Electronic Supplementary Material (ESI) for Chemical Science.

Improvement of enzyme-linked immunosorbent assay for multicolor detection of biomarkers

Chao Li,[a] Yucai Yang,[b] Dan Wu,[a] Yongmei Yin,[b] Genxi Li [ac*]

[a] State Key Laboratory of Pharmaceutical Biotechnology and Collaborative Innovation Center of Chemistry for Life Sciences, Department of Biochemistry, Nanjing University, Nanjing 210093, China

[b] Department of Oncology, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China

[c] Laboratory of Biosensing Technology, School of Life Sciences, Shanghai University, Shanghai 200444, China

EXPERIMENTAL METHODS

Materials and Instruments. Carboxyl graphene oxide (cGO) was purchased from Nanjing XFNANO Materials Tech Inc. Malachite green carbinol base (MGCB), methyl red (MR), phenolphthalein (PP) and thymolphthalein (TP), N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), Tween 20 were purchased from Sigma-Aldrich and were used as received. Phosphate-buffered saline (PBS, 10 ×, pH 8.0) was purchased from Sangon Biotech Inc. and was diluted 10-fold when used. Monoclonal primary antibodies (Ab1) and secondary antibodies (Ab2) were purchased from Abcam. Human immunoglobulin G (IgG) ELISA kit was obtained from Bethyl Laboratories, Inc. The 96-well PS plate was purchased from Corning Corporation. All the other reagents/materials required for the experiments were of analytical grade and used as received. Deionized water (Milli-Q grade) with a resistivity of 18.2 MΩ-cm was used throughout this study. The UV-vis spectra of cGO, MGCB, MR, PP, and TP solutions were recorded with a UV-1800 spectrophotometer (Shimadzu, Japan). The absorption intensities in the 96-well plates were collected by a Safire2 microplate reader (Tecan Group Ltd., Switzerland). Atomic force microscope (AFM) studies were performed on a Dimension Icon AFM (Bruker, Germany) under ambient conditions. X-ray photoelectron spectroscopic (XPS) measurements were performed usinga PHI 5000 VersaProbe (UlVAC-PHI, Japan). Fourier transform infrared spectroscopy (FTIR) spectra of synthesized nanomaterials on silicon substrates were measured at a resolution of 2.0 cm^{-1} using a Bruker IFS66 V/S spectrometer coupled with a HYPERION 3000 microscope (Bruker Optics Inc., Germany).

Preparation of MGCB-Ab2-cGO. The procedure of preparing all four allochroic nanoprobes was similar, so we herein only take MGCB-Ab2-cGO as an example. First, the pure cGO solution (0.1 mg mL^{-1}, 1 mL) was adjusted by K_2CO_3 to be around pH 6.0 under vigorous ultrasonication for 3 h to break down oversize layers. Then, the cGO was activated by the addition of an aqueous mixture of EDC (2 mg mL^{-1}) and NHS (4 mg mL^{-1}) with gently shaking for 20 min at room temperature. After centrifugation (13 000 rpm, 15 min) for three runs, Ab2 (0.5 mg mL^{-1}) in a PBS buffer was added to the solution. The sample was shaken at 4 °C for 6 h for sufficient immobilization. Remaining NHS-active sites of cGO were passivated with 1% BSA solution for another 3 h. The solution was centrifuged at 13 000 rpm for 20 min at 4 °C to remove any unbound biomolecules and was re-suspended with 900 µL PBS buffer. After that, a stock solution of MGCB in DMSO (25 mM, 100 µL) was added into the as-prepared Ab2-cGO solution dropwise with gently shaking to allow adsorption of MGCB onto the cGO surfaces. The mixture was shaken at room temperature for 2 h. MGCB-Ab2-cGO conjugates were formed, and an excess amount of free MGCB was removed by centrifugation (13 000 rpm, 15 min) for two runs. The resulting precipitate was dispersed in 1 mL of PBS and the solution was stored at 4 °C for further use.

The preparation of MR-Ab2-cGO, PP-Ab2-cGO, and TP-Ab2-cGO for human IgG detection was similar with MGCB-Ab2-cGO. The differences are the concentration of three stock solutions added into the as-prepared Ab2-cGO solution, which the

concentration of MR, PP, and TP was changed to 3 mM, 2 mM and 1.5 mM, respectively. Notably, higher concentration of dye molecules will lead to obvious aggregation.

Procedures of the Immunoassays. For human IgG allochroic-cGO linked immunosorbent assay (ALISA), we carried out the sandwich-type immunoassay as follows. 100 μL of the Ab1 solution (2 μg/mL) was added into each well of the 96-well PS plate. The plate was incubated at 4 °C overnight. After discarding the solutions, we washed the plate with 0.05% Tween-20 in PBS buffer (PBST) and blocked it with 5% BSA (150 μL) for 1 h at 37 °C, followed by copious rinsing with PBST solutions for three runs. After passivation, PBST solutions containing varying concentrations of protein targets were added to the Ab1-modified wells to incubate at 37 °C for 1 h. The PBST-only solution was set as the blank. Then the plate was washed with PBST for another 3 runs. In a typical ALISA, MGCB-Ab2-cGO probes were diluted with PBS containing 1% BSA to a final concentration of 50 μg mL^{-1}. A total of 50 μL MGCB-Ab2-cGO solution was added to each well. The plate was covered with a plate sealer and incubated at 37 °C for 1 h with vigorous shaking. After that, the unbound MGCB-Ab2-cGO was washed away by PBST solutions for two runs. Finally, each well was added release reagents (acidic water, pH 3.0), and the plate was shaken at 600 rpm for 3 min. The absorbance intensities were recorded by Safire2 microplate reader at 617 nm.

As for the conventional ELISA for human IgG, the detection procedure was performed according to the manufacturer's instructions. The main difference between ALISA is addition of horseradish peroxidase-labeled antibody for final detection. The absorbance intensities were recorded by Safire2 microplate reader at 450 nm.

ALISA for the Real Clinical Samples. Sera from patients who suffered from lung cancer were provided by local hospital. For single and multiplex tumor marker detection, some adjustments of nanoprobes were made for better visual discrimination. In this study, MR (or TP, or PP)-Ab2-cGO was prepared with the similar procedures of MGCB-Ab2-cGO that only changed Ab2 molecules and stock solutions of MR (30 mM), PP (20 mM), and TP (10 mM) added into the synthesized Ab2-cGO conjugates. The absorbance intensities were recorded by Safire2 microplate reader at 434 nm (MR), 552 nm (PP) and 593 nm (TP) after introducing of basic water (pH 12.0). Other processes were the same with the human IgG detection.

As for the multicolor detection of three tumor biomarkers, we performed the ALISA as follows. 100 μL of the Ab1 mixture solution (2.5 μg/mL of NSE, CEA, and Cyfra21-1 monoclonal antibodies in PBST buffer containing 0.1% BSA) was added into each well of the 96-well PS plate. The plate was incubated at 4 °C overnight. After discarding the solutions, we washed the plate with PBST buffer and blocked it with 5% BSA (150 μL) for 1 h at 37 °C, followed by copious rinsing with PBST solutions for three runs. After passivation, 100 μL patients' sera were added to the Ab1 mixture-modified wells to incubate at 37 °C for 1 h. Then the plate was washed with PBST for another 3 runs. Afterward, a cocktail of three nanoprobes solution (50 μL, 30 μg mL^{-1}) was added to each well. The plate was coveredwith a plate sealer and

incubated at 37 °C for 1 h with vigorous shaking. After that, the unbound nanoprobes were washed away by PBST solutions for two runs. Finally, each well was added release reagents (basic water, pH 12.0), and the plate was shaken at 600 rpm for 3 min. The absorbance intensities were recorded by Safire2 microplate reader at 434, 552, and 593 nm, respectively.

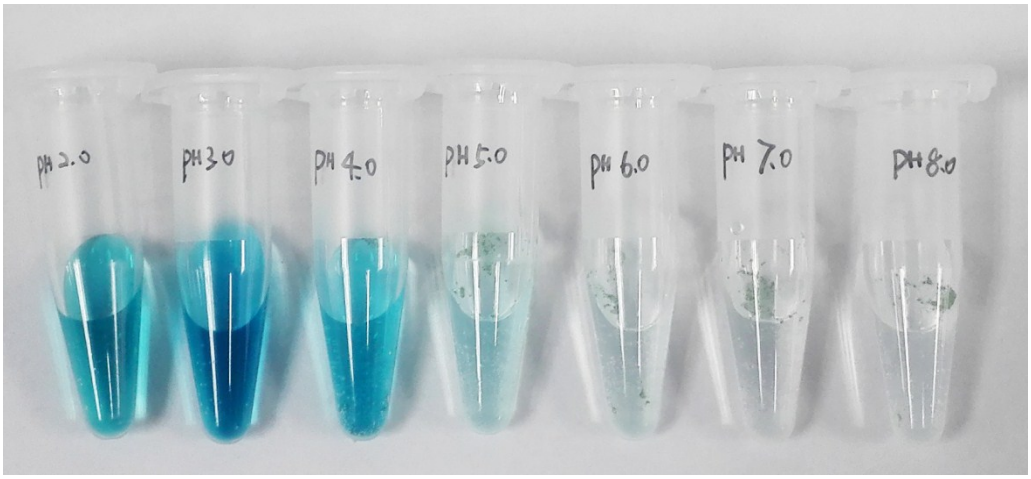

Figure S1. Photographs of MGCB solutions with pH intensities from 2.0 to 8.0 (left to right).

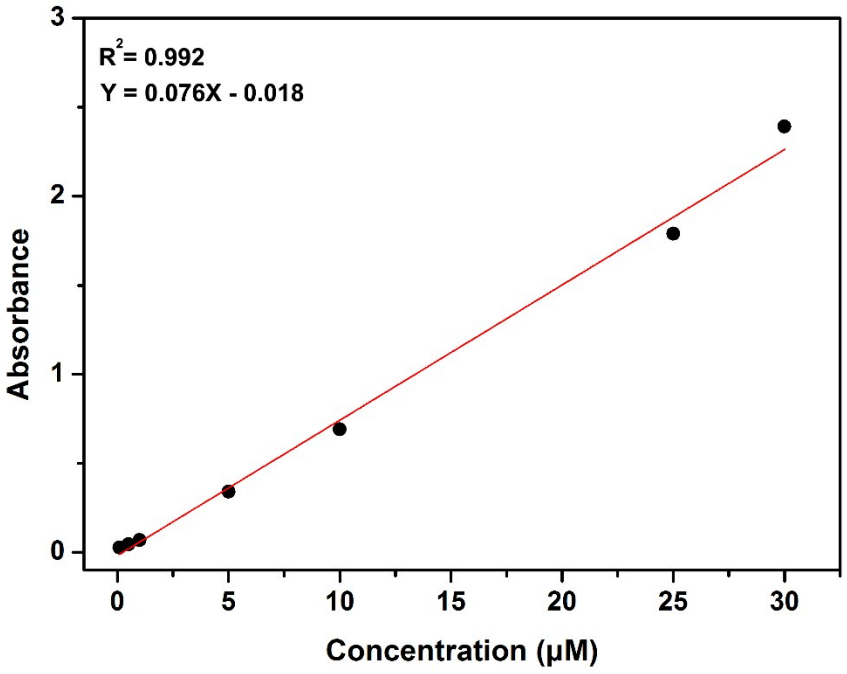

Figure S2. Plot of absorbance at 617 nm versus varying concentrations of MGCB from 0.1 to 30 µM.

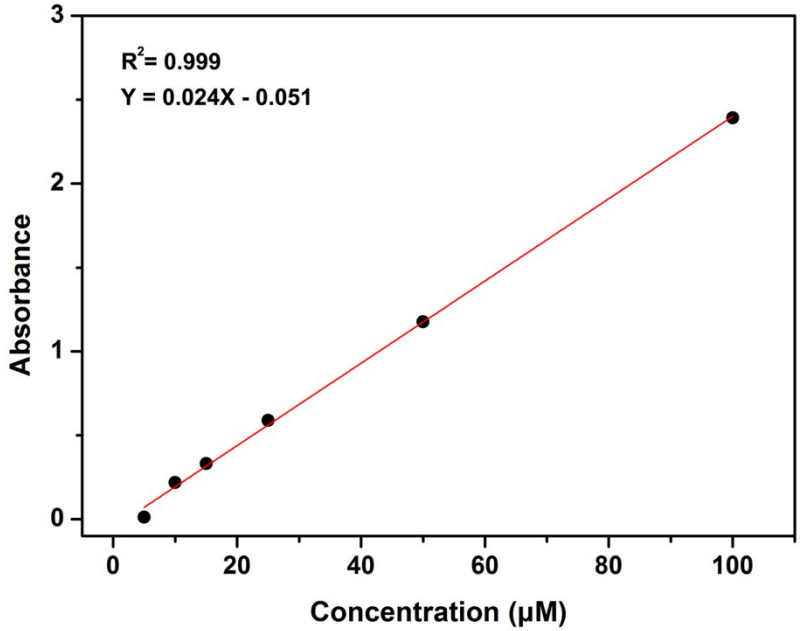

Figure S3. Plot of absorbance at 429 nm versus varying concentrations of MR from 1 to 100 µM.

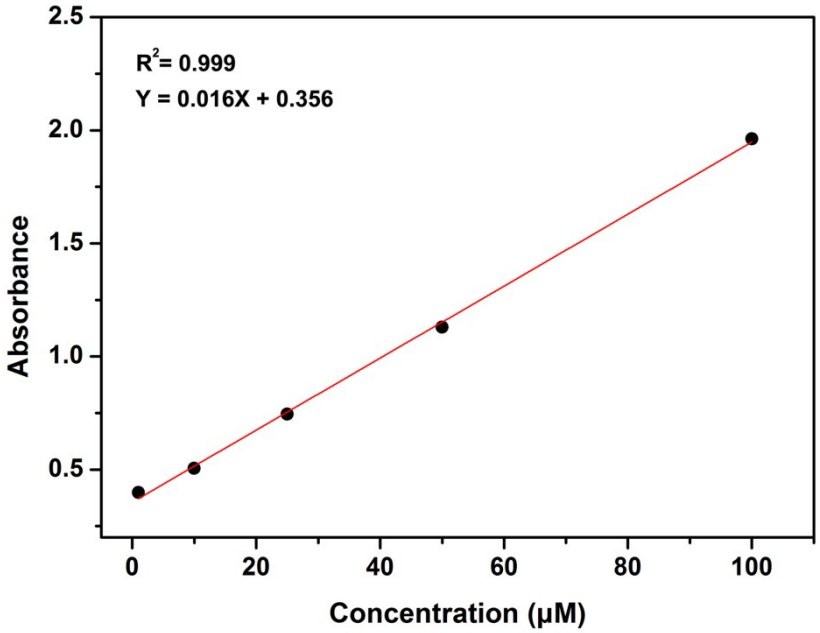

Figure S4. Plot of absorbance at 552 nm versus varying concentrations of PP from 5 to 100 µM.

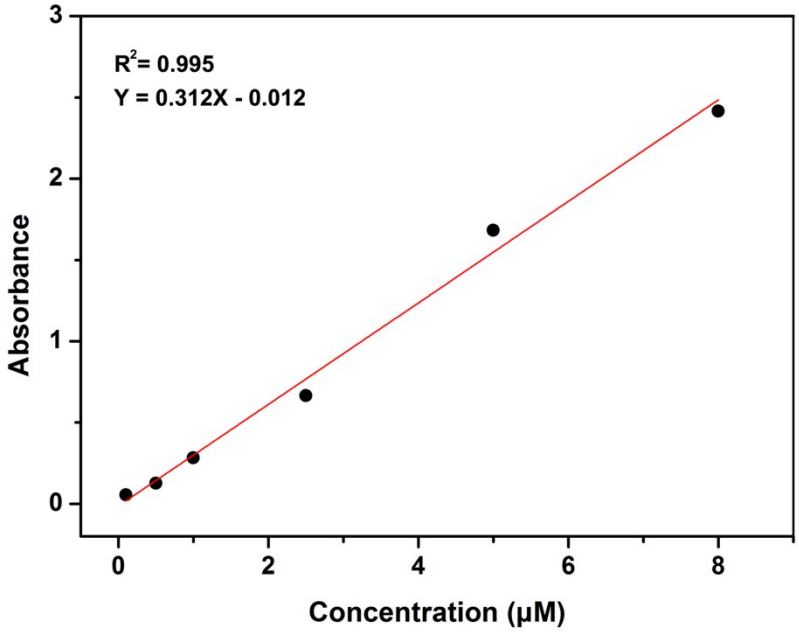

Figure S5. Plot of absorbance at 594 nm versus varying concentrations of TP from 0.1 to 8 μM.

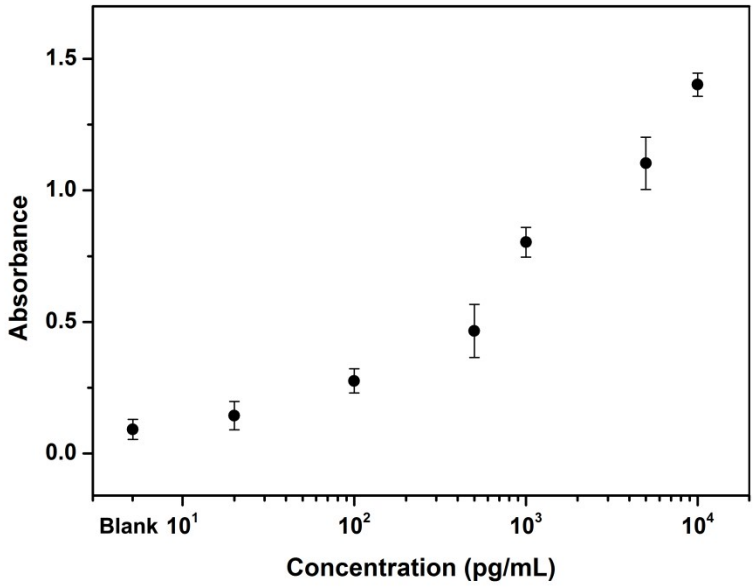

Figure S6. Detection performance of ALISA based on MR-Ab2-cGO for human IgG.

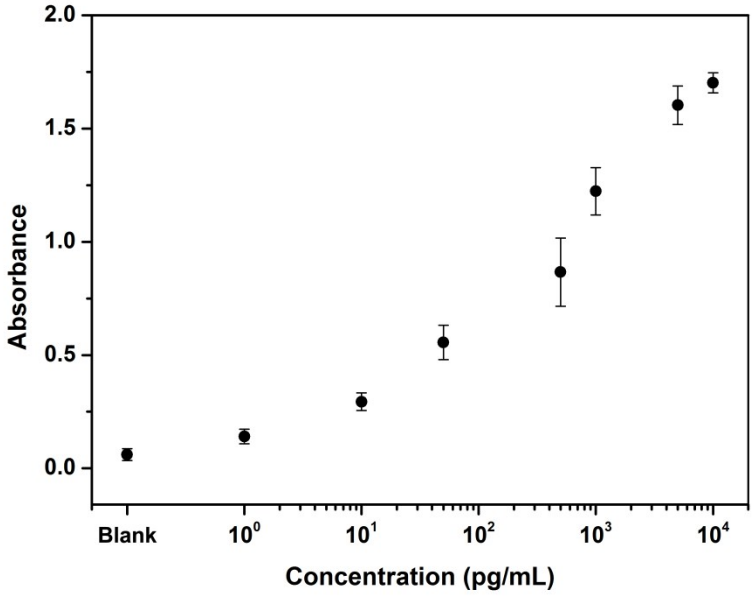

Figure S7. Detection performance of ALISA based on PP-Ab2-cGO for human IgG.

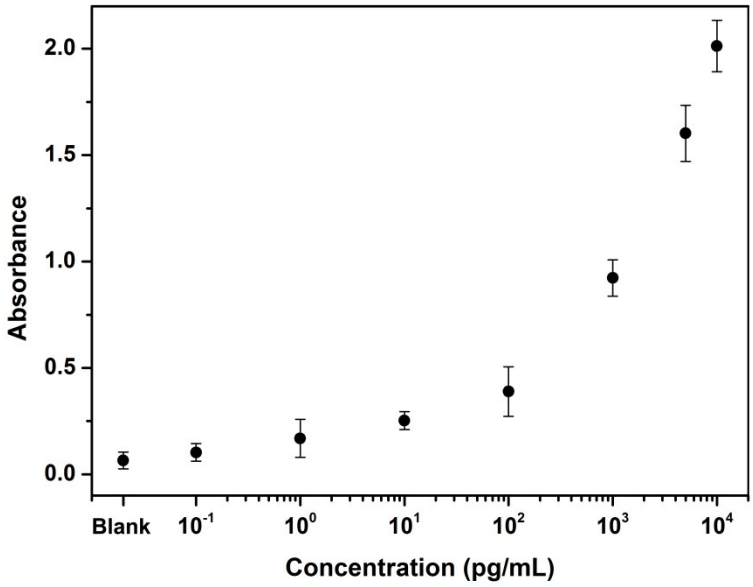

Figure S8. Detection performance of ALISA based on TP-Ab2-cGO for human IgG.

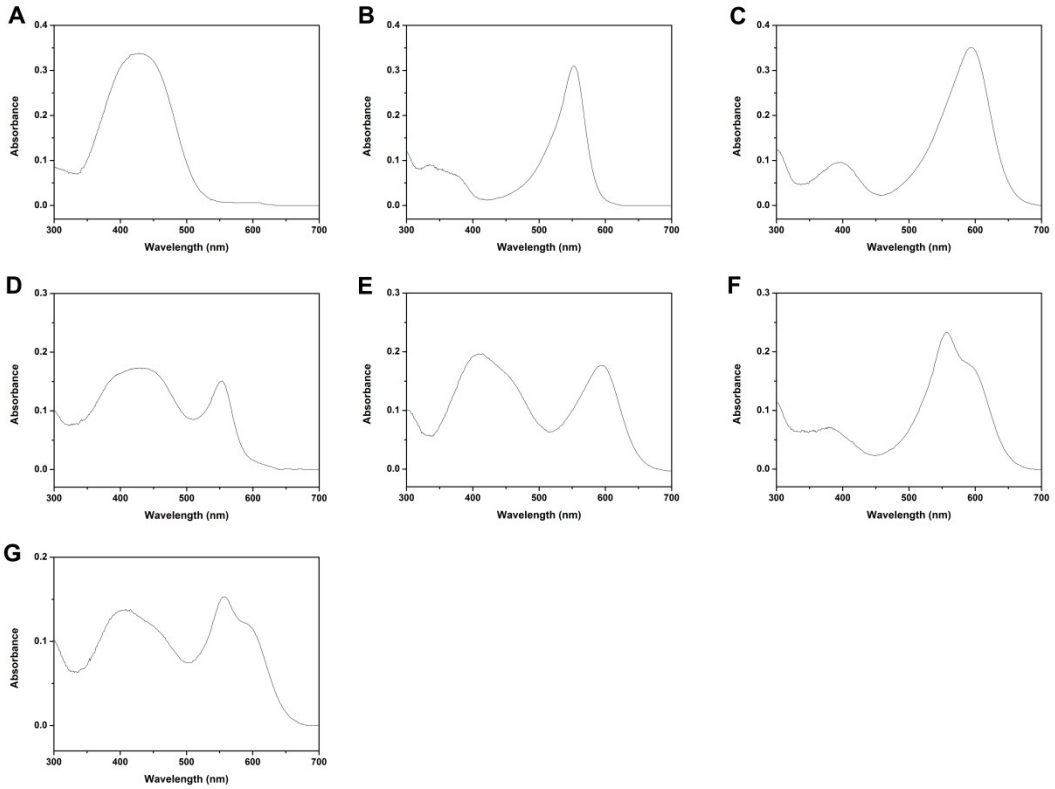

Figure S9. Absorption spectra of MR (A), PP (B), TP (C), MR+PP (D), MR+TP (E), PP+TP (F), and MR+PP+TP (G) solutions.

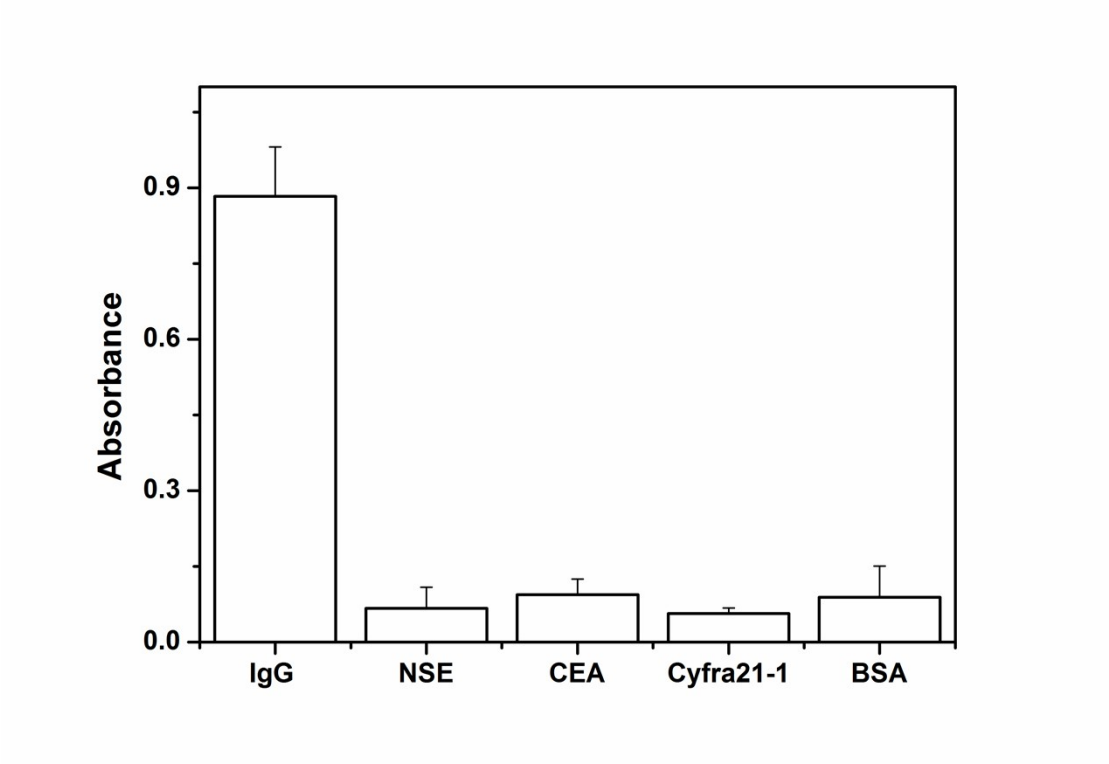

Figure S10. Specificity of the ALISA using MGCB-Ab2-cGO nanoprobe for human IgG detection. Protein concentration: 1 ng/mL.

Table S1. Information of lung cancer samples collected from 24 adenocarcinoma (AC) patients, 24 squamous carcinoma (SC) patients, 24 small cell lung carcinoma patients (SCLC) patients, and 24 healthy individuals.

AC Patients sera			
No.	Age	Gender	Hospital
1	62	M	The First Affiliated Hospital of Nanjing Medical University
2	70	F	
3	83	F	
4	66	F	
5	58	M	
6	69	F	
7	43	F	
8	77	M	
9	86	M	
10	66	M	
11	79	F	
12	58	M	
13	43	M	
14	59	F	
15	72	F	
16	48	F	
17	65	F	
18	49	M	
19	45	M	
20	38	F	
21	70	F	
22	42	M	
23	74	F	
24	59	F	
SC Patients sera			
1	78	F	The First Affiliated Hospital of Nanjing Medical University
2	68	F	
3	74	M	
4	57	M	
5	59	F	
6	60	F	
7	69	M	
8	72	M	
9	45	F	
10	38	M	
11	56	F	
12	48	M	
13	42	F	

14	78	M	
15	80	F	
16	73	F	
17	73	M	
18	74	F	
19	76	M	
20	63	F	
21	55	M	
22	70	F	
23	75	F	
24	54	M	
SCLC sera			
1	42	F	The First Affiliated Hospital of Nanjing Medical University
2	84	F	
3	67	M	
4	70	M	
5	63	M	
6	59	M	
7	55	F	
8	48	F	
9	77	M	
10	82	F	
11	80	F	
12	78	F	
13	48	F	
14	57	M	
15	69	F	
16	77	F	
17	73	M	
18	80	M	
19	71	F	
20	75	F	
21	63	M	
22	66	F	
23	70	F	
24	70	F	
Healthy sera			
No.	Age	Gender	Hospital
1	30	F	
2	24	F	
3	36	F	
4	42	M	
5	45	M	

6	54	M	The First Affiliated Hospital of Nanjing Medical University
7	58	F	
8	71	M	
9	26	M	
10	43	F	
11	57	F	
12	47	M	
13	62	F	
14	65	F	
15	26	F	
16	29	F	
17	35	F	
18	49	M	
19	36	M	
20	25	F	
21	41	M	
22	42	F	
23	28	F	
24	65	F	

Table S2. Detection results by ALISA for single tumor biomarker assay.

AC Patients	OD values at 429 nm for CEA detection	OD values at 552 nm for NSE detection	OD values at 594 nm for Cyfra21-1 detection
1	0.865	0.212	0.212
2	0.721	0.154	0.075
3	0.143	0.381	0.254
4	1.176	0.154	0.154
5	1.212	0.176	0.176
6	0.987	0.212	0.213
7	0.525	0.276	0.135
8	0.921	0.487	0.128
9	0.674	0.176	0.516
10	0.383	0.126	0.426
11	0.412	0.115	0.365
12	0.484	0.301	0.321
13	0.333	0.241	0.141
14	0.065	0.399	0.199
15	0.634	0.219	0.219
16	0.399	0.2	0.4
17	0.763	0.482	0.181
18	0.221	0.535	0.532
19	0.232	0.148	0.387
20	0.154	0.175	0.086
21	1.403	0.128	0.107
22	0.111	0.167	0.105
23	0.845	0.145	0.281
24	0.687	0.115	0.115
SC Patients			
1	0.964	0.193	0.393
2	1.178	0.478	0.418
3	0.093	0.186	0.496
4	0.192	0.443	0.329
5	0.212	0.127	0.227
6	0.634	0.211	0.289
7	1.365	0.18	0.098
8	0.087	0.187	0.117
9	0.109	0.465	0.365
10	0.455	0.17	0.57
11	0.189	0.238	0.438
12	0.263	0.145	0.513
13	0.745	0.145	0.115
14	0.443	0.399	0.243

15	0.213	0.213	0.273
16	0.154	0.154	0.444
17	0.678	0.678	0.178
18	0.606	0.476	0.076
19	0.106	0.278	0.178
20	0.145	0.563	0.145
21	0.306	0.418	0.458
22	0.892	0.192	0.192
23	0.327	0.327	0.327
24	0.527	0.227	0.227
SCLS patients			
1	0.092	0.531	0.131
2	1.24	0.279	0.212
3	0.198	0.46	0.26
4	0.742	0.749	0.149
5	0.69	0.59	0.19
6	0.453	0.428	0.088
7	0.86	0.487	0.087
8	0.288	0.689	0.189
9	0.327	0.997	0.197
10	0.086	1.074	0.218
11	0.912	0.411	0.111
12	0.145	0.281	0.105
13	0.315	0.265	0.065
14	0.455	1.176	0.176
15	0.413	0.33	0.13
16	0.321	0.508	0.308
17	0.625	0.365	0.365
18	0.395	0.578	0.178
19	0.243	0.843	0.143
20	0.587	0.568	0.208
21	0.489	0.488	0.188
22	0.202	0.402	0.102
23	0.19	0.422	0.422
24	0.239	0.222	0.322
Healthy individuals			
1	0.234	0.184	0.134
2	0.123	0.153	0.153
3	0.354	0.254	0.154
4	0.456	0.166	0.165
5	0.231	0.102	0.102
6	0.312	0.212	0.212

7	0.289	0.189	0.139
8	0.282	0.282	0.182
9	0.273	0.273	0.173
10	0.105	0.105	0.115
11	0.214	0.214	0.114
12	0.443	0.238	0.238
13	0.189	0.189	0.18
14	0.196	0.196	0.061
15	0.221	0.3	0.31
16	0.301	0.271	0.171
17	0.421	0.13	0.095
18	0.645	0.252	0.102
19	0.212	0.212	0.212
20	0.167	0.167	0.168
21	0.094	0.301	0.101
22	0.243	0.234	0.384
23	0.059	0.156	0.116
24	0.128	0.129	0.139

Yellow: positive sample, red: false positive sample, green: cut-off values.

Table S3. Detection results by ALISA for multiple tumor biomarkers assay.

AC Patients	OD values at 429 nm for CEA detection	OD values at 552 nm for NSE detection	OD values at 594 nm for Cyfra21-1 detection
1	0.691	0.182	0.159
2	0.584	0.121	0.101
3	0.107	0.432	0.211
4	0.743	0.145	0.15
5	0.85	0.158	0.142
6	0.791	0.178	0.2
7	0.423	0.198	0.117
8	0.843	0.464	0.072
9	0.551	0.212	0.483
10	0.213	0.098	0.404
11	0.305	0.107	0.316
12	0.416	0.251	0.288
13	0.139	0.222	0.16
14	0.066	0.439	0.154
15	0.433	0.242	0.162
16	0.321	0.174	0.426
17	0.743	0.477	0.192
18	0.132	0.635	0.372
19	0.2	0.111	0.405
20	0.144	0.155	0.113
21	1.308	0.108	0.097
22	0.077	0.133	0.136
23	0.632	0.178	0.242
24	0.422	0.092	0.125
SC Patients			
1	0.768	0.172	0.407
2	0.856	0.452	0.342
3	0.101	0.2	0.496
4	0.144	0.457	0.3
5	0.185	0.126	0.165
6	0.432	0.183	0.303
7	1.029	0.199	0.092
8	0.071	0.145	0.113
9	0.103	0.505	0.292
10	0.345	0.153	0.566
11	0.136	0.223	0.436
12	0.223	0.162	0.577
13	0.645	0.145	0.129
14	0.37	0.334	0.183

15	0.182	0.173	0.283
16	0.148	0.151	0.478
17	0.521	0.708	0.114
18	0.496	0.477	0.092
19	0.077	0.22	0.153
20	0.132	0.587	0.14
21	0.266	0.423	0.452
22	0.721	0.205	0.192
23	0.226	0.289	0.265
24	0.46	0.183	0.226
SCLS patients			
1	0.074	0.533	0.126
2	0.728	0.306	0.187
3	0.122	0.502	0.223
4	0.652	0.774	0.137
5	0.621	0.587	0.163
6	0.422	0.431	0.089
7	0.707	0.488	0.092
8	0.226	0.639	0.174
9	0.288	0.803	0.186
10	0.087	0.921	0.205
11	0.775	0.435	0.119
12	0.128	0.256	0.108
13	0.249	0.232	0.097
14	0.445	1.027	0.176
15	0.366	0.323	0.13
16	0.302	0.527	0.268
17	0.587	0.409	0.313
18	0.315	0.583	0.165
19	0.202	0.821	0.121
20	0.532	0.575	0.226
21	0.399	0.477	0.142
22	0.167	0.416	0.101
23	0.155	0.479	0.382
24	0.164	0.222	0.344
Healthy individuals			
1	0.183	0.165	0.113
2	0.106	0.156	0.141
3	0.321	0.223	0.152
4	0.346	0.184	0.16
5	0.096	0.142	0.087
6	0.261	0.172	0.184

7	0.232	0.139	0.143
8	0.217	0.25	0.165
9	0.233	0.243	0.172
10	0.067	0.081	0.109
11	0.158	0.206	0.117
12	0.373	0.214	0.212
13	0.182	0.138	0.155
14	0.19	0.176	0.103
15	0.226	0.278	0.261
16	0.222	0.246	0.155
17	0.341	0.121	0.126
18	0.525	0.25	0.123
19	0.197	0.172	0.174
20	0.136	0.159	0.168
21	0.114	0.294	0.101
22	0.192	0.196	0.312
23	0.119	0.182	0.079
24	0.104	0.098	0.125

Yellow: positive sample, red: false positive sample, green: cut-off values.

Table S4. The positive rates of combined detection of multiple tumor biomarkers.

Tumor biomarker	AC	SC	SCLC
CEA	58.3%	37.5%	33.3%
NSE	20.8%	25%	83.3%
Cyfra21-1	37.5%	54.2%	20.8%
CEA + NSE	70.8%	50%	87.5%
CEA + Cyfra21-1	83.3%	79.2%	50%
NSE + Cyfra21-1	50%	66.7%	87.5%
CEA + NSE + Cyfra21-1	87.5%	83.3%	91.7%