Fast immunofluorescence lateral flow test strip approach for detection of homocysteine

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Published in Micro & Nano Letters; Received on 11th April 2018; Revised on 23rd July 2018; Accepted on 30th August 2018

The study developed a novel quantitative detection method of homocysteine (Hcy), an independent risk factor for cardiovascular disease, based on an immunofluorescent test strip. The fluorescent nanospheres (F-NSs) were prepared by embedding fluorophores (Cy5) into poly (styrene-acrylate) copolymer nanospheres, then conjugated with streptavidin (SA) to obtain SA/F-NSs as a signal amplification label stored in sample diluents. Biotin–antibody was fixed on the conjugate pad to specifically capture *S*-adenosyl Hcy (SAH) in the sample, where SAH was transformed from *S*-adenosyl methionine (SAM) by Hcy *S*-methyltransferase catalysis using Hcy as a substrate, the formed SAH could be trapped by SA/F-NSs and biotin–antibody conjugates in a competitive way with SAH–bovine serum albumin fixed on the test line, a detection limit of 0.27 μM Hcy was achieved. The fluorescence intensity of F-NSs remained stable during 273 days of storage, the bioactivity of the test strip was stable during 12 months of storage, and the strip possessed good reproducibility (intra-assay variability of 5.8%). Furthermore, other structural analogues SAM and cysteine showed negative results, validating the excellent specificity of the strips.

1. Introduction: Homocysteine (Hcy) is a by-product of transmethylation reactions and is detoxified by methionine synthetase, which depends on vitamin B_{12} and folate as coenzymes for proper function [1–3]. Hcy is a well-known independent risk factor for cardiovascular disease (CVD) [4, 5], hyperhomocysteinaemia (Hhcy) is widely recognised as a risk factor of acute ischaemic stroke and is listed in the Chinese guidelines for the management of stroke. In general, the cut-off value for high Hcy is 10 μmol/l, any higher Hcy level is defined as Hhcy [6, 7]. The patients with homocystinuria have dramatically increased plasma Hcy levels [8], it is found that elevated levels of plasma Hcy may cause accelerated atherosclerosis and premature CVD, in particular, coronary heart disease [9–11]. Therefore, rapid, sensitive, and quantitative detection of Hcy is becoming crucial for clinical application, which can assist early diagnosis of CVD and determine the extent of diseases [12]. It is imperative to develop a more rapid and convenient point-of-care assay [13, 14]. The most well-known one is the lateral flow strip assay, whose unique advantage lies in speediness, also it is simple to operate and needs no large instruments and professional operators [15, 16]. To our knowledge, there is no any study about Hcy detecting on lateral flow test strips for now.

Herein, a rapid and sensitive assay for quantitative detection of Hcy was developed for the first time, based on the benefits inherited from both fluorescent nanospheres (F-NSs) as a signal amplification label and a lateral flow test strip. As expected, experimental results demonstrated that the assay can be used to rapidly and quantitatively detect Hcy with a good linear relationship and a low detection limit, the assay was robust with high specificity, strong anti-interference ability, and good reproducibility, indicating that the test strip for Hcy detection is promising in practice.

2. Experimental section

2.1. Reagents and instruments: Hcy, dithiothreitol (DTT) and foetal bovine serum (FBS) were from Sangon Biotech Co., Ltd (Shanghai, China). *S*-Adenosyl Hcy (SAH) and *N*-(3-dimethylaminopropyl-*N*′-ethylcarbodiimide hydrochloride (EDC) were from Sigma-Aldrich (USA). Rabbit monoclonal antibody against SAH, goat anti-rabbit IgG antibody and horseradish peroxide (HRP)-labelled were purchased from Abcam (British). 3,3′,5,5′-Tetramethylbenzidine (TMB) chromogenic substrate was from CWBIO Co., Ltd (Beijing, China), bovine serum albumin (BSA) was ordered from Yeasen Biotech Co., Ltd (Shanghai, China), *S*-adenosyl methionine (SAM), 2-(*N*-morpholino)ethanesulfonic acid (MES) and cysteine (Cys) were from Aladdin (Shanghai, China). Hcy *S*-methyltransferase (HMT) was obtained from Ambition Biotechnology Co., Ltd (Beijing, China). Carboxyl nanospheres were from JSR Life Sciences Corporation (Japan). Sulpho-*N*-hydroxysulphosuccinimide (NHS), *N*-hydroxysuccinimide-polyethylene glycol 4-biotin and near-infrared indocyanine fluorophores (Cy5) were from Thermo Fisher Scientific (USA), core streptavidin 2 (cSA2) was from NeuroPeptide Biotech Co., Ltd (Hangzhou, China). A nitrocellulose membrane was supplied by Millipore. Conjugate pad, absorbent pad, polyvinyl chloride board and a complete set of equipment for preparation of strips were obtained from Shanghai Kinbio Tech. Co., Ltd and used after treatment. Pure water was used for the preparation of all solutions.

SAH–BSA was characterised by using an ultraviolet–visible (UV–vis) spectrophotometer (Shimadzu Corporation). SA/F-NSs were characterised by using a particle size analyser (Malvern Instruments Ltd.). The size distributions and surface morphology were observed by using a scanning electron microscope from ZEISS. A fluorescent signal was read by using a fluorescent strips reader Nanoeasy 1700 (NE 1700) (Nanjing Nanoeast Biotech Co., Ltd).

2.2. Fabrication of streptavidin (SA)-coated F-NSs: First, F-NSs were prepared by embedding Cy5 into poly(styrene-acrylate) copolymer nanospheres with surface carboxyl groups

(NSs–COOH) according to the reported literature [17], then the F-NSs were coated with cSA2, as shown in Fig. 1. In brief, 5 mg F-NSs were dispersed in the MES buffer (0.01 M MES, pH 5.5), 3 mg core SA were added into the system at room temperature with continuous shaking for 45 min, then EDC was added for the further combining of core SA for 2 h, afterwards, the core SA-coated F-NSs (SA/F-NSs) were separated by centrifugation and washed with phosphate buffered saline (0.01 M PBS, pH 7.2) three times. After that, SA/F-NSs were blocked with BSA (2%, pH 8.3) for about 1 h at room temperature, and then washed five times with PBS (0.01 M, pH 7.2); finally, SA/F-NSs were dispersed in 1 ml of PBS (0.01 M, pH 7.2) and stored at 4°C until use.

2.3. Fabrication of biotin–antibody conjugates: One hundred micrograms of anti-SAH antibody were diluted to 1–2 mg/ml by carbonate buffer (0.1 M CB, pH 9.0), then the biotin (1 mg/ml) was added into the system at 37°C with continuous shaking for 45 min. Afterwards, biotin–antibody and free biotin were separated by ultrafiltration with PBS (0.02 M, pH 7.4) four times. The final concentration of the biotin–antibody conjugates was 1 mg/ml and stored at 4°C before using.

2.4. Fabrication of SAH–BSA conjugates: One hundred microlitres of SAH (3.5 mg/ml) and 200 µl of BSA (10 mg/ml) were mixed in MES buffer solution (0.01 M, pH 5.5), and then 25 µl EDC (10 mg/ml) and 25 µl NHS(10 mg/ml) were added with continuous shaking at 37°C for 1 h. Afterwards, the free SAH and the residual EDC/NHS were cleared away by ultrafiltration, finally, the SAH–BSA conjugates were dispersed in PBS (0.01 M, pH 7.2) and stored at 4°C until use.

2.5. Fabrication of lateral flow immunofluorescence test strip: As shown in Fig. 2*a*, a test strip consisted of five parts, including a sample pad, conjugate pad, nitrocellulose membrane, absorption pad, and plastic adhesive card. Biotin–antibody (1 mg/ml) was fixed on the conjugate pad by a scribing instrument with 2 µl/cm scribing amount, SAH–BSA (2.5 mg/ml) and goat anti-rabbit IgG (1 mg/ml) were on the nitrocellulose membrane as test line and control line, respectively. The scribing amount of both was 1 µl/cm, and the scribing speed was 50 mm/s for all. After that, the obtained conjugate pad and nitrocellulose membrane were placed in a drying cupboard overnight. Then assemble and stick the sample pad, conjugate pad, nitrocellulose membrane, and absorption pad onto the plastic adhesive card. Finally, the whole pad was cut into test strips of 4 mm width and assembled in the strip shells, and the made strips were stored in a drying cupboard sealed until use.

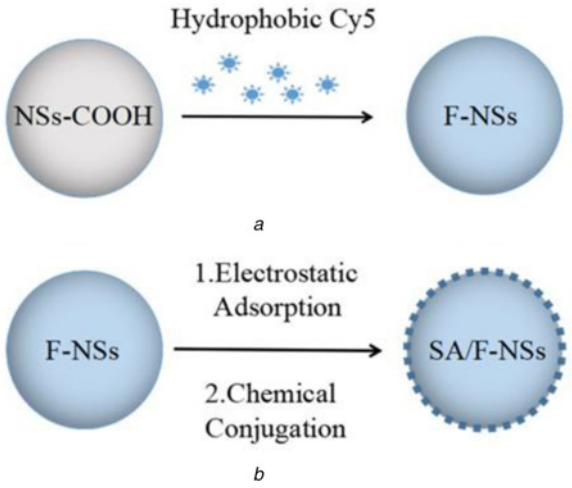

Fig. 1 *SA/F-NS formation process*
a F-NS formation by embedding fluorophores into NSs–COOH
b SA-coating by electrostatic adsorption and chemical conjugation

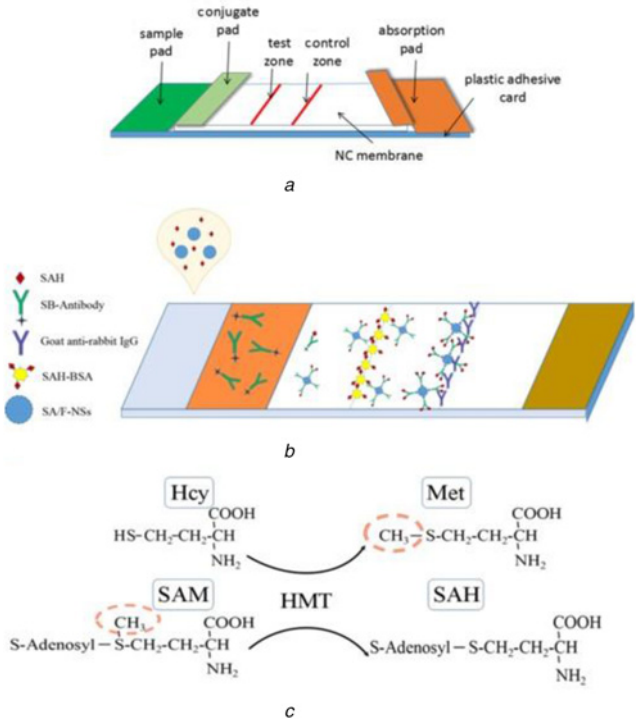

Fig. 2 *Detection schematic of strip*
a Configurations of lateral flow test strip
b Detection principle of Hcy
c Illustration of Hcy transformation

2.6. Detection of Hcy based on lateral flow test strip assay: The samples with a different concentration of Hcy, such as 50, 40, 30, 20, 10, 5 and 1 µM, were treated with 10 mM DTT, 200 µM SAM and HMT for 20 min at 37°C. After that, the treated sample was diluted 50 times with the sample diluent containing 0.5 mg/ml SA/F-NSs in a centrifuge tube. Then the mixer (the sample volume was 100 µl) was loaded onto the sample pad of anti-SAH lateral flow test strip with SAH–BSA and goat anti-rabbit IgG antibody on the nitrocellulose membrane as test line and control line separately. Eight minutes later, the fluorescence intensity of the test line and control line can be read out by an immunofluorescence reader.

3. Results and discussion

3.1. Fabrication of lateral flow immunofluorescence test strip: The SA/F-NSs were firstly synthesised and characterised with a uniform size distribution and good dispersion, as shown in the scanning electron microscopy (SEM) image (Fig. 3*a*), the mean diameter of SA/F-NSs was 208 nm. Cy5 was embedded in NSs through physical swelling and adsorption process, and the obtained F-NSs were coated with SA by EDC cross-linking, ensuring the fluorescence stability (Fig. 1). Dynamic light scattering (Fig. 3*D*) characterisation showed the average hydrodynamic size of the SA/F-NSs in aqueous solution was 220 nm, showing an increase compared with the NSs–COOH (209 nm, Fig. 3*b*) due to the SA coating. At the same time, the dispersity of SA/F-NSs was demonstrated by the polydispersity index (0.06), consistent with the result in Fig. 3*a*. The stability of the SA/F-NSs was also investigated (Fig. 3*c*), further demonstrating that SA/F-NSs were well dispersed with a perfectly uniform size even after 273 days' storage.

doi: 10.1049/mnl.2018.5040

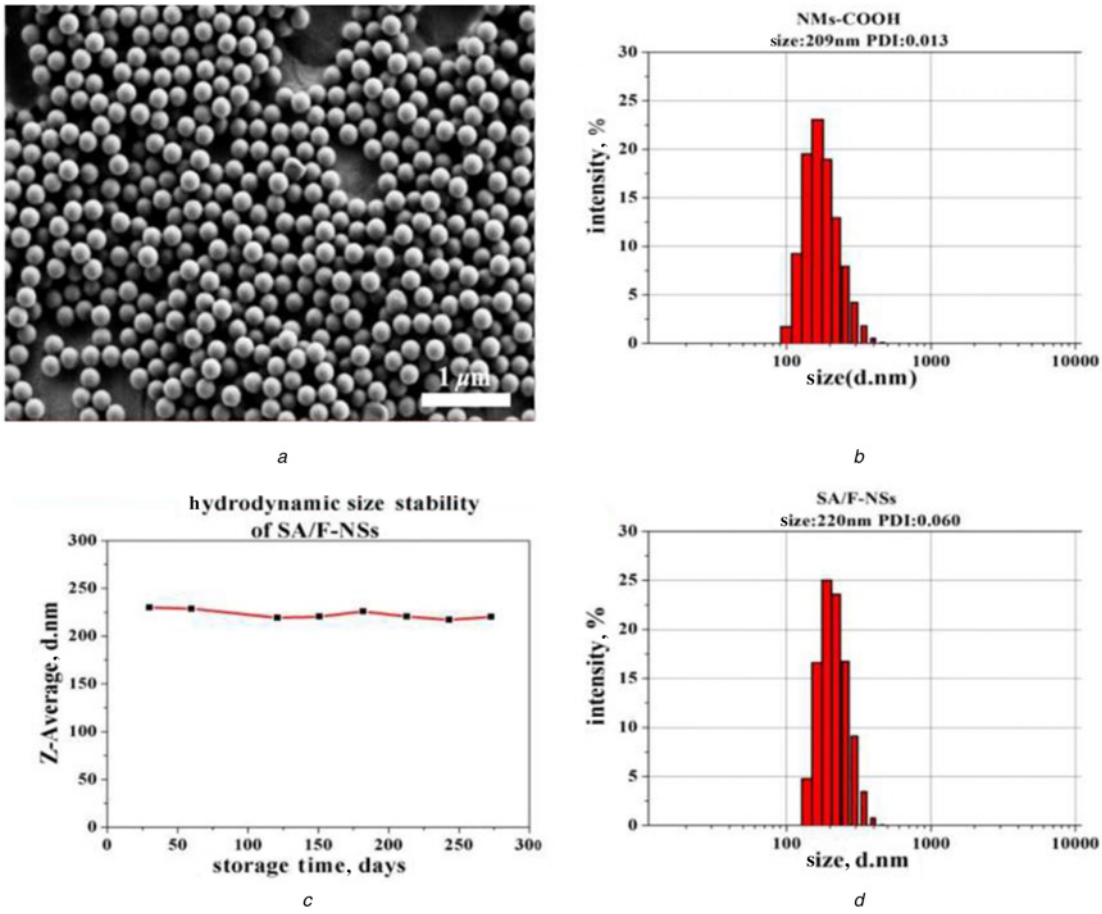

Fig. 3 *Characterisation of SA/F-NSs*
a SEM image of SA/F-NSs
b, *d* Are the hydrodynamic size distribution of NSs–COOH and SA/F-NSs respectively
c Hydrodynamic sizes of SA/F-NSs at different storage times

The UV–vis spectrophotometer analysis (Fig. 4*a*) showed that compared with the pure BSA, the absorption peaks of SAH–BSA shifted from 278 to 276 nm, also, the ratio of A_{280}/A_{260} shifted from 1.65 to 1.15. These changes indicated that a certain amount of SAH had been coupled to BSA. Furthermore, the activity of SAH–BSA conjugates was measured by the enzyme linked immunosorbent assay (ELISA) method. 1, 0.5, 0.2, 0.1, 0.05 mg/ml of SAH–BSA was coated in 96-well plates followed by BSA blocking, then reacting with rabbit monoclonal antibody against SAH was performed, and goat anti-rabbit IgG antibody with HRP was further used to catalyse TMB colouration at 450 nm. The results (Fig. 4*b*) proved that the conjugates had strongly concentration-dependent immunogenic activity.

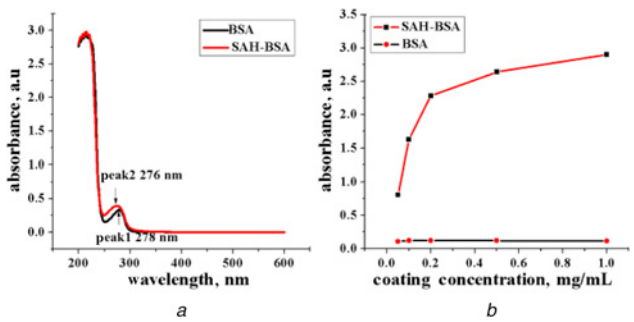

Fig. 4 *Characterisation analysis of SAH-BSA and BSA*
a UV–vis absorption spectra of BSA and SAH-BSA
b ELISA results of SAH-BSA and BSA

The assay employed a competitive immunofluorescence lateral flow test approach (Fig. 2*b*). SA/F-NSs as reporters because of their bright fluorescence and high signal-to-noise ratio were stored in a diluent and used simultaneously to dilute the enzyme treated samples. Biotin–antibody was fixed on the conjugate pad to specifically capture SAH in the sample where SAH was from SAM by HMT catalysis using Hcy as a substrate and the concentration of SAH was proportional to that of Hcy (Fig. 2*c*).

The test was started by loading 100 µl mixer of the diluted sample onto the sample pad and all liquid was absorbed and migrated along the strip by capillary forces. In this process, the SAH was captured by biotin–antibody in a competitive way with SAH–BSA on the test zone, at the same time, biotin–antibody or biotin–antibody–SAH composites were labelled by the SA/F-NSs due to the strong affinity between avidin and biotin. If no SAH was present in the sample, the antibody would be trapped by the SAH–BSA in the test zone, displaying the highest signal. When the biotin–antibody combined with SAH in the sample, it would flow past the test line and continue migrating until to be trapped by the goat anti-rabbit IgG to form a control zone. Therefore, the intensity of the fluorescence of the test zone is negatively related to the concentration of SAH, and the signal can be read out quantitatively with a portable immunofluorescence reader.

3.2. Sensitivity of the lateral flow strip assay for quantification of Hcy: The samples with different concentrations of Hcy, 50, 40, 30, 20, 10, 5 and 1 µM, were treated as experimental section and diluted 50 times with the sample diluent. Then 100 µl of the mixer was loaded onto the sample pad of the anti-SAH lateral

flow test strip with SAH–BSA and goat anti-rabbit IgG antibody on the nitrocellulose membrane as test line and control line separately. Eight minutes later, the fluorescence intensity can be read out by using an immunofluorescence reader. As expected, the brightness of the test zone decreased with increasing concentration of Hcy (Fig. 5*a*). For the better observation of changes in fluorescent signal values, the fluorescence signal values were normalised by the max, as shown in Fig. 5*b*, a linear relationship with a correlation coefficient (R^2) 0.9894 was obtained in a range of 1–50 µM Hcy, and the detection limit was calculated to be 0.26 µM. Compared with the clinical threshold value of 10 µM, the lateral flow test strip exhibited higher sensitivity and could predict the risk of cardiovascular and many other diseases. Moreover, compared with other methods of Hcy detection, such as chromatography detection and enzymatic cycling assay, the strip was easy to operate and timesaving, hence, had great potential in clinical applications.

3.3. Specificity of the lateral flow strip assay: The specificity was tested by comparing SAH with structural analogues as negative controls, including SAM and Cys, as shown in Fig. 6. The fluorescence intensity of Hcy (0 to 50 µM) decreased deeply with the increase of concentration, while there was no change of fluorescence intensity in control groups, suggesting the high specificity and anti-interference of the lateral flow test strip.

To verify the feasibility for clinical application, FBS samples containing Hcy were applied to the assay. The results proved that it was similar to that in the buffer, and we obtained a good calibration curve (Fig. 7) with a detection limit of 0.27 µM, which was a little different from that in the buffer, demonstrating that the strips we developed with high anti-interference were practicable in a complex matrix.

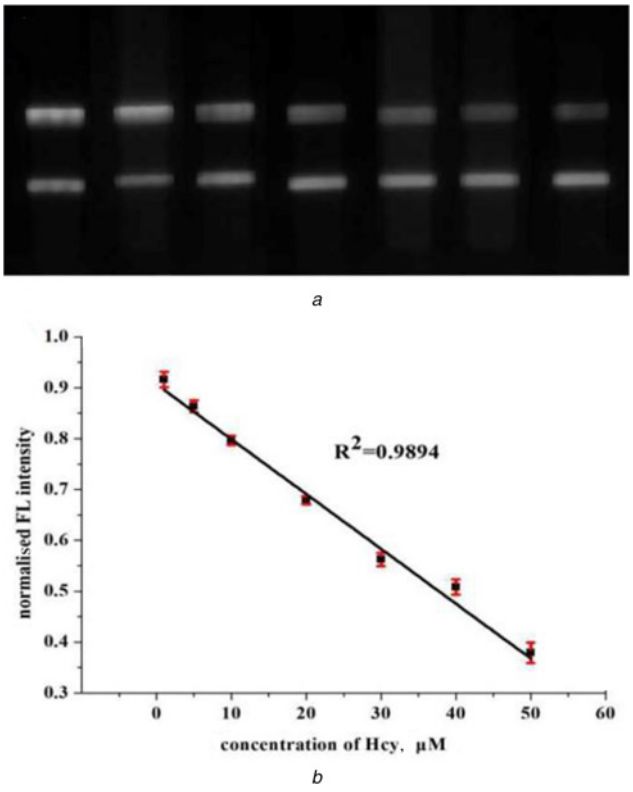

Fig. 5 *Detection results for samples with different concentrations*
a Fluorescence pictures of the test zone (top line) and control zone (bottom line) for the detection of Hcy in the concentration range of 1–50 µM
b Normalised fluorescence intensity as a function of Hcy concentration from 1 to 50 µM in the buffer

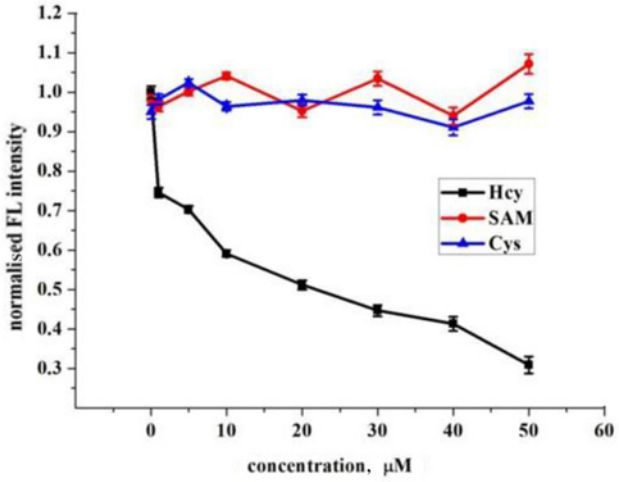

Fig. 6 *Normalised fluorescence intensity was detected for Hcy and different structural analogues in the concentration range of 0–50 µM in the buffer*

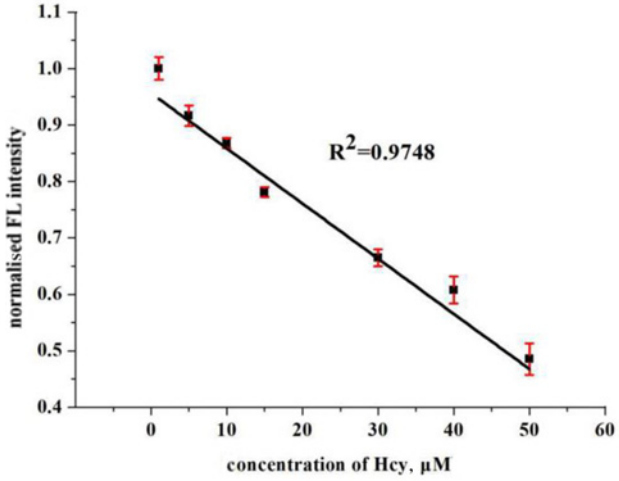

Fig. 7 *Immunofluorescence based lateral flow test strip for Hcy detection ranged from 1 to 50 µM in FBS*

3.4. Precision and stability: Precision and reproducibility were of great significance for strips further application, the intra-assay coefficient of variance (CV) using 5, 15 and 40 µM Hcy standard samples was evaluated. As shown in Table 1, the CV < 10% was obtained which indicated the good performance matching with the requirement of practical applications. The stability of the lateral flow test strips was also evaluated during the period of

Table 1 Reproducibility analysis of the Hcy test strip

Hcy concentration, µM	Intra-assay		
	Mean[a]	SD[b]	CV[c], %
5	4596.5	277.8	6.04
15	3752.3	259.8	6.93
40	2790.7	124.8	4.47

[a]Values represent the average of detected fluorescence intensity of parallel samples ($n = 10$).
[b]Values represent the standard deviation of parallel results ($n = 10$).
[c]CV = SD/mean.

doi: 10.1049/mnl.2018.5040

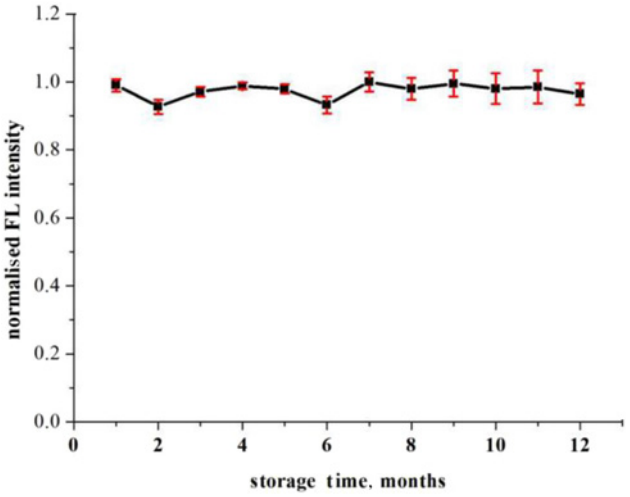

Fig. 8 *Stability test of the strips at different storage times*

storage of 12 months (Fig. 8), indicating excellent detection stability.

4. Conclusions: A novel immunofluorescence lateral flow test strip approach was developed to detect Hcy with the good linear relationship and a detection limit of 0.26 μM in the buffer and 0.27 μM in serum. The assay is sensitive and specific with good reproducibility and stability and can be completed in a short time (<30 min). The assay needed no expensive equipment and highly trained personnel, it was a breakthrough for Hcy detection with an immunofluorescence lateral flow test strip and provided a new method for the detection of many other risk factors of humans in clinical applications.

5. Acknowledgment: This work was supported by the National Key Research and Development Program of China (grant no. 2017YFA0205502), the National Natural Science Foundation of China (grant nos. 81571806 and 81671820), the Science and Technology Support Project of Jiangsu Province (grant no. BE2017763), the Jiangsu Provincial Special Program of Medical Science (grant no. BL2013029) and the Fundamental Research Funds for the Central Universities.

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