Thermal-induced Immuno-nephelometry Using Gold Nanoparticles Conjugated with a Thermoresponsive Polymer for the Detection of Avidin

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Thermoresponsive immunonephelometry was achieved with biotinylated poly(acrylate) and thermoresponsive gold nanocomposites composed of 13-nm gold nanoparticles and thermoresponsive polymers containing triethylenetetramine and biotin groups. The avidin-biotin interaction was used to model an immunoreaction in order to demonstrate thermoresponsive immunonephelometry. In the absence of avidin, positively charged gold nanocomposites electrostatically interacted with biotinylated poly(acrylate) to form binary complexes, in which the charges canceled each other out. The charge cancelation resulted in the binary complexes precipitating when the solution was heated above the phase-transition temperature. However, adding avidin formed ternary sandwich complexes through the avidin-biotin interaction. The ternary complexes remained sufficiently soluble above the phase-transition temperature because of the spatial isolation of the positive and negative charges. The transmittance of the solution containing the thermoresponsive gold nanocomposites and biotinylated poly(acrylate) at 37°C increased as the avidin concentration increased. A sigmoidal profile was observed from 10^{-6.5} to 10^{-5.5} mol/L. The concentration of avidin spiked in bovine serum was determined by our method.

Keywords Thermoresponsive polymer, gold nanoparticles, immunonephelometry, avidin

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Introduction

Immunooassays are simple and highly sensitive, and as such are widely used to analyze toxic compounds, drugs, hormones, and allergenic substances.1,2 Immunoassays can be categorized as homogeneous or heterogeneous. Although homogeneous methods, which do not require physical separation, are rapid and simple, their sensitivity is low, limiting their application for trace analyses. In contrast, heterogeneous methods are highly sensitive, although they require a suitable physical separation, which can result in a laborious, time-consuming protocol. Thermoresponsive polymers are a promising method for overcoming the shortcomings of both homogeneous and heterogeneous immunoassays.3,4 Thermoresponsive polymers containing antibodies have been used as immunoreaction carriers to improve the immunoassay performance. The immunoassay proceeds rapidly between antigens and antibodies bound to the thermoresponsive polymers below the phase-transition temperature, because of the homogeneity. Subsequently, the solution was heated to precipitate the antigen-antibody binary complex, allowing it to be separated from coexisting substances, and enriching the antigen analytes. These systems are a hybrid of homogeneous and heterogeneous methods and can overcome the low sensitivity of homogeneous methods and the time-consuming protocols of heterogeneous methods.

Magnetic beads have been widely investigated for heterogeneous immunoassays.2,4 Nano-sized magnetic particles in immunoassays serve as quasi-homogeneous media to facilitate immunoreactions and to increase the antibody load. However, the particles tend to aggregate easily, which has limited their practical application to bioassays. Adding thermoresponsive polymers can overcome these problems because the polymers disperse the nano-sized magnetic particles below the phase-transition temperature. When a dispersion is heated above the phase-transition, the dispersiveness decreases, resulting in the aggregation of nanoparticles due to the magnetic force. Thus, the conjugation of the thermoresponsive polymer with magnetic nanoparticles allows the conjugates to be controlled by thermal stimuli. Based on this principle, a thermoresponsive immunoassay was developed with thermoresponsive magnetic nanoparticles and polyanions.5 Immunoprobes were fabricated from thermoresponsive magnetic nanoparticles, polyanions, and antibodies. In the absence of the antigen, the thermoresponsive magnetic nanoparticles did not interact with the polyanions, so the magnetic nanoparticles formed aggregates and precipitated. The aggregates could be separated by an external magnetic field when the solution was heated above the phase-transition temperature. However, when the antigen was added, it combined with the magnetic nanoparticles and the polyanions through an immunoreaction to form ternary sandwich complexes. The negative charges in the poly(acrylate) polyanion maintained the
solubility of the ternary complexes above the phase-transition temperature and resisted the external magnetic force to keep the complex dispersed. Because the magnetic nanoparticles were orange, the absorbance of the solution containing the ternary complex above the phase-transition temperature was related to the concentration of the antigens. Thermoresponsive polymers and magnetic nanoparticles were combined, and both components were used as immunocarriers. Several analytes were sequentially separated by external magnetic fields and temperature control, and then analyzed.

Thus, using thermoresponsive polymers in immunocarriers is a promising approach for improving immunoassays. However, thermoresponsive polymers have not been investigated as carriers for immunonephelometry, which is a typical homogeneous method. Latex agglutination assays are commonly used in immunonephelometry, and they output changes in the turbidity of the solution in response to the immunoreaction induced by antigens. The trade-off between assay sensitivity and stability of the latex particles has hampered the improvement of the assay. The ease of latex agglutination favors sensitivity, although it reduces the dispersiveness of the latex during long-term storage, which decreases the reproducibility of the assay. Thermoresponsive polymers are expected to prevent this trade-off. The thermoresponsive polymer produces a homogeneous solution below the phase-transition temperature, whereas they form globules that behave as small insoluble particles above the phase-transition temperature. Although the thermoresponsive polymer globules cause turbidity, the scattering effect is not sufficient to achieve the sensitivity required for practical immunonephelometry.

In this study, thermoresponsive gold conjugates were prepared by conjugation with thermoresponsive polymers and gold nanoparticles (AuNPs), and were examined as a probe for immunonephelometry. The resulting solution of AuNPs was characterized by an absorption maximum at 520 nm. Transmission electron microscopy (JEM-2010, JEOL, Tokyo, Japan) indicated the globule size of the AuNPs. TEM observations revealed that the AuNPs were monodisperse with an average particle size of 13 ± 1.7 nm (100 particles sampled). The avidin–biotin interaction was used as a model immunoreaction to develop thermoresponsive immunonephelometry because it is one of most well characterized bio-reactions and it has been used in many bio-assays. Positively charged thermoresponsive gold nanoconjugates that contain biotin groups formed a ternary complex with anionic biotinylated poly(acrylate) via avidin. Because the positive and negative charges were spatially isolated in the ternary complex, the complex remained soluble above the phase-transition temperature. However, in the absence of avidin, positively charged thermoresponsive gold conjugates interacted with biotinylated polyanions to cancel charges. The resulting binary complex precipitated above the phase-transition temperature because of charge cancelation. The transmittance of the solution, which is a measure of turbidity, was related to the concentration of avidin above the phase-transition temperature. The assay was applied to bovine serum samples spiked with avidin.

Experimental

Reagents and chemicals
Hydrogen tetrachloroaurate(III) tetrahydrate and triethylene-tetramine (TETA) were obtained from Kanto Chemical (Tokyo, Japan), and used without further purification. N-Isopropylacrylamide and avidin were obtained from Wako Pure Chemicals (Osaka, Japan). N-Isopropylacrylamide was recrystallized twice with hexane before use. N-Methacryloyl-\(N^\prime\)-(5-biotinyl-n-propanoyl)-1,3-propanediamine (a biotin monomer) and biotinylated poly(acrylate) sodium salt (a vinylated biotin, \(M_w = 28000\)) were kindly donated by JNC Petrochemical Corporation (Tokyo, Japan). Methanol was distilled before use. All other reagents and solvents were of analytical reagent grade and used without further purification.

Highly purified water (>18 MΩ) was made by an Organo Pure Lab Ultra (Tokyo, Japan) water supply system and used throughout the study. Phosphate-buffered saline (PBS) was prepared by dissolving 0.014 mol/L sodium chloride, 0.003 mol/L potassium chloride, 0.008 mol/L disodium hydrogen phosphate, and 0.002 mol/L potassium dihydrogen phosphate in water.

Preparation of gold nanoparticles
All glassware was thoroughly cleaned in aqua regia (HCl/HNO₃, 3:1), rinsed in deionized and doubly distilled water, and oven-dried prior to use. AuNPs were prepared according to a method reported by Grabar and Sutherland with slight modifications. Briefly, in a 1 L round-bottom flask equipped with a condenser, 1 × 10⁻³ mol/L hydrogen tetrachloroaurate(III) tetrathiate (500 mL) was boiled with vigorous stirring. The addition of 38.8 × 10⁻³ mol/L sodium citrate (50 mL) to the solution immediately changed the solution from pale yellow to dark red. After boiling for 10 min, the heating mantle was removed, and stirring was continued for 15 min. After the solution reached room temperature, it was filtered through a cellulose acetate membrane filter with a pore size of 0.4 μm. The resulting solution of AuNPs was characterized by an absorption maximum at 520 nm. Transmission electron microscopy (JEM-2010, JEOL, Tokyo, Japan) indicated the particles were monodisperse with an average particle size of 13 ± 1.7 nm (100 particles sampled).

Preparation of thermoresponsive copolymers
A biotinylated poly(N-isopropylacrylamide) terpolymer was synthesized by a radical polymerization according to a previous report with slight modifications. Prior to the radical polymerization, the N-acryloyl TETA precursor was synthesized from acryloyl chloride and TETA. Acryloyl chloride (1.63 mL, 0.01 mol) in 1,4-dioxane (25 mL) was added to 1,4-dioxane (100 mL) containing TETA (14.6 g, 0.1 mol). The white precipitate was filtered and then suspended in methanol (100 mL) containing potassium hydroxide (0.59 g, 0.01 mol). The precipitated potassium chloride was filtered off, and the filtrate, which contained N-acryloyl TETA, was copolymerized immediately without purification.

The methanol solution of N-acryloyl TETA was transferred into a 500 mL round-bottom separable flask equipped with a condenser, N-isopropylacrylamide (10.2 g, 0.09 mol), vinylated biotin (N-methacyrloyl-\(N^\prime\)-(5-biotinyl-n-propanoyl)-1,3-propanediamine) (0.0184 g, 5 × 10⁻³ mol/L), 3-mercaptopropionic acid (0.5 mL), and azobisisobutyronitrile (0.82 g) were added to the flask. The mixture was kept at 60°C under nitrogen. After cooling, the solution was poured into an equal volume of cooled diethyl ether. The crude copolymer precipitate was recrystallized with methanol and diethyl ether. The purified precipitate was then dissolved in water (100 mL) and the polymer solution was dialyzed using an ultrafiltration membrane tube (>5000 Da) for 7 days with water that was changed daily. The resulting polymer
solution was freeze-dried. Elemental analysis was performed to confirm the introduction of biotin and to estimate the molar content of TETA groups in the polymer. A 20 g/L polymer solution was prepared and stored at 4°C in the dark for at least 1 week before use.

**Preparation of thermoresponsive gold nanocomposites**

Thermoresponsive gold nanocomposites were prepared by conjugating AuNPs with thermoresponsive polymers. Briefly, the gold nanoparticle solution (6.25 mL) and the 20 g/L polymer solution (15 mL) were placed in a centrifugal tube and shaken gently by hand. The mixture was heated at 90°C for 30 min in a water bath, and was allowed to cool at room temperature. This thermal treatment was repeated three times. The resulting solution was dialyzed with an ultrafiltration membrane (>10000 Da) with water.

**Protocol of avidin assay**

Avidin was detected by using the following protocol. To a semi-micro cuvette (2 mL, 1 cm path length), 0.3 mL of the gold nanocomposite solution was taken; then, 0.1 mL of a sample solution containing avidin was transferred into the cuvette. After 0.1 mL of a PBS solution was added, the resulting solution was mixed by gently shaking the cuvette. Then, the cuvette was placed in a spectrophotometer (UV-VIS 650, JASCO) equipped with a temperature-control unit. When the solution in the cuvette reached the target temperature, the transmittance of the solution was measured at 850 nm, where none of the constituents in the solution exhibited any absorption.

**Results and Discussion**

**Principle of thermoresponsive immunonephelometry**

Figure 1 shows a schematic of thermoresponsive immunonephelometry, where the avidin-biotin interaction is used as a model immunoreaction to assay avidin as an analyte. Two components containing biotin groups function as probes in the system. The first is the cationic thermoresponsive gold nanocomposites prepared by the conjugation of 13 nm AuNPs with the thermoresponsive polymer containing biotin and TETA groups, which introduced positive charges to the nanocomposites. The second is biotinylated poly(acrylate), which has a negative charge because of the dissociation of carboxylate. Avidin reacts with the gold nanocomposites and poly(acrylate) to form ternary sandwich complexes through the avidin-biotin interaction. The avidin-biotin interaction spatially isolates the positively charged nanocomposites and negatively charged poly(acrylate) in the ternary complexes and maintains their solubility. Owing to the solubility, the ternary complexes do not precipitate when heated above the phase-transition temperature. However, in the absence of avidin, the cationic thermoresponsive gold nanocomposites interact with anionic biotinylated poly(acrylate) to form uncharged binary complexes, in which the positive and negative charges cancel. Above the phase-transition temperature, the binary complex precipitates and increases the turbidity of the solution due to the lack of charge. Thus, the turbidity or transmittance of a solution above the phase-transition temperature is related to the concentration of avidin in the solution.

The AuNPs in the gold nanocomposites play an important role in the sensitivity of the assay. AuNPs serve as colloidal cores in the ternary complexes and increase light scattering. The effect of the solution temperature on the transmittance of a solution containing thermoresponsive gold nanocomposites and biotinylated poly(acrylate) was investigated at various avidin concentrations because the solution temperature is important for controlling the phase-transition of thermoresponsive gold nanocomposites. The profile in Fig. 2(a) shows the phase-transition behavior of the gold nanocomposites, which had an
identical phase-transition temperature to poly(acrylamide-co-TETA) at any avidin concentration. These profiles indicate that the formation of ternary complexes had little macroscopic effect on the phase-transition properties of the thermoresponsive polymers tethered to AuNPs. As the avidin concentration increased, the transmittance of the solution increased above the phase-transition temperature (Fig. 2(a)). A typical relationship between the transmittance of the solution at 35°C and the avidin concentration is plotted in Fig. 2(b). Sigmoidal profiles were observed at any temperature above the phase-transition temperature. The experiment at 35°C gave the most sensitive profile, where a substantial change in the transmittance was observed at between 10⁻⁷ and 10⁻⁶ mol/L of avidin.

The schematic shown in Fig. 1 was confirmed by dynamic light scattering (DLS) measurements. Table 1 summarizes the hydrodynamic radii of the thermoresponsive gold nanocomposites and their complexes below (30°C) and above (40°C) the phase-transition temperature. The instrumental parameters of DLS were tuned to measure AuNPs, so the values given in Table 1 should be considered as relative values because the polymer moieties surrounding the AuNPs were not estimated exactly under the conditions. The results for the thermoresponsive gold nanocomposites in the presence and absence of avidin given in Table 1 show that the radii of the nanocomposites increase substantially as a result of the phase-transition owing to their aggregation. The addition of avidin suppressed aggregation because it interacted with the nanocomposites to form binary complexes that were soluble even above the phase-transition temperature. In the absence of avidin, the electrostatic interaction between the cationic gold nanocomposites and anionic poly(acrylate) forms binary complexes and leads to partial aggregation that increases the hydrodynamic radii below the phase-transition temperature. However, avidin repelled the gold nanocomposites from poly(acrylate) in the ternary complex and maintained the solubility, as shown in Fig. 1.

The working range of the present thermoresponsive immunonephelometry is affected by the chemical structure of the thermoresponsive gold nanocomposites, the concentration of biotinylated polyanions and PBS, and the order of addition of the reagents. These factors are discussed in the following section.

**Preparation of thermoresponsive nanocomposites**

The structures of the thermoresponsive gold nanocomposites were affected by protocols for the preparation of the nanocomposites. We conjugated 13 nm AuNPs with N-isopropylacrylamide terpolymers that contained TETA groups and biotin groups by thermal redispersion. Briefly, when a solution of N-isopropylacrylamide copolymers containing TETA was mixed with a solution of 13 nm AuNPs, the color of the mixture changed from red to blue-purple. This indicated the aggregation of AuNPs due to the interaction between the AuNPs and the TETA groups on the thermoresponsive polymers. Heating the mixture above the phase-transition temperature shrunk the thermoresponsive polymers tethered to the AuNPs, and expanded the distances between the AuNPs, with the polymers acting as if they were wedges. After the solution was cooled below the phase-transition temperature, the AuNPs were redispersed and the solution turned red. The thermal treatment created individually dispersed AuNPs coated with thermoresponsive polymers, which made the nanoparticles very soluble and kept them dispersed even in saline.

![Fig. 2](image)

**Table 1** Hydrodynamic diameters of thermoresponsive gold nanocomposites and their complexes

<table>
<thead>
<tr>
<th>Temperature/°C</th>
<th>diameter/nm (Average ± deviation)a</th>
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<tbody>
<tr>
<td>Au composites</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>124.4 ± 1.9</td>
</tr>
<tr>
<td>40</td>
<td>1946 ± 80</td>
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<tr>
<td>Au composites + Avidin</td>
<td></td>
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<tr>
<td>30</td>
<td>120 ± 2.6</td>
</tr>
<tr>
<td>40</td>
<td>237 ± 52</td>
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<tr>
<td>Au composites + poly(acrylate)</td>
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</tr>
<tr>
<td>30</td>
<td>1517 ± 42</td>
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<tr>
<td>40</td>
<td>2610 ± 253</td>
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<tr>
<td>Au composites + Avidin + poly(acrylate)</td>
<td></td>
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<tr>
<td>30</td>
<td>1113 ± 4.3</td>
</tr>
<tr>
<td>40</td>
<td>136.1 ± 2.1</td>
</tr>
</tbody>
</table>

a. Three replicate measurements.
We examined another way to prepare thermoresponsive gold nanocomposites. First, the N-isopropylacrylamide copolymer containing TETA groups was conjugated and then thermally redispersed. Subsequently, biotin groups were introduced to the nanocomposites through peptide condensation. However, the gold nanocomposites fabricated by this method were less sensitive than the nanocomposites composed of the terpolymers and AuNPs.

The effect of the gold nanoparticle size was investigated with 13 and 30 nm AuNPs. The responses of the conjugates to avidin concentration were similar, suggesting that the size of AuNPs did not affect the sensitivity. Therefore, 13 nm AuNPs were used to prepare the nanocomposites because they were easy to prepare and had a high monodispersity.

Effect of the concentration of biotinylated poly(acrylate) and PBS

The concentration of biotinylated poly(acrylate) is an important parameter for the response of the system because the polyanions introduce anionic charges in the binary complexes without avidin and the ternary complexes with avidin. Figure 3 shows the relationship between the transmittance and temperature as a function of the concentration of poly(acrylate) in a solution containing thermoresponsive gold nanocomposites. In the absence of poly(acrylate), the transmittance decreased slightly when the solution was heated above the phase-transition temperature because the solubility of avidin suppressed a decrease of whole solubility of the binary complexes, even though the nanocomposites in the complex become less hydrophilic. The addition of more than 0.2 μmol/L of biotinylated poly(acrylate) also suppressed the decrease in the transmittance because the excess of anionic charges in the ternary complex increased its solubility. The response of avidin is good over a biotinylated poly(acrylate) concentration range of 0.025 – 0.0125 μmol/L because of the cancelation of charges in the ternary complexes.

The concentration of PBS, which controls the ionic intensity of the solution, also affected the sensitivity of the assay. Increasing the PBS concentration decreased the transmittance of the solution in both the absence and presence of avidin above 35 °C.

**Fig. 3** (a) Effect of the temperature and concentration of poly(acrylate) on the transmittance of a solution containing 0.0162 g/L Au, 0.0625 wt% poly(N-isopropylacrylamide-TETA), and 7.0 μmol/L avidin. The measurement wavelength was 850 nm. The broken line denotes the transmittance at 35 °C for (b). (b) Transmittance at 35 °C vs. the logarithm of avidin concentration. Data were taken from (a).

**Fig. 4** Effect of the temperature and concentration of the PBS on the transmittance of a solution containing 0.0162 g/L Au, 0.0625 wt% poly(N-isopropylacrylamide-TETA), 0.40 μmol/L poly(acrylate) in (a) the presence of 7.0 μmol/L avidin and (b) the absence of avidin. The standard PBS concentration is given in the experimental section.
the phase-transition temperature (Fig. 4). The decrease in the transmittance is explained by salting out of the thermoresponsive polymers.\textsuperscript{17–20} Maximizing the difference in transmittance between the absence and presence of avidin improves the sensitivity of the assay. We chose the standard concentration of PBS for the following experiments because it produced the maximum difference in transmittance. Half of the standard concentration of PBS produced insufficient precipitation of the binary complex, consisting of gold nanocomposites and poly(acrylate). However, in the presence of 7.4 μmol/L avidin, PBS above the standard concentration caused precipitation of the ternary complex containing avidin.

PBS is also important because of its pH buffering. The solution pH controls the protonation of TETA groups in the polymers and deprotonation of the carboxyl groups in polyanions. Under acidic conditions, the TETA groups are protonated and the deprotonation of the carboxyl groups is suppressed, whereas the opposite is true under basic conditions. Because of their potential use in biological applications, the pH controlled by PBS was used, and the effects of the pH were not investigated further.

Reagent order of addition

The schematic shown in Fig. 1 suggests that the structures of the ternary complexes of gold nanocomposites, avidin, and poly(acrylate) should be affected by the order of addition of the reagents. Therefore, the order of addition should affect the response to the avidin concentration. Figure 5, which compares the orders of addition of the reagents, indicates that the addition of avidin before poly(acrylate) gave a superior response to the opposite order. When avidin was added to the solution of the gold nanocomposites before poly(acrylate), binary complexes of the nanocomposites and avidin formed. This favored the spatial isolation of positive and negative charges in the ternary complexes resulting from the addition of poly(acrylate). In contrast, adding poly(acrylate) before avidin produced binary complexes of cationic gold nanocomposites and anionic poly(acrylate). The subsequent addition of avidin did not isolate the charges in the binary complexes. Thus, this order of addition produced an insufficient difference in solubility between the presence and absence of avidin, leading to the inferior response to avidin concentration.

Analytical performace and application

The practical application of the assay was tested with bovine serum samples. Figure 6 shows the relationship between the avidin concentration and the transmittance in standard PBS solutions with and without bovine serum. Although both profiles have a similar sigmoidal shape, the curve obtained with the bovine serum sample was shifted toward higher avidin concentration compared with the standard PBS solution. In addition, the transmittance of the solution with the bovine serum exhibited a significant deviation near the inflection point. The bovine serum probably caused shifts of the profiles and the deviation. Therefore, recovery tests with 1.0 mol/L avidin were also conducted with the bovine serum sample after a deproteinizing treatment. The recoveries showed high reproducibility and repeatability, although the average recovery values were overestimated (Table 2). This overestimation comes from a steep curve near an inflection point in the sigmoidal profile. Thus, the recovery tests demonstrate that the
assay could be used for the threshold detection or semi-quantification of avidin in bovine serums, and suggest that thermoresponsive gold nanocomposites provide a practical platform for thermoresponsive immunonephelometry.

Conclusions

The development of thermoresponsive immunonephelometry relying on the avidin-biotin interaction was demonstrated using thermoresponsive gold nanocomposites and biotinylated poly(acrylate). The thermoresponsive gold nanocomposites were prepared by conjugating 13 nm AuNPs with thermoresponsive polymers containing TETA and biotin groups. Avidin bound to the gold nanocomposites and poly(acrylate) and formed ternary complexes, in which positive charges on the polymers and negative charges on the polyanions were isolated. The isolated charges maintained the solubility of the ternary complexes above the phase-transition temperature. The transmittance of a solution increased as the concentration of avidin increased. The sigmoidal response of the transmittance was in the range of $10^{-6.5}$ to $10^{-5.5}$ mol/L.

References


<table>
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<th>Sample No.</th>
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<th>II</th>
<th>III</th>
<th>IV</th>
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<td>Transmittance (Average ± deviation)</td>
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<td>86.5 ± 0.6</td>
<td>87.8 ± 2.8</td>
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<tr>
<td>Recoverya</td>
<td>127</td>
<td>127</td>
<td>129</td>
<td>125</td>
</tr>
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Average recovery with deviation 127.0 ± 1.1

a. Relationship depicted in Fig. 6 was used as a calibration curve.