

Sensitive NMR Sensors Detect Antibodies to Influenza**

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Point of care (POC) sensors for the determination of immune statuses are an important part of the preparation for possible pandemics such as that created by avian influenza.^[1,2] NMR-based magnetic-relaxation switches (MRSw) are attractive for this application because they are indifferent to light and involve no immobilization of materials on vessel walls.^[3] MR relaxometers are practical as POC sensors because their requirements for magnetic-field strength, volume, and homogeneity are minimal.^[4–6]

To date, MRSws have used magnetic nanoparticles (NPs) that react with molecular targets to form aggregates and decrease the transverse relaxation time (T_2). Although MRSws detect highly multivalent viruses or bacteria with high sensitivity,^[7,8] their sensitivity for proteins is far lower.^[9,10] Our goal was to improve the sensitivity of MRSw sensors for divalent antibodies so that the immune status of birds or humans might eventually be determined by using a POC MR relaxometer. A monoclonal antibody recognizing the Tag peptide from a hemagglutinin of a human influenza virus was used as a target to assess strategies for improving MRSw sensitivity.

An obvious strategy was to employ the equivalence principle of antibody–antigen reactions involved in precipitin formation,^[11–13] by reducing the concentration of Tag peptide–magnetic particles (a synthetic multivalent antigen) so that lower concentrations of anti-Tag antibodies might achieve aggregation. The Tag peptide from the influenza hemagglutinin was therefore conjugated to nanoparticles (NPs) and to far larger micrometer-sized magnetic particles (MPs) to yield Tag–NPs and Tag–MPs. As indicated in Table 1, a Tag–MP contained 350 000 times more iron than a Tag–NP and had a correspondingly higher magnetic moment per particle. With a typical initial T_2 value for MRSw assays (100 ms), the concentration of particles decreased from 2.8×10^{-9} M with Tag–NPs to 5.1×10^{-15} M by using Tag–MPs.

Attempts to use Tag–MPs for MRSw assays indicated that Tag–MPs differ from the earlier Tag–NPs in two key respects. First, as depicted in Figure 1, Tag–MPs undergo a reversible increase in the T_2 value in the 0.47 T relaxometer magnet. Micrographs indicated that the T_2 increase was associated

Table 1: Properties of magnetic particles (MPs) and nanoparticles (NPs).

	MP	NP
size [nm]	1000	30
settling	< 5 %	none
peptides per particle	3.0×10^5	20–30
R_2 [s^{-1} mM Fe]	43	50
M [emu g^{-1} Fe]	105	86.6
Fe atoms per particle	2.8×10^9	8000 ^[a]
particle concentration at $T_2 = 100$ ms [M]	5.1×10^{-15}	2.8×10^{-9}

[a] Taken from reference [22].

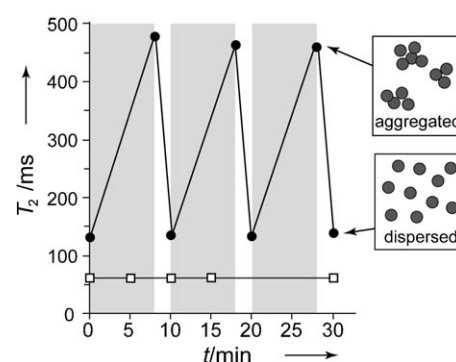


Figure 1. Magnetic particles respond to a homogeneous magnetic field. The T_2 value of an MP solution (●), but not of an NP solution (□), increases when in a 0.47 T field (gray). White areas indicate periods when no field was applied.

with MP aggregation resulting from magnetic attraction between the Tag–MPs (see Figure 2 of reference [14]), with the randomizing effects of thermal energy causing dispersion when the field was removed. Second, Tag–MP aggregation results in a T_2 increase whereas Tag–NP aggregation resulted in a T_2 decrease,^[3,15] observations consistent with the outer-sphere-diffusion theory used to describe the effects of magnetic particles on the T_2 value.^[16–19] This theory employs two parameters, τ_d , the diffusion time for water, and $\Delta\omega$, the difference in angular frequency between the magnetic field experienced by a proton at the particle (or aggregate) surface and that experienced by a proton in the bulk. Outer-sphere-diffusion theory predicts that the T_2 value will decrease as Tag–NPs aggregate, since the motional averaging condition is fulfilled with both dispersed and aggregated materials ($\Delta\omega \times \tau_d < 1$). With Tag–MP aggregation, the resulting magnetic-field inhomogeneities become so few and infrequent that water molecules must traverse long distances to encounter them. Here, the effects on the T_2 value become diffusion-limited ($\Delta\omega \times \tau_d > 1$). The effects of NP and MP aggregation on the T_2 value have been discussed elsewhere.^[14]

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Three techniques were then explored separately and in combination to increase the sensitivity of MRSw assays (Figure 2). First, a decrease in particle concentration was achieved by replacing the magnetic nanoparticle (NP) with a

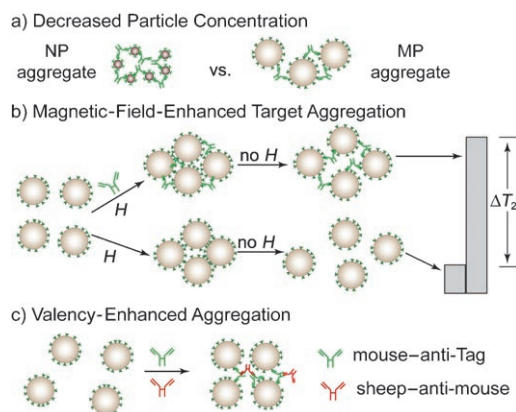


Figure 2. Methods of increasing MRSw sensitivity. a) Decrease in particle concentration. Anti-Tag antibodies form bridges between larger MPs. b) Magnetic-field-enhanced target aggregation. A magnetic field (H) produces magnetic attractions between MPs that result in aggregation. In the presence of anti-Tag antibodies (top), the aggregate is maintained when the field is removed (no H) and a high T_2 value results. c) Valency-enhanced aggregation. The addition of an anti-Fc antibody permits the anti-Tag antibodies to bind more than two MPs simultaneously.

micrometer-sized particle (MP) (Figure 2a). The second technique, magnetic-field-enhanced molecular-target aggregation, exploited the reversible magnetic-field-induced MP aggregation observed in the absence of a target ligand (Figure 1). We hypothesized that the magnetic-field aggregation of Tag-MPs might enhance anti-Tag-mediated aggregation (Figure 2b). Therefore, solutions of Tag-MPs were exposed to anti-Tag antibodies in the relaxometer magnet, which resulted in both magnetic-field-induced and anti-Tag-mediated Tag-MP aggregation. Samples were then removed from the magnetic field, to allow the Tag-MPs to disaggregate; this occurred only in the absence of particle crosslinking by the anti-Tag antibodies. The T_2 value was determined by placing samples in the relaxometer for less than 30 s so that magnetic-field-induced aggregation during T_2 measurement was minimal. Finally, we employed valency enhancement, where the valency of the monoclonal anti-Tag antibodies was increased above the normal two antigen-combining sites per immunoglobulin G (IgG) by adding a sheep F(ab')₂ antibody to the Fc fragment of the mouse anti-Tag monoclonal antibody (Figure 2c).

We first measured the T_2 value as a function of anti-Tag concentration by using Tag-NPs and Tag-MPs (that is, by decreasing particle concentration but without magnetic-field aggregation or valency enhancement; Figure 3a). Data were fitted using a four-parameter equation to obtain the EC_{50} value (midpoint), the Hill coefficient (curve slope), and the computer-generated maximum and minimum T_2 values. Table 2 provides EC_{50} values, Hill coefficients, and ΔT_2 values for the curves shown in Figure 3a. The value of ΔT_2 ,

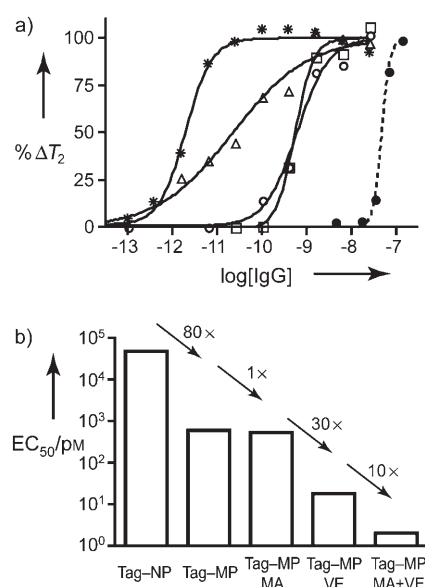


Figure 3. Results of the techniques for increasing the response to anti-Tag antibodies. a) The initial reference assay with Tag-NPs is indicated by a dotted line and $\bullet$. To obtain a decrease in particle concentration, Tag-MPs ($\circ$) were used. The Tag-MPs were then used with magnetic-field aggregation ($\square$), with valency enhancement ($\triangle$), and with both magnetic aggregation and valency enhancement ($*$). $[IgG]$ indicates the molar concentration of IgG. b) EC_{50} values obtained by decreasing particle concentration, magnetic-field aggregation (MA), and valency enhancement (VE). Additional curve parameters are provided in Table 2.

Table 2: Sensitivity of NP- and MP-based MRSw sensors.

	with magnetic aggregation	with valency enhancement	EC_{50} [nM]	Hill slope	ΔT_2 [ms]	PSC [nM]
Tag-NP	no	no	49	4.7	+110	26
Tag-MP	no	no	0.60	1.4	-20	0.41
Tag-MP	yes	no	0.55	2.1	-140	0.060
Tag-MP	no	yes	0.017	0.55	-64	0.00020
Tag-MP	yes	yes	0.0020	1.5	-250	0.00014

the T_2 value for the fully dispersed state minus the T_2 value for the fully aggregated state, is positive for anti-Tag-induced Tag-NP aggregation but negative for anti-Tag-induced Tag-MP aggregation. The $\% \Delta T_2$ value is the difference in the T_2 value in the presence and absence of anti-Tag antibodies divided by the ΔT_2 value and expressed in percent.

With Tag-NPs, increased concentrations of anti-Tag antibodies gave an EC_{50} value of 49 nM, versus an EC_{50} value of 0.60 nM for Tag-MPs. Further reductions in the EC_{50} values of the MP-based MRSw assays were obtained by the use of magnetic-field aggregation and valency enhancement, as shown in Figure 3b. Through a combination of all three techniques, the EC_{50} values for anti-Tag antibodies were

progressively decreased from a starting value of 49 nm with Tag-NPs to 0.0020 nm (2.0 pm) with decreased particle concentration, magnetic-field aggregation, and valency enhancement (Figure 3b).

To obtain a detection limit from these results, we determined a “projected sensitivity concentration” (PSC). Five milliseconds were added to (or subtracted from) the value of T_2 in the absence of anti-Tag antibodies and the concentration at this T_2 value was determined from the curve parameters (Table 2). The PSC value for anti-Tag antibodies with the Tag-NP sensor, the starting point of our investigation, was 26 nm. By using our three techniques for enhancing sensitivity, the PSC value was reduced to 0.00014 nm, or by 186000-fold. The PSC value is discussed further in the Supporting Information. This detection sensitivity is comparable with those of the most-advanced nanostructure-based antibody assays.^[20]

We obtained a high-sensitivity MRSw sensor for influenza antibodies by using MPs and exploiting a previously unrecognized feature of their response to a magnetic field. Application of a homogeneous magnetic field enhanced the antibody-based crosslinking between particles and provided a novel method of increasing the sensitivity of a homogeneous particle-aggregation-based immunoassay. Together with high-throughput methods of peptide and peptide-MP conjugate synthesis,^[21] MRSw sensors might be used both to analyze the immune response to mutating viruses in laboratory settings and for an MRSw-based POC antibody sensor.

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