Electronic Supporting Information (ESI)

Development of immunoblot assay for carcinoembryonic antigen (CEA) in human serum using portable UV illuminator

Raghuraj S. Chouhan,[1]* Javed H. Niazi [2] and Anjum Qureshi

[1] Department of environmental sciences, Jožef Stefan Institute, Jamova cesta 39, 1000 Ljubljana

[2] Sabanci University Nanotechnology Research and Application Center, Orta Mah. 34956, Tuzla, Istanbul, Turkey

* Corresponding author

email: raghuraj.singh@ijs.si

Experimental methods

Reagents and Materials

Human serum used in present study was procured from EMD Millipore. Whatman Protran nitrocellulose membranes circles (BA85) with pore size 0.45 μm and diam of 25 mm was purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-Mouse IgG (H+L) Cross Adsorbed Secondary Antibody, HRP conjugate was obtained from Thermo scientific, USA. N-hydroxy-succinimide (NHS), 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), CEA antigen, Mouse monoclonal CEA antibody and 3', 3', 5', 5', Trimethyl benzidine (TMB) were purchased from Sigma-Aldrich (St. Louis, MO). Nitrocellulose membrane was purchased from Millipore, Germany. Qdots 625 ITK carboxyl QDs was purchased from Thermo scientific, USA. All chemicals used in this study were analytical reagent grade. All stock solutions were prepared using deionized water purified with 0.22 μm filters.

Conjugation of QDs with anti-CEA antibody

QD-Mab conjugates were prepared using a carbodiimine chemistry involving EDC as cross-linkers. Carboxylic acid groups of amphiphilic polymer displayed on the QDs surface bound with the amino-groups of antibody. The optimized parameters and amount of reagents used for conjugation was presented in (Table S1). The final QD-Mab bioconjugates were purified by centrifugation and washed with PBS solution (pH 7.4).

QDs based NCs assay

Preparation of CEA-spiked human serum samples

The background CEA from the 10-folds diluted human serum was removed by suspending the CEA-antibodies functionalized magnetic beads followed by magnetic separation. The CEA-free dilute human serum was used for spiking. The preservative-free and standard CEA (Fitzgerald Ind.) was then spiked in CEA-free serum with a series of different concentrations. The CEA-free dilute human serum served as a control.

Immobilization of CEA on NCs

NCMs were dot blotted with pure CEA or CEA spiked serum of different concentrations (0, 6.25, 12.5, 25, 50 and 100 ng mL^{-1}). The sample blotted NCM area was marked with a pencil and dried at room temperature for 15 min. The dot blotted NCMs were later blocked by 5% skimmed milk solution at 37 °C for 1 h followed by washing with PBS solution.

Immunoblot assay for CEA detection

The dot blotted NCMs in which each blotted area corresponding to CEA concentration of 6.25, 12.5, 25, 50 and 100 ng mL^{-1}, were were incubated for 15 min with consistent 0.25 ng mL^{-1} of QDs-Mab conjugates and the NCMs were rinced with PBS. The development of intense red color corresponding to the CEA levels was monitored with a brief illumination with a hand-held portable UV-illuminator. The

color intensity was directly proportional to the amount of CEA present on the NCMs. Schematic diagram of the QD-based immunoblot assay is shown in Scheme 1.

Analysis of CEA by indirect ELISA

Analysis of CEA by immunoblot assay was compared and validated with most sensitive ELISA assay. ELISA wells were coated with different concentrations of CEA (0, 6.25, 12.5, 25, 50 and 100 ng mL^{-1}) using bicarbonate buffer pH 8.4. After overnight incubation the wells were blocked using 5 % skimmed milk in PBS (Phosphate Buffer Saline pH 7.4) for one hour followed by washing of the wells using PBST (phosphate buffer saline pH 7.4 Tween-20). After 1 h incubation, primary antibody (0.25 µg mL^{-1}) were added and allowed to incubate at 37 °C for 1 h. The plate was then washed twice with PBST and incubated with secondary antibody labelled HRP (1: 5000) for 1 h. The plate was further washed twice and tapped dry on blotting paper. TMB substrate solution was added and the reaction was stopped by the addition of 1N H_2SO_4. The absorbance was measured at 450 nm using a Synergy HTX-multimode microplate reader.

Table S1. Reaction conditions for preparation of QDs-Mab bio-conjugates. It was estimated that, 8 nM QDs was required for 0.25 µg of antibody for stable bioconjugation that yield detectable fluorescence signals (fluorescence pixels intensity of spots under UV 385 nm = 255).

Reaction mixture	Antibody (µg)	QDs (nM)	EDC (mM)	NHS (mM)	Total Volume (µL)	Fluorescence pixels intensity
1	0.0625	2	50	5	100	100
2	0.0625	4	50	5	100	110
3	0.0625	8	50	5	100	123
4	0.125	2	50	5	100	134
5	0.125	4	50	5	100	140
6	0.125	8	50	5	100	177
7	0.25	2	50	5	100	208
8	0.25	4	50	5	100	213
9	0.25	8	50	5	100	255
10	0.5	2	50	5	100	212
11	0.5	4	50	5	100	235
12	0.5	8	50	5	100	197

Optimization parameters for QDs-Mab bioconjugates

Carboxyl-QDs (2, 4 and 8 nM) were activated by adding EDC and NHs at the optimized concentrations shown in Table S1. The mixtures were incubated for 15 min at ambient temperature after which Mab (0.25 to 0.5 µg) was added to a total volume of 100 µL. The reaction was then incubated at ambient temperature for 1 h on a rotating wheel in the dark. For optimization, 2 µL of CEA (12.5 ng/mL) was applied directly to a methanol-activated PVDF membrane and dried at 37^0 C for 30 min. Dried membranes were then blocked with 5 %w/v skimmed milk powder for 1 h with gentle agitation followed by 3 washes in PBST, 10 min each. Washed membranes were then spotted (2 µL each) with the above prepared QDs-Mab mixtures and incubate at ambient temperature for 15 min under dark. The membranes were dried and placed under hand held UV detector to observe the color intensity. The developed colored on the image were analyzed using ImageJ software (http://rsb.info.nih.gov/ij/). According to the image intensity it was found that reaction mixture (9) showed best combination with 255 pixel intensity. This concentration was selected and used for the detection of CEA in human serum.

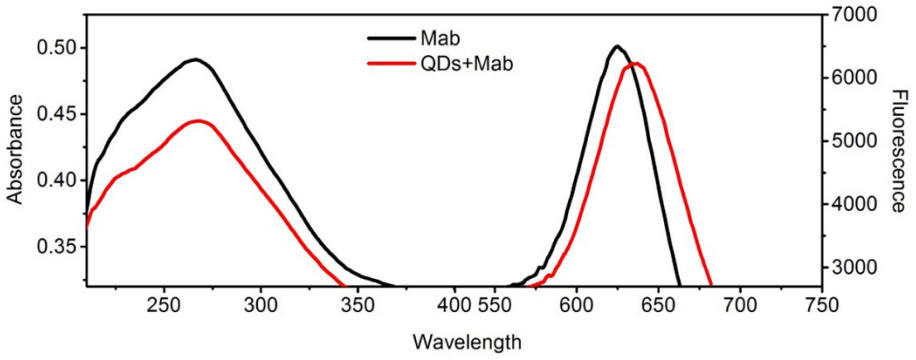

Fig. S1. Absorbance and fluorescence spectra of CEA monoclonal antibody (Mab) and QDs+Mab conjugates.