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Supplementary Information for

Three-Dimensional Protein Microarray Fabricated on Reactive Microspheres Modified COC Substrate

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Experimental section S1. Calculation of the grafting density of PGMA

The grafting density can be defined as the mass of grafted polymer per unit area, which can be calculated by the following formula:

$$\text{Grafting density } (\mu g/cm^2) = (M_1 - M_0) / S \qquad (1)$$

Where M_0 and M_1 are the mass of the pristine COC film before and after photografting, respectively; S is the surface area of COC film.

Experimental section S2. Synthesis of poly(styrene-*alt*-maleic anhydride) (PSM) copolymer microspheres

The PSM copolymer microspheres with different particle sizes were synthesized through the self-stabilized precipitation (2SP) polymerization. Typically, equimolar styrene and maleic anhydride monomers were added into a flask containing isoamyl acetate with the AIBN as the initiator. The reaction system was purged with N_2 for 30 minutes to removal inhibitor and then was placed at a certain temperature for a certain time. In the final system, the solid components in the suspension are the PSM microspheres we need, and we do not have to separate them out immediately due to the subsequent operations. The PSM microspheres with different particle sizes could be obtained by adjusting the formula and conditions according to following table S1.

Table S1. Reaction formula for synthesizing microspheres of different particle sizes.

Particle size of obtained microspheres	Styrene	Maleic anhydride	AIBN	Isoamyl acetate	Reaction temperarure	Reaction time
350 nm	0.8238 g	0.7762 g	0.008 g	80 mL	75 ℃	1.5 h
460 nm	2.471 g	2.3285 g	0.024 g	80 mL	75 ℃	3 h
630 nm	5.2 g	4.93 g	0.0506 g	100 mL	80 ℃	2 h

Experimental section S3. Preparation of the flat poly(styrene-*alt*-maleic anhydride) brush modified COC (COC-PSM brush) substrate

The COC-PSM brush substrate was prepared by UV-induced grafting polymerization. Firstly, equimolar styrene and maleic anhydride were dissolved in acetone to form the reaction solution with a mass fraction of 70%, which contains the initiator BP with a mass fraction of 2%. Then, 5μL of the reaction solution was injected on the pristine COC film (2 cm×2 cm), covered by a piece of BOPP film (4 cm×4 cm) to form a "sandwich" structure. Lastly, the "sandwich" structure was irradiated under a high-pressure mercury lamp for 5 min at 254 nm, and the COC-PSM brush substrate was obtained after being extracted in acetone for 30 min.

Other Supplementary Figures and Tables

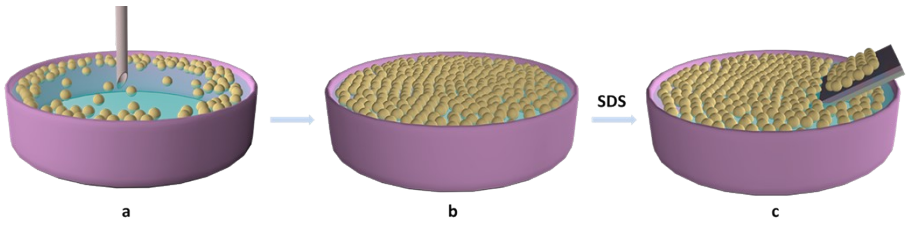

Fig. S1 Schematic illustration of preparation of single layer PSM microspheres on the modified COC films based on simplified needle tip flow method.

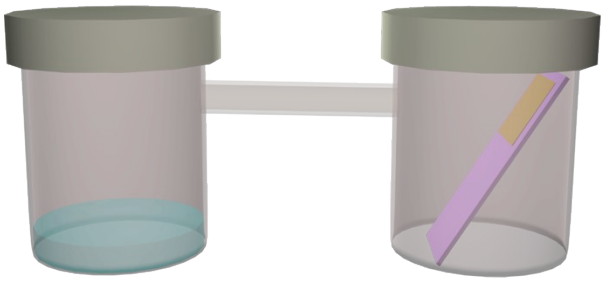

Fig. S2 Schematic illustration of blocking the residual anhydride groups by gas-solid reaction strategy based on ethylenediamine.

Table S2 Influence of different solvents and initiators on grafting density.

Initiators (2 wt%)	Monomer (10 wt%)	Solvent (88 wt%)	Grafting density (μg/cm²)
ITX	GMA	Acetone	16.06
BP	GMA	Acetone	24.56
BP	GMA	Ethyl acetate	30.64

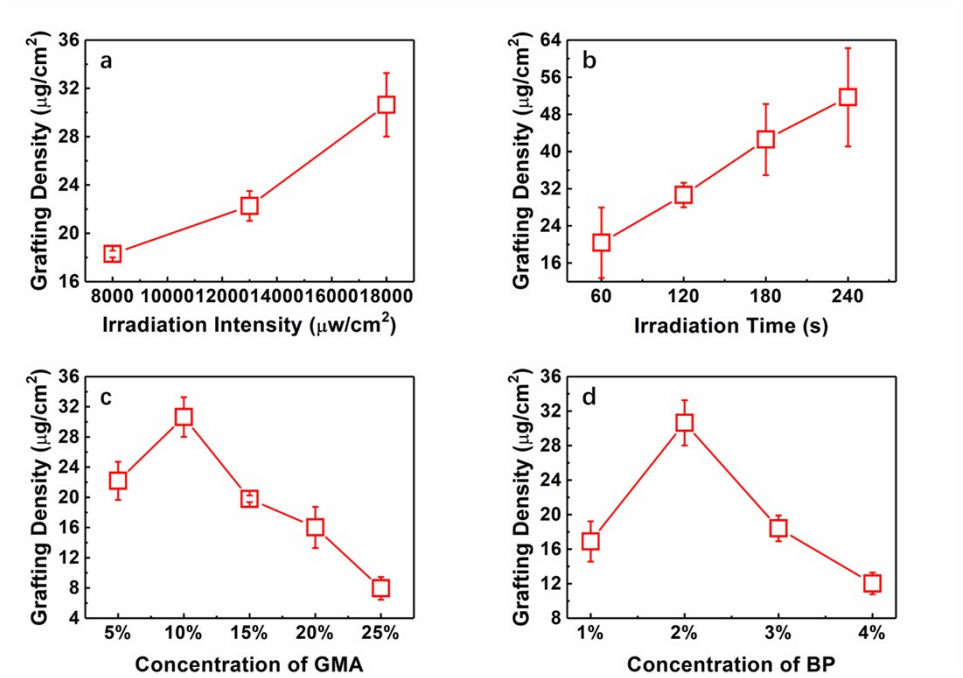

Fig. S3 Relationship between grafting density and (a) light intensity, (b) irradiation time, (c) monomer concentration and (d) initiator concentration.

Table S3 The element contents of COC films before and after modification.

Sample	C element	O element	N element
Original COC	97.56%	2.44%	-
COC-PGMA	73.24%	26.76%	-
COC-NH_2	71.14%	25.15%	3.71%

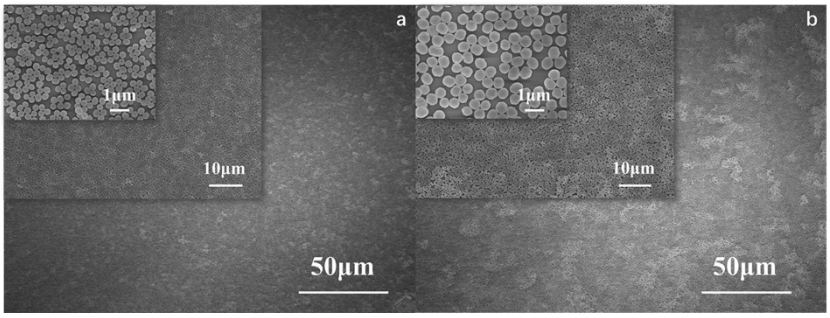

Fig. S4 The surface morphology of the modified COC film arranged with single-layer PSM microspheres with a particle size of (a) 350 nm and (b) 630 nm.

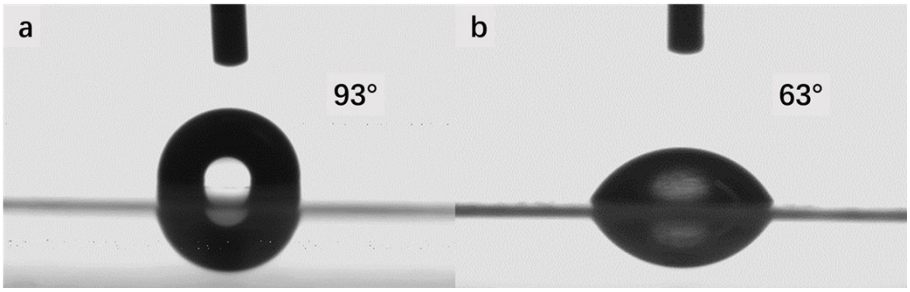

Fig. S5 The water contact angle of (a) original COC film and (b) COC-PSM film.

Table S4 Z-factor of the probe protein immobilization.

Concentration of probe protein	Z-factor
20 μg/mL	0.70
40 μg/mL	0.82
60 μg/mL	0.70
80 μg/mL	0.96
100 μg/mL	0.66
120 μg/mL	0.96
140 μg/mL	0.83
160 μg/mL	0.74
180 μg/mL	0.56
200 μg/mL	0.63

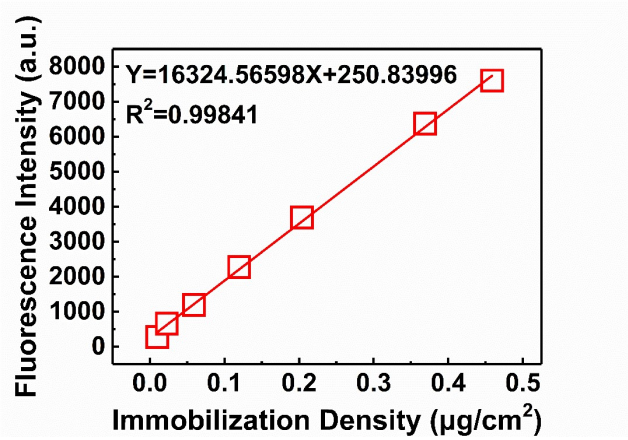

Fig. S6 The calibration curve for calculation of immobilization density of AF647 IgG.

Table S5 The RSD values of the immobilization efficiency under different probe protein concentrations.

Concentration of probe protein	Immobilization Efficiency			Mean value	RSD value
	Batch 1	Batch 2	Batch 3		
20 μg/mL	69.03%	78.30%	72.14%	73.16%	6.45%
40 μg/mL	76.63%	71.18%	79.09%	75.63%	5.35%
60 μg/mL	68.11%	73.00%	64.26%	68.45%	6.40%
80 μg/mL	67.05%	65.12%	62.14%	64.77%	3.82%
100 μg/mL	60.89%	66.91%	66.48%	64.76%	5.19%
120 μg/mL	62.79%	63.18%	61.79%	62.58%	1.15%
140 μg/mL	58.88%	64.66%	65.23%	62.92%	5.58%
160 μg/mL	61.09%	63.44%	61.62%	62.05%	1.99%
180 μg/mL	58.57%	63.81%	64.55%	62.31%	5.23%
200 μg/mL	61.55%	62.68%	53.50%	59.24%	8.46%

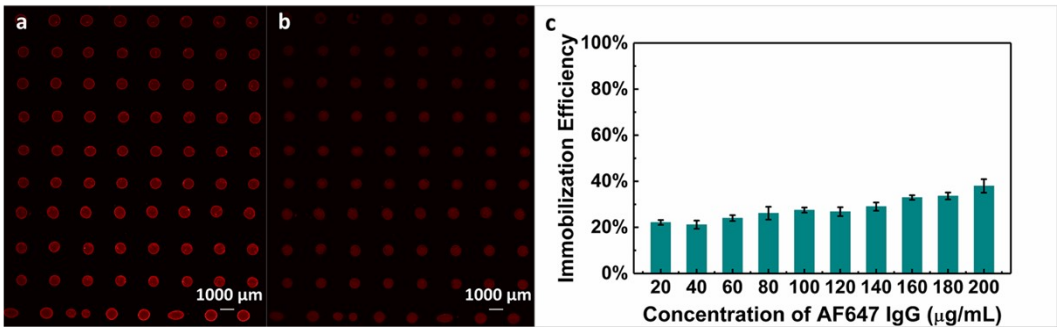

Fig. S7 Fluorescence images of flat COC-PSM brush microarray (a) before and (b) after washing; The (c) immobilization efficiency of flat COC-PSM brush substrate under different probe protein concentrations.

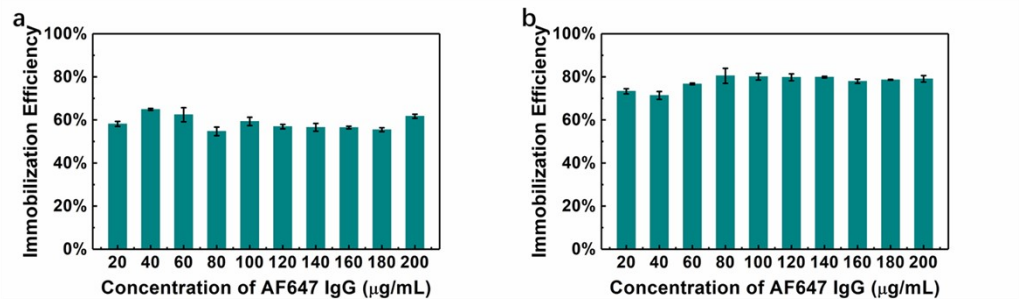

Fig. S8 The immobilization efficiency of AF647 IgG on COC-PSM substrate with a particle size of (a) 350 nm and (b) 630 nm.

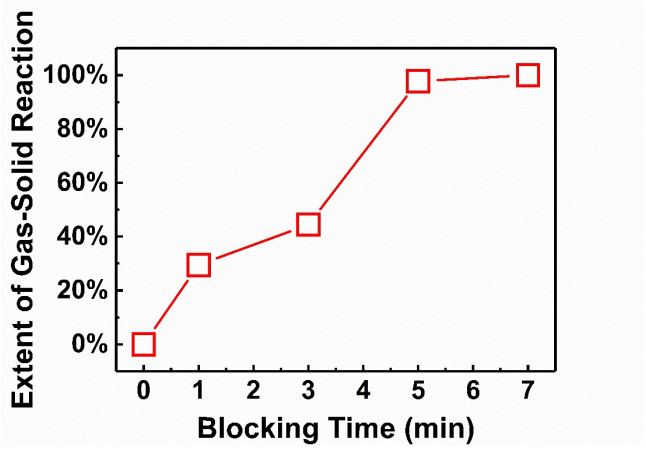

Fig. S9 The relationship between the extent of gas-solid reaction and blocking time.

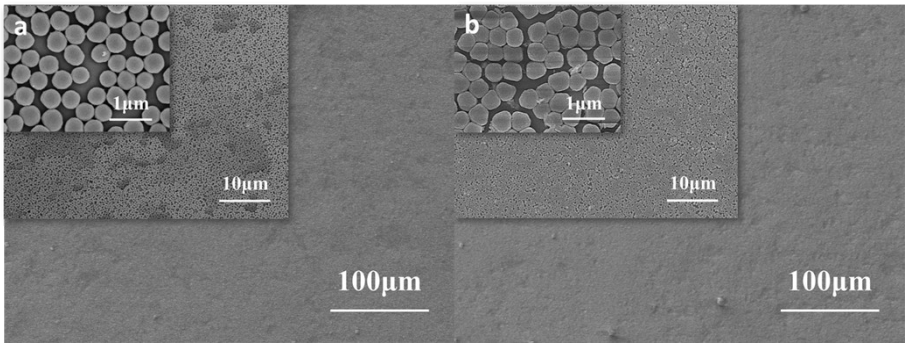

Fig. S10 The SEM images of COC-PSM-IgG (a) before and (b) after blocking.

Table S6 Z-factor of the immunoassay of microarray.

Concentration of probe protein	Z-factor					
	Concentration of target protein (ng/mL)					
	250 ng/mL	100 ng/mL	50 ng/mL	25 ng/mL	12.5 ng/mL	6.25 ng/mL
120 μg/mL	0.53	0.55	0.46	0.35	0.28	0.34
140 μg/mL	0.61	0.46	0.72	0.54	0.43	0.16
160 μg/mL	0.57	0.57	0.77	0.43	0.58	0.36
180 μg/mL	0.60	0.80	0.63	0.55	0.51	0.55
200 μg/mL	0.51	0.70	0.80	0.53	0.64	0.72

Table S7 The RSD values of the fluorescence intensity in the immunoassay of microarray.

Concentration of target protein	Fluorescence intensity			Mean value	RSD value
	Batch 1	Batch 2	Batch 3		
6.25 ng/mL	1970.5	1885.8	1824.2	1893.50	3.88%
12.5 ng/mL	1115.4	1037.7	918.102	1023.73	9.71%
25 ng/mL	751.4	689.5	691.518	710.81	4.95%
50 ng/mL	555.9	485.9	457.135	499.65	10.17%
100 ng/mL	389.6	410.7	399.5875	399.96	2.64%
250 ng/mL	378.9	380.8	357.296	372.33	3.51%