Click Chemistry

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Copper-Mediated Amplification Allows Readout of Immunoassays by the Naked Eye**

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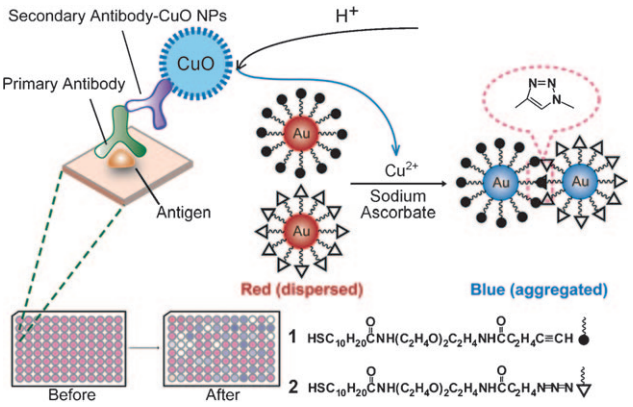
A new method of labeling antibodies for the colorimetric detection of immunoassays without using advanced equipment is described. Colorimetric detections are convenient and effective in many applications because the readout requires only human eyes. Gold nanoparticles (Au NPs) are useful in colorimetric assays because the aggregation of solutions with low concentrations of Au NPs displays a clear color change. The aggregation of Au NPs in solution has yielded many assays,[1] including those for ions,[2,3] small molecules,[4] DNA,[5,6] proteins,[7] and cancerous cells.[8,9] Advances in Cu^{I}-catalyzed tethering reactions, such as "click chemistry"[10,11] in aqueous solutions at room temperature, have allowed for new types of chemistry for Au NP-based biochemical analysis.

Our previous work shows that Cu^{I}-based click chemistry allows high sensitivity and selectivity[2] for chemical analysis. The fact that copper is used as a catalyst (a small amount of it is required for the reaction) ensures the sensitivity, and the fact that the reaction is orthogonal to most known chemical reactions ensures the selectivity. In the presence of Cu^{II} with sodium ascorbate as the reductant, Au NPs that have azide- and alkyne-terminated groups undergo aggregation as the result of Cu^{I}-catalyzed click chemistry. At ambient temperature, this process can be monitored by the naked eye. This method is highly specific even in the presence of high concentrations of mixtures of other cations and interfering molecules. We reasoned that if such assays can be extended to detect species other than Cu^{II}, it might be generally useful for many different types of highly sensitive and selective assays. We now report a colorimetric immunoassay based on detecting Cu^{II} released from copper monoxide nanoparticle (CuO NP)-labeled antibodies as the secondary antibody, in place of the fluorescent dye- or enzyme-labeled secondary antibodies traditionally used in immunoassays (Scheme 1).



Scheme 1. Immunoassay based on CuO-labeled antibody and click chemistry.

One of the bottlenecks for developing "point of care" (POC) detection[12,13] is the readout method. With the rapid development of nanoscience, many nanomaterials have been widely applied in bioanalysis as the label of probe molecules, to realize various signal output modes.[14] Because of the size effects and optical properties of nanomaterials, many of these methods showed good sensitivity, but many of them relied on bulky and complex equipment. Generally applicable readout methods by the naked eye alone for immunoassays have the potential to revolutionize analytical sciences based on the lab-on-a-chip format.[15] Naked-eye-based results simply need to match those from instruments, such as fluorescence assays, to be widely applicable.[16]

Herein, we provide a method of labeling antibodies for the highly sensitive and selective colorimetric detection of immunoassays without the use of advanced equipment. Human immunodeficiency virus (HIV) is the cause of acquired immune deficiency syndrome (AIDS), a worldwide pandemic. Because there is no cure for AIDS or effective vaccine against HIV, clinical assays for HIV are one of the major tools for prevention. Amongst all the methods for detecting HIV,[17] the detection of virus-elicited antibody (in human blood serum) is most widely applied.[18,19] Because AIDS affects many resource-poor regions, simple and sensitive assays for the detection of infection by HIV are essential for controlling this pandemic.[20]

The classical method for HIV detection is based on enzyme-linked immunosorbent assay (ELISA) in microwells, with the most current forms employing enzyme-labeled, fluorescent or chemiluminescent antibodies for readout. This type of ELISA needs instruments for detection, and hence is costly and requires hours of work and skilled labor.

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New chemical methods that allow sensitive detection of HIV infection are therefore particularly urgently needed. In developed economies, such tests are typically carried out in centralized laboratories. Instrument-free detection for HIV may help dramatically reduce soaring medical costs and enable the general public to easily test for such diseases. Our method can rapidly analyze HIV antibodies without using advanced instruments, by detecting copper through a colorimetric approach based on the aggregation of Au NPs and click chemistry.

Our approach relies on antibodies modified by CuO NPs. When Cu^{II} is released into the solution by HCl, it can be assayed by the detection mixture (DM, comprising azide- and alkyne-functionalized Au NPs and sodium ascorbate) as a naked-eye-based readout (Scheme 1). It can be applied to essentially any biochemical assay relying on immunoreactions that take place on a solid/liquid interface. Instead of an enzyme or a fluorophore, CuO NPs are the label that modifies the secondary antibody. After immobilization of the antibodies, we added acid to dissolve the CuO NPs to produce copper ions, which in turn can be detected with high sensitivity and specificity by click chemistry in which copper acts as a catalyst, thereby inducing aggregation of Au NPs functionalized with azide and alkyne groups (the components of click chemistry[2]). Since the aggregation of Au NPs can be monitored with the naked eye alone (dispersed Au NPs show a red color while aggregated Au NPs result in a purple or blue color), no instrument is needed for the readout. Because the method can detect the antibody without equipment, we applied it to the diagnosis of diseases relying on immunoassays, such as HIV.

To enhance the sensitivity of the DM and decrease the time for the detection compared with our earlier work, we optimized the conditions that would influence the limit of detection (LOD) for the DM, such as the ratio between the ligand (alkyne/azide-terminated thiol) and the stabilizing agent (polyethylene glycol-terminated thiol[21]; for details of optimization of the conditions, see the Supporting information, Table S1). Based on our early work, we synthesized a new alkyne ligand (compound **1** in Scheme 1; for the synthetic protocol and characterization, see the Supporting Information) to improve the reactivity of alkyne toward the azide. The LOD for Cu^{II} is 1 μM and the assay takes about 10 minutes for the color change of the DM to be observed by the naked eye.

To show that our method can be useful for ELISA-type assays, we first investigated the detection of the antibody of a model protein, ovalbumin (OVA). In this assay, the antigen was OVA, the primary antibody was rabbit anti-OVA, and the secondary antibody was goat anti-rabbit immunoglobulin G (IgG) labeled with CuO NPs. We mainly tested whether factors that would normally affect such experiments might adversely influence the selectivity of our assay (Figure 1). We incubated OVA in a 96-well plate, blocked the wells with 5% fetal bovine serum (FBS), and incubated the primary antibody. CuO NP-labeled secondary antibody was used as the probe. After immobilization of these molecules on the wells, hydrochloric acid released copper ions from the immobilized antibodies; we added the DM to every well and observed the color change after 10 minutes (for the details of optimizing the immunoassay conditions, see the Supporting Information, Figures S5 and S6).

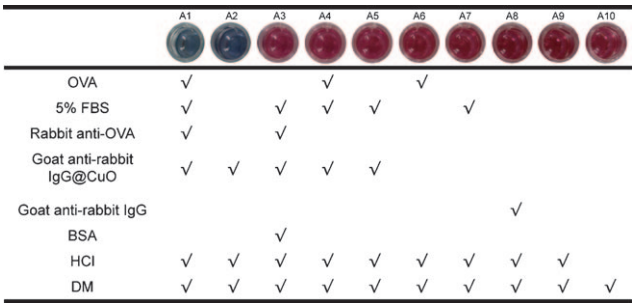

Figure 1. Detection of a model antibody (the antibody of OVA). Photographs taken 10 min after adding the DM to microwells are shown for each sample. The reagents added to each well are indicated below the images.

Only wells A1 and A2 showed color changes. Well A1 had CuO NP-labeled secondary antibody immobilized on the well by the immunoreaction. Well A2 only had secondary antibody labeled with CuO NPs. The reason for the color change was that this antibody was nonspecifically adsorbed on the well. Well A3 had no OVA; instead it had bovine serum albumin (BSA), where neither the primary antibody nor the secondary-antibody-labeled CuO NPs could adsorb on the well. Well A4 had no primary antibody, so the secondary-antibody-labeled CuO NPs could not adsorb on the well. Well A5 was negative because CuO NP-labeled antibody could not adsorb on the well as a result of effective blocking that prevented nonspecific adsorption after. Well A6 had only OVA to exclude the influence of antigen. Well A7 had only FBS to exclude the influence of the blocking reagent. Well A8 had goat anti-rabbit IgG without CuO NPs to exclude the influence of secondary antibody. Well A9 had only hydrochloric acid and well A10 had only the DM. Wells A1 and A2 showed positive results, while wells A3 to A10 showed negative results, as expected. We carried out the experiment to exclude the possible factors affecting the results, and verified that CuO NPs were labeled with the antibody successfully.

To apply our method of detection to a model HIV diagnosis, we assayed rabbit serum with anti-gp41 IgG (Figure 2). The HIV-1 gp41 antigen was incubated in the 96-well plate and the wells were blocked with 5% FBS. Rabbit serum containing rabbit anti-gp41 IgG of serially diluted concentration (from 1/200 to 1/25 600) was added to the wells. The goat anti-rabbit IgG labeled with CuO NPs by nonspecific adsorption was added last. The control experiments included a nonspecific rabbit IgG as the negative control, blank control, and BSA as the unrelated protein control. After incubation, HCl was used to produce copper ions and the DM was added to every well. Some wells had a color change from red to purple or blue. In Figure 2a, it can be seen that the color of the liquids from wells B3 to B8 changed 10 minutes after adding the DM (the corresponding time is shown in the Supporting Information, Figure S6, Table S2). The higher the concentration of the primary antibody, the faster the color change of the solution, so the rabbit anti-gp41

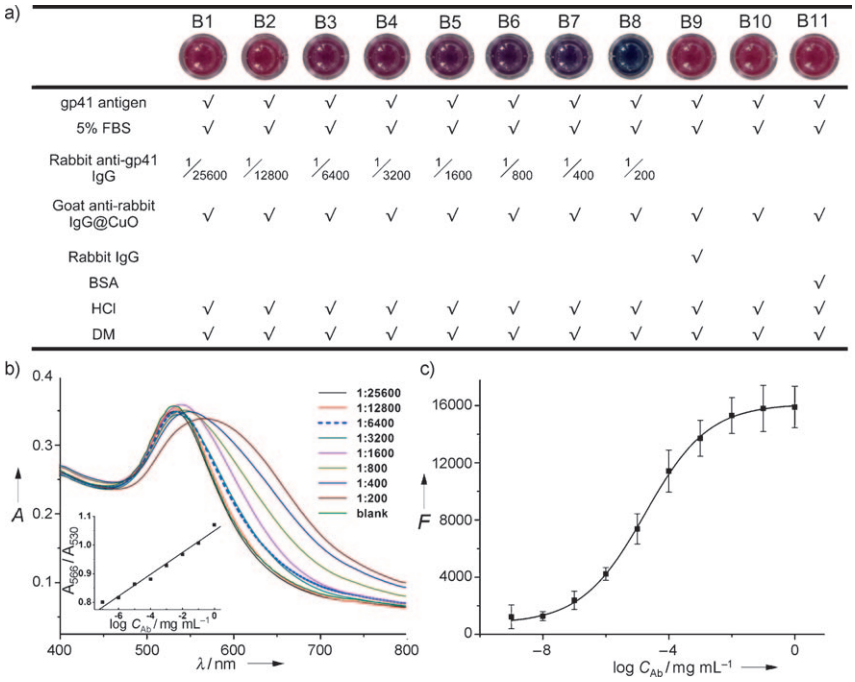

Figure 2. Model HIV assay. a) An immunoassay was performed on a 96-well plate that detected anti-HIV IgG with serially diluted concentrations from wells B8 to B1. Well B9 had nonspecific rabbit IgG as negative control, well B10 was the blank control, and well B11 had BSA as the unrelated protein control. Photographs of the DM on microwells are shown for each sample and the reagents added to every well are indicated below. b) The absorbance (*A*) of each microwell was measured by a UV/Vis microplate reader (at wavelength λ). Inset: plot of A_{566}/A_{530} against $\log C_{Ab}$ for rabbit IgG assay. c) Response curve of the fluorescence signals (*F*) versus different concentrations of rabbit anti-gp41 IgG under the FITC-goat anti-rabbit IgG as secondary antibody. Error bars indicate one standard deviation from the mean of three assays. Photographs were taken 10 min after adding the DM.

IgG diluted 6400 times could be detected by the naked eye and its concentration was about 150 ng mL^{-1}.

We next compared the sensitivity of the method with UV/Vis absorption (for detecting the aggregation of Au NPs) and fluorophore-labeled secondary antibody (which is the current industry standard for clinical immunoassays of HIV). We measured the UV/Vis absorption in each well by a microplate reader (Figure 2b). To our amazement, the sensitivity for immunoassay readout by the naked eye was at the same level as that measured by UV/Vis spectrometry.

In addition, as shown in Figure 2b, increasing the concentration of primary antibody in the well also results in a clear increase in the absorbance at 566 nm (A_{566}) and a decrease in the absorbance at 530 nm (A_{530}). The ratio between A_{566} and A_{530} was linear with the logarithm of primary antibody concentration C_{Ab} ($r = 0.991$), which demonstrated that the assay described here could be used for visual detection of HIV at high sensitivity. When comparing the analytical performance of our method with that of immunoassays that measure the fluorescence of secondary antibody labeled by fluorescein isothiocyanate (FITC, Figure 2c; we carried out the same immunoassay using FITC-labeled secondary antibody instead of the CuO NP-labeled secondary antibody), the LOD for the concentration of the primary antibody, at a signal-to-noise ratio of 3, is approximately at the primary antibody diluted 12800 times (the highest detectable dilution in our assay is 6400 times by naked-eye readout). The LOD for our method rivals that of absorbance or fluorescence, two commonly used reporting systems for microplate readers. Contrary to existing reporting systems, the new method requires no equipment at all.

To test the utility of our approach for real samples, and to explore the possible interference of endogenous Cu present in blood plasma, we used this method to distinguish the serum of HIV-infected patients from that of uninfected patients (Figure 3). Wells C1 to C3 had serum samples of different HIV-infected patients diluted 40 times and the secondary antibody was rabbit anti-human IgG labeled with CuO NPs. The other conditions were the same as those for the HIV model experiment. Because different patients have different concentrations of anti-HIV antibody in the blood serum, the wells showed different color changes, while the color of control wells had no change. We can distinguish the positive blood serum from the negative by the naked eye, because the serum sample of the HIV-negative samples did not cause a color change of the DM. This experiment indicates that endogenous Cu in the serum does not affect our approach.

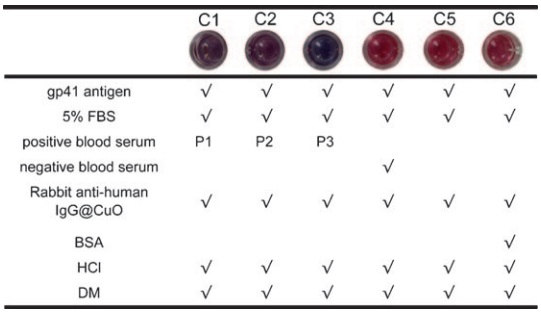

Figure 3. Detection of real HIV-1 samples. Wells C1 to C3 had blood serum from different HIV-1-infected patients. Well C4 had blood serum from uninfected individuals. Well C5 had no blood serum as blank control. Well C6 had BSA as the unrelated protein control. The reagents added to every well are indicated below the photographs. P1, P2, and P3 indicate different HIV-1-infected patients. Photographs were taken 10 min after adding the DM.

The high selectivity and sensitivity of the immunoassay result from the highly selective and sensitive nature of the copper-catalyzed reaction. This new immunoassay may therefore be useful for many applications, including clinical assays in resource-poor settings. Since immunoassays comprise the largest class of assays for proteins (and are becoming more

important for the detection of small molecules[22]), we expect our approach to have wide-ranging applications from analytical biochemistry and clinical chemistry to biodefense-related assays. Such types of assays may pave the way for highly sensitive and selective detection of molecules through the indirect detection of metal ions.

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