Chemiluminescence enzyme immunoassay based on magnetic nanoparticles for detection of hepatocellular carcinoma marker glypican-3

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\textbf{Abstract} Glypican-3 (GPC3) is reported as a great promising tumor marker for hepatocellular carcinoma (HCC) diagnosis. Highly sensitive and accurate analysis of serum GPC3 (sGPC3), in combination with or instead of traditional HCC marker alpha-fetoprotein (AFP), is essential for early diagnosis of HCC. Biomaterial-functionalyzed magnetic particles have been utilized as solid supports with good biological compatibility for sensitive immunoassay. Here, the magnetic nanoparticles (MnPs) and magnetic microparticles (MmPs) with carboxyl groups were further modified with streptavidin, and applied for the development of chemiluminescence enzyme immunoassay (CLEIA). After comparing between MnPs- and MmPs-based CLEIA, MnPs-based CLEIA was proved to be a better method with less assay time, greater sensitivity, better linearity and longer chemiluminescence platform. MnPs-based CLEIA was applied for detection of sGPC3 in normal liver, hepatocirrhosis, secondary liver cancer and HCC serum samples. The results indicated that sGPC3 was effective in diagnosis of HCC with high performance.

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\textbf{1. Introduction}

Great progress has been made in clinical therapeutic strategies, but patients with cancer are still undergoing poor effects of therapy and high mortality rate\textsuperscript{[1–3]}. Patients with cancer are often asymptomatic in early stages\textsuperscript{[4–6]} and consequently are frequently diagnosed late. Prevention is one of the most effective cancer-fighting tools. Early diagnosis may allow for timely prevention of malignancy and initiation of appropriate therapies. Fortunately, the use of biomarkers in predicting disease holds considerable promise and has played an important role in early diagnosis\textsuperscript{[7]}. Hepatocellular carcinoma (HCC) is the third most common cancer because of cancer-related death.
Alpha-fetoprotein (AFP), commonly regarded as a tumor marker of HCC, has encountered challenges from other HCC markers because of the low sensitivity of AFP. For example, glypican-3 (GPC3) [8], reported to be overexpressed in HCC, is considered as an early tissue tumor marker for HCC [9–12]. Additionally, it was reported that GPC3 was secreted into blood and could be a serological tumor marker with higher sensitivity than AFP [13–15]. In our previous work [16], competitive radioimmunoassay for detection of GPC3 had been developed, and tentative clinical application showed that GPC3 presented higher sensitivity and specificity than AFP in diagnosis of HCC.

In recent years, functionalized magnetic particles (MPs) [17] have attracted tremendous interests in many biological applications, such as biomedicine [18], isolation of specific DNA [19], mRNA subtraction [20,21] and manipulation cells [22]. Functionalized MPs are first modified with amino or carboxyl groups, and then proteins (e.g. antibodies/antigens) can be covalently bond to MPs-carboxyl or MPs- amino [23,24]. However, biological activity of proteins might be affected by steric hindrance and non-specific adsorption on the modified MPs surface [25]. Therefore, some attempts have been made to further modify MPs-carboxyl or MPs- amino with functional ligands (e.g. streptavidin, second antibodies) [26,27] in order to reduce the effect of steric hindrance and preserve the bioactivity of proteins. Functionalized MPs, with the diameter in the range from nanometers to micrometers, have been applied for immunoassay with higher sensitivity [28–31] and wider detection range [32]. However, to our knowledge, the influence of the diameters of the particles and the role of MPs in immunoassay have not been discussed in detail up to now.

Herein, we developed magnetic particles-based chemiluminescence enzyme immunoassay (CLEIA) for detection of serum GPC3 (sGPC3). Magnetic nanoparticles (MnPs) and microparticles (MmPs) with carboxyl group were both modified with streptavidin, and further used as solid phase for coating GPC3 antibodies. MnPs-based CLEIA (MnPs-CLEIA) was compared with MmPs-CLEIA in developing sensitive and fast immunoassay. Finally, MnPs-CLEIA was applied to the detection of GPC3 in normal liver, hepatocirrhosis, secondary liver cancer and HCC serum samples for evaluating its sensitivity and specificity.

2. Experimental

2.1. Materials and apparatus

MnPs-carboxyl (100 nm in diameter, 10 mg/mL stock solution) and MmPs-carboxyl (2 μm in diameter, 10 mg/mL stock solution) suspended in buffer solution were purchased from Shanghai Allrun Nano Science & Technology Co., Ltd. (Shanghai, China). Sheep immunized anti-human GPC3 antibody and horseradish peroxidase (HRP) labeled recombinant human GPC3 were from R&D systems Inc. (Minneapolis, USA). Streptavidin and biotin were from Vector Laboratories Inc. (Burlingame, USA). Chemiluminescent substrate (luminol, H2O2 and chemiluminescence enhancer) was obtained from Monobind Inc. (America).

Blood samples were collected from Beijing Tumor Hospital (Beijing, China). Approval was obtained from the Regional Ethics Committee, and all subjects gave informed consent to participate. HCC patients (30 males and 30 females) were at the mean age of 50.7 ± 15.5 without any therapeutic approach. Hepatocirrhosis patients (40 males and 20 females) were at the mean age of 45.5 ± 20.5. Secondary liver cancer patients with the mean age of 50.5 ± 12.5 were 15 males and 15 females. Normal liver serum samples were collected from 60 persons (30 males and 30 females) of the mean age of 40.5 ± 10.5.

A universal luminometer (Hamatsu Photonics, Beijing, China) with 60 test tubes (12-mm in diameter × 60-mm in length) was used for the chemiluminescence detection. A test tube rack equipped with a samarium–cobalt magnet (4000 G) was used for the magnetic separation. ZHWY-100 thermostatic culture oscillator (Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd., Shanghai, China) was used for incubation and shaking procedures.

2.2. Preparation of biotin labeled polyclone anti-GPC3 antibody

Anti-GPC3 antibody (dissolved in 0.05 M carbonate buffer, pH 8.0) was incubated with biotin-7-NHS (dissolved in DMSO) at room temperature. After 2 h, the mixture was dialyzed in carbonate buffer (0.05 M, pH 9.6) for 2 h to discharge unbound biotin-7-NHS, and the repeated dialysis treatment was carried out three times. Finally, biotin labeled polyclonal anti-GPC3 antibody (B-GPC3 Ab) was stored in borate buffer (pH 8.0) at −20 °C for further use.

2.3. Preparation of streptavidin modified magnetic particles

The protocol for the preparation of streptavidin modified magnetic particles (SA-MPs) is briefly summarized as follows. The following operations were all performed at room temperature. MPs-carboxyl was washed three times with 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES, pH 5.0) buffer. After that, MPs-carboxyl was activated by directly suspending 2.1. Materials and apparatus

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the activated MPs-carboxyl was incubated with streptavidin on a shaker for 2 h. Finally, streptavidin modified magnetic particles (SA-MPs) were washed three times with 0.1 M phosphate buffer solutions containing 0.05% (v/v) Tween-20 (PBST).

Anti-GPC3 antibodies coated MPs (anti-GPC3 Abs-MPs) were simply prepared by incubating B-GPC3 Abs with SA-MPs at 37 °C for 15 min. The prepared anti-GPC3 Abs-MPs were suspended in 0.2 M borate buffer (pH 8.0) containing 2% (w/v) BSA, 0.3% (v/v) Tween-20 and 0.1% (w/v) biologic preservatives for further use.

2.4. Immunoassay procedures of the chemiluminescence enzyme immunoassay based on MnPs and MmPs

One-step competitive immunoassay was carried out by incubating GPC3 calibrators (or serum samples), anti-GPC3 Abs-MPs and HRP-GPC3 at 37 °C. After immunoreaction, the whole mixture was washed four times with PBST buffer to remove unbound immunoreagents. Luminescent substrate (100 μL) was introduced into the immuno-complex, and incubated for 5 min at room temperature. Finally, the emitted photons were measured as relative luminescence unit (RLU).

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**Figure 1** Influence of the concentration of magnetic particles (MPs) on chemiluminescence. (A) Comparison of RLUs with corresponding concentration of MnPs or MmPs; (B) optimization of the concentration of MnPs depending on chemiluminescence intensity and duration. The experimental conditions: 37 °C, 50 μL HRP-GPC3 (1:2000), 100 μL of chemiluminescent substrate, a serial concentration of anti-GPC3 Abs-MPs.
2.5. Data analysis

Standard curves were obtained by plotting the log of RLU against the logarithm of GPC3 concentration and fitting to a linear curve. Sensitivity of sGPC3 in diagnosis of HCC was defined by true positive/(true positive + false negative results). The specificity was defined by true negative/(true negative + false positive results). Fisher’s exact test and student’s $t$-test were used for categorical variables analysis between groups. Significant difference was defined as $P < 0.05$.

![Figure 2](image.png)  
**Figure 2** Influence of the MPs size on immunoreaction time. (A) Variation of $RLU_{s1}$, $RLU_{s0}$ and $RLU_{s1}/RLU_{s0}$ upon immunoreaction time with MmPs. (B) Variation of $RLU_{s1}$, $RLU_{s0}$ and $RLU_{s1}/RLU_{s0}$ upon immunoreaction time with MnPs. Detection conditions: $37 ^\circ C$, $50 \mu$L HRP-GPC3 (1:2000), $50 \mu$L anti-GPC3 Abs-MPs, $100 \mu$L of chemiluminescent substrate.
3. Results and discussion

3.1. Optimization of the concentration of B-GPC3 antibody and HRP-GPC3

Concentrations of B-GPC3 Abs and HRP-GPC3, which mainly determine the sensitivity and working range in an immunoassay, were optimized according to inhibition ratios. Inhibition ratios ranging from 85% to 15% were well acceptable in competitive immunoassay for a good separation and even distribution calibrator points in calibration curve. Stock solutions of B-GPC3 Abs and HRP-GPC3 were step-wise diluted to three titters, respectively. Orthogonal test with two factors and three levels was carried out (Table 1). RLUs decreased with the increasing concentration of HRP-GPC3 and B-GPC3 Abs. RLU_{S1}/RLU_{S0} (86%) and RLU_{S5}/RLU_{S0} (21%) in MnPs-CLEIA were acceptable at 1:1000 of HRP-GPC3 and 1:120 of B-GPC3 Abs, respectively. RLU_{S1}/RLU_{S0} (85%) and RLU_{S5}/RLU_{S0} (21%) in MmPs-CLEIA were acceptable at 1:2000 of HRP-GPC3 and 1:240 of B-GPC3 Abs, respectively. The results indicated that immunoreagents might have a higher bioactivity with MnPs than with MmPs, and the steric hindrance effect could be lower with MnPs than with MmPs.

3.2. Optimization of the concentration of MPs

As shown in Fig. 1, the influence of the concentration of MPs and MPs on chemiluminescence was investigated. RLUs were decreased with the increasing concentration of MPs, but RLUs by using MnPs were much higher than by MmPs with the same concentration (Fig. 1A). Furthermore, the concentration of MPs was extensively optimized. RLUs decreased greatly after a short chemiluminescence platform (within 10 min) when too low concentration (0.125 mg/mL) of MnPs was used (Fig. 1B). Considering an appropriate intensity of RLUs attained, 0.25 mg/mL of MnPs was settled for MnPs-CLEIA. Similar results were obtained for optimization of the concentration of MmPs. The difference was that 0.5 mg/mL of MmPs was settled for MmPs-CLEIA. Obviously, the concentration of MnPs was one fourth of MmPs by developing CLEIA, which might be due to a larger effective area of MnPs than MmPs with the same concentration of MPs.

3.3. Influence of the diameter of MPs on immunoreaction time

The influence of the diameter of MPs on immunoreaction time was studied and the results are shown in Fig. 2. RLU_{S0} and RLU_{S1} enhanced with immunoreaction time increasing. Interestingly, the inhibition ratio of RLU_{S1}/RLU_{S0} decreased with immunoreaction time increasing, and reached 84% at 70 min with MmPs and 85% at 40 min with MnPs. Immunoreaction time of 70 min was consequently selected for further developing MmPs-CLEIA, and 40 min for MnPs-CLEIA. The less immunoreaction time might be due to better reservation of heterogeneous phase for MnPs holding in the immunoreaction solution than MmPs, and increased collision reaction probability by MnPs compared with MmPs.

| Table 2 | Comparison of analytical parameters. |
|---------|--------------------------------------|
| Method  | Calibration curve | Linear range (ng/mL) | LOD (ng/mL) | Total assay time (min) |
| MnP-CLEIA | log Y = 0.2819 -0.2498 | 0 - 2500 | 0.38 | 50 |
| MmP-CLEIA | log Y = 1.6972 -0.2618 | 0 - 2500 | 1.05 | 100 |
| RIA [25] | log Y = 1.2632 -0.6466 | 0 - 500 | 0.5 | 150 |

Figure 3 Chemiluminescence kinetics corresponding to the two kinds of MPs (MnPs and MmPs).
3.4. Influence of the diameter of MPs on chemiluminescence kinetics

Furthermore, the influence of the diameter of MPs on chemiluminescence kinetics was studied (Fig. 3). Although RLU s with MnPs and MmPs both increased immediately with time increasing, RLU s in MnPs-CLEIA were much higher than in MmPs-CLEIA. In addition, RLU s in MnPs-CLEIA preserved a longer platform (from 3 to 40 min) than in MmPs-CLEIA (from 9 to 20 min). The reasons might be a higher bioactivity of HRP-GPC3 on MnPs surface than on MmPs, and a better reservation of heterogeneous phase for MnPs suspended in chemiluminescent substrate than that for MmPs. Additionally, a smaller dosage of MnPs used would give a lower absorbency of photo counts compared with MmPs.

Figure 4 Calibration curve and linear dilution curve. (A) Calibration curve was obtained by plotting the logistic of chemiluminescence intensity against the logarithm of concentration of calibrators; (B) linear dilution curve was obtained by concentration of serial diluted samples against dilution ratio.
3.5. Analytical parameters

On the basis of the above optimized conditions, we constructed MnPs-CLEIA. Analytical parameters were further evaluated for detection of sGPC3 and compared with our previous radioimmunoassay (RIA) [16]. As shown in Table 2, MPs-CLEIA exhibited high performances than RIA in terms of linear range, limit of detection (LOD) and total assay time. This obviously resulted from greater sensitivity of counting photo counts equipped with photomultiplier for chemiluminescence detection than counting γ rays in RIA. In addition, MPs introduced to the immunoassay were another key factor for improving sensitivity and accelerating immunoreaction.

3.6. Calibration curve and validity

Linearity of the calibration curve, displayed in Fig. 4A, was obtained in the range of 2.0–2500 ng/mL (correlation coefficient $r = 0.9973$).

Validity was evaluated to study the effects of calibrator matrix on the accuracy of the detection method. An HCC serum sample was stepwise diluted (two-, four-, eight- and 16-fold) by calibrator matrix, and GPC3 in these five samples was determined by calibration curve. The relationship between GPC3 concentration in the serial diluted HCC samples and the dilution factors gave a high linearity ($r = 0.9999$) (Fig. 4B). The results confirmed that the calibrator matrix had a similar biochemical characteristic to human serum for detection of sGPC3.

3.7. Determination of the cut-off value of sGPC3 for HCC diagnosis

GPC3 in serum samples collected from patients with normal liver (NL) was evaluated with our proposed method. When we adopted the cut-off points distinguishing normal liver and other liver diseases at $\tau + 3\sigma$ (according to student’s $t$-test), sGPC3 in about ninety-eight percents of the total samples was below 21.10 ng/mL ($\tau + 3\sigma$). So we set the cut-off point of sGPC3 at 21.10 ng/mL ($\tau + 3\sigma$) to distinguish normal liver and benign liver diseases.

3.8. Determination of sGPC3 in HCC, hepatocirrhosis and secondary liver cancer serum samples

GPC3 in HCC serum samples was detected by MnPs-CLEIA and compared with the results obtained by RIA (proposed method of our previous work). As shown in Fig. 5, there was a good agreement between the two methods in the range of 0–1400 ng/mL ($r^2 = 0.9582$), and also in the range of 0–300 ng/mL ($r^2 = 0.9203$). Hence, the present proposed method was reliable and could be used in the clinical diagnosis.

GPC3 levels in normal samples (NL), hepatocirrhosis and HCC serum samples were compared by statistical method (Fig. 6). As can be seen, sGPC3 in normal liver ($3.81 \pm 5.77$ ng/mL) was much lower than in HCC [(148.29 ± 215.76) ng/mL], and the results showed a significant difference between the two groups ($P < 0.001$). So using sGPC3 as an HCC tumor marker and our proposed method, HCC could be well differentiated from normal liver. Moreover, sGPC3 in hepatocirrhosis [(18.69 ± 17.47) ng/mL] tended to be slightly elevated compared with in normal liver, but still at a significantly lower level than in HCC samples. Moreover, sGPC3 in all of the HCC samples was above 29.01 ng/mL (higher than the cut-off value), and in 38.3% (23 case) of the hepatocirrhosis samples was above the cut-off value. From the data we found that the sensitivity of sGPC3 reached 100% and the specificity of screening HCC from hepatocirrhosis reached 61.7%. Therefore, we could confirm that sGPC3 was an effective marker to distinguish HCC from healthy individuals. What should be mentioned is that sGPC3 in hepatocirrhosis patients might elevate above cut-off value.

![Figure 5](image_url) Correlation between results measured by the proposed MnPs-CLEIA and RIA. Inset is the enlarged diagram with abscissa between 0 and 300 ng/mL.
Secondary liver cancer serum samples were also put into consideration to evaluate specificity of GPC3. Among 30 secondary liver serum samples, sGPC3 in 90% of the total samples had no elevation, and only in three samples had slight elevation (26.36, 18.83 and 12.27 ng/mL, respectively). So our proposed method with specificity of 90% was a useful tool for screening HCC from secondary liver cancers.

4. Conclusion

In this work, MnPs-based CLEIA with high performance was successfully proposed for detection of the promising HCC tumor marker sGPC3. MnPs-CLEIA exhibited smaller dosage of MPs, shorter immunoassay time, longer chemiluminescence platform and greater sensitivity than MnPs-CLEIA. The bioactivity of anti-GPC3 antibody and HRP-GPC3 binding to MnPs remained higher than binding to MmPs. Besides, by successfully applying MnPs-CLEIA to detect sGPC3, the cut-off value of sGPC3 in diagnosis of HCC was determined at 21.01 ng/mL. More importantly, our results confirmed that sGPC3 could well differentiate HCC patients from ones with normal liver and secondary liver cancers. However, our results suggest that the elevation of sGPC3 in hepatocirrhosis patients may increase the difficulty in screening HCC from hepatocirrhosis, and should be paid attention to with further clinical supervision.

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