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Detection of hepatitis B virus markers using a biosensor based on imaging ellipsometry

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SUMMARY. A biosensor based on imaging ellipsometry (BIE) has been developed and validated in 169 patients for detecting five markers of hepatitis B virus (HBV) infection. The methodology has been established to pave the way for clinical diagnosis, including ligand screening, determination of the sensitivity, set-up of cut-off values (CoVs) and comparison with other clinical methods. A matrix assay method was established for ligand screening. The CoVs of HBV markers were derived with the help of receiver operating characteristic curves. Enzyme-linked immunosorbent assay (ELISA) was the reference method. Ligands with high bioactivity were selected and sensitivities of 1 ng/mL and 1 IU/mL for hepatitis B surface antigen (HBsAg) and surface antibody (anti-HBs) were obtained respectively. The CoVs of HBsAg, anti-HBs, hepatitis B e antigen, hepatitis B e antibody and core antibody were as follows: 15%, 18%, 15%, 20% and 15%, respectively, which were the percentages over the values of corresponding ligand controls. BIE can simultaneously detect up to five markers within 1 h with results in acceptable agreement with ELISA, and thus shows a potential for diagnosing hepatitis B with high throughput.

Keywords: hepatitis B, imaging ellipsometry, label-free biosensor, microfluidic.

INTRODUCTION

The detection of hepatitis B virus (HBV) markers is important clinical data for the diagnosis of infection with this virus [1]. Several methods, including enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction, chip-based detection methods and surface plasmon resonance, have been used to detect HBV markers; however, they suffer from inherent limitations [2–5], such as expensive equipment, specialist skills and complicated sample preparation processes. Conventional ELISA is still the main diagnostic test for hepatitis B [6], which has, however, some shortcomings, such as need of tracer labelling, indirect format of detection and length of testing time. Thus, a rapid, simple and direct method is an urgent need.

Compared with previous methods, biosensor based on imaging ellipsometry (BIE) offers several advantages such as a label-free rapid test, intuitionistic image, multiplexed analysis and low cost. The concept of biosensor usage in diagnosis was proposed in 1995 [7], and since then it has been successfully applied in the biomedical field, for example, in cancer marker detection, bacteria or virus detection, SARS antibody identification and so on [8–12]. Some initial results of hepatitis B detection have been reported, which show that BIE is feasible for clinical diagnosis of disease [13,14].

We report here on the BIE detection procedure for hepatitis B markers, including modifications and substrate, screening and immobilization of hepatitis B ligands, sensitivity, cut-off value (CoV) criteria, as well as testing of patient sera. A systematic hepatitis B markers detection method by means of BIE was thus established.

Abbreviations: anti-HBc, hepatitis B core antibody; anti-HBe, hepatitis B e antibody; anti-HBs, hepatitis B surface antibody; APTES, 3-aminopropyltriethoxy-silane; BIE, biosensor based on imaging ellipsometry; BSA, bovine serum albumin; CoVs, cut-off values; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; ELISA, enzyme-linked immunosorbent assay; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; IE, imaging ellipsometry; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 1% Tween 20; ROCC, receiver operating characteristic curves.

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Table 1 Clinical information of hepatitis B patients from Shandong Provincial Hospital

Patients' no.	Age	Sex	HBV markers ('+'is positive; '–' is negative: '+/–' is uncertain)					Time of sampling	Time of ELISA detection
			HBsAg	HBsAb	HBeAg	HBeAb	HBcAb		
P001	47	Woman	+	–	+	–	+	14 December 2006	14 December 2006
P002	34	Woman	+	–	–	+	+	13 December 2006	14 December 2006
P003	22	Woman	+	–	+	–	+	13 December 2006	14 December 2006
P004	12	Man	+	–	–	–	+	14 December 2006	14 December 2006
P005	50	Man	+	–	–	+	+	14 December 2006	14 December 2006
P006	22	Woman	+	–	+	–	–	14 December 2006	14 December 2006
P007	27		+	–	+	–	+	14 December 2006	14 December 2006
P008	43	Woman	+	–	–	+	–	13 December 2006	14 December 2006
P009	23	Woman	+	–	+	–	–	14 December 2006	14 December 2006
P010	25	Woman	+	–	+	–	–	14 December 2006	14 December 2006
P011	50	Woman	+	–	–	+	+	14 December 2006	14 December 2006
P012	21	Woman	+	–	+	–	+	15 December 2006	15 December 2006
P013	38	Man	+	–	–	–	+	15 December 2006	15 December 2006
P014	30	Man	+	–	–	+	+	15 December 2006	15 December 2006
P015	20	Man	+	–	–	+	+	15 December 2006	15 December 2006
P016	48	Man	+	–	–	+	+	15 December 2006	15 December 2006
P017		Woman	+	–	+	–	+	15 December 2006	15 December 2006
P018	44	Woman	+	–	–	+	+	15 December 2006	15 December 2006
P019		Woman	+	–	–	+	+	15 December 2006	15 December 2006
P020	62	Man	+	–	–	+	+	19 December 2006	19 December 2006
P021	56	Woman	+	–	–	+	+	19 December 2006	19 December 2006
P022	18	Man	+	–	–	+	+	19 December 2006	19 December 2006
P023	47	Man	+	–	–	+	+	19 December 2006	19 December 2006
P024	44	Man	+	–	+	–	+	19 December 2006	19 December 2006
P025	53	Man	+	–	–	+	+	19 December 2006	19 December 2006
P026	35	Man	+	–	–	+	+	19 December 2006	19 December 2006
P027	21	Woman	+	–	–	+	+	19 December 2006	19 December 2006
P028	52	Man	+	–	–	–	+	19 December 2006	19 December 2006
P029	66	Woman	+	–	–	+	+	19 December 2006	19 December 2006
P030	40	Man	+	–	–	+	+	20 December 2006	20 December 2006
P031	24	Woman	+	–	–	–	+	20 December 2006	20 December 2006
P032	20	Woman	+	–	–	+	+	20 December 2006	20 December 2006
P033	17	Man	+	–	–	–	+	20 December 2006	20 December 2006
P034	30	Woman	+	–	–	+	+	20 December 2006	20 December 2006
P035	31	Woman	+	–	+	–	+	20 December 2006	20 December 2006
P036	50	Woman	+	–	–	+	+	20 December 2006	20 December 2006
P037	42	Man	+	–	–	+	+	20 December 2006	20 December 2006
P038	34	Woman	+	–	+	–	+	20 December 2006	20 December 2006
P039	72	Man	+	–	–	–	+	20 December 2006	20 December 2006
P040	32	Woman	+	–	–	+	+	20 December 2006	20 December 2006
P041	60	Man	+	–	–	–	+	20 December 2006	20 December 2006
P042	32	Man	+	–	–	+	+	21 December 2006	21 December 2006
P043	20	Woman	+	–	+	–	+	21 December 2006	21 December 2006
P044	20	Woman	+	–	+	–	+	21 December 2006	21 December 2006
P045	30	Man	+	–	–	+	+	21 December 2006	21 December 2006
P046	38	Man	+/–	–	–	+	+	21 December 2006	21 December 2006
P047	48	Man	+	–	+	–	+	21 December 2006	21 December 2006
P048	41	Man	+	–	–	–	+	21 December 2006	21 December 2006
P049	19	Woman	+	–	+	–	+	21 December 2006	21 December 2006
P050	18	Woman	+	–	+	–	+	21 December 2006	21 December 2006

Table 1 (Continued)

Patients' no.	Age	Sex	HBV markers ('+'is positive; '–' is negative: '+/–' is uncertain)					Time of sampling	Time of ELISA detection
			HBsAg	HBsAb	HBeAg	HBeAb	HBcAb		
P051	32	Woman	+	–	–	+	+	21 December 2006	21 December 2006
P052	67	Woman	+	–	–	+	+	21 December 2006	21 December 2006
P053	38	Man	+	–	+	–	+	22 December 2006	22 December 2006
P054	25	Woman	+	–	–	+	+	22 December 2006	22 December 2006
P055	26	Man	+	–	+	–	+	22 December 2006	22 December 2006
P056	58	Man	+	–	–	–	+	22 December 2006	22 December 2006
P057	53	Man	+	–	+	–	+	22 December 2006	22 December 2006
P058	37	Woman	+	–	–	+	+	22 December 2006	22 December 2006
P059	48	Man	+	–	–	+	+	26 December 2006	26 December 2006
P060	48	Man	+	–	–	+	+	1 January 2007	1 January 2007

60 patients were detected with ELISA kit produced by Beijing Wantai Co Ltd.

Table 2 Clinical information of hepatitis B patients from Tientsin Blood Disease Hospital

Patients' no.	Age	HBV markers (ELISA OD/CoV)					Time of sampling	Time of ELISA detection
		HBsAg	HBsAb	HBeAg	HBeAb	HBcAb		
1	52	0.36	33.70	0.08	0.29	0.02	21 December 2006	22 December 2006
2	40	0.41	11.10	0.14	1.73	0.30	21 December 2006	22 December 2006
3	25	7.51	0.14	0.13	0.01	0.10	21 December 2006	22 December 2006
4	53	5.71	0.08	0.08	0.73	0.02	21 December 2006	22 December 2006
5	58	0.15	30.90	0.08	0.70	0.03	21 December 2006	22 December 2006
6	61	0.10	21.70	0.13	0.13	0.03	21 December 2006	22 December 2006
8	63	0.25	3.77	0.14	0.45	0.06	21 December 2006	22 December 2006
9	46	43.80	0.08	0.08	1.52	0.01	22 December 2006	25 December 2006
10	75	0.21	41.70	0.98	0.03	0.42	23 December 2006	25 December 2006
11	54	37.50	0.11	31.90	1.69	0.01	23 December 2006	25 December 2006
12	51	42.90	0.10	0.10	0.02	0.01	23 December 2006	25 December 2006
13	30	38.60	0.98	30.70	1.01	0.01	23 December 2006	25 December 2006
14	49	42.70	0.10	0.10	2.11	0.01	23 December 2006	25 December 2006
15	64	23.70	0.24	6.87	2.87	0.03	23 December 2006	25 December 2006
16	69	38.40	0.18	0.65	1.83	0.01	23 December 2006	25 December 2006
17	40	40.90	0.12	0.11	0.17	0.01	23 December 2006	25 December 2006
19	46	48.50	0.17	37.90	1.80	0.03	24 December 2006	25 December 2006
20	69	48.90	0.21	0.05	0.60	0.01	24 December 2006	25 December 2006
21	39	45.30	0.08	0.32	1.03	0.01	24 December 2006	25 December 2006
22	60	0.11	3.47	0.19	0.69	0.05	25 December 2006	26 December 2006
23	57	24.20	0.07	0.20	0.02	0.42	25 December 2006	26 December 2006
24	19	13.60	0.06	18.90	1.01	0.02	25 December 2006	26 December 2006
25	41	25.30	0.74	19.20	1.01	0.01	26 December 2006	27 December 2006
26	47	0.11	20.20	0.24	0.55	0.99	26 December 2006	27 December 2006
27	50	0.11	34.90	0.03	0.02	0.02	26 December 2006	27 December 2006
28	56	15.50	0.01	0.20	0.51	0.01	26 December 2006	27 December 2006
29	32	0.49	26.50	0.10	0.47	0.01	26 December 2006	27 December 2006
30	64	27.50	0.23	0.16	0.02	0.02	28 December 2006	28 December 2006
31	34	35.40	0.13	0.21	0.01	0.01	28 December 2006	28 December 2006
32	54	39.70	0.21	8.35	1.24	0.02	28 December 2006	28 December 2006
33	56	0.11	2.79	0.88	0.49	0.19	28 December 2006	28 December 2006
34	50	0.11	21.90	0.08	0.02	0.02	28 December 2006	28 December 2006

Table 2 (Continued)

Patients' no.	Age	HBV markers (ELISA OD/CoV)					Time of sampling	Time of ELISA detection
		HBsAg	HBsAb	HBeAg	HBeAb	HBcAb		
35	61	41.30	0.08	0.09	0.01	0.01	28 December 2006	28 December 2006
36	41	38.90	0.29	0.10	0.02	0.03	28 December 2006	29 December 2006
37	49	0.08	7.50	0.01	1.16	0.82	28 December 2006	29 December 2006
38	21	26.80	0.18	7.28	1.01	0.03	28 December 2006	29 December 2006
39	51	0.06	0.14	0.01	0.34	0.03	28 December 2006	29 December 2006
40	28	0.04	5.83	0.06	0.01	0.02	29 December 2006	29 December 2006
41	23	37.70	0.12	0.11	0.70	0.02	30 December 2006	30 December 2006
42	70	0.24	37.20	0.18	0.05	0.99	30 December 2006	30 December 2006
43	42	0.11	0.18	0.12	0.58	0.05	29 December 2006	29 December 2006
44	30	0.09	41.60	0.15	0.15	0.48	30 December 2006	30 December 2006
45	73	0.19	1.85	0.04	0.77	0.01	30 December 2006	30 December 2006
46	54	35.80	0.08	0.08	0.01	0.01	31 December 2006	31 December 2006
47	41	19.20	0.10	6.32	1.01	0.11	31 December 2006	31 December 2006
48	49	39.80	0.16	0.11	0.02	0.01	31 December 2006	31 December 2006
49	41	0.01	4.38	0.04	0.60	0.01	31 December 2006	31 December 2006
50	42	0.05	0.45	0.03	0.01	0.39	1 January 2007	1 January 2007
51	41	43.40	0.04	0.19	0.51	0.01	1 January 2007	1 January 2007
52	36	0.03	14.70	0.01	1.02	0.07	1 January 2007	1 January 2007
53	60	0.19	3.01	0.12	0.97	0.48	4 January 2007	4 January 2007
54	50	0.21	21.70	0.14	0.02	0.01	4 January 2007	4 January 2007
55	42	32.00	0.10	0.04	0.01	0.01	4 January 2007	4 January 2007
56	55	45.60	0.19	0.32	0.62	0.01	4 January 2007	4 January 2007
57	50	36.90	0.06	0.44	0.83	0.02	4 January 2007	4 January 2007
58	27	39.50	0.13	0.13	0.01	0.02	4 January 2007	4 January 2007
59	28	38.90	0.08	0.10	0.01	0.01	4 January 2007	4 January 2007
60	23	13.10	0.05	13.30	1.01	0.02	4 January 2007	4 January 2007
61	52	0.26	4.66	0.04	0.01	0.01	4 January 2007	4 January 2007
62	44	0.00	21.40	0.05	0.49	0.01	1 January 2007	1 January 2007
63	48	0.04	20.80	0.07	0.07	0.01	4 January 2007	4 January 2007
64	34	12.80	0.09	7.67	1.01	0.01	4 January 2007	4 January 2007
65	44	41.30	0.08	0.48	0.62	0.01	4 January 2007	4 January 2007
66	62	38.90	0.06	0.10	0.14	0.01	4 January 2007	4 January 2007
67	54	0.12	25.80	0.03	0.48	0.01	4 January 2007	4 January 2007
68	50	0.21	31.60	0.12	0.02	0.03	4 January 2007	4 January 2007
69	45	0.07	19.20	0.06	0.34	0.03	4 January 2007	4 January 2007
70	46	0.10	30.40	0.12	0.01	0.01	4 January 2007	4 January 2007
71	60	0.12	37.90	0.14	0.02	0.01	4 January 2007	4 January 2007
72	65	0.08	14.10	0.04	0.43	0.59	4 January 2007	4 January 2007
73	58	0.11	45.30	0.08	0.37	0.01	4 January 2007	4 January 2007
74	45	39.00	0.17	0.10	0.09	0.01	5 January 2007	5 January 2007
75	52	0.12	23.20	0.13	0.05	0.02	5 January 2007	5 January 2007
76	50	0.10	2.90	0.11	1.01	0.35	5 January 2007	5 January 2007
77	/	0.08	3.06	0.09	0.32	0.38	5 January 2007	5 January 2007
78	46	44.60	0.08	0.08	0.01	0.01	5 January 2007	5 January 2007
79	61	0.01	6.70	0.01	0.01	0.01	5 January 2007	5 January 2007
80	58	44.30	0.03	0.07	0.01	0.01	5 January 2007	5 January 2007
81	43	0.30	30.00	0.04	0.02	0.37	5 January 2007	5 January 2007
82	44	0.15	6.63	0.13	0.67	0.04	4 January 2007	4 January 2007
83	62	0.14	7.33	0.15	1.56	0.04	5 January 2007	5 January 2007
84	/	0.16	0.37	0.10	0.35	0.22	5 January 2007	5 January 2007
85	49	0.17	0.42	0.06	0.04	0.44	5 January 2007	5 January 2007

Table 2 (Continued)

Patients' no.	Age	HBV markers (ELISA OD/CoV)					Time of sampling	Time of ELISA detection
		HBsAg	HBsAb	HBeAg	HBeAb	HBcAb		
86	40	0.06	40.60	0.11	0.02	0.14	5 January 2007	5 January 2007
87	47	0.06	32.70	0.09	1.10	0.01	8 January 2007	8 January 2007
88	36	0.09	37.30	0.10	0.05	1.78	8 January 2007	8 January 2007
89	45	49.10	0.10	0.09	0.01	0.16	8 January 2007	8 January 2007
90	70	17.90	0.15	0.11	0.06	0.03	8 January 2007	8 January 2007
92	73	51.30	0.04	0.38	0.99	0.01	8 January 2007	8 January 2007
93	79	0.14	11.30	0.06	0.11	0.14	8 January 2007	8 January 2007
94	37	0.10	27.30	0.08	1.14	0.07	8 January 2007	8 January 2007
95	54	0.41	39.50	0.13	0.01	0.01	8 January 2007	8 January 2007
96	47	0.19	32.20	0.08	0.05	0.02	8 January 2007	8 January 2007
97	55	0.25	37.60	0.10	0.56	0.05	8 January 2007	8 January 2007
98	44	0.14	20.10	0.18	0.76	0.22	8 January 2007	8 January 2007
99	30	22.40	0.30	46.80	1.01	0.02	8 January 2007	8 January 2007
100	66	33.60	0.30	0.23	0.01	0.01	8 January 2007	8 January 2007
101	46	0.13	37.10	0.25	0.38	0.02	9 January 2007	9 January 2007
102	31	18.70	0.22	37.40	1.01	0.17	9 January 2007	9 January 2007
103	34	31.20	0.13	0.20	0.45	0.01	9 January 2007	9 January 2007
104	36	0.11	0.98	0.20	0.01	0.31	9 January 2007	9 January 2007
105	59	33.60	0.09	0.18	0.88	0.06	9 January 2007	9 January 2007
106	59	30.80	0.13	0.07	0.04	0.02	9 January 2007	9 January 2007
107	43	0.25	34.50	0.07	0.44	0.01	11 January 2007	11 January 2007
108	24	35.90	0.13	20.50	0.41	0.90	11 January 2007	11 January 2007
109	65	0.25	25.40	0.21	0.60	0.22	11 January 2007	11 January 2007
110	55	64.00	0.18	0.14	0.01	0.01	11 January 2007	11 January 2007
111	33	39.00	0.25	0.02	0.01	0.01	11 January 2007	11 January 2007
112	53	0.06	1.02	0.01	0.10	0.71	10 January 2007	10 January 2007

109 patients (absence of patients' no.: 7, 18, 91) were detected by ELISA kit produced by Shanghai Rongsheng Biotech CoLtd. The absence of information was marked as '/'. ELISA OD/CoV of HBV markers was presented. If the value of HBeAg, HBsAb and HBsAg detected with noncompetitive ELISA method was equal or greater than 1, the result was considered as positive. If the value of HBcAb and HBeAb detected with competitive ELISA method was equal or lesser than 1, the result was considered as positive.

PATIENTS AND METHODS

Study subjects, sample collection and reagents

Silicon wafers were purchased from the Luoyang Monocrystalline Silicon Factory (Luo, China). H_2O_2 (30%), H_2SO_4 (98%) and absolute ethanol were purchased from Beijing Bei Hua Fine Chemicals Co Ltd (Beijing, China). 3-Aminopropyltriethoxy-silane (APTES, 99%, v/v), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from ACROS (Acros Organics, Geel, Belgium). Succinic anhydride was purchased from Beijing Hengye Zhongyuan Chemical Co Ltd (Beijing, China). All chemicals were of analytical grade. Hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (anti-HBs), hepatitis B core antigen (HBcAg), hepatitis B core antibody (anti-HBc), hepatitis B e antigen (HBeAg) and hepatitis B e antibody (anti-HBe) ligands were purchased from Beijing Hotgen Biotechnology Co Ltd (China). Anti-HBs and HBsAg national reference samples (1 and 2 ng/mL respectively) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijin, China). All sera with available ELISA results were from Shandong Provincial Hospital and Tientsin Blood Disease Hospital (China). ELISA kits were purchased from Beijing Wantai Biological Pharmacy Enterprise Co Ltd (authorized no.: S10980089) and Shanghai Rongsheng Biotech Co Ltd (S10950044) (China). Patient demographic data can be found in Tables 1 and 2. Tween 20 and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St Louis, MO, USA), phosphate-buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), PBS with 1% Tween 20 (PBST) and NE (0.05 mol/mL NHS, 0.2 mol/mL EDC) were prepared

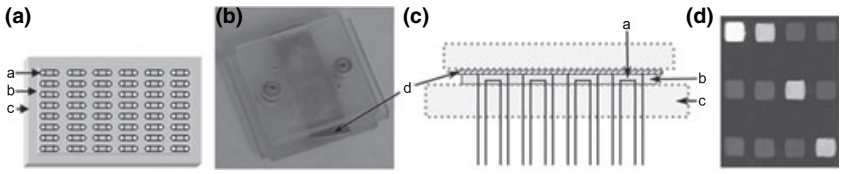


Fig. 1 Microfluidic system. (a) Micro-reaction interface; (b) module for microarray; (c) schematic illustration of the microfluidic system; (d) a sample of protein microarray prepared by using the microfluidic system and grey-scale image obtained using IE. 'a' is small cell; 'b' is polydimethyl-siloxane film; 'c' is organic glass with small holes; 'd' is silicon wafer.

in deionized water (18.3 MΩcm, Milli-Q plus system; Millipore, Bedford, MA, USA).

BIE detection procedure and principle

First, silicon wafers were chosen as the substrate and modified by chemical reagents forming reactive groups on its surface. Second, the modified substrate was put in a microfluidic system and its surface was patterned in an array format. Hepatitis B ligands were immobilized in different areas. Third, sera were passed through the ligand areas and hepatitis B markers were captured. Finally, the wafer was taken from the microfluidic system and placed in the imaging ellipsometry (IE) apparatus to analyse the results.

Imaging ellipsometry is a display technique for ultrathin film and surface characterization [15,16]. The incident wave of polarized light beam used as probe irradiates the substrate and is thus modified, which makes the reflective or transmission beam carry relevant sample information, for example, protein surface concentration. When IE detects a biomolecule layer, the value of reflection intensity is presented in greyscale. The variation of surface concentrations causes changes in the grey-scale value, which reflects directly the molecule mass surface concentration on the substrate. To visualize its variation across layers, the result can also be transformed to represent the corresponding distribution in three dimensions.

Substrate surface modification

The hydrophilic and hydrophobic modifications were the same as in previous studies [10]. After hydrophobic modification with APTES, a layer of densely packed amino groups is formed on the substrate [11]. After rinsing in ethanol, the

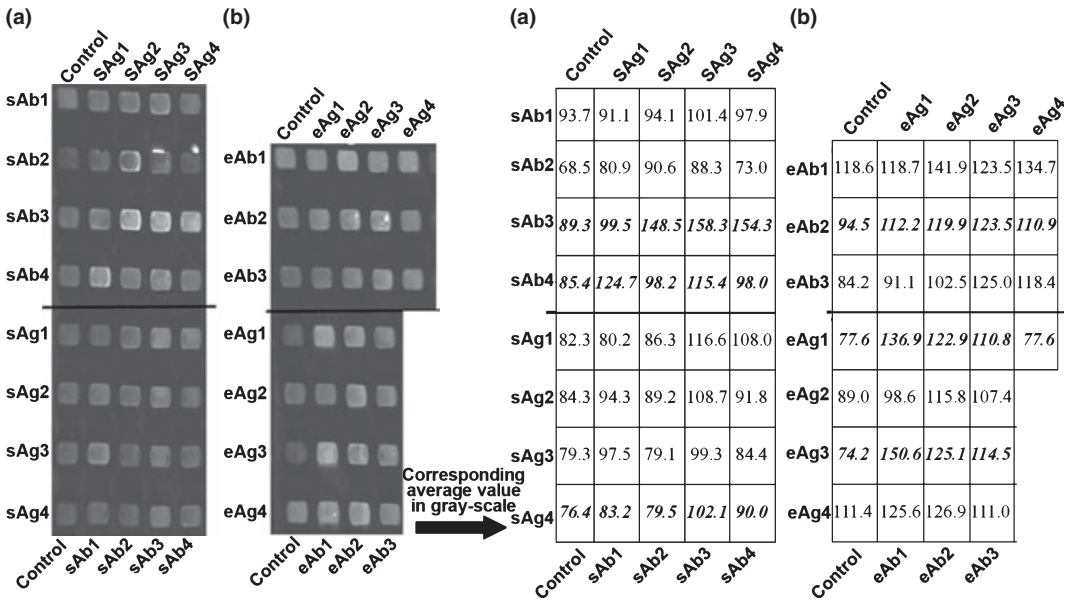


Fig. 2 Screening of hepatitis B ligands. (a) Screening of anti-HBs and HBsAg. Different lots of anti-HBs and HBsAg ligands were firstly immobilized in different lines. After blocking with BSA, the first row was used as control. Different lots of HBsAg and anti-HBs markers were detected in different rows. (b) Screening for anti-HBe and HBeAg. Anti-HBe and HBeAg ligands were immobilized in different lines. After blocking with BSA, the first row was used as control. Different lots of HBeAg and anti-HBe markers were detected in different rows. The *italics* indicate results with the largest variation in grey-scale value, which in turn indicate that the ligands had higher bioactivity.

substrate is incubated in saturated succinic anhydride solution in ethanol for 3 h. The CH_2CH_2COOCO of succinic anhydride reacts with the -NH_2 groups immobilized on the substrate and generates -$(CH_2)_3$NH-CO$(CH_2)_2$-COOH. After rinsing in ethanol, the substrate is kept in ethanol. Some carboxyl groups are formed on the substrate, which could be activated by NE (NHS and EDC) for ligand or protein immobilization. In the presence of NHS, EDC can transfer carboxyl groups to Sulfo-NHS ester, which can react with the -NH_2 groups of protein to immobilize hepatitis B ligands covalently.

Substrate pattern and immobilization of hepatitis B ligands

Hepatitis B ligands can be patterned homogeneously and simultaneously on the substrate in an array format by the microfluidic system (Fig. 1). The physical size of the patterned site is about 1.5×1 mm^2. With simple microfluidic channel junction, the microfluidic array can be used in serial or parallel formats to analyse single or multiple samples simultaneously [14].

Hepatitis B ligand screening with matrix assay

Hepatitis B ligands were immobilized on the substrate in an array format, and then their bioactivities were checked with corresponding hepatitis B markers. The process for checking the bioactivities of anti-HBs and HBsAg ligands was as follows. After carboxyl activation with NE (10 μL/area), the anti-HBs and HBsAg ligands were added to the microfluidic system (0.1 mg/mL, 10 μL/area, passing the substrate with a flowrate of 2 μL/min for 5 min). All areas were blocked with 10 mg/mL BSA for 30 min, and then some areas were chosen as control to add PBST buffer. In other areas, the corresponding pure HBsAg and anti-HBs markers were added to interact with the corresponding ligands immobilized on the substrate. These areas were rinsed with deionized water between consecutive operation steps. Then, the substrate was taken out of the microfluidic system and its surface was rinsed with deionized water. After blowing nitrogen, the result was read and analysed by IE. When the same hepatitis B marker reacted with different ligands, the results exhibited different values in the greyscale, which indicated that the ligands had different bioactivities.

Sensitivity

Anti-HBs was immobilized in six areas. All areas were blocked for 30 min with a negative serum sample (diluted 1:1 v/v with PBST). Sensing surface areas with anti-HBs were formed for HBsAg marker detection. Two areas were chosen to detect HBsAg from the national positive reference sample (1 ng/mL), and two other areas were chosen to detect the national HBsAg-negative reference sample as negative control. The remaining two areas were chosen to add PBST as blank control. National positive, negative reference samples and PBST were added (20 μL/area, 2 μL/min and 10 min respectively) and the results were read by IE.

Serum detection and CoVs

Five hepatitis B ligands (HBsAg, HBeAg and HBcAg: 0.3 mg/mL, anti-HBs and anti-HBe: 0.1 mg/mL) were immobilized in five rows. All areas were blocked with negative serum reference sample for 30 min. Different lines were chosen to detect different sera (diluted 1:15 with PBST, 20 μL/area). CoV of each marker was analysed by referring to receiver operating characteristic curves (ROCC) [17], and ELISA was used as reference test criterion. Each 96-well micro-titre ELISA plate was coated with a particular kind of hepatitis B ligand. Two wells were used as positive and negative controls, respectively, and one well was used as blank control. Other wells were used to detect sera. The remaining steps were as in the instruction manual of the ELISA kit.

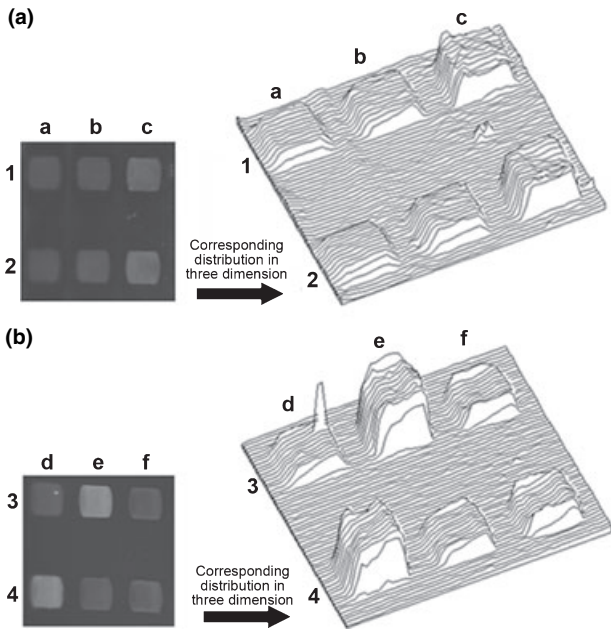


Fig. 3 Sensitivity of BIE. (a) Sensitivity in detecting HBsAg. The anti-HBs ligand was immobilized in six areas. After blocking with the national HBsAg reference negative sample, HBsAg-positive reference samples with 1 ng/mL were detected in area 'c1' and 'c2', and HBsAg reference negative samples used as negative control were detected in 'b1' and 'b2'. PBST, used as blank control, was added to 'a1' and 'b1'. (b) Sensitivity in detecting anti-HBs. The anti-HBs positive reference sample with 1 IU/mL was detected in 'd4' and 'e3', whilst the negative samples were detected in 'd3' and 'e4'. PBST was added to 'f3' and 'f4'.

RESULTS

Hepatitis B high bioactive ligand screening

To achieve high detection rates, we selected ligands that could interact with most of the corresponding markers. From the analysis of grey-scale values, high bioactive anti-HBs ligands are in lines 3 and 4, and HBsAg in line 8 (Fig. 2a). A high bioactive anti-HBe ligand is seen in line 10, and HBeAg in lines 12 and 14 (Fig. 2b). HBcAg ligand can also be screened in a similar fashion.

Sensitivity

The sensitivity in detecting HBsAg reached 1 ng/mL (Fig. 3a). The value in greyscale for the blank control (area a1 and a2) was 63.3 ± 0.1. The value for the negative control (area b1 and b2) was 66.7 ± 1.7, about 5% higher than that of the blank control. The value for the positive sample (1 ng/mL, subtype ad, area c1 and c2) was 83.4 ± 0.4, about 31.8% and 25.0% higher than the blank and negative control respectively, which indicated that a sample of the order of 1 ng/mL could be detected. The sensitivity of anti-HBs was also tested (Fig. 3b). The values for the blank control, negative control and positive sample were 69.5 ± 1.0, 70.0 ± 0.2 and 113.9 ± 1.3 respectively, suggesting that the sensitivity might be better than 1 IU/mL.

Clinical hepatitis B serum detection

Sixty hepatitis B patients (Table 1) with qualitative results by ELISA were tested with BIE and an automatic BIE operation process was established as follows: immobiliza-

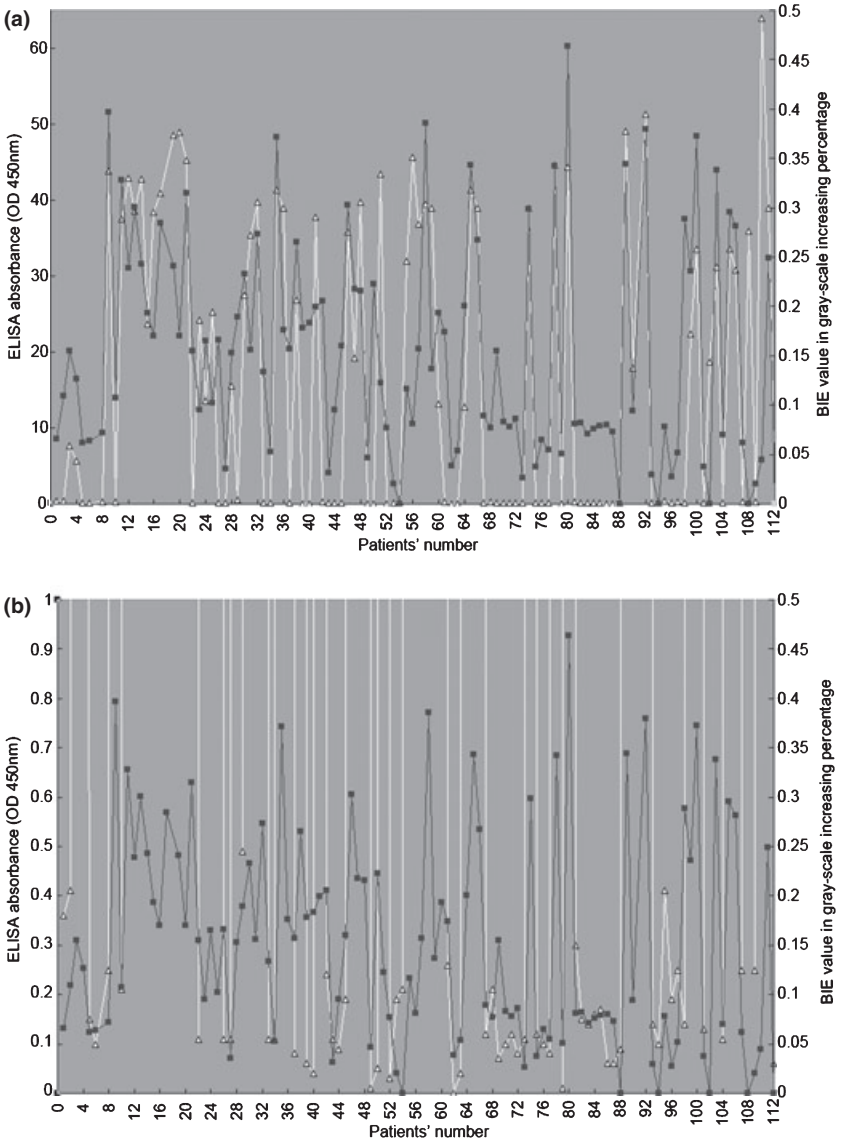


Fig. 4 Comparison of HBsAg detection by BIE (- ■ -) and by ELISA (- Δ -). (a) Original image; (b) enlarged image of the lowest part of image (a).

Table 3 HBsAg detection results of BIE and ELISA. Data of BIE were the variation of value in greyscale above the corresponding ligand control. Data of ELISA were the variation of OD above the corresponding ligand control

BIE	ELISA	BIE	ELISA	BIE	ELISA	BIE	ELISA	BIE	ELISA
0.07	0.36	0.10	25.3	0.22	19.2	0.16	0.07	0.38	51.3
0.11	0.41	0.17	0.11	0.22	39.8	0.08	0.1	0.03	0.14
0.16	7.51	0.04	0.11	0.05	0.01	0.08	0.12	0	0.1
0.13	5.71	0.15	15.5	0.22	0.05	0.09	0.08	0.08	0.41
0.06	0.15	0.19	0.49	0.12	43.4	0.03	0.11	0.03	0.19
0.06	0.1	0.23	27.5	0.08	0.03	0.30	39	0.06	0.25
0.07	0.25	0.16	35.4	0.02	0.19	0.04	0.12	0.29	0.14
0.40	43.8	0.27	39.7	0	0.21	0.07	0.1	0.24	22.4
0.11	0.21	0.13	0.11	0.12	32	0.05	0.08	0.37	33.6
0.33	37.5	0.05	0.11	0.08	45.6	0.34	44.6	0.04	0.13
0.24	42.9	0.37	41.3	0.16	36.9	0.05	0.01	0	18.7
0.30	38.6	0.18	38.9	0.39	39.5	0.46	44.3	0.34	31.2
0.24	42.7	0.16	0.08	0.14	38.9	0.08	0.3	0.07	0.11
0.19	23.7	0.27	26.8	0.19	13.1	0.08	0.15	0.30	33.6
0.17	38.4	0.18	0.06	0.17	0.26	0.07	0.14	0.28	30.8
0.28	40.9	0.18	0.04	0.04	0	0.08	0.16	0.06	0.25
0.24	48.5	0.20	37.7	0.05	0.04	0.08	0.17	0	35.9
0.17	48.9	0.21	0.24	0.20	12.8	0.08	0.06	0.02	0.25
0.31	45.3	0.03	0.11	0.34	41.3	0.07	0.06	0.04	64
0.15	0.11	0.09	0.09	0.27	38.9	0	0.09	0.25	39
0.09	24.2	0.16	0.19	0.09	0.12	0.34	49.1	0	0.06
0.16	13.6	0.30	35.8	0.08	0.21	0.09	17.9		

tion of ligand (10 μL, 2 μL/min, 5min), blocking of substrate (40 μL, 2 μL/min, 20 min), sample detection (20 μL, 2 μL/min, 10 min) and rinsing with deionized water between consecutive operation steps (60 μL, 20 μL/min, 3 min). Five markers from seven patients could be detected on a single substrate in 1 h or less. With this operation process, 109 samples (Table 2) were detected. The HBsAg detection results using BIE were compared with those of ELISA, as shown in Fig. 4, and Table 3. Regression analysis showed that the results were in quite good agreement between the two methods ($r = 0.67 > r_{0.01} = 0.247$). The degree of agreement between BIE and ELISA in detecting HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc was 90.3%, 90.2%, 71.3%, 90.6% and 94.4% respectively.

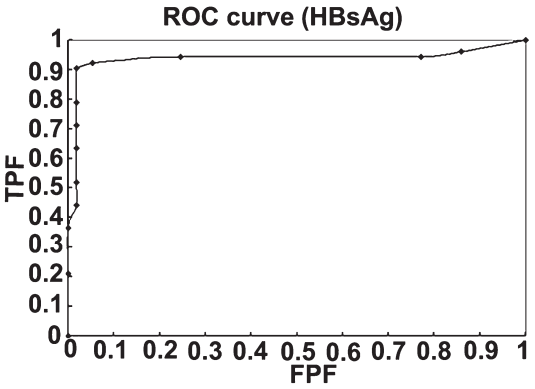
Cut-off values for HBsAg and anti-HBs were derived from ROCC (Fig. 5). The CoV was determined by the value in greyscale above the corresponding ligand control. When both the positive likelihood ratio and Youden's index reached their maxima, the corresponding variation in grey-scale value was taken as the best CoV point [17], as shown in Fig. 5. If there were two maxima of the same value, the bigger variation in grey-scale value was taken as the best CoV point to avoid false positives. The values of CoV for HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc were 15%, 18%, 15%, 20% and 15% respectively.

DISCUSSION

Ligand screening has applications in clinical diagnosis of hepatitis B and is also helpful for developing BIE. At present, various kinds of ligands have been produced by various factories, but their bioactivity has large diversity even among different lots of the same products. Most ligands are suited to conventional methods only, and few ligands match BIE. When the same marker is detected, different ligands on the substrate exhibit different bioactivity. Polyclonal antibody ligands cannot guarantee full sensitivity [18], and artificial ligands cannot exhibit entirely natural bioactivity. If a ligand has high bioactivity, it is possible to achieve higher sensitivity and efficiency, which can be verified by the HBsAg Elecsys assay [19]. If ligands were not screened, marker detection would have high false positive rates and low sensitivity, and thus ligand screenings are necessary for successful BIE.

Cut-off values and sensitivity are important for hepatitis B marker detection. CoVs can help us to distinguish strong positive, near cut-off and negative samples. ELISA kits have different CoVs for different markers and BIE also gives different CoVs, which are helpful in distinguishing each marker accurately. ELISA has detection limits as low as 1 ng/mL of HBsAg [20] and 10 mIU/mL of anti-HBs [21]. However, it must be noted that sensitivity for HBsAg detection has not been evidently improved in the last 10 years, and the most

HBsAg detection variation value in gray-scale	TPF	FPF	Positive likelihood ratio	Youden's idex
3%	0.9615	0.8596	1.1185	0.1019
5%	0.9423	0.7719	1.2207	0.1704
8%	0.9423	0.2456	3.8365	0.6967
10%	0.9231	0.0526	17.5385	0.8704
13%	0.9038	0.0175	51.5192	0.8863
15%	0.9038	0.0175	51.5192	0.8863
18%	0.7885	0.0175	44.9423	0.7709
20%	0.7115	0.0175	40.5577	0.6940
23%	0.6346	0.0175	36.1731	0.6171
25%	0.5192	0.0175	29.5962	0.5017
28%	0.4423	0.0175	25.2115	0.4248
30%	0.3654	0.0000		0.3654
35%	0.2115	0.0000		0.2115



HBsAb detection variation value in gray-scale	TPF	FPF	Positive likelihood ratio	Youden's idex
3%	1.0000	1.0000	1.0000	0.0000
5%	1.0000	0.9310	1.0741	0.0690
8%	0.9804	0.8276	1.1846	0.1528
10%	0.9608	0.6724	1.4289	0.2884
13%	0.9412	0.3966	2.3734	0.5446
15%	0.9216	0.2414	3.8179	0.6802
18%	0.9020	0.1379	6.5392	0.7640
20%	0.8431	0.1034	8.1503	0.7397
23%	0.7255	0.0690	10.5196	0.6565
25%	0.6863	0.0517	13.2680	0.6346
28%	0.6275	0.0345	18.1961	0.5930
30%	0.5882	0.0345	17.0588	0.5538
35%	0.4706	0.0345	13.6471	0.4361

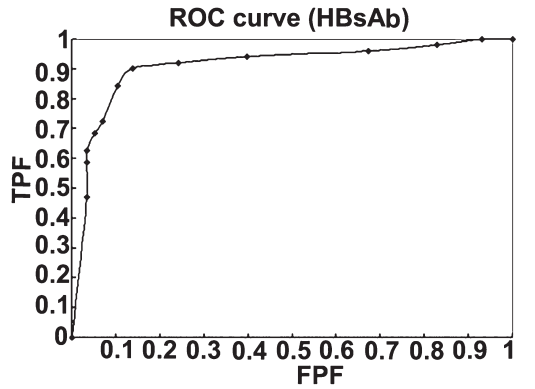


Fig. 5 CoVs for HBsAg and anti-HBs detection. The variation in grey-scale values for HBsAg and anti-HBs detection is above the blank control of ligand. TPF is true positive fraction/rate; FPF is false positive fraction/rate; Positive likelihood ratio is TPF divided by FPF (TPF/FPF); Youden's index is TPF minus FPF (TPF − FPF). The CoV is indicated by the lines with dark background.

sensitive detection gained market approval in 1995 [22,23]. The HBsAg detection limit of newly licensed tests in Europe is now under 0.15 ng/mL (enzyme-linked fluorescent immunoassay) [24]. Compared with the label methods, although the sensitivity of BIE has already reached clinical standard, it is still at a low level, and further improvements in sensitivity are still needed.

Biosensor based on imaging ellipsometry is able to realize multiplexed analysis, a simplified process and short test time. It can detect five markers of several patients simultaneously in about 1 h and higher throughput is possible with an improved setup, whereas ELISA can detect only one marker on each plate over a longer test time (ligand immobilizing and assaying lasts 1–2 days). BIE allows us to test crude samples in a label-free method, with a simple process. Furthermore, contaminating spots can also be easily discerned with the help of visual BIE images to avoid false signals. Thus, BIE has some merits over ELISA.

In conclusion, BIE has potential in the detection of hepatitis B markers with an acceptable accuracy.

ACKNOWLEDGEMENTS

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