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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 350000 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 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]

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
$R2$ [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].

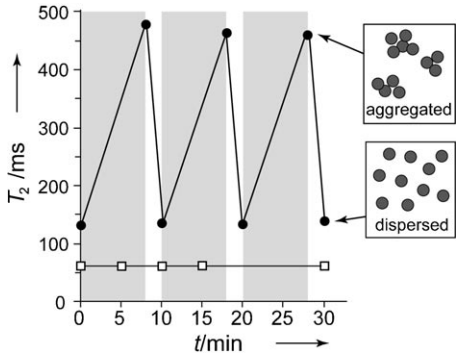

Figure 1. Magnetic particles respond to a homogeneous magnetic field. The T_2 value of an MP solution (●), but not of an NP solution (□), increases when in a 0.47 T field (gray). White areas indicate periods when no field was applied.

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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 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).

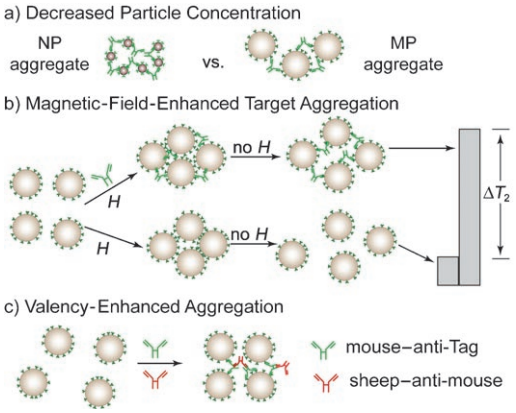

Figure 2. Methods of increasing MRSw sensitivity. a) Decrease in particle concentration. Anti-Tag antibodies form bridges between larger MPs. b) Magnetic-field-enhanced target aggregation. A magnetic field (*H*) produces magnetic attractions between MPs that result in aggregation. In the presence of 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.

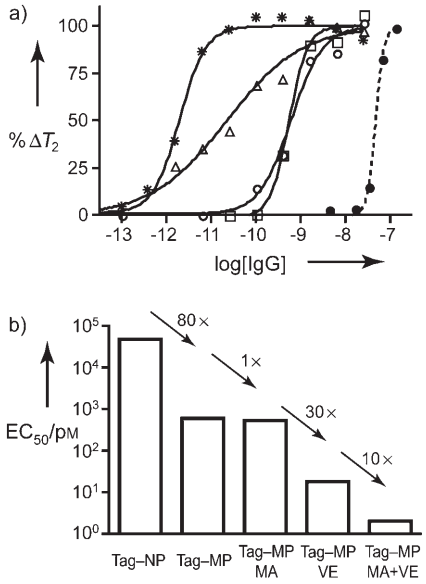

Figure 3. Results of the techniques for increasing the response to anti-Tag antibodies. a) The initial reference assay with Tag–NPs is indicated by a dotted line and ●. To obtain a decrease in particle concentration, Tag–MPs (○) were used. The Tag–MPs were then used with magnetic-field aggregation (□), with valency enhancement (△), 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

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, 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 % ΔT_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

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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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