One-step, Wash-free, Nanoparticle Clustering-based Magnetic Particle Spectroscopy (MPS) Bioassay Method for Detection of SARS-CoV-2 Spike and Nucleocapsid Proteins in Liquid Phase

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S1. Static magnetic hysteresis loops and magnetic properties of IPG30 MNPs.

The static magnetic hysteresis loops are collected from air-dried 10 μL IPG30 MNP and the magnetizations are calculated based on its weight concentration. As shown in Figure S1, magnetic fields are swept from -5000 Oe to +5000 Oe for Figure S1(a), and from -500 Oe to +500 Oe for Figure S1(b). The IPG30 MNPs show a saturation magnetization of 30.04 emu/g and specific magnetization of 22.3 emu/g at 500 Oe. The magnetic coercivity is around 15 Oe. Due to the surface coating layer of protein G, the inter-particle distance is increased thus, this small coercivity field does not cause the clustering or sedimentation of MNPs.

Figure S1. Static magnetic hysteresis loops of air-dried IPG30 MNPs measured by a PPMS. The field range is (a) -5000 – 5000 Oe and (b) -500 – 500 Oe, respectively.
S2. The minimum detectable amount of IPG30 by homebuilt MPS system.

The minimum detectable amount of IPG30 MNPs by our homebuilt MPS system is firstly investigated by two-fold dilutions of IPG30. There are a total of 13 vials prepared each contains 80 μL IPG30 of varying degrees of dilutions, and vial #13 is a blank sample for control purpose, as shown in Table S1. Three independent MPS readings are taken on each sample and the higher harmonics (the 3rd, 5th, 7th, and 9th) are summarized in Figure S2. Overall, the harmonic signal amplitudes decrease linearly as we decrease the amount of IPG30 MNPs in the vial. The figure insets zoom in the harmonic amplitudes of vials that contain less than 1 μg of IPG30 MNPs. All of the 3rd, the 5th, and the 7th harmonics show the detection limit of IPG30 MNPs is at 265 ng (vial #10, 512-fold dilution), which equals to 9 fmole of IPG30 nanoparticles. In order to cut down the cost per assay and make MPS an inexpensive platform for high volume bioassays in the future, we explored the feasibility of using 20-fold diluted IPG30 for detection of SARS-CoV-2 nucleocapsid and spike proteins in this paper.

Table S1. IPG30 Samples Prepared by Two-fold Dilutions.

| Vial Index | IPG30 Concentration (mg/mL) | IPG30 Amount (μg) | Dilution (fold) |
|------------|-----------------------------|-------------------|-----------------|
| 1          | 1.7                         | 136               | 1               |
| 2          | 0.85                        | 68                | 2               |
| 3          | 0.425                       | 34                | 4               |
| 4          | 0.2125                      | 17                | 8               |
| 5          | 0.10625                     | 8.5               | 16              |
| 6          | 0.053125                    | 4.25              | 32              |
| 7          | 0.0265625                   | 2.125             | 64              |
| 8          | 0.01328125                  | 1.0625            | 128             |
| 9          | 0.006640625                 | 0.53125           | 256             |
| 10         | 0.003320313                 | 0.265625          | 512             |
| 11         | 0.001660156                 | 0.1328125         | 1024            |
| 12         | 0.000830078                 | 0.06640625        | 2048            |
| 13         | 0                           | 0                 | NA              |
Figure S2. The higher harmonic amplitudes collected from vials 1-13 with varying amount of IPG30 MNPs. Error bars represent standard errors.
S3. Hydrodynamic size, zeta potential, the 3rd harmonic amplitudes of bare MNPs and MNPs functionalized with different amount of pAbs.

To confirm the successful functionalization of different numbers of pAb on each MNP, the hydrodynamic sizes of MNPs are characterized by Dynamic Light Scatter (DLS, Microtrac NanoFlex). As shown in Figure S3, the bare MNPs show smallest averaged hydrodynamic size of 41.4 nm, followed by MNP:pAb=1:1 and MNP:pAb=1:2. With MNP:pAb=1:3 and MNP:pAb=1:4 showing largest hydrodynamic sizes.

The functionalization of different amount of pAbs will inevitably hinder the Brownian relaxation of MNPs, thus, with more pAbs functionalized on each MNP, the weaker magnetic responses (reflected in the MPS harmonic amplitudes) will be observed. As summarized in Table S2, the 3rd harmonic amplitudes of vials #9 (bare MNP), #IV-8 (MNP:pAb=1:4), #III-8 (MNP:pAb=1:3), #II-8 (MNP:pAb=1:2), and #I-8 (MNP:pAb=1:1) are 22.76 mV, 21.33 mV, 20.58 mV, 19.95 mV, and 18.18 mV, respectively.

![Figure S3](image.png)

**Figure S3.** The hydrodynamic size distributions of bare MNPs, MNPs each functionalized with one, two, three, and four pAbs.

The colloidal stability of bare MNPs is compared with MNPs functionalized with pAbs. It is confirmed that the MNP suspensions show a neutral to slightly alkaline pH of 7.0 - 7.1. As shown in Table S2, with the functionalization of pAbs, the zeta potential barely changes. Thus, functionalizing pAbs to MNPs won’t affect the stability of MNPs.
Table S2. Average hydrodynamic sizes, zeta potentials, and 3\textsuperscript{rd} harmonic amplitudes of bare MNPs and MNPs functionalized with different amount of pAbs.

| Sample          | Average Hydrodynamic Size (nm) | Zeta Potential (mV) | The Averaged 3\textsuperscript{rd} Harmonic Amplitude (mV) |
|-----------------|--------------------------------|---------------------|-------------------------------------------------------------|
| Bare MNP        | 41.4                           | -0.34               | 22.76                                                       |
| MNP:pAb=1:1     | 44                             | -0.33               | 21.33                                                       |
| MNP:pAb=1:2     | 44.5                           | -0.33               | 20.58                                                       |
| MNP:pAb=1:3     | 46.4                           | -0.34               | 19.95                                                       |
| MNP:pAb=1:4     | 47.8                           | -0.34               | 18.18                                                       |
S4. Grayscale heatmaps of higher harmonic signal drop $\Delta$ (in %) compared to bare MNPs for SARS-CoV-2 spike proteins.

The 3rd, 5th, 7th, 9th, 11th, 13th, and 15th harmonics of each sample is compared with the corresponding harmonics from bare MNP sample (vial #9) and the grayscale heatmaps of harmonic signal drop (defined as $\Delta=\frac{A_{i9}-A_{X-j}}{A_{i9}} \times 100\%$, where $i$ is the harmonic index, the subscripts are sample indexes, X=I, II, III, and IV, $j=1, 2, 3, \ldots, 8$) are plotted. In each row of Figure S4(a – g), by adding the same amount of spike protein molecules, the harmonic signal drop $\Delta$ decreases from I to IV, which agrees with the results in Figure 4(e – i). Thus, ideally, the color becomes darker from left column to right column in each grayscale heatmap (as schemed in Figure S4(h)). In each column of Figure S4(a – g), with IPG30 MNPs surface functionalized with an identical number of pAbs, adding more spike protein causes larger harmonic signal drop. Thus, ideally, the color becomes darker from the bottom row (vials #X-1, X=I, II, III, and IV) to the top row (vials #X-8, X=I, II, III, and IV). Again, this ideal trend is schemed in Figure S4(h).

Figure S4. Grayscale heatmaps of higher harmonic signal drop $\Delta$ (in %) compared to bare MNPs for SARS-CoV-2 spike proteins. (a) – (g) are the 3rd, 5th, 7th, 9th, 11th, 13th, and 15th harmonic signal drop from 32 samples in Group I – IV compared to bare MNPs, respectively. (a1 - a4) are the harmonic signal drop, $\Delta$, plotted as a function
of spike protein amount/concentration for groups I – IV, respectively. (h) is the grayscale heatmap showing the ideal color trend regarding different amount of pAbs functionalized on MNPs as well as different scenarios of extra and insufficient target analytes (i.e., SARS-CoV-2 spike protein).

The 3\textsuperscript{rd} harmonics give highest signal to noise ratio (SNR), and we can clearly see this trend from Figure S4(a). Figure S4(a1 – a4) plots the 3\textsuperscript{rd} harmonic signal drop \( \Delta \) curves for samples from Groups I – IV, where Group II shows the best monotonic concentration-response curve. Figure S4(b: 1 – 3) and Figure 6(c: 1 – 3) plot the 5\textsuperscript{th} and the 7\textsuperscript{th} harmonic signal drop \( \Delta \) curves for samples added with same amount of spike protein molecules, across Groups I – IV. Figure S4(h) schematically draws the scenarios where extra and insufficient target analytes (i.e., spike protein) are added. As a result, the number of target analytes directly affects the degree of nanoparticle clustering as well as the dynamic magnetic responses.
Figure S5. Grayscale heatmaps of higher harmonic signal drop $\Delta$ (in %) compared to bare MNPs for SARS-CoV-2 nucleocapsid proteins.
S6. The ratios of higher harmonics to the 3rd harmonics recorded from groups I to IV for SARS-CoV-2 nucleocapsid protein.

The ratios of higher harmonics (from the 5th to the 15th harmonics) to the 3rd harmonics collected from each sample for SARS-CoV-2 nucleocapsid protein detection. As shown in Figure S6, for Group I, the harmonic ratio curves from samples #I-1 to #I-8 are sparsely distributed. The significant differences in harmonic ratio curves from vials added with different concentration/amount of nucleocapsid protein molecules allow us to analyze and collect meaningful concentration-response profiles as shown in Figure 7(a). However, for groups II and III, the harmonic ratio curves are tightly distributed with very narrow gaps or even overlapping. Indicating very small differences in harmonic signals for samples in Groups II and III, as shown in Figure 7(b) & (c). Interestingly, Group IV shows relatively sparse distributions of harmonic ratio curves compared with Groups II & III, while it’s denser than Group I. The harmonic ratio curves from Group IV can be divided into three factions: vials#IV-1 to #IV-4 showing lower harmonic ratios are overlapping, vials #IV-6 to #IV-8 showing higher ratios are overlapping, and the harmonic ratio of vial#IV-5 (blue curve) is in between. Thus, Group IV is also not practical for the detection of nucleocapsid proteins.

![Figure S6](image-url)  
Figure S6. Ratios of higher harmonics to the 3rd harmonics recorded from Groups (a) I, (b) II, (c) III, and (d) IV. For SARS-CoV-2 nucleocapsid protein detection.
S7. Specificity of SARS-CoV-2 spike and nucleocapsid pAbs confirmed by ELISA.

As shown below, ELISA was performed using polyclonal antibody (pAbs) to spike or nucleocapsid as capture antibody and corresponding biotinylated pAbs as detection antibody. Microtiter wells were coated with Rabbit anti-Spike-RBD (for Spike ELISA) or rabbit anti-Nucleocapsid (for nucleocapsid ELISA) as capture antibody and detected using corresponding biotin conjugated rabbit polyclonal antibodies and streptavidin-HRP. Different isolates of heat inactivated SARS-CoV-2 (USA-WA 1/2020, Hong Kong, Italy, USA-IL 1/2020), heat inactivated human corona viruses hCoV-NL63, hCoV-OC43, and hCoV-229E, porcine epidemic diarrhea virus (PEDV) and recombinant influenza A virus nucleoprotein (IAV NP) were tested using this ELISA. These pAbs to spike and nucleocapsid did not bind to other human corona viruses- hCoV-NL63, hCoV-OC43, and hCoV-229E and porcine epidemic diarrhea virus (PEDV), a corona virus causing diarrhea in pigs.

Figure S7. The binding specificities of pAbs are confirmed by testing four different SARS-CoV-2 isolates and four other coronaviruses using ELISA.