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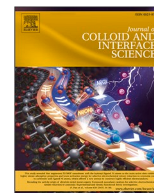

Regular Article

Synthesis of eco-friendly multifunctional dextran microbeads for multiplexed assays

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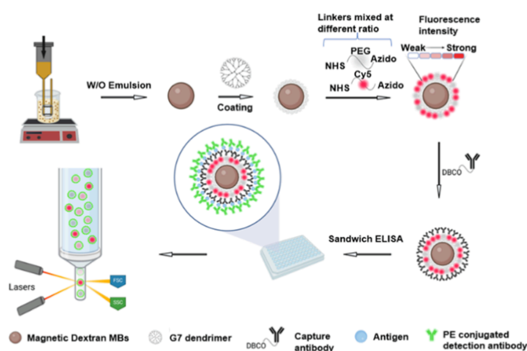
GRAPHICAL ABSTRACT

A new type of eco-friendly multifunctional dextran microbeads are synthesized for multiplexed assays.



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ABSTRACT

There has been an increasing demand for simultaneous detection of multiple analytes in one sample. Microbead-based platforms have been developed for multiplexed assays. However, most of the microbeads are made of non-biodegradable synthetic polymers, leading to environmental and human health concerns. In this study, we developed an environmentally friendly dextran microbeads as a new type of multi-analyte assay platform. Biodegradable dextran was utilized as the primary material. Highly uniform magnetic dextran microspheres were successfully synthesized using the Shirasu porous glass (SPG) membrane emulsification technique. To enhance the amount of surface functional groups for ligand conjugation, we coated the dextran microbeads with a layer of dendrimers via a simple electrostatic adsorption process. Subsequently, a unique and efficient click chemistry coupling technique was developed for the fluorescence encoding of the microspheres, enabling multiplexed detection. The dextran microbeads were tested for 3-plex cytokine analysis, and exhibited excellent biocompatibility, stable coding signals, low background noise and high sensitivity.

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1. Introduction

Measurement of analytes of interest in biological samples is essential for both life science research and clinical diagnostics [1–3]. Traditional methods such as enzyme-linked immunosorbent assay (ELISAs) are single assay techniques, whereby only one analyte is measured per test [4,5]. At certain circumstances, several biomarkers need to be monitored simultaneously in order to understand the complex underlying biochemical mechanisms or diagnose diverse disease states. Multiplexed analysis thus holds great potential in these cases [6–8].

One popular multiplexed analysis strategy is microbead-based suspension arrays. In contrast to ELISAs that generally rely on flat surfaces of well plates to capture targets, the bead arrays capture targets onto spherical beads suspended in liquid solutions [9–13]. The microbeads are encoded with varying fluorescent colours or intensities, which define 'spectral addresses' for each bead population. In a multiplex assay, the barcoded microbeads are coated with designated biorecognition elements to bind specific analytes. After incubation with the sample, the microbeads are read using flow cytometry to identify the fluorescent signature of the microbeads as well as quantify the amount of analyte bound on each bead population. As a result, multiple detection reactions can be carried out on the various bead populations in very small sample volumes, which greatly saves time and expense whilst reducing the risk of error when interpreting data [14–16].

In recent years, both academia and industry have devoted enormous efforts to develop microbead-based assays. Polystyrene (PS), PS copolymers and silica are the most common materials. Both organic and inorganic fluorophores have been used to optically encode microbeads [10,17–19]. Various methods are available to synthesize the microbeads. For instance, the commercial Luminex multi-analyte profiling (xMAP) technology uses classifying PS microbeads dyed with distinct proportions of red and near-infrared fluorophores. The PS seed particles are first prepared by dispersion polymerization of styrene monomers, then a swelling procedure is followed to dope the fluorescent labels to the seed PS microspheres [20,21]. Another example, Zhang et al. created a type of hybrid PS-silica microbeads with a host − guest structure. The microbeads consist of two building blocks: a host PS particle deposited with a layer of quantum dots (QDs) and a set of FITC-doped guest silica nanoparticles with different fluorescent intensities. After chemically assembling the two building blocks together, a library of microbeads is formed, encoded by the combination of size, fluorescence emission wavelength, and intensity of the fluorescence color [17]. Alternatively, Leng et al. prepared near infrared (NIR)-emitting QDs, and applied emulsification technique to incorporate the QDs into polystyrene–maleic anhydride (PSMA) microbeads to obtain fluorescent barcoded microbead array [22].

Although these microbeads have shown favourable features such as high stability, and high multiplex capabilities, several outstanding issues remain to be addressed. One primary concern of microbead arrays is their toxicity [23]. Most synthetic polymers and semi-conductor QDs are not biodegradable, and their environmental persistence could reach hundreds of years. The widespread use of microbeads will cause a long-term damaging effect on marine life, the environment and human health [22–25]. Eco-friendly materials like cellulose, starch, and aliphatic polyesters would become ideal substitutes for plastic. Whereas, very few research work has been carried out to synthesize microbeads using these biodegradable materials [17,26,27].

Secondly, current methods to fabricate barcoded microbeads are lengthy and complex. The swelling technique requires two steps of polymerization, the host–guest assembling technique involves multiple layer-by-layer charged polymer coatings, while encapsulation of the QDs during emulsification needs considerable pre-synthetic QD surface modification [16,28–31]. Other encoding techniques, such as color coding and binary encoding, require the application of complex and expensive photolithography techniques [32–34]. Shape coding necessitates 3D printing technology, which is costly, time-consuming, and requires specialized technical personnel [35]. As a result, difficulties are encountered in mass production of robust and reproducible barcoded materials. Facile and rapid methods to construct barcodes are much needed [36,37].

Moreover, obtaining enough functional groups on the microbeads is another challenge. In the abovementioned examples, PS microbeads could be modified by copolymerization of styrene with acrylic acid during seeded polymerization; the silanol groups on the silica particle can react with a modifying agent to graft carboxylic groups; and hydrolysis of the anhydride groups on the PSMA bead leaves carboxyl groups exposed on the surface. Despite the fact that these modification strategies are able to generate active surface groups for biomolecule attachment, it is frequently observed that the density of immobilized ligands is not sufficiently high, thereby significantly comprising the sensitivity and the linear range of the assays [16,28–31].

Since natural polysaccharides such as dextran are well known for their superior biodegradability and biocompatibility, in this study, we hypothesize that the microbeads made of dextran could be a novel sustainable alternative to plastic particles, and they could be used as multiplexed assay platforms after proper surface modification. As shown in Fig. 1, we employed a Shirasu-porous-glass (SPG) membrane emulsification process to generate stabilized dextran microdroplets, and subsequent crosslinking of dextran polymer with adipic dihydrazide (ADH) resulted in the formation of solid spherical and uniform dextran microbeads. To create abundant functional groups on the microbead surface, we innovatively coated the dextran microbeads with dendrimer nanoparticles via electrostatic interaction. Dendrimers are three-dimensional globular macromolecules with a perfectly branched tree-like structure. They provide a large number of amine functional groups at the end terminal that are easily accessible, ensuring high efficiency of further ligand conjugation [38,39]. Last but not least, we showed a very simple and unique way to encode the microbeads. Fluorescently labelled *N*-hydroxysuccinimide (NHS) ester linkers were utilized to react with the primary amine groups on the dendrimers [40]. By adjusting the color of fluorescent labels on the NHS ester linkers and by changing the mixing ratio of these NHS ester linkers, we easily obtained barcoded dextran microbeads with different fluorescence colours and intensities. Finally, we tested the feasibility of the dextran microbeads for multiplexed assay. Human IFN-gamma, IL-4 and IL-6 were successfully detected in a 3-plex cytokine immunoassay, with the limits of detection (LODs) down to 2–10 pg/mL. The new type of dextran microbeads are environmentally friendly, facile to construct and highly sensitive and stable. It holds great potential to become a valuable high-throughput analysis platform.

2. Experimental section

2.1. Reagents

Dextran (molecular weight: 500 kDa) and pullulan were obtained from Pharmacosmos A/S (Denmark). Human IFN gamma Matched Antibody Pair Kit, Human IL-6 Matched Antibody Pair Kit and Human IL-4 Matched Antibody Pair Kit were bought from Antibodies-online (Sweden). Nhydroxysuccinimidyl ester-Cy5-azide (NHS ester-Cy5-azide) and Nhydroxysuccinimidyl ester-(polyethylene glycol)-azide (NHS ester-PEG-azide) linkers were provided from Click Chemistry Tools (Scottsdale, AZ). Bovine serum albumin (BSA), glycine, glutaraldehyde, polyethylenimine (PEI 25KDa), ADH, G7 dendrimer, hydrochloric acid, glutaraldehyde solution, acetoacetanilide and magnetic nanoparticles solution were purchased from Sigma-Aldrich (Germany). Sodium periodate, span 80, tween 80, phosphate-buffered saline containing 0.05 wt% Tween-20 (PBST), anhydrous dimethyl sulfoxide (DMSO), cyclohexane, regenerated cellulose membrane, fluorescent antibody, streptavidin-labeled PE, Pierce™ Protein-Free (PBS) Blocking Buffer and phosphate buffered saline (PBS) (pH 6.5–7.5) were provided from Thermal Fisher (USA). Millipore-purified water was used during

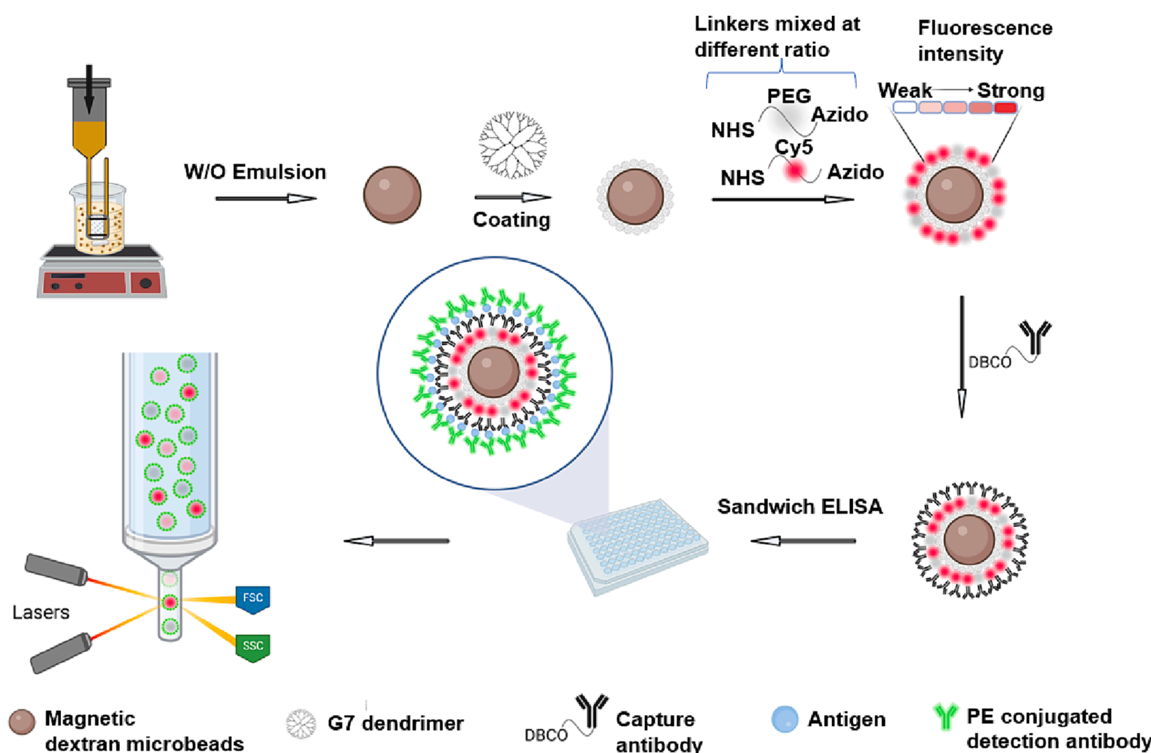

Fig. 1. Schematic illustration of the synthesis of multifunctional dextran microbeads for multiplexed immunoassays.

the whole experiment.

2.2. Apparatus

SPG glass membranes (pore size is 5.9 μm) and SPG emulsification device were purchased from SPG Technology Co., Ltd (Japan). The particle size and PDI of dextran microbeads were determined by a dynamic light scattering (DLS) analyzer (Malvern Panalytical, United Kingdom). The hydrogen-1 spectroscopy of oxidized dextran was recorded with a nuclear magnetic resonance spectrometer (NMR, Bruker 700 MHz, UK). The morphology of the microspheres was evaluated using the EVOS M5000 Imaging System (Invitrogen by Thermo Fisher Scientific, USA), where filters for transmitted light phase-contrast and fluorescence were selected as detectors (Filters: DAPI, Excitation: 357 nm, Emission: 447 nm). Flow cytometry analysis was carried out using a BD Biosciences Immunocytometry System (BD FACS Calibur™, SanJose, CA). A violet laser (405 nm) was used to stimulate the Brilliant Violet™ 421 (BV421) channel (450/50 nm). Qsonica Q700 Sonicator was purchased from Avanto (Denmark). Electric stirrer was obtained from Steinberg Systems (Germany). IKA Shakers (MS 3 basic) was bought from IKA. Hand-Held Magnetic Plate Washer was provided from Thermal Fisher (USA). NucleoCounter® NC-200™ was purchased from ChemoMetec A/S (Denmark). Gel permeation chromatography (GPC) was purchased from Shimadzu (Japan).

2.3. Synthesis of oxidized dextran

Before the preparation of dextran microbeads, partially oxidized dextran was synthesized. In order to obtain as many aldehyde groups as possible, the dextran with a larger molecular weight (molecular weight: 500 kDa) was selected. Ten gram of dextran was dissolved in 40 mL of Milli Q water by magnetic stirring for 5 min. The solution was stirred for five hours at room temperature after adding sodium periodate (6 g, 28.51 mmol). The product was purified by dialysis of the solution against distilled water using a regenerated cellulose membrane having a molecular weight cut-off (MWCO) of 7000 g/mol. After changing the water five times, the sample was lyophilized to yield a white powder (4.82 g).

2.4. Determination of aldehyde content in oxidized dextran

The test sample (oxidized dextran) was diluted to a concentration of 3.5 mg/mL using MQ water. Glutaraldehyde stock solution (0.1 M) was prepared by diluting an appropriate amount of 25 % aqueous glutaraldehyde solution with MQ water. The standard glutaraldehyde solutions were further diluted with MQ water to a series of concentrations (1×10^{-6}, 2×10^{-6}, 4×10^{-6}, 6×10^{-6}, 8×10^{-6}, 10×10^{-6} M). A 0.2 M acetoacetanilide stock solution was prepared by dissolving 88.5 mg of acetoacetanilide in 1 mL of ethanol and diluted to 2 mL with MQ water. A 4.0 M ammonium acetate stock solution was prepared by dissolving 1.54 g of ammonium acetate in 5 mL MQ water. A mixture of 100 μL of standard glutaraldehyde working solution or the test sample, 500 μL of 4.0 M acetic ammonium, 200 μL of 0.2 M acetylacetone benzylamine and 200 μL of ethanol was prepared. As the excitation and emission wavelengths of aldehyde groups in the solution are 370 nm and 470 nm respectively, the relative fluorescence intensity of the standard solution and sample solution was measured at 470 nm to characterize the amount of aldehyde groups after standing for 10 min. Each measurement was repeated three times.

2.5. Determination of molecular weight of oxidized dextran

Gel permeation chromatography (GPC) was utilized to determine the molecular weight of the oxidized dextran. Pullulan with different molecular weights (750000, 212000, 10175, 6255, 1145 Da) was dissolved separately in a phosphate buffer solution (pH 7.4, 0.1 M) to prepare standard samples with a final concentration of 2.5 mg/mL. Similarly, oxidized dextran was dissolved in a phosphate buffer solution to prepare test samples with the same concentration. Due to the relatively uniform mass sensitivity provided by GPC, TSK gel column was used to determine the retention time of pullulan, and calibration curves were subsequently prepared. The corresponding molecular weights of oxidized

dextran were determined by the retention times of dextran derivatives. In this case, 100 mM of NaCl was used as the mobile phase (flow rate = 1.0 mL/min), and the column temperature was set at 40 °C.

2.6. Preparation of magnetic dextran microbeads

SPG emulsification device was used to prepare the magnetic microbeads from partially oxidized dextran. In the first step, 200 mL of cyclohexane containing 10.0 g of Span 80 and 3.0 g of Tween 80 were separated by ultrasonication for 10 min. Then, 1 mL of 1 % oxidized dextran (Dex-CHO), 40 μL of magnetic nanoparticles, and 100 μL of ADH solution (2 %) were mixed and added into the SPG emulsification device and emulsified for 10 min. Following mechanical stirring at room temperature for 4 h, microgel particles were collected by centrifugation (4700 rpm, for 8 min) and washed two times with ethanol and PBS, respectively, to remove excess surfactants (Span 80/Tween 80).

2.7. Coating the dextran microbeads with PEI and dendrimers

The prepared microbeads were mixed with 2 mL of PEI or dendrimer (0.5 mg/mL). The mixture was shaken at room temperature for 1 h. The excess dendrimer was then removed by washing three times with mili-Q water.

2.8. Encoding by coupling of fluorescent compounds with magnetic dextran microbeads

The two dual-purpose linkers were mixed and applied to dextran microbeads for encoding. The detailed procedure was as follows. NHS ester-Cy5-azide was dispersed in PBS (200 μL) with a final concentration of 0.02 to 50 μg/mL. The 200 μL of corresponding dilution of NHS ester-Cy5-azide and 3 μL of NHS ester-PEG-azide (1 mg/mL) were then mixed together. Eight thousand microbeads were added into the mixture and shaken for 1 min, forming a homogeneous solution. The reaction was carried out 2 h at room temperature. The resulting Q-MPS were isolated by magnet and washed three times with mili-Q water.

2.9. Bioconjugation with antibodies

For multiplexed detection of cytokines, three barcodes with coding addresses of Cy5 dye in different fluorescent intensities were conjugated with specific capture antibodies corresponding to IFN-γ, IL-4, and IL-6, respectively. Typically, 100 μL of 0.5 mg/mL capture Abs was reacted with 4 μL of NHS ester-DBCO solution for 2 h at room temperature, and then quenching buffer was added to block the reaction. The modified capture Abs was then added to 250 μL of microbeads after centrifugal washing, and the reaction was incubated with rotation for 2 h in 0.5 mL of PBS, followed by washing with PBS buffer three times. Afterwards, the antibody-conjugated dextran microbeads were added into 300 μL of pierce™ protein-free (PBS) blocking buffer for further incubation at 37 °C for 1 h. Finally, the obtained dextran microbeads were washed with mili-Q water three times and stored in 1 mL of PBST (pH = 7.4, 0.5 wt% BSA).

2.10. Multiplexed detection of cytokines

The three-plexed detection of cytokines was performed in a 200 μL of PBS assay system by using a 96-well plate. Before the test, the corresponding antigen was prepared in gradient concentrations (2 pg/mL – 10 ng/mL) and stored at 4 °C for further use. For each multiplexed test, 4000 microbeads conjugated with each capture antibody in 2 μL of buffer were mixed with 100 μL of a testing sample containing antigen with various concentrations. After incubation at 25 °C for 1 h, 50 μL of mixed biotin-labeled detection antibodies were added and the reaction system was further incubated at 25 °C for 30 min. Then 100 μL of PE-labeled streptavidin was added into each well and incubate at 25 °C for 30 min. All incubation steps were accompanied by shaking and followed by washing with washing buffer and a magnetic separator. Finally, the multiplexed detection results were analyzed by flow cytometry via FSC, SSC (515 ± 12.5 nm), Alexa Fluor 647 (670 ± 30 nm), and PE (586 ± 15 nm) channels. The signals from FSC and SSC channels were excited by the first laser of 488 nm. The signals from Alexa Fluor 647 channel were excited by the second laser of 640 nm, which were utilized to recognize the type of barcodes. Meanwhile, the signals from the PE channel, which were excited by the third laser of 532 nm, was used to determine the concentration of the corresponding analytes.

2.11. Stability test

In order to investigate the influence of long-term storage on the stability and reproducibility of our fluorescent suspension array results, a batch of synthesized dextran microspheres conjugated IFN-γ capture antibodies was stored at 4 °C for four months. Different concentrations of IFN-γ (0.01, 2, 3.9, 7.8, 15.6, 32.5, 125, 500 pg/mL, and 1, 2, 5 ng/mL) were selected for ELISA measurements as described above, with each concentration repeated three times.

2.12. Biodegradability test

10 μg of dextran microspheres were each weighed and mixed with hydrochloric acid solutions at pH levels of 2, 3, 4, 5, and 6, resulting in microsphere suspensions with a concentration of 0.1 mg/mL. Additionally, an equal mass of dextran microspheres was dissolved in MQ water as a control group. All six samples were then placed at 50 °C and left undisturbed for 24 h, after which their morphology was observed under a microscope.

3. Results and discussion

3.1. Synthesis of magnetic dextran microbeads

Most of the traditional or commercial microbeads are made of synthetic polymers, such as polystyrene, and the fabrication methods often require the use of harsh solvents or strict synthetic conditions, such as an oxygen-free environment. Here, we presented a simple way to synthesize microbeads in eco-friendly oxidized dextran polymer. To obtain uniform and solid dextran microbeads, we developed a SPG membrane-based emulsification process to generate homogenous water-in-oil dextran microdroplets. A small amount of crosslinker was incorporated in the droplet, so that stable dextran microbeads can be formed.

We initially oxidized dextran to create aldehyde groups (Fig. 2a) through the classical periodate oxidation method, which provided the necessary functional groups for crosslinking during the emulsification process. The presence of aldehyde groups was confirmed through ^{1}HNMR analysis, showing a weak peak at a chemical shift of 9.5 (Fig. S1). Subsequently, we extruded mixtures containing oxidized dextran, carboxyl acid-modified magnetic nanoparticles (to provide magnetic separation properties for the microbeads) and the crosslinker ADH through a hydrophobic SPG membrane under nitrogen pressure, generating water-in-oil droplets (Fig. 2b). The SPG membrane has a very narrow pore size distribution and high mechanical strength, therefore this membrane emulsification method makes it possible to produce monodispersed emulsions. Moreover, the throughput of the SPG membrane emulsion is extremely high, and 1 mL of dextran solution could be processed in just 10 min. The crosslinker ADH contains two amine groups, which can form covalent bonds with the aldehyde groups on the dextran polymer, thereby crosslinking the polymer. After solvent evaporation, the resulting solidified dextran microbeads were then collected using a magnet and re-suspended in PBS. As shown from the SEM image and size measurement (Fig. 2c and d), the produced dextran microbeads were highly homogeneous and monodispersed. The average

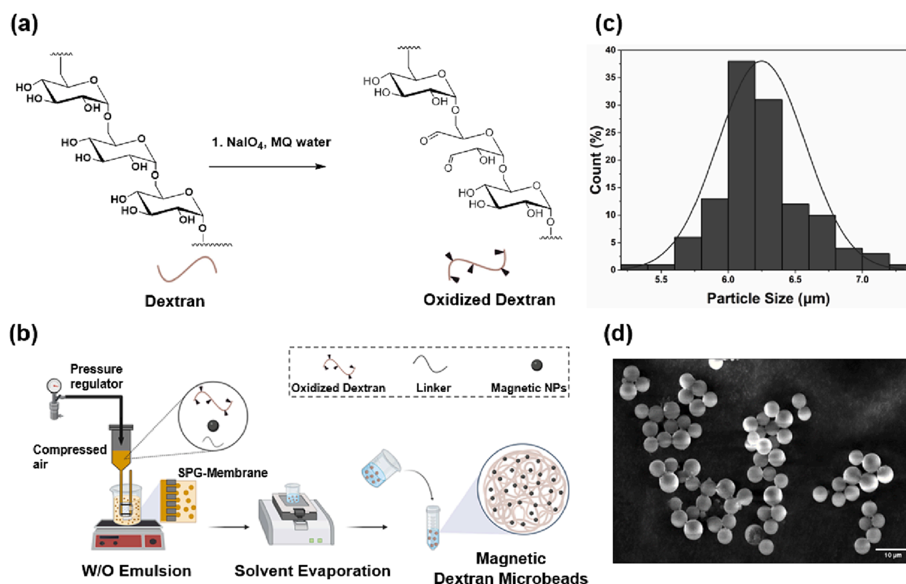

Fig. 2. (a) Schematic synthesis reactions of oxidized dextran. (b) Schematic diagram of the synthesis of the magnetic dextran microbeads. (c) Particle size distribution of the magnetic dextran microbeads. (d) The SEM image of the magnetic dextran microbeads.

size is 6.04 μm and the coefficient of variation (CV) values of size is less than 5 % (Tables S2 and S3). After comparing with several commonly used commercial microspheres, it can be concluded that the uniformity of our beads meets the market standards (Fig. S2 and Table S3).

To our best knowledge, this is the first demonstration of highly uniform dextran-based microbeads. The use of SPG technology offers numerous advantages in terms of mass production and easy operation. Although SPG membrane method has been used to prepare microspheres in other polymer materials (Table S1), it is not trivial to develop the protocol for the dextran polymer. Parameters including the charge and hydrophobicity of the modified dextran, the selection and amount of crosslinker, the size and charge of the magnetic nanoparticles, as well as the selection of solvent and surfactant, were all critical for the successful synthesis of magnetic dextran microbeads. The protocol developed in this work could be inspirational for synthesizing other types of polymer microbeads using SPG membrane. Moreover, the solvents used in the manufacturing process are non-corrosive and easily volatile, and the surfactants are also considered safe and green.

3.2. Determination of aldehyde content in oxidized dextran

To quantify the content of aldehyde groups on oxidized dextran, the fluorescence intensity of aldehyde groups in the samples was measured using a microplate reader. The specific method employed was based on the reaction of acetoacetanilide with glutaraldehyde in the presence of ammonia, as reported by Nonsuwan *et al.* [41]. Under optimal conditions, a series of standard solutions of glutaraldehyde were measured to construct the calibration plot (as shown in Fig. S3). The fluorescence response exhibited linearity within the range of glutaraldehyde concentrations from 0 to 10×10^{-7} M. The linear regression equation was y = 13496x + 212, where x and y represent the concentration of glutaraldehyde in 10^{-7} M and the fluorescence response intensity, respectively, with a correlation coefficient of 0.9965.

The concentration of aldehyde groups in the test sample was determined to be 2.5×10^{-8} M based on the generated calibration curves and the detected fluorescence intensity of oxidized dextran. The degree of oxidation in oxidized *gluco*-polysaccharides was assessed by calculating the percentage of oxidation per glucose unit, defined as the number of C—C bonds in the 1,2-glycol cleavage of each glucose unit. Here, the degree of oxidation for oxidized dextran was found to be 32 %, indicating the high content of aldehyde groups in the functionalized dextran.

3.3. Determining molecular weight of oxidized dextran

GPC as a type of size exclusion chromatography (*SEC*), is a commonly used method for the separation of linear polymers and measurement of their molecular weights. It is capable of detecting polymer samples with molecular weights ranging from approximately 100 to several million Daltons. As illustrated in Fig. S4, GPC was applied for the generation of a calibration curve for the polymer standard pullulan. The final fitted exponential function is represented as exp(x) = 4E + $10e^{-1.743x}$, with a correlation coefficient of 0.9979. The retention time of the synthesized oxidized dextran was determined to be 6.48 min. Therefore, the corresponding molecular weight was calculated to be 498 kDa through the obtained calibration curve. Since the oxidation process involves only the dehydrogenation reaction of some glucose units, the molecular weight shows no significant change compared to its original state.

3.4. Magnetic property of dextran microbeads

Due to their safety and excellent biocompatibility, magnetic nanoparticles have been widely applied in various fields such as disease diagnosis and targeted drug delivery [42]. To evaluate their magnetic performance, 40 μL/mL of magnetic nanoparticle were added to a 1 mL solution during the synthesis process. As shown in Fig. S5a-b, a comparison was made to the microbeads without magnetic nanoparticles, which appeared as a milky white liquid. Upon the addition of iron oxide nanoparticles, the color of the microemulsion changed from white to dark brown. This change in color indicated the successful incorporation of magnetic nanoparticles. Furthermore, as shown in Fig. S5c, the magnetic dextran microbeads exhibited exceptional magnetic responsiveness, as they could be collected by a magnet within 2 min. The result highlighted the effectiveness of the microbeads' magnetic properties.

3.5. Surface modification of dextran microbeads

The above synthesized magnetic dextran microbeads have aldehyde groups on the surface, therefore they are negatively charged. In order to introduce high amount of functional amine groups on the surface for ligand conjugation, we innovatively coated the microbeads with a layer

of cationic dendrimers via electrostatic interaction. The polymer coating is a frequently employed method to modify the surface of microbeads. PEI is one of the most widely used cationic polymers for this purpose. However, the number of amino groups provided by linear or branched PEI is relatively low, which negatively affects the density of immobilized ligands and subsequently the sensitivity of the whole assay. In contrast, dendrimers are three-dimensional branched tree-like structures, consisting of an ethylenediamine core, a repetitive branching amidoamine internal structure and a large amount of primary amines at terminals. For instance, G7 PAMAM dendrimer particle has a diameter of 20 nm and contains 512 surface primary amine groups. These amine functional groups are easily accessible, ensuring highly efficient ligand conjugation. In addition, the dendrimers are biodegradable and biocompatible as they have been used as drug delivery systems.

In this study, we compared the dextran microbeads coated with PEI and dendrimers, respectively. Zeta potential measurements (Fig. 3a) revealed that with the PEI coating, the zeta potential of dextran microbeads changed from −23.0 mV to +22.0 mV, while with dendrimer coating, the zeta potential increased significantly to +38.1 mV, indicating that dendrimer coating provided a higher number of surface amine groups. SEM investigation of the morphology of dextran microbeads after cationic polymer coating (Fig. 3b-d) demonstrated that the surface modification with either PEI or dendrimer did not cause any significant changes in the morphology. The average microbead size and CV after cationic polymer coating were calculated, as shown in Table S4. These findings suggest that dendrimers could function as an efficient coating material to modify the surface of dextran microbeads.

3.6. Encoding the dextran microbeads

After obtaining the dendrimer-coated magnetic dextran microbeads, we then added optical barcodes to the microbeads. Here we presented a new encoding strategy. Unlike conventional ways that dope fluorescent dyes or quantum dots inside the microbeads, we encoded the microbeads by simply reacting the primary amine groups on the surface of microbeads with fluorescently labelled NHS ester linkers (Fig. 4a). The NHS linkers have dual functions: as a linker for subsequent antibody conjugation, and at the same time, labeling the dextran microbeads with different fluorescence colors and intensities. Two types of linkers were used: linker 1 (NHS ester-Cy5-Azido) contains the fluorescent molecule Cy5, while linker 2 (NHS ester-PEG-Azido) incorporates non-fluorescent PEG molecule.

As shown in Table 1, we designed buffer solutions with seven-level fluorescence intensities by adjusting the molar ratio of the two linkers from 1:150 to 1:6000. The microbeads were incubated in the solutions for 30 min. The microscopic image in Fig. 4a illustrates the fluorescent color after coupling the linkers with dendrimer-modified microspheres, demonstrating the successful covalent conjugation of the linkers.

After encoding, the microspheres were mixed in a single tube and characterized for decoding accuracy using flow cytometry. As shown in Fig. 4b, seven clusters were distinctly identified through the fluorescence intensity in the Alexa 647 channel, corresponding to the seven encoded fluorescent microspheres. Fig. 4c displays a histogram of the population, where each individual peak presents a specific fluorescence intensity. No overlap was observed, indicating clear barcode classification and minimal interference between signals, enabling accurate decoding of the optical barcodes. As shown in Table 1, the fluorescence CV of seven-plex encoded dextran microspheres was less than 15 %, confirming the excellent fluorescence dispersion and encoding accuracy of the microspheres. The encoding capacity could be easily expanded via adjusting the color of fluorescent labels on the NHS ester linkers. Compared to the previously reported fluorescence barcoding methods for microbeads, our approach is much simpler, more flexible and cost-effective. Therefore, it offers powerful alternative to barcode microbeads libraries for high-throughput analysis.

3.7. Application of dextran microbeads in multiplexed cytokine detection

To demonstrate the applicability of the encoded microbeads in multiplexed detection, three barcoded microbead groups (group B, C and E) were randomly selected to perform immunoassays to simultaneously detect three cytokines, IFN-γ, IL-4, and IL-6. Immobilization of corresponding capture antibodies on the microbeads is a critical step, as the density and bioactivity of the immobilized antibodies directly determines the sensitivity and specificity of the immunoassay. Here, the click chemistry was utilized for antibody conjugation. Since during the

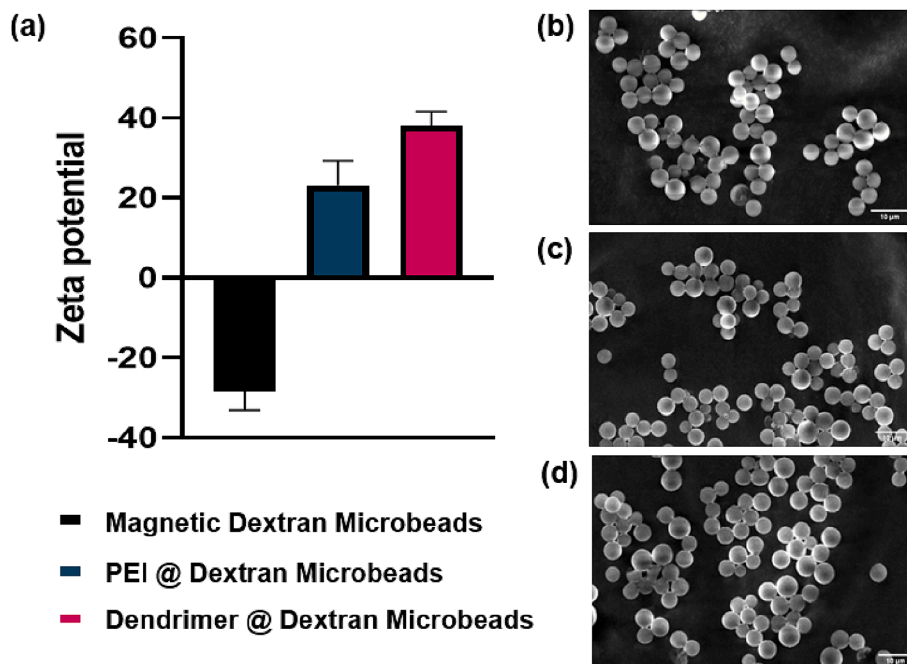

Fig. 3. (a) Zeta potential of microbeads with different surface modifications. Representative SEM images of (b) magnetic dextran microbeads, (c) magnetic dextran microbeads with PEI modification, and (d) magnetic dextran microbeads with dendrimer modification. Scale bar: 10 μm.

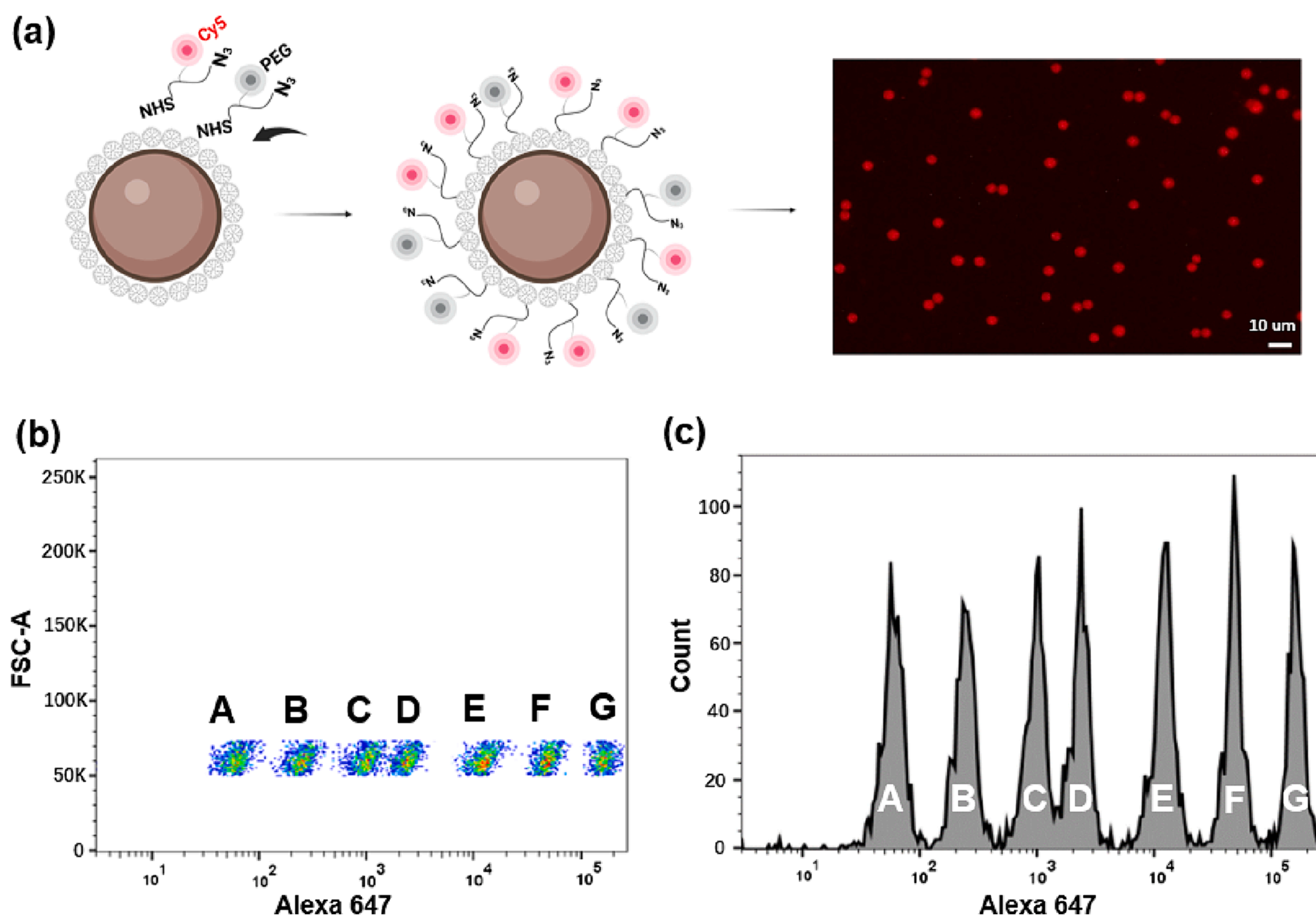

Fig. 4. (a) Schematic illustration of linkers coupling on the dendrimer-modified microsphere surface, along with the corresponding microscopy image. (b) Fluorescence intensity scatter plots of the mixed samples after gating under the Alexa Fluor 647 channel (668 nm). (c) The encoded signals of dextran microbeads with corresponding concentrations of Cy5 in the histogram mode.

Table 1
The molar ratio of the two linkers for the seven-plex fluorescent encoding and their corresponding fluorescence CV.

	Linker 1 (NHS Ester-Cy5-Azido)	Linker 2 (NHS Ester-PEG-Azido)	Mixed Ratio (Linker 1/Linker 2)	Fluorescence CV (%)
1 (A)	1 μL (1 μg/ml)	6 μL (1 mg/ml)	1:6000	14.19
2 (B)	1 μL (2 μg/ml)	6 μL (1 mg/ml)	1:3000	12.9
3 (C)	1 μL (5 μg/ml)	6 μL (1 mg/ml)	1:1200	13.3
4 (D)	1 μL (7.5 μg/ml)	6 μL (1 mg/ml)	1:800	12.26
5 (E)	1 μL (10 μg/ml)	6 μL (1 mg/ml)	1:600	12.08
6 (F)	1 μL (30 μg/ml)	6 μL (1 mg/ml)	1:200	10.93
7 (G)	1 μL (40 μg/ml)	6 μL (1 mg/ml)	1:150	9.61

* Total reaction volume is 500 μL after adjusting by PBS.

encoding step, the surface of microbeads has already been modified with azido group by attaching the azido-containing linkers, we only needed to modify the antibodies with the DBCO linker. When brought together, Huisgen cycloaddition rapidly formed between the azido and alkyne groups, allowing the antibodies to be covalently attached to the microbeads. We chose the click chemistry approach is because the reaction is fast, efficient and occurs at high conversion rates. In addition, it works in aqueous solvent systems and requires relatively low temperatures, making the reaction conditions milder more environmentally friendly. Consequently, high amount of antibodies with good bioactivities could be immobilized on the microbeads.

To develop the immunoassays, we optimized the number of capture antibodies on the dextran microbeads conjugated with NHS ester-PEG-Azido linker. We labelled the DBCO-linked capture antibody with PE molecule for signal measurement. The number of microbeads was fixed at 4000, while the antibody was increased from 0.1 to 6 μg. After immobilization and washing, the microbeads were run in flow cytometer. The fluorescence signal reached saturation when 5.1 μg ($\approx 2 \times 10^{10}$ molecules) antibody was added, showing that at this condition, the microbeads were fully covered by the antibody. The density of the immobilized antibody was calculated to be $\approx 5 \times 10^6$ molecules/bead), which demonstrated significant improvement compared to previous studies [43,44], where the antibody density achieved with surface immobilization was around $\sim 10^5$ molecules/bead.

Subsequently, three different sets of fluorescently encoded microspheres carrying specific capture antibodies were constructed for cytokine immunoassays. As depicted in Fig. 5a, the capture antibody-conjugated microbeads were incubated with samples containing

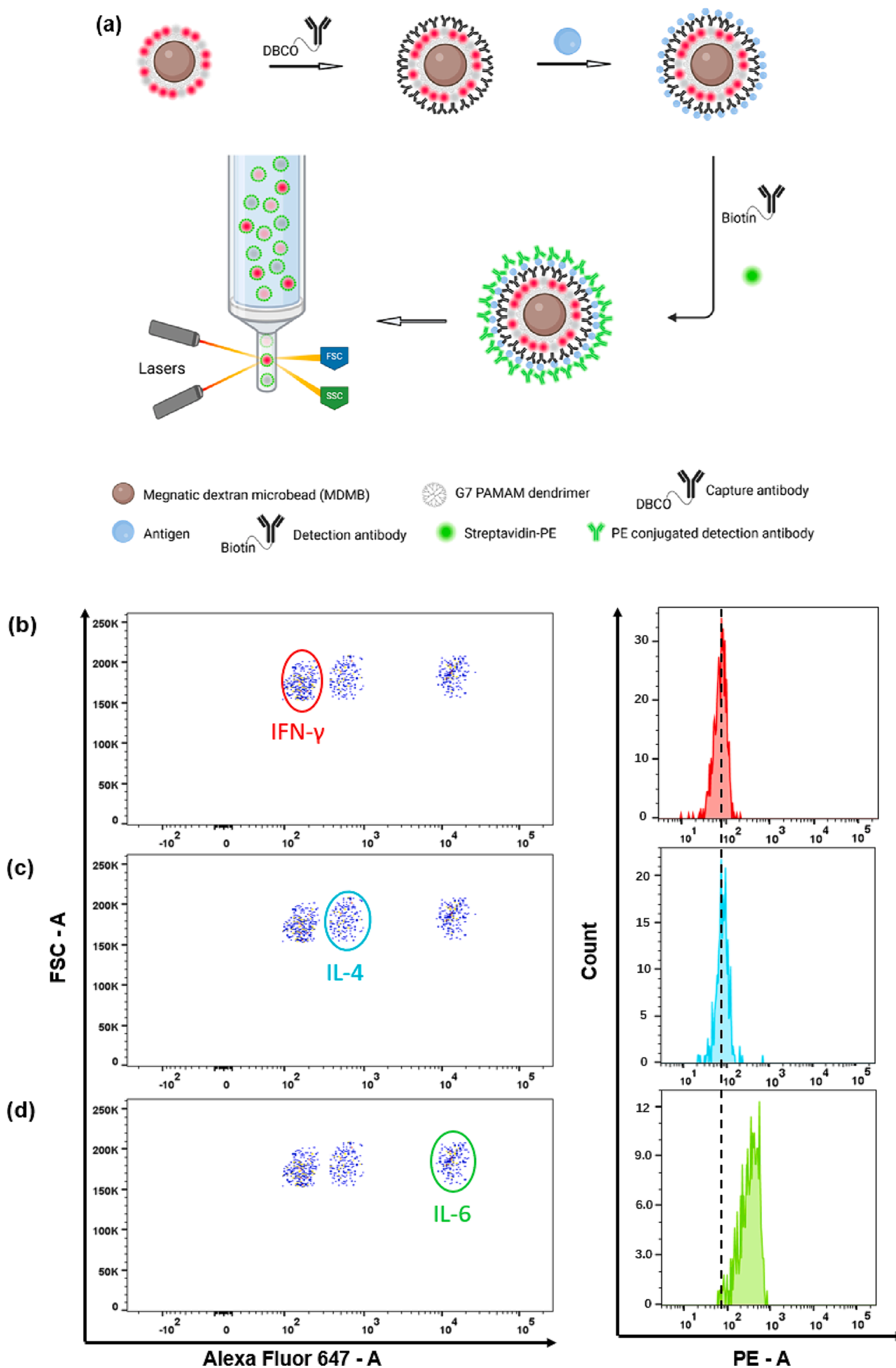

Fig. 5. (a) Schematic diagram of immunoassay with flow cytometry. (b-d) Specificity test for the three cytokines: IL-4, IFN-γ, and IL-6 respectively. (e-g) Linearity test for IFN-γ, IL-4, and IL-6 respectively. (h) Standard curves obtained from 3-plex (IFN-γ, IL-4, and IL-6) multiplexed assays.

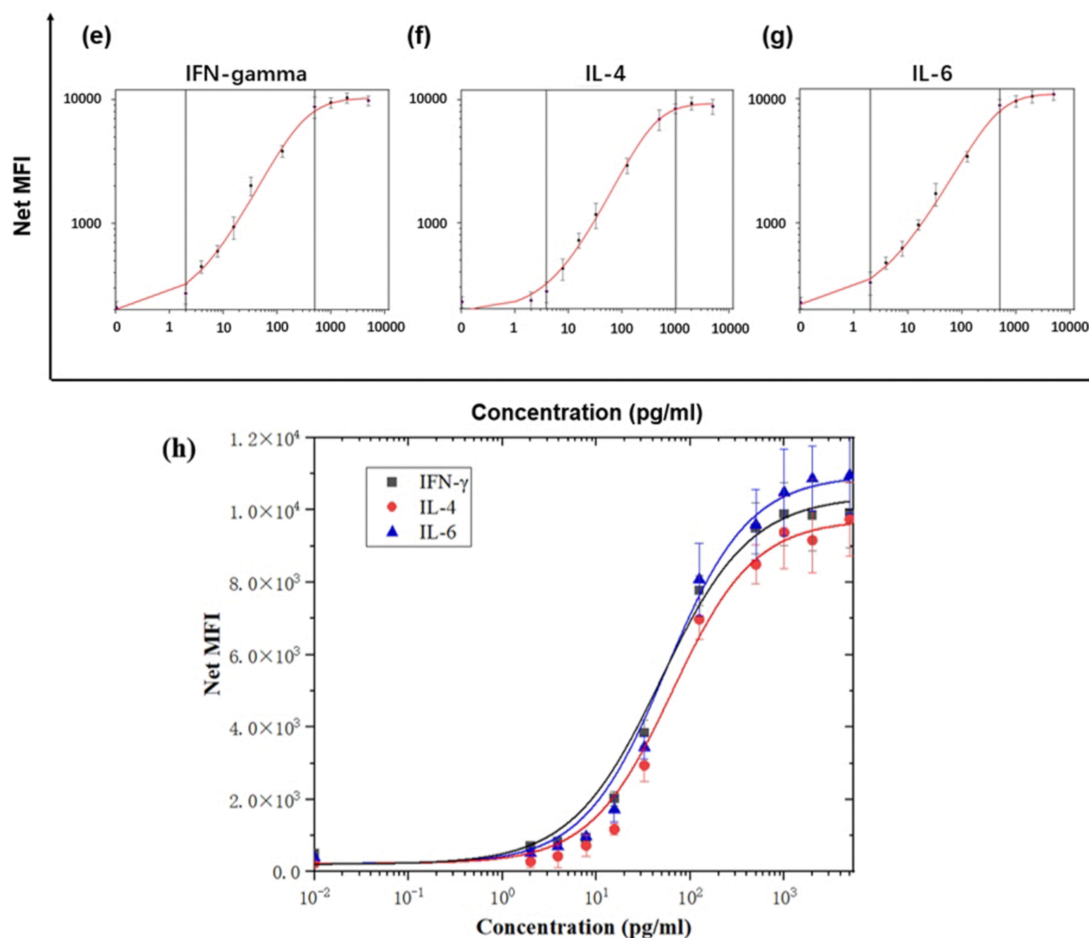

Fig. 5. (*continued*).

cytokines, then detection antibodies and PE-labelled streptavidin were added, forming sandwich immunocomplexes. After the reactions, the microbeads were measured one by one by flow cytometry. The Alexa 647 channel was used to decode the microbeads based on the Cy5 intensity, identifying which antigen the microbead targets for; while the PE channel was used to quantify the amount of antigen bounds to the microbead. It is worth noting that the multiplex immunoassay only required a small amount of sample (25 μL) and microbeads (approximately 4000 beads for each target). To achieve fast reaction kinetics, the reactions were carried out under constant shaking. All the washing steps were rapidly done by magnetic manipulation, owning to the paramagnetic property of the microbeads. The entire immunoassay took less than two hours, and we did not observe bead aggregation or close packing effects during the process. Fig. 5b-d show one representative data, where 2 pg/mL of IL-6 was present in the sample. It can be seen that only the group of microbeads coated with IL-6 capture antibody produced signals in PE channel, whereas the PE signals for the other two groups of microbeads are similar to the negative control, demonstrating no interferences or cross-reaction in the multiplexed assay.

Next, we performed linearity test for all the three cytokines. For each cytokine, ten different concentrations were prepared, ranging from 2 pg/mL to 5 ng/mL. As shown in Fig. 5e-g, by plotting the mean fluorescent intensity (MFI) of the PE signal from corresponding groups of microbeads, the calibration curves for the three cytokines were obtained.

The LODs for IFN-γ, IL-4, and IL-6 were determined to be 2, 3.9, and 2 pg/ml, respectively. The LODs for different cytokines were calculated by adding 3 × standard deviation (SD) to the fluorescence intensity of the corresponding control group, with R^2 values of 0.994, 0.999, and 0.993, respectively. The linear ranges for the three cytokines were 2–1000, 3.9–1000, and 2–1000 pg/mL, respectively, which meets the requirements for clinical applications. [45,46]. The LODs obtained using the dextran microbeads were compared with those of commercial cytokine assay kits, demonstrating similar detection limit (Table S5). We also tested the repeatability of the multiplexed immunoassays by running the assays in 20 repetitions, and inter-assay CV was found to be less than 10 %, demonstrating the reliability of detections using encoded dextran microbeads.

Subsequently, we performed multiplex immunoassays with three cytokines added simultaneously in a single reaction. The experimental steps were identical to those of single-analyte experiments. The analytes were captured by corresponding barcoded microbeads, followed by decoding and quantitative analysis using flow cytometer. Fig. 5h illustrates the fitted standard curves for the 3-plex cytokine detection. The measured net mean fluorescence intensity (MFI) were similar to those of single-analyte assays at all concentration levels (Fig. 5e-g). There was no apparent increase in background noise, and no cross-reactions were observed. The results demonstrated the feasibility and reliability of the fluorescent-coded dextran microbeads for multiplexed detection.

3.8. *Stability test*

To evaluate the stability of the dextran microbeads, we stored the microbeads for four months, and performed immunoassay for IFN-γ with concentrations ranging from 0.01 to 5 ng/ml. As shown from Fig. 6, the LOD remained at 2 pg/ml, with R^2 values of 0.981, and a linear range of 2–1000 pg/ml. This indicates that there was no significant change in the detection ability of the dextran microspheres after four months of

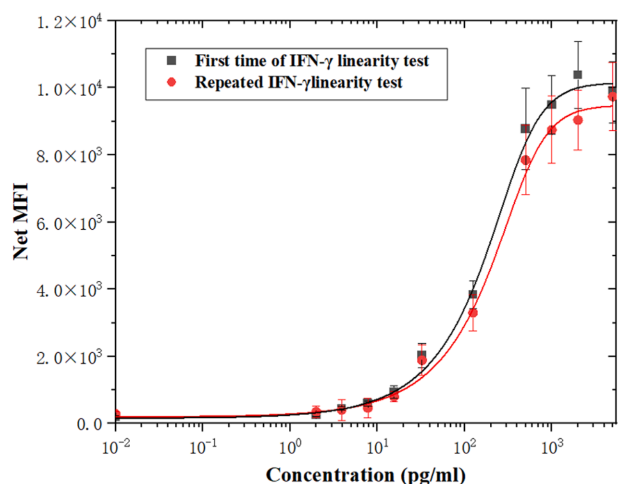

Fig. 6. Stability test results of dextran microspheres coupled with IFN-γ capture antibodies.

storage, so when the microspheres were not in use, they can be temporarily stored in PBS (pH 7.4) at 4 °C, and the detection reproducibility of the suspension array is acceptable. Therefore, the proposed immune assay system demonstrates excellent stability for practical applications.

3.9. *Biodegradability test*

To test whether the dextran microbeads can be degraded by acids, we dissolved the microbeads in hydrochloric acid solutions with pH ranging from 2 to 6, for 24 h at 50 °C. The microscopic examination (Fig. 7b-f) of the dextran microspheres shows that, the lower the pH, the more reduction of microbeads compared to the control group (Fig. 7a). For the samples in the hydrochloric acid solution at pH 2, the microspheres were completely degraded. This demonstrates the superior degradability of the dextran microspheres.

4. Conclusion

In this paper, we present a new type of dextran microbeads for

Fig. 7. (a) The morphology of dextran microspheres after being placed in MQ water at 50 °C for 24 h. (b-f) The morphology of dextran microspheres placed in hydrochloric acid solutions with pH values of 6, 5, 4, 3, and 2, respectively, at 50 °C for 24 h.

multiplexed immunoassays. Dextran as the material for microspheres is more advantageous in terms of biocompatibility and eco-friendliness when compared to the other commonly used polymer materials. Three novel techniques have been developed, including the simple SPG-based microsphere preparation method, the efficient dendrimer coating process and the facile fluorescent encoding approach. With the technologies, it is possible to produce the dextran microbeads in large scale for industrial applications. We have successfully prepared distinctly encoded dextran microbeads and employed them for the detection of cytokines in a multiplexed immunosensing system. The dextran microbeads-based suspension array exhibits high sensitivity, a wide linear range (2–1000, 3.9–1000, and 2–1000 pg/mL for IFN-γ, IL-4, and IL-6, respectively), excellent reproducibility, and high stability. Our work shows that the dextran microbeads hold enormous potential as a sustainable alternative for multiplexed assays.

CRediT authorship contribution statement

Jing Zhang: Methodology; Investigation; Validation; Data curation; Formal analysis; Visualization; Writing - original draft; and Writing - review & editing. **Tao Zheng:** Conceptualization; Data curation; Methodology; Writing - review & editing. **Seyed Hossein Helalat:** Methodology; Writing - review & editing. **Murat Nulati Yesibolati:** Methodology. **Yi Sun:** Conceptualization; Funding acquisition; Investigation; Methodology; Supervision; Writing - review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Yi Sun reports financial support was provided by Novo Nordisk Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcis.2024.04.061.

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