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Natural phage nanoparticle-mediated real-time immuno-PCR for ultrasensitive detection of protein marker†

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Current immuno-PCR methods either use bare or nanostructured DNA as a reporter or combine phage display for antigen binding and reporting, however, they are often complex to carry out and lack universality. We present a novel and universal design of immuno-PCR termed natural phage nanoparticle-mediated real-time immuno-PCR.

Many disease markers at low levels in body fluids are particularly significant for clinical cases. However, methods for protein detection are not as sensitive as those for specific DNA detection because the latter can be amplified exponentially by polymerase chain reaction (PCR). Sano *et al.*[1] introduced PCR into immunoassays by using a reporter DNA that is amplified to a diagnostic concentration by PCR after immune reaction and established the immuno-PCR method to detect proteins of low abundance. In the initial immuno-PCR method, a protein A-streptavidin chimera bound to a biotinylated linear plasmid[1] was used to bridge the detection antibody and the reporting DNA. Later, nanostructured networks self-assembled based on biotin–streptavidin interaction[2,3] were proposed and are now widely used for qualitative testing in both fundamental and applied immunological research.[4–8] However, the difficult and complex preparation of chimera and the nanostructured networks has hindered immuno-PCR from wide acceptance. Alternative reporting systems were also reported. For example, liposomes embedded with DNA reporters in the bilayer were proposed to be universal reporting systems for immuno-PCR.[9] Another strategy takes advantage of the phage display technique to introduce single chain variable fragments (scFv) for antigen binding while inherent phage DNA was used as a reporter template for PCR.[10] However, scFv molecules normally have an apparent affinity that is typically 10- to 1000-fold lower than their parent monoclonal antibody.[11] Furthermore, acquisition of such phages with scFv avidity involves a complex and time-consuming procedure that needs repeated screening by bio-panning. More recently, Hee-Joo Kim *et al.*[12] exploited phagemid DNA contained in M13 bacteriophage displaying peptides that bind to an analyte–antibody immune complex to develop non-competitive immuno-PCR for small molecules. This method, however, like the phage display strategy, requires a specific scFv display and cannot be applied to different analytes.

To construct an ease-of-use and universal reporting system for immuno-PCR, we hereby introduce the use of natural phage nanoparticles (T7 phage) for immuno-PCR assay. We tested this idea by using the human hepatitis B surface antigen (HBsAg) as a model analyte. HBsAg is a viral protein marker for hepatitis B virus (HBV) infection and its highly sensitive detection allow early diagnosis of HBV infection and confirmation of virus clearance. Currently, the most widely used enzyme-linked immunosorbent assay (ELISA) for HBsAg has a limit of detection (LOD) of *ca.* 0.2 ng ml^{-1}, which is often not low enough to meet the clinical requirements.

Details of preparation and characterization of the T7-antibody complex, optimization of immuno-PCR conditions, and real-time sandwich immuno-PCR for human HBsAg are provided in the data supplement that accompanies the online version of this article (see Materials and methods, ESI†). The assay procedure is illustrated in Fig. 1. A monoclonal capture antibody against HBsAg was coated onto the inner surface of the PCR tubes, which were previously coupled with glutaraldehyde. The target antigen in the sample was captured by the immobilized capture antibody. Then, the T7 phage–antibody complex bound with the antigen through the monoclonal detection antibody in the complex, thereby forming the immune complex containing the T7 phage. Because T7 could be easily lysed by heating, it could directly serve as the template for PCR, which was initiated by a denaturation step at 95 °C for 6 min.

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† Electronic supplementary information (ESI) available: Experimental details, sequences of primers and probes, photograph of T7 phage-FITC, and results of optimization of immuno-PCR conditions using the different washing buffer. See DOI: 10.1039/c3cc40688a

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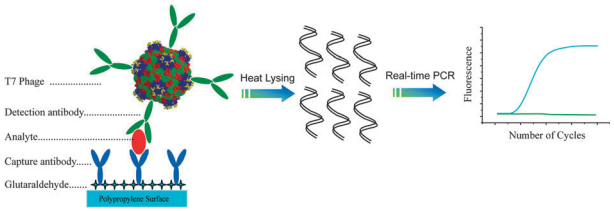

Fig. 1 Schematic diagram of immuno-PCR using the T7 phage–antibody complex for detection of human HbsAg.

The real-time PCR based on displacing probes[13] was then performed to quantify the DNA template of T7, which in turn would reflect the amount of antigen to be detected.

Preparation of the T7–antibody complex relies on the assumption that T7, as a natural phage nanoparticle, should have a sufficient number of free amino groups on the surface. We tested this assumption by chemical staining of T7 using fluorescein isothiocyanate (FITC), which could react with an amino group and make it fluorescent. As expected, T7 phages became strongly fluorescent after being stained with FITC (see Fig. S1, ESI†). The number of amino groups on the surface of T7 phages were determined to be $(1.492 \pm 0.201) \times 10^3$ per phage by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method.[14] The T7 phage was conjugated to the detection antibody by coupling its amino groups with a thiolated antibody using a crosslinker, sulfosuccinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC), *via* an ester reaction. The T7 phage–antibody complex was characterized using a JEOL JEM-2100 (100 kV) transmission electron microscope. As shown in Fig. 2, the surface of free T7 phages was sleek whereas the surface of the T7 phage–antibody complex was obviously blurred, confirming that the antibodies were present on the T7 phage surface.

Regular ELISA plates cannot be directly used in a PCR machine because of their flat bottoms and different sizes from those of the PCR blocks, therefore hindering immuno-PCR from being conducted with simple handling and ordinary apparatus.[15] To address this problem, we modified the inner surface of regular polypropylene PCR tubes by immobilizing glutaraldehyde, which could attach the captured antibody through chemical linking and allow both immune reaction and PCR to be performed in a single module. Another problem with the immuno-PCR approach is the nonspecific binding, which can cloud positive results with high background signals. Sufficient blocking, washing and pre-PCR sample preparation are preferable ways to overcome this problem. After extensive optimization, we concluded that satisfactory results could be obtained by including Tween-20 in the blocking buffer and sodium dodecyl sulfate (SDS) in the washing buffer (see Fig. S2, ESI†). Upon combining immune reaction and PCR in a single reaction tube, immuno-PCR became simple and straightforward and the whole procedure, starting from antigen capture, could be finished within 4 hours for a batch of 10 samples. The manipulations also became simple as the multiple washing steps could be carried out using an automatic washing machine and the PCR step only involved a single step of reagent addition.

We then evaluated the analytical performance of T7 phage-mediated real-time immuno-PCR. We first prepared a series of standard HBsAg solutions with concentrations ranging from 1000 ng ml^{-1} to 0.01 ng ml^{-1} and detected each of them in triplicate. The typical real-time PCR profile could be obtained for each concentration. After the cut-off fluorescence intensity value was set in the exponential amplification region automatically using the machine, the threshold cycle numbers (C_t) could be obtained for each concentration (Fig. 3). By plotting these averaged C_t values against the logarithmic value of each concentration, a linear calibration curve ($y = 30.72 - 2.79x$, $y = C_t$ value, x = log[HBsAg] in ng ml^{-1}, $R = 0.988$) was obtained in the whole concentration range with the coefficient of variation (CV) being between 6.2% and 15.1%. To determine the LOD of this immuno-PCR, another series of solutions of 0, 0.1, 1, 10, 100, 1000 pg ml^{-1} of HBsAg were prepared and repeatedly detected 10 times. By setting $C_t \pm 3$SD from the blank (0 ng ml^{-1}) as the cut-off value, we observed that the lowest concentration that could always be distinctively detected as positive was 0.1 pg ml^{-1}, which was therefore set as the LOD of this assay (Fig. S3, ESI†). Such a LOD value was approximately 2000-fold lower compared with that obtained from a regular ELISA for HBsAg. These results evidenced that real-time immuno-PCR using a natural T7 phage is a highly sensitive method.

We then evaluated this method by detecting HBsAg in human serum samples. The 74 serum samples were collected

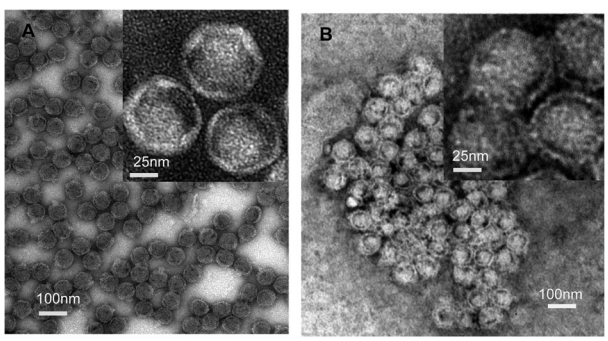

Fig. 2 TEM images of uranyl acetate negatively stained T7 bacteriophage (A) and T7–antibody complex. (B) The insets show higher resolution images of the same sample.

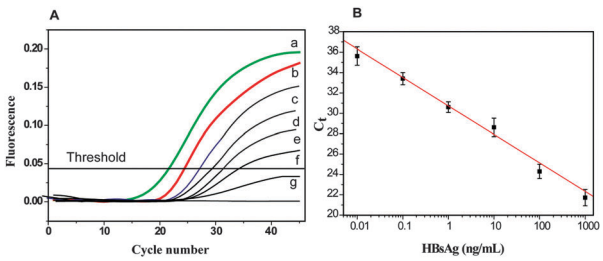

Fig. 3 Quantitative detection of standard human HBsAg solutions by phage-mediated immuno-PCR. (A) Real-time PCR amplification curves. Curves a–f represent serial 10-fold dilutions of HBsAg from 1000 ng ml^{-1} to 0.01 ng ml^{-1}. Curve g stands for the negative control that had no HBsAg antigen added, and the fluorescent signal was observed because of non-specific absorption. (B) Calibration plot of log HBsAg concentration *versus* threshold cycles (C_ts). The results obtained from three individual experiments were averaged.

from both HBV-infected and non-HBV-infected persons. All of the samples were previously analysed by ELISA for HBsAg and real-time PCR for the HBV DNA. All the samples were renumbered and the detection result for each sample was blinded to the person who conducted real-time immuno-PCR. Our detection results showed that of the 74 samples, 29 were HBsAg positive and 45 were HBsAg negative. By referring to the results of ELISA and real-time PCR, a second person found that our result for each sample was completely concordant with the ELISA result and the HBV DNA status detected by real-time PCR was also in line with the two immunoassays. These results supported the feasibility of using this immuno-PCR method in clinical settings.

Recent advances in the field of clinical diagnostics have been incorporated successfully for detection of numerous infectious agents including human and animal viruses with significant improvement in sensitivity and specificity.[7] Owing to the low level of transcription of the viral gene as one of the persistence mechanisms of the virus in its host, resulting in difficulties in detection, HBsAg is still a challenging target for immuno-PCR.[5,16] The hereby described immuno-PCR method based on the natural phage offers several advantages over the current immuno-PCR methods. First, the natural phage nanoparticle is a universal and two-functional reagent in immuno-PCR. The T7 phage is a widely available carrier for molecular cloning and it can be conjugated with any type of antibody regardless of the antigen. The large number of amino groups on the surface makes the T7 phage a convenient carrier for antibody labelling. Meanwhile, the template DNA is naturally encapsulated inside the phage nanoparticles, which offer a safe shelter for DNA against chemical or enzymatic degradation by impurities present in the sample. Second, phage-mediated immuno-PCR facilitated prevention of PCR product contamination problems. Because contaminated DNA from the environment, carryover by pipette tips or genomic DNA contamination remaining in the samples can all be eliminated by DNase I while the template DNA remains intact inside the phage, false-positive results would be substantially decreased. Third, the use of ordinary PCR tubes for both immune reaction and PCR renders this method compatible with all current real-time PCR machines, thereby simplifying the operation and reducing the cost as well.

In conclusion, we have described a novel version of immuno-PCR based on the natural phage nanoparticles for the first time. The protocol for using a prepared T7 phage–antibody complex as a detection reagent for immuno-PCR was straightforward and practical as shown in the detection of HBsAg. Additional merits such as compatibility with real-time PCR machines, easy preparation and purification of detection reagents, simple protocol, low cost, high sensitivity, and low false positive rate, *etc.*, were also revealed by our preliminary experimental data. Improved sensitivity and lower nonspecific binding could be achieved through further optimization of experimental conditions. As the technique matures through continued research, this new model will likely be a potential toolkit for immuno-PCR in the detection of a variety of protein biomarkers.

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