (recovery rate = 95.5%) and 30 (recovery rate = 10,292.5%) minutes. For the 100-pg/mL Tau PBS samples, nonsignificant changes in measured concentration were found for the 20- (recovery rate = 102.0%), 30- (recovery rate = 94.8%), and 40-min (recovery rate = 90.2%) durations. A nonsignificant change in measured concentration for the 1,000-pg/mL Tau PBS samples was observed for only the duration of 20 min (recovery rate = 99.0%). Regardless of Tau concentration, nonsignificant changes in measured concentration were observed for mixing period durations of 20 min and below.

### Discussion

The recovery rate as a function of the duration of the mixing period is plotted in Figure 2a–c for Aβ1–40, Aβ1–42 and Tau. The data for the 10-pg/mL biomarker PBS samples are plotted as dots (●). The rectangular symbols (■) denote the recovery rates for the 100-pg/mL biomarker PBS samples. The recovery rates for the 1,000-pg/mL PBS samples are plotted as triangular symbols (▲).

It is clear from Figure 2a–c that the recovery rates are approximately 100% for short mixing periods and lower at longer time intervals. The range from 90% to 110% recovery rate is marked in Figure 2a–c by the gray areas. The data points within the gray areas correspond to nonsignificant changes in the measured concentration from that obtained for the 0-min mixing period. All the data points for the 20-min period are located within the gray areas. For the 30-min mixing period, the 1,000-pg/mL Aβ1–40, 10-pg/mL Aβ1–42, and 1,000-pg/mL Tau PBS samples show significant changes in measured concentration from that obtained for the 0-min mixing period. For durations longer than 30 min, the recovery rates are lower than 90%, indicating significant reductions in the measured concentrations relative to those for the 0-min mixing period once the mixing period is not shorter than 30 min duration. These results evidence the underestimation of biomarker concentration by IMR once the mixing period becomes too long. The results in Figure 2a–c also suggest that the mixing period duration should be within 20 min to obtain accurate IMR measurements of Aβ1–40, Aβ1–42, and Tau concentrations.

For a given biomarker, two typical differences are apparent in the real-time signals of ac magnetic susceptibility of the reagent-sample mixture between samples of lower biomarker concentration and those of higher concentration, as illustrated in Figure 3a. The samples with

| Biomarker | Spiked concentration, pg/mL | Mixing period, min | Measured concentration | Recovery rate, % |
|-----------|-----------------------------|-------------------|------------------------|-----------------|
|           | 1st measurement, pg/mL      | 2nd measurement, pg/mL | mean, pg/mL | SD, pg/mL | CV, % |
| Tau 10    | 0                           | 10.85             | 9.40                  | 10.13          | 1.03  | 101.7 | 100 |
|           | 20                          | 9.74              | 9.60                  | 9.67           | 0.10  | 1.02  | 95.5 |
|           | 30                          | 9.06              | 9.67                  | 9.36           | 0.43  | 4.57  | 92.5 |
|           | 40                          | 8.29              | 8.36                  | 8.32           | 0.05  | 0.65  | 82.2 |
|           | 120                         | 4.70              | 3.86                  | 4.28           | 0.59  | 13.85 | 42.2 |
| Tau 100   | 0                           | 95.98             | 93.65                 | 94.82          | 1.64  | 1.73  | 100 |
|           | 20                          | 97.25             | 96.10                 | 96.67          | 0.82  | 0.84  | 102.0 |
|           | 30                          | 93.06             | 86.64                 | 89.85          | 4.54  | 5.05  | 94.8 |
|           | 40                          | 88.32             | 82.79                 | 85.55          | 3.91  | 4.57  | 90.2 |
|           | 120                         | 52.08             | 43.83                 | 47.96          | 5.83  | 12.16 | 50.6 |
| Tau 1,000 | 0                           | 1,009.9           | 978.9                 | 994.4          | 21.91 | 2.20  | 100 |
|           | 20                          | 1,012.0           | 956.7                 | 984.3          | 39.11 | 3.97  | 99.0 |
|           | 30                          | 741.1             | 662.3                 | 701.7          | 55.73 | 7.94  | 70.6 |
|           | 40                          | 542.3             | 578.1                 | 560.2          | 25.29 | 4.52  | 56.3 |
|           | 120                         | 170.5             | 216.0                 | 193.3          | 32.19 | 16.66 | 19.4 |

Mean = (1st measurement + 2nd measurement)/2. SD, standard deviation; CV, coefficient of variation.
higher concentrations show higher values of IMR (%). The time at which the ac magnetic susceptibility of mixture starts to descend, marked with arrows in Figure 3a, is earlier for the samples with higher concentrations of biomarker. In samples with higher biomarker concentrations, once the mixing period exceeds 20 min, for example, once it reaches 120 min, most of the data points within the initial interval for obtaining $\chi_{ac,0}$ lie within the descending interval, as shown in Figure 3b, c. Thus, the suppression in IMR (%) is enhanced for higher concentration samples, which results in lower measured concentrations and recovery rates.

Figure 4 plots the recovery rate as a function of biomarker concentration for the 120-min mixing-period duration for $\text{A}\beta_{1–40}$, $\text{A}\beta_{1–42}$, and Tau. The recovery rate tends to be lower at higher biomarker concentrations. This finding implies that IMR-measured concentrations of samples with higher concentrations of biomarker would more readily be underestimated at mixing-period durations longer than 20 min. Inspection of Figure 2a–c reveals that for durations longer than 20 min, the lowest recovery rates tend to correspond to the samples of the highest concentration, i.e., 1,000 pg/mL (▲).

In summary, in practice, IMR assays of biomarkers in biofluid samples are performed with a batch of several vials, wherein the reagent is mixed with the samples sequentially from the 1st to last vial. This period of sequential mixing of reagent and sample is referred to as the mixing period. After the mixing period, the real-time signals of ac magnetic susceptibility of all of the reagent-sample vials are recorded simultaneously; this period is referred to as the recording period. The recording period is divided into several segments. This operation may lead to unreliable estimates of concentration when the mixing period is too long. In the present study, $\text{A}\beta_{1–40}$, $\text{A}\beta_{1–42}$, and Tau, which are associated with AD, were used as biomarkers, and the effect of mixing period duration on the concentrations measured via IMR was investigated. It was found that the mixing period for IMR measurement should not exceed 20 min to avoid underestimating concentration. Thus, the real-time ac magnetic susceptibility of a reagent-sample mixture should be recorded no later than 20 min after mixing the reagent with the sample.

**Fig. 2.** Recovery rate as a function of the mixing period duration for $\text{A}\beta_{1–40}$ (a), $\text{A}\beta_{1–42}$ (b), and Tau (c).
**Fig. 3.** Schematic of real-time signals of ac magnetic susceptibility of reagent-sample mixtures of differing biomarker concentrations, i.e., $\chi_{ac}$-t curves (purple: lower concentration; light purple: higher concentration; a), and a comparison of the $\chi_{ac}$-t curves between the 1st vial and the last vial of the same mixing period duration for samples of lower concentration (b) or higher concentration (c).

**Fig. 4.** Recovery rate versus biomarker concentration for the 120-min mixing period for Aβ1-40 (●), Aβ1-42 (▲), and Tau (■).
Statement of Ethics

There is no human subjects or animals enrolled in this study. Ethical approval is not necessary.

Conflict of Interest Statement

All the authors are employees of MagQu Co. Ltd. S.Y. Yang is a shareholder of MagQu Co. Ltd.

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